Chapter 8

Western Blotting Analysis as a Tool to Study Receptor Tyrosine Kinases

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Abstract

Receptor tyrosine kinases (RTKs) are involved in critical aspects of cell physiology ranging from cell survival, proliferation, growth, migration, and differentiation. A tight control of the extent and duration of signals elicited by activated RTKs is crucial for preventing over-stimulation, which can ultimately lead to unrestrained proliferative ability and neoplastic growth. Ligand-induced downregulation of RTKs has emerged as a key negative regulatory mechanism that can accomplish signaling attenuation, by removing activated receptors from the cell surface and committing them to degradation. The ability of RTKs to escape from ligand-induced downregulation has been reported as a recurrent mechanism of oncogenic deregulation in cancer.

Western blotting procedures have been extensively proven as straightforward assays to evaluate protein expression levels and have been widely applied to study RTKs downregulation.

Key words: Protein, Western blotting, Receptor tyrosine kinases

1. Introduction

Deregulated activation of receptor tyrosine kinases (RTKs) has been extensively documented in different types of human tumors (1) and frequently correlates with poor responsiveness to conventional therapies. The tight control of signals elicited by activated RTKs is critical for preventing over-stimulation and receptor downregulation has been shown to play a key role in this process (2, 3).

The product of the proto-oncogene RON is one such RTKs, identified as the receptor for macrophage stimulating protein (MSP) belonging to the subfamily of the Hepatocyte Growth Factor (HGF) receptor (4, 5). On engagement by its cognate ligand, Ron activates multiple intracellular signaling pathways

including Ras/MAPK, PI-3K/Akt, JNK/SAPK, β -Catenin, and NF- κ B (6) and mediating cellular proliferation, survival, migration, and differentiation. In physiological conditions, Ron activation is a transient event, whereas its deregulated activation is involved in cancer progression and metastasis in humans (6, 7) and in murine models (8). The RON gene is normally transcribed at relatively low levels in cells of epithelial origins, while overexpression and subsequent aberrant activation have been observed in epithelial cancers (9–11). Downregulation of this receptor, therefore, critically contributes to the maintenance of normal cell phenotype. Moreover, targeting Ron expression by forcing its downregulation has been proposed as suitable tool for therapy of cancers where altered Ron signaling is involved (12). All these studies involve an extensive use of western blotting technique to monitor Ron receptor expression levels.

Widely used in research and clinical laboratories, western blotting is an analytical method that involves the immobilization of proteins on membranes before detection with monoclonal or polyclonal antibodies. As it is based on a good resolution protein separation procedure such as sodium dodecylsulphate polyacrylamide denaturing gel (SDS-PAGE) and on high affinity antibody-antigen interactions, western blotting is a very sensitive and specific technique. Western blotting can provide qualitative information about the presence, the size, and post-translational modifications of proteins, but can also be used in a quantitative way to detect the relative abundance of a given protein in a sample. Since its original invention (13), this technique has improved and vast information exists on troubleshooting and improving the sensitivity, speed, and quantification. A typical western blotting procedure includes the following steps: (1) extraction and quantification of protein samples; (2) resolution of protein samples by electrophoresis on SDS-PAGE; (3) transfer of the separated polypeptides to a membrane support; (4) blocking nonspecific protein binding sites; (5) addition of antibodies; and (6) detection.

A detailed protocol to quantitatively study Ron ligand-dependent and ligand-independent downregulation by western blotting analysis and general methodological conditions optimized for the detection of RTKs expression are reported in this chapter.

2. Materials

2.1. Cell Culture, Extraction, and Quantification of Protein Samples

1. FG2 pancreatic carcinoma cells – RMPI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. NIH-3T3 fibroblast stably expressing Ron (3T3-Ron)-DMEM medium supplemented with 10% FBS and 2 mM L-glutamine.

2. Trypsin solution: 0.5 g/L porcine trypsin and 0.2 g/L EDTA-4Na in Hank's Balanced Salt Solution with phenol red.
3. Phosphate-buffered saline (PBS): sodium chloride 8 g/L, potassium chloride 0.2 g/L, di-sodium hydrogen phosphate 1.15 g/L, and potassium dihydrogen phosphate 0.2 g/L, pH 7.3 at 25°C.
4. MSP (R&D System, Minneapolis, MN), geldanamycin (GA) (Alexis, Montreal, Canada), and 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Alexis).
5. Radioimmune precipitation buffer (RIPA): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS. Protease and phosphatase inhibitors (10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 mM sodium fluoride) to be added immediately before use.
6. Micro-BCA protein assay kit (ThermoFisher Scientific Inc, Waltham, MA).
7. 6-Well cell culture clusters and polystyrene cell scrapers 18-mm blade.

2.2. SDS-Polyacrylamide Gel Electrophoresis

1. Laemmli sample buffer (2×): 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris-HCl, pH 6.8. Stored at -20°C.
2. Resolving buffer (4×): 1.5 M Tris-HCl, pH 8.8, 0.4% SDS. Stored at room temperature.
3. Stacking buffer (4×): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS. Stored at room temperature.
4. Acrylamide/bis-acrylamide, 30% solution (Sigma). Stored at 4°C.
5. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED) (Sigma). Stored at 4°C.
6. Ammonium persulfate: 10% solution in double-distilled water, freshly prepared.
7. Running buffer (10×): 250 mM Tris, 1.92 M glycine, and 1% (w/v) SDS. Stored at room temperature.
8. Prestained Molecular weight markers: PageRuler™ Prestained Protein Ladder (Fermentas, Burlington, Canada).
9. 1-D Electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA).

2.3. Western Blotting

1. Blotting buffer: 25 mM Tris-HCl pH 8.3, 192 mM glycine and 20% (v/v) methanol. Stored at 4°C. Methanol to be added immediately before use.

2. Immun-Blot PVDF membrane and extra thick blot paper (Bio-Rad Laboratories Inc.).
3. Tris-buffered saline (TBS, 10×): 100 mM Tris-HCl pH 7.5, 1.5 M NaCl. Stored at room temperature.
4. Blocking buffer: 5% (w/v) bovine serum albumin (BSA) solution in TBS. Single aliquots frozen at -20°C.
5. Washing buffer (TBS-T): 1× TBS solution supplemented with 1% Tween-20. Stored at room temperature.
6. Antibody dilution buffer: 1× TBS supplemented with 3% (w/v) BSA and 1% Tween-20. Single aliquots frozen at -20°C.
7. Primary antibodies: Polyclonal antibody against Ron C-terminal domain (C-20) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:200 in antibody dilution buffer and monoclonal anti- α -tubulin (B-5-1-2) (Sigma) diluted 1:5,000 in antibody dilution buffer before use.
8. Secondary antibodies: Anti-rabbit and anti-mouse IgG HRP-linked (Cell Signaling Technologies Inc., Danvers, MA). To be diluted 1:1,000 in antibody dilution buffer before use.
9. Enhanced chemiluminescent (ECL) reagents: Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).
10. Stripping buffer: 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS: Stored at room temperature. Warm to 70°C and add 100 mM β -mercaptoethanol before use.
11. Wet electroblotting system (Bio-Rad Laboratories Inc.).
12. Imaging system: Molecular Imager Versa-Doc MP system (Bio-Rad Laboratories Inc.).

3. Methods

3.1. Cell Lysis and Samples Preparation

The effectiveness of the western blot analysis is strictly dependent on the quality of protein lysates prepared for polyacrylamide gel electrophoresis (SDS-PAGE). There are a number of available cell lysis buffers (14) that can be used depending on whether an antigen is primarily extracellular, cytoplasmic, or membrane-associated. The use of the appropriate detergent for protein extraction is, therefore a key step to obtain reliable results. RIPA lysis buffer is one of the most used buffers to lyse cultured mammalian cells from both plated cells and cells pelleted from suspension cultures. It contains two ionic detergents, SDS and sodium deoxycholate, as well as a non-ionic detergent Triton X-100, critical for denaturing proteins, breaking many protein-protein interactions and enabling

the extraction of cytoplasmic, membrane, and nuclear proteins. To obtain reliable and reproducible results, quantification of protein content in cell extracts with a good precision is mandatory to proceed with quantitative studies. Indeed, even if it is possible to normalize for small differences in total protein by the use of housekeeping genes (e.g., α -tubulin), significant discrepancies in protein loading results in defective results of the western blotting analysis. It is thus of great importance to select an appropriate protein quantification assays. The procedure based on the use of bicinchoninic acid (BCA) has proved to be particularly suitable to use with cell lysates, given its compatibility with most ionic and non-ionic detergents.

1. For ligand-dependent downregulation studies, pancreatic carcinoma FG2 cells are seeded at sub-confluent density on 6-well plates (one well is required for each experimental data point) in normal growth medium and incubated for 24 h. The cultures are then rinsed twice with PBS and incubated for a further 24 h in medium without serum (see Note 1). Treatments with vehicle (-) or different concentration of MSP are performed for the indicated times (see Fig. 1a, b) and the cultures are then lysed.
2. For drug-induced downregulation studies, NIH-3T3 fibroblasts (3T3-Ron) and FG2 cells, expressing recombinant and endogenous Ron, respectively, are seeded at sub-confluent density on 6-well plates (one well is required for each experimental data point) in normal growth medium and incubated for 24 h. The cultures are then treated with vehicle (-), GA or 17-AAG (see Fig. 2) for the indicated times and then lysed.
3. For cell lysis, the cultures are carefully rinsed twice with PBS and ice-cold RIPA buffer, containing protease and phosphatase inhibitors (see Note 2), is added directly on cultured cells (150 μ L RIPA buffer are required for each well). Plates are incubated on ice for 5 min with occasional swirl.
4. Bottom of wells is gently scraped with clean cell scrapers and cell lysates are collected in microcentrifuge tubes.
5. Lysates are then cleared by centrifugation at $13,000 \times g$ for 15 min at 4°C and supernatants are transferred to fresh tubes. This separates total proteins (supernatant) from the cellular debris (pellet).
6. Five microliter aliquots are used for protein quantification. The lysates are stored at -80°C, if not used immediately (see Note 3).
7. Total protein content of the samples is determined using the Micro BCA Protein Assay kit (see Note 4). Before starting, preheat a hot-block or water bath to 60°C.

Fig. 1. Ligand-induced Ron downregulation. (a) FG2 cells endogenously expressing Ron are treated with vehicle (-) or with the indicated amount of human recombinant MSP for 8 h. Cell lysates are analyzed by western blotting with a polyclonal antibody against Ron C-terminal domain, recognizing both the 170-kDa precursor and the 150-kDa β -chain of the receptor. α -Tubulin blotting is used as loading control. In the lower panel, densitometry of Ron immunoblotting is shown and values, normalized to the relative α -tubulin control bands, are plotted as percentages of vehicle-treated cells. Each data point represents the mean $\pm$ SE of three independent experiments. (b) Time course of MSP-induced Ron downregulation. FG2 cells are treated with 100 ng/ml. MSP for the indicated times or left untreated (0 h). Cell lysates are analyzed by western blotting as described in panel (a). In the lower panel, densitometry of Ron is shown and values, normalized to the relative α -tubulin control bands, are plotted as percentages of unstimulated cells. Each data point represents the mean $\pm$ SE of three independent experiments.

8. Calculate the total volume (TV) of BCA Working Solution for the unknowns samples, six standards and 1 mL excess [TV = (# unknowns + 6 standards + 1) $\times$ 0.5 mL]. Use 25 parts of reagent A, 24 parts of reagent B, and 1 part of reagent C, using the following formula: $V_A = TV/50 \times 25$; $V_B = TV/50 \times 25$; $V_C = TV/50$. Mix the reagents thoroughly.
9. Prepare the test samples: dispense 495 μ L of water in 1.5-mL test tubes and then add the 5 μ L cell extract aliquots from step 6 to each tube.
10. Prepare the BSA standards at the following final concentrations: 0 μ g/mL (blank), 1, 2.5, 5, 10, and 20 μ g/mL. Dispense the following amounts of water in 1.5-mL test tubes: 500, 499, 497.5, 495, 490, and 480 μ L. Then add to the tubes 0, 1, 2.5, 5, 10, and 20 μ L of 1 μ g/ μ L BSA solution, respectively.
11. Add 500 μ L of the working solution to each test tube, mix thoroughly and incubate the tubes in the heat-block or water bath at 60°C, with the caps closed.

Fig. 2. (a) Geldanamycins induce degradation of both precursor and mature cell surface Ron. 3T3-Ron and FG2 cells are treated with vehicle (-) or 1 μ M GA or 1 μ M 17-AAG for the indicated times. Cell lysates are analyzed by western blotting with a polyclonal antibody against Ron C-terminal domain. α -Tubulin blotting is used as loading control. (b) Densitometry of Ron immunoblotting is shown and values, normalized to the relative α -tubulin control bands, are plotted as percentages of vehicle-treated cells. White columns: GA; black columns: 17-AAG. Each data point represents the mean $\pm$ SE of three independent experiments.

12. Cool the tubes at room temperature for 5 min, then transfer the content in plastic cuvettes and immediately read absorbance at 562 nm, with the spectrophotometer zeroed using a cuvette containing the 0 μ g/mL standard.
13. Prepare a standard curve by plotting the Abs for each standard against its concentration. Use the standard curve to calculate the protein concentration of each unknown sample.
14. Preheat the hot-block to 95°C. Transfer 25–30 μ g of total protein samples from step 5 to new tubes and add an equal amount of Laemmli sample buffer (2 $\times$) (see Note 5). Close the lids and incubate the samples at 95°C for 5 min to denature the proteins. Cool down to room temperature before loading the gel (see Note 6).

3.2. SDS-PAGE

The SDS-PAGE procedure utilizes the strongly anionic detergent sodium dodecyl sulfate with or without a reducing agent and heat to denature proteins. Proteins bind to SDS within and as a result become negatively charged. The amount of SDS that binds to the denatured proteins is approximately proportional to the molecular mass of the polypeptides (i.e., all proteins acquire the same charge/mass ratio) facilitating directional migration of the highly negatively charged SDS-protein complexes in the polyacrylamide gel based on polypeptide size.

1. The protocol here reported refers to the use of mini gels 1-D Electrophoresis system (Bio-Rad Laboratories), but can be adapted to other formats.
2. Carefully scrub the clean glass plates with 95% (v/v) ethanol and assembly the front and back glasses in the apposite clamps. It is advisable to check that there is no leakage from the bottom by pouring water inside the plates. Then pour off the water.
3. Prepare a 7.5% gel solution by mixing 2 mL of resolving buffer (4×), 2 mL of 30% acrylamide/bis-acrylamide solution, and 4 mL of water. Then add 100 μ L ammonium persulfate solution and 40 μ L TEMED, mix and immediately pour the gel, leaving enough space for the stacking gel. Overlay with ethanol and allow to polymerize (see Note 7).
4. Pour off the ethanol and rinse with water. Then pour the stacking gel solution prepared by mixing 1 mL stacking buffer (4×), 0.5 mL of 30% acrylamide/bis-acrylamide solution and 2.5 mL of water. Immediately insert the combs and allow to polymerize (see Note 7).
5. Remove the glasses from the holder, then assemble the gasket with the electrodes. Remove the comb, fill the gasket and the outer chamber with running buffer (1×), and then wash the wells with a syringe fitted with a thin gauge needle.
6. Load the samples and the molecular markers (see Note 8) in the wells.
7. Assembly the unit and connect to the power supply. Use constant voltage up to 130 V through the stacking gel, then up to 150 V in the running gel. Turn off the power supply immediately after the bromophenol blue dye has run off the gel and disconnect the power supply.

3.3. Western Blotting

This section describes the electrotransfer of proteins separated from SDS-PAGE gels via a step-by-step procedure.

1. The procedure here described assumes the use of a wet transfer method (see Note 9) employing a Bio-Rad Trans-Blot system.

2. Wet a sheet of PVDF paper, cut slightly larger than the gel size, in a tray containing methanol for 1 min to activate the membrane. Then transfer it in another tray filled with blotting buffer. Wet two sheets of extra thick paper and two sponges in blotting buffer.
3. Disassemble the gel unit, cut and remove the stacking gel with a blade, and then transfer the resolving gel in a tray containing blotting buffer. If desired, a corner of the gel can be cut to allow the tracking of the gel orientation.
4. Assemble the transfer cassette by lying a sheet of paper onto a sponge and the PVDF membrane on the top. The gel is then carefully laid in top of the membrane. Then another sheet of thick paper and a sponge are positioned on top of the gel and the transfer cassette is locked. Make sure that no air bubbles remain between the gel and the membrane.
5. Insert the cassette in the transfer tank, carefully checking the orientation, the membrane must be oriented towards the anode, while the gel towards the cathode (see Note 10). Put an iced freezer pack in the tank, add enough blotting buffer to cover the cassette and activate a magnetic stir-bar in the tank to avoid heating of the buffer.
6. Close the lid, connect the unit to the power supply and begin transfer, with a constant current of 200 mA for 1.5 h.
7. Disconnect the power supply; then disassemble the transfer cassette. Remove the sponge, the paper, and the gel. If using pre-stained markers, check that their corresponding bands are clearly visible on the membrane.
8. Add to the membrane 10 mL of Ponceau S staining solution and incubate the membrane for 2 min. The Ponceau S solution can be reused several times.
9. Wash the membrane briefly with TBS to remove excess staining. Continue washing the membrane until the staining is gone. If stains persist, wash the membrane TBS containing 0.02% NaAzide for 1–3 min and then rinse once with TBS.
10. The membrane is then incubated in 15 mL blocking buffer for 1 h at room temperature on a rocker with gentle shaking (i.e., 20 rpm).
11. After blocking, the membrane is rinsed twice with TBS and then incubated for 3 h with primary antibody solution at room temperature with gentle shaking. Alternatively, the incubation can be performed overnight at 4°C to enhance the signal.
12. The primary antibody is then removed (see Note 11) and the membrane is washed three times for 10 min each with 15 mL of TBS-T with vigorous shaking (i.e., 40 rpm).

13. Freshly prepared secondary antibody solution is then added to the membrane for 1 h at room temperature with gentle shaking.
14. The secondary antibody solution is discarded and three washes for 10 min each with TBS-T are performed with vigorous shaking.
15. The ECL reagents are mixed together in the ratio 1:1 immediately before use and evenly added to the blot for 3 min.
16. The excess of ECL is removed and the membrane is put in a tray. Proceed with image acquisition as detailed in the next section.
17. Once a satisfactory signal has been obtained, wash the membrane and then proceed with the stripping procedure to clear the membrane before reprobing for a housekeeping gene (see Note 12).
18. For the stripping procedure, warm 30 mL stripping buffer at 70°C and then add. Incubate the membrane in this solution for 30 min and then perform extensive washes with TBS. Repeat the blocking step again before reprobing with the primary antibody solution.

3.4. Quantification by Image Densitometry

Digital imaging systems are preferably used in protein expression analysis. Indeed, they display a higher dynamic range when compared to autoradiography films (15). Moreover, digital imaging allows rapid data digitization and monitoring of image saturation immediately after the acquisition. Systems equipped with flat field correction give best results, due to a lower coefficient of variation along the image.

1. A suitable exposure for reliable data quantification must not present saturated signals. This could be easily checked when using digital imaging system is a quantitative imaging system (see Note 13). If using autoradiography sheets, acquire the image by scanning (a scanner that has a transparency mode gives better results).
2. Once a satisfactory image has been obtained, proceed with receptor bands quantification using an Image Analysis Software.
3. Subtract background signals from all bands (see Note 14).
4. Repeat steps 3 and 4 quantifying the housekeeping gene bands (i.e., α -tubulin).
5. Calculate, using background-subtracted data, normalized values by dividing receptor bands densities by their respective housekeeping gene bands densities (i.e., Ron/α -tubulin).
6. Set normalized values obtained from the unstimulated cells as the calibrator.

7. Calculate the fraction of residual receptor expression after stimulation: divide all normalized values by the calibrator value (i.e., MSP-stimulated cells/unstimulated cells).
8. Alternatively, the percentage of receptor reduction can be easily calculated by the following formula: $100 \times (\text{calibrator} - \text{sample}) / \text{calibrator}$ (i.e., $100 \times (\text{unstimulated cells} - \text{MSP-stimulated cells}) / \text{unstimulated cells}$).

4. Notes

1. Serum starvation is required when studying ligand-induced receptor downregulation, as growth factor contained in serum can bias the results obtained.
2. Proteases can be released during lysis, as well as phosphatases, and act on the target proteins. Therefore, to prevent proteolysis, protease and phosphatase inhibitors should be included in the lysis buffer and added immediately before applying it to the cultures. Inhibitors are available separately or can be purchased as cocktails in tablet form.
3. To avoid protease degradation of proteins in the samples, cell lysis should be performed quickly on ice and cell lysates should be immediately stored at -80°C and further freeze and thaw should be avoided.
4. The linear working range of this procedure is $0.5\text{--}20\text{ }\mu\text{g/mL}$. Several kits based on BCA, with different linearity and volume formats, are available on the market.
5. Note that up to $70\text{ }\mu\text{L}$ can be accommodated in each well of the gel, when using 1.5-mm spacers and 10-well combs, so consider lowering the total amount of proteins to be loaded in case of low concentration samples (but avoid quantity lower than $10\text{--}15\text{ }\mu\text{g}$).
6. Boiled samples can be stored at -80°C or -20°C . Boil again at 95°C for 1 min when thawing, to facilitate SDS resolubilization.
7. Polymerization is complete when the extra gel solution that has not been poured and has been kept in the tube is completely polymerized.
8. Molecular markers based on recombinant proteins have the advantage of lot-to-lot consistency. Prestained molecular weight marker standards are available from several companies. Markers containing protein conjugated to different dyes (multicolor markers) allow an easier identification of the bands.
9. Transfer tank blot systems (also known as wet transfer) offer a more efficient transfer of high molecular weight proteins (16),

such as RTKs. Moreover, with the semi-dry system, some molecules will not transfer quantitatively.

10. The Trans-Blot system from Bio-Rad is endowed with a black and white color code to check the correct orientation of the cassette.
11. Primary antibody solutions can be used several times. It is possible to store them up to 1 month at 4°C upon addition of 0.02% of NaAzide. Nevertheless, it should be taken into consideration a progressive loss of signal due to antibody depletion after each incubation step.
12. When using a housekeeping gene with a molecular weight significantly different from the gene of interest (as in the case of α -tubulin and Ron), it is possible to proceed with the second staining step without stripping the membrane.
13. When using the Quantity One Software with a GelDoc or VersaDoc System, verify that the "highlight saturated pixel" option is ticked: saturated pixels will turn red.
14. Image analysis software offers many options to perform background calculation and subtraction. An easy way to do it, is to select an empty area of the membrane of the same size of an average band, and use as background reference.
15. This protocol can be adapted for many other protein quantification issues. For instance, it can be used to monitor protein overexpression/silencing in gain-of- or loss-of-function experiments.

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