

Techniques to Study FcεRI Signaling

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Summary

Mast cells are the crucial effector cells for allergic reactions. They are activated through the aggregation of the high-affinity immunoglobulin E (IgE) receptor (FcεRI) with allergen and allergen-specific IgE. Tyrosine phosphorylation of FcεRI subunits and various signaling proteins is an initial triggering event, leading to the activation of several signaling pathways in mast cells. Much has been learned from analysis of mast cells derived from gene-targeted mice. Therefore, in this chapter we will first describe how to generate mast cells from mouse bone marrow cells and how to correct the genetic defect by retroviral transduction. Then, we will describe how to assess early activation events by measuring several protein-tyrosine kinases and serine/threonine kinases such as protein kinase B (= Akt), protein kinase C, and JNK. As signal transduction is highly dependent on protein-protein interactions, we will describe experimental details of co-immunoprecipitation methods that are used to confirm such interactions.

Key Words: Mast cell; FcεRI; Lyn; Fyn; Src; Syk; Btk; JNK; PKC; Akt; co-immunoprecipitation; retroviral transduction; in vitro kinase assay.

1. Introduction

Despite a recent flurry of articles indicating roles for mast cells in innate immunity, autoimmune disease models, angiogenesis, and chronic cardiac failure, immediate hypersensitivity and allergic diseases remain the most important focus in mast cell research (1). Because allergic reactions in these diseases are largely dependent on allergen and allergen-specific immunoglobulin E (IgE), the study of the effector cells that are activated by these allergy-triggering agents is central to our understanding of allergy. Mast cells (and basophils) are considered to be the major effector cell type. IgE is bound to the high-affinity IgE receptor, FcεRI, on the surface of mast cells, and IgE-bound FcεRI molecules are aggregated with multivalent allergen. The FcεRI expressed on mast cells consists of IgE-binding α subunit, a signal-amplifying β subunit, and two disulfide-bonded γ subunits with a signal-initiating capability (2).

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Upon receptor aggregation, receptor-bound Src family protein tyrosine kinases (PTKs), such as Lyn and Fyn, are activated. Activated Src PTKs phosphorylate tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) in the β and γ subunits. Phosphorylation of ITAMs in the β and γ subunits creates the binding sites for Lyn and Syk (another PTK with two tandem SH2 domains upstream of its catalytic domain), respectively. These PTKs are activated and phosphorylate a variety of substrates, including adaptor proteins, enzymes (kinases, phosphatases, phospholipases), transcription factors, and cytoskeletal proteins. These phosphorylations eventually lead to the activation of several signaling pathways, such as phospholipase C/ Ca^{2+} , Ras/mitogen-activated protein kinase, nuclear factor- κB , AP1, and NF-AT. Finally, coordinate activation of these pathways results in degranulation, synthesis and release of lipid mediators, and synthesis and secretion of cytokines and chemokines (3,4).

Much of signaling networks has been figured out by analysis of mast cells derived from gene-targeted mice. Therefore, this chapter will begin with a description of how to generate mast cells from bone marrow cells (5). Biological and biochemical phenotypes found in mast cells derived from gene-targeted mice can most definitively be ascribed to the lack of the gene by reconstituting the mutant cells with wild-type gene or complimentary deoxyribonucleic acid (cDNA). The current standard method of gene transduction in mast cells is retroviral transduction (6). Therefore, we will describe our standard procedures for retroviral transduction. Although the research in Fc ϵ RI signaling deals with many classes of molecules, our description here will be focused on early activation events, that is, activation of PTKs of the Src, Syk, and Tec families and several serine/threonine kinases (PS/TKs). Because signal transduction often depends on protein–protein interactions, experimental details in co-immunoprecipitation will be depicted as well.

2. Materials

2.1. The Generation of Bone Marrow-Derived Mast Cells (see Note 1)

1. BMMC medium (600 mL): 500 mL RPMI1640 (GIBCO, Carlsbad, CA), 10% FCS (60 mL, not heat-inactivated, GIBCO), 40 mL D11* (~7%, IL-3), 2 mM L-Glutamine (6 mL of 200 mM solution, Calbiochem, La Jolla, CA), 0.1 mM non-essential amino acids (6 mL of 10 mM solution, GIBCO), 50 $\mu\text{g}/\text{mL}$ Gentamycin sulfate (0.6 mL of 50 mg/mL solution, Calbiochem), 50 μM 2-mercaptoethanol (0.6 mL of 50 mM solution, Sigma, St. Louis, MO).

2.2. Retroviral Transduction

1. sBMMC medium (600 mL): 438 mL RPMI1640, 15% FCS (90 mL, not heat-inactivated), 60 mL D11*, 2 mM L-Glutamine, 0.1 mM nonessential amino acids, 50 $\mu\text{g}/\text{mL}$ Gentamycin sulfate, 50 μM 2-mercaptoethanol, 50 ng/mL recombinant mouse stem cell factor (SCF).

2.3. Fc ϵ RI Stimulation

1. Tyrode's buffer: 112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4 , 1.6 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.94, 0.05% gelatin, and 0.1% glucose.
2. Anti-DNP IgE.
3. DNP₂₃-HSA.

2.4. Kinase Assays

1. Lysis buffer: 1% Nonidet-P40 (NP-40) lysis buffer: 1% Igepal CA-630 (Sigma; see Note 2), 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% NaN_3 .
2. Inhibitors (all from Sigma): add inhibitors to the lysis buffer right before use. 1 mM Na_3VO_4 , 1 mM phenylmethyl sulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 μM pepstatin, 25 μM *p*-nitrophenyl *p*'-guanidinobenzoate, 2 mM NaF.
3. 5X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 0.5 M dithiothreitol (DTT), 10% SDS, 0.4 M Tris-HCl, pH 6.8, and 50% glycerol.
4. PTK kinase buffer:
 - a. –ATP buffer: 20 mM HEPES, pH 7.5, 10 mM MgCl_2 , and 10 mM MnCl_2 .
 - b. +ATP buffer (for autophosphorylation): –ATP buffer, 0.1 mM cold ATP, and 10 μCi [γ - ^{32}P] ATP (ICN; Irvine, CA)/reaction.
 - c. +ATP buffer with substrate: the above buffer + 2 mg enolase/reaction.
5. Preparation of acid-denatured enolase:
 - a. Thaw 100 μL aliquot of frozen enolase (2 mg/mL in H_2O ; keep at -20°C).
 - b. Add 100 μL of 50 mM acetic acid.
 - c. Incubate at room temperature for 5 min.
 - d. Add 50 μL of 1 M HEPES, pH 7.0.
 - e. Keep at room temperature until use.
6. Akt kinase buffer:
 - a. –ATP buffer: 20 mM Tris-HCl, pH 7.5, 50 mM MgCl_2 , 1 mM DTT, 25 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM Na-o-vanadate, and 1 mg/mL microcystin.
 - b. +ATP buffer: –ATP buffer, 2 μg of crosstide (Upstate, Charlottesville, VA)/reaction, 50 μM cold ATP, 10 μCi [γ - ^{32}P] ATP/reaction.

2.5. Immunoprecipitation

1. Antibodies:
 - Anti-Lyn: Lyn (44)
 - Anti-Btk: Btk (M138)
 - Anti-Akt: Akt1 (C-20)
 - Anti-JNK1: JNK1 (C-17)
 - Anti-PKC α : PKC α (C-20)
 - Anti-PKC β I: PKC β I (C-16)
 - Anti-PKC β II: PKC β II (C-18)

(All antibodies are from Santa Cruz Biotechnology, Santa Cruz, CA).

2. Pansorbin (Calbiochem; La Jolla, CA).
3. Protein G PLUS-Agarose (Santa Cruz Biotechnology).
4. High salt wash buffer for coimmunoprecipitation: 1 M NaCl, 1% Triton X-100, and 10 mM Tris-HCl, pH 7.2.

2.6. Equipment

1. Culture flasks and tubes; all from BD Falcon.
2. Dissection kit: forceps, scissors, 10-mL syringes, and 26-gauge needles.
3. SDS-PAGE apparatus (Biometra/Labrepco, Horsham, PA).
4. Gel transfer apparatus (Amersham; Piscataway, NJ).
5. Liquid scintillation counter (Beckmann).

3. Methods

The methods described in **Subheading 3.1–3.4.** will outline; (1) the generation of BMMCS from mouse bone marrow cells; (2) the genetic manipulation of BMMCS by retroviral transduction; (3) the measurement of PTKs and PS/TKs; and (4) a detailed procedure to detect protein–protein interactions by co-immunoprecipitation.

3.1. Generation of BMMCS

Here we describe our standard procedures for BMMC generation, which usually takes 4–6 wk (*see Note 1*). After this period, mast cells constitute more than 95% of live cells, as assessed by flow cytometry for positive expression of FcεRI and c-Kit. With this high percentage, the cells are ready for biological (e.g., assays for degranulation, leukotriene, and cytokine secretion), biochemical (e.g., kinase assays, phosphatase assays, GTPase measurement, and so on), genetic (e.g., cDNA transfection, siRNA, and so on), and pharmacological (e.g., inhibitors and activators) experimentations.

3.1.1. Day-by-Day Procedures

DAY 1

1. Euthanize a mouse with carbon dioxide (CO₂). Drench the mouse in 70% alcohol to sterilize it.
2. Strip off the skin from one leg.
3. Holding the femur with the forceps, cut away as much of the quadriceps muscle as possible. Then cut the muscles behind the knee joint.
4. Bend the knee joint in the “unnatural” or “incorrect” direction to dislocate it. Cut off the lower leg at the broken knee joint.
5. Snip off a small amount of bone from each end to expose the bone marrow.
6. While holding the femur above the open small flasks (25 cm²), take a syringe (with a 26-gauge needle containing 10 mL of BMMC media) and insert the tip of the needle into the exposed bone marrow.

7. Push the full 10 mL of media through the femur slowly and into the flask to wash out the cells and collect the drops in the flask.
8. Repeat **steps 2–7** with the other leg.
9. Store the flasks in a 37°C, 5% CO₂ incubator for 2 d.

DAY 3

1. Transfer each 10-mL culture from the small flask to a medium flask (75 cm²) containing 40 mL of BMDMC medium.

DAY 8

1. Transfer the cultures to 50-mL tubes.
2. Centrifuge the cells at 300g for 5 min and aspirate supernatant.
3. Resuspend the cells in an optimum volume of BMMC medium and transfer to a new flask (*see Note 3*).
4. Store the flasks in the incubator for 1 wk.

DAY 15 (1 Wk LATER)

1. Change the media and flasks again.
2. Store the flasks in the incubator for 1 wk.

DAY 22

1. Change the media and flasks again.
2. Store the flasks in the incubator for 1 wk.

DAY 29

1. Change the media and flasks again.
2. The cells are now ready to use. At this point, BMMCs can be used for approx 2–3 more weeks.

3.2. Retroviral Transduction of BMMCS

This section can be divided into: (1) the construction of retroviral expression vector; (2) the generation of recombinant retrovirus; and (3) the infection of replicating mast cells with recombinant retrovirus (*see Note 4*).

3.2.1. Construction of Retroviral Vector

A Moloney murine leukemia virus-based vector, pMX-puro (8,9), has been extensively used for transfection of mouse BMMC. As almost all gene-targeted mice have a “neo” gene cassette in their genome, the retroviral vector must have a different drug-resistance gene, such as the puromycin resistance gene or another way of selection, for example, a green fluorescent protein gene in the bicistronic gene expression allele. The expression of the latter can be detected by flow cytometry or fluorescent microscopy. The most popular vector, pMX-puro (**Fig. 1**), can accommodate a gene or cDNA in the region

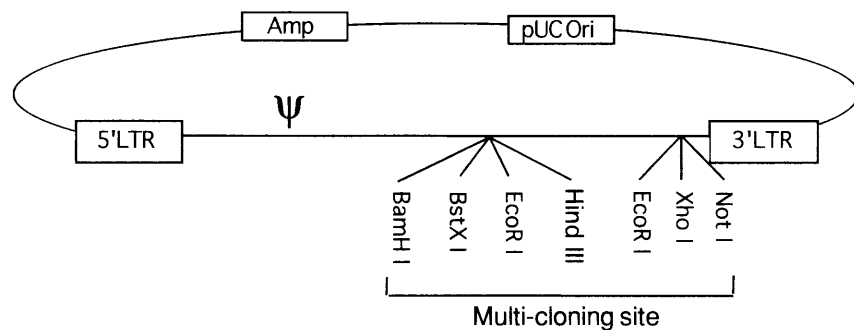


Fig. 1. The most popular vector, pMX-puro (the original figure was kindly provided by Dr. Toshio Kitamura of the University of Tokyo), can accommodate a gene or cDNA in the region between the BamHI (nucleotide 1884) and NotI (nucleotide 3161) sites.

between the Bam HI (nucleotide 1884) and Not I (nucleotide 3161) sites. Standard molecular biological techniques are used to construct recombinant vectors.

3.2.2. Generation of Recombinant Retrovirus

Recombinant retroviral vectors can be transfected into a packaging cell line to generate infectious virus particles. There are several packaging cell lines available for this purpose: for example, BOSC 23 (*10*), Phoenix (*11*), and Plat-E (*12*). Our experience indicates that all of these packaging cell lines yield titers of viruses high enough to produce transduced BMMC in a scale of 5 to 20×10^7 cells after puromycin selection. These numbers of transfectants allow for most of biological and some biochemical analyses. In this section, we will describe our standard protocol using BOSC 23 cells.

3.2.3. Infection of Mast Cells and Selection of Transgene-Expressing Cells

Retroviral genomes can integrate into a host genome only when host cells are replicating. Although IL-3 usually is used as a growth factor to generate BMMC, IL-3 alone is not strong enough to induce vigorous cell cycling for efficient retroviral transduction in BMMC. For this purpose we and others feed bone marrow cells in IL-3 and SCF to generate BMMC that are ready for retroviral infection (hereafter abbreviated as sBMMC).

3.2.4. Day-by-Day Procedures

APPROXIMATELY 10 DAYS BEFORE TRANSFECTION

1. Reconstitute a vial of frozen BOSC 23 cells in DMEM/10% FCS/Gln.

2 DAYS LATER

1. Change medium to GPT selection medium. GPT selection medium: Dulbecco's modified Eagle's medium (DMEM), 10% FCS/Gln, and GPT Selection Reagent (the kit contains 500X mycophenolic acid and 100X aminopterin solution; Specialty Media, Phillipsburg, NJ).

BOSC 23 CELLS PASSAGE

1. After BOSC 23 cells become confluent, passage every 3 or 4 days by three- to fivefold dilution.
2. Aspirate medium very carefully with Pasteur pipet and wash once with 5 mL of PBS.
3. Add 1 mL of trypsin-EDTA and incubate at room temperature for 1–2 min.
4. Add 3 mL of DMEM and pipet well to separate individual cells. It is very important to prevent cells from making cramps.
5. Centrifuge the cells.
6. Suspend in 8 mL of GPT medium per 100-mm dish.
7. Plate BOSC 23 cells homogeneously. Pipet BOSC 23 cells well to prevent them from making cramps.

2 DAYS BEFORE TRANSFECTION

1. Remove GPT selection medium from BOSC 23 cells 48 h before transfection and add DMEM/10% FCS/Gln.

1 DAY BEFORE TRANSFECTION

1. Plate $5.5\text{--}6.5 \times 10^6$ cells per 100-mm dish or 2×10^6 cells per 60-mm dish. For each transfection, prepare three plates/cell densities of 5.5×10^6 , 6×10^6 , and 6.5×10^6 .

DAY 1

1. Make DNA/Lipofectamine mixtures:
 - a. Mix 10 μ g of DNA with 0.75 mL of OPTI-MEM (Gibco) in a Falcon 2059 tube. Add 20 μ L of Plus Reagent (Invitrogen) and incubate at room temperature (RT) for 15 min.
 - b. Mix 30 μ L of Lipofectamine reagent (Invitrogen) with 0.75 mL of OPTI-MEM (Lipofectamine cocktail).
 - c. Transfer Lipofectamine cocktail to the DNA tube, mix, and incubate at room temperature for 15 min.
2. Add 4.5 mL of OPTI-MEM (final total volume: 6 mL/transfection).
3. Pick up BOSC cell plates of the best condition (~60% confluent).
4. Rinse BOSC cells once with 5 mL of OPTI-MEM and overlay the above described DNA-Lipofectamine complex solution (6 mL) onto the rinsed BOSC cells.
5. Incubate at 37°C for 5 h in a CO₂ incubator.
6. Add 6 mL of DMEM/20% FCS/Gln.

DAY 2

1. Replace the medium with 9 mL (per 100-mm dish) of fresh complete medium (DMEM/10% FCS/Gln) at 24 h after transfection.

DAY 3

1. At 48 h after transfection, collect the supernatant of BOSC cells and centrifuge for 5 min at 350g at 4°C to remove living cells.
2. Infection cocktail (10 mL): Supernatant of BOSC cells (8 mL), D11 (1 mL), FCS (1 mL), 0.15 mg/mL SCF (3.3 μ L), and 4 mg/mL polybrene (freshly prepared; 25 μ L).
3. Centrifuge 1×10^7 cells/tube sBMMC, remove supernatant, and suspend the cells in the 10 mL of infection cocktail.
4. Plate sBMMC cells into a 100-mm dish. After 8 h, add 10 mL of fresh sBMMC medium with 10 μ g/mL polybrene.
5. Incubate 16 h in 5% CO₂ incubator.

DAY 4

1. At 24 h after infection, wash the cells, suspend in 25 mL of fresh sBMMC medium, and culture them in a 75-cm² flask (Falcon cat. no. 3111).

DAY 5

1. At 48 h after infection, start to select the cells with puromycin. Add 10 mL of puromycin-containing sBMMC medium to 25 mL of cell culture fluid (final puromycin concentration: 1.5 μ g/mL).

DAY 8

1. At 3 d after selection, add 15 mL of sBMMC medium.

DAY 15

1. At 10 d after selection, remove 15–20 mL of supernatant and add 15–20 mL of fresh medium, including puromycin.

DAY 19

1. After 14–16 d of selection, the cells usually start to grow well, then select living cells using Ficoll as follows.
2. Collect the cells in a 50-mL tube. Spin down at 300g for 5 min.
3. Resuspend the cells in 6 mL of BMMC medium.
4. Overlay the cell suspension in a 15-mL tube containing 6 mL of Ficoll (Roche).
5. Spin at 300g for 15 min.
6. Save the live cells packed between Ficoll and medium and transfer to a 50-mL tube.
7. Wash the cells twice with 30 mL of BMMC medium.
8. Transfer the cells to a 75-cm² flask. Keep incubate for several more days in the selection medium.

2 DAYS BEFORE USING THE CELLS FOR EXPERIMENT

1. Wash the cells twice with BMMC medium and remove puromycin.
2. Suspend the cells in sBMMC medium, and keep incubating until the day the experiment is performed.

THE DAY OF EXPERIMENT

1. Wash the cells twice with BMMC medium to remove SCF from medium.
2. Incubate the cells in BMMC medium with or without IgE for 6–8 h (*see Note 5*).
3. Stimulate cells with antigen as described in **Subheading 3.3.2**.

3.3. Fc ϵ RI Stimulation in BMMC

3.3.1. Sensitization

1. Count mast cell numbers.
2. Transfer the cells to 200-mL centrifuge tubes or 50-mL tubes.
3. Spin down the cells at 300g for 5 min.
4. Resuspend the cells in BMMC medium to a density of 2×10^6 /mL.
5. Add IgE to a final concentration of 0.5 μ g/mL.
6. Incubate overnight in a CO₂ incubator.

3.3.2. Stimulation

1. Spin down the cells in a 50-mL tube for 5 min at 300g.
2. Wash once in Tyrode's buffer.
3. Resuspend the cells in Tyrode's buffer to 2×10^7 cells/mL.
4. Make 1-mL aliquots.
5. Add antigen (DNP-HSA) to a final concentration of 10–100 ng/mL.
6. Incubate at 37°C for predetermined periods (depending on the purpose of the experiment).
7. Spin down the cells and aspirate sup.
8. If you continue experiment, add an appropriate buffer to the pellet. Otherwise, freeze down the cells in dry ice and keep frozen at –80°C until use.

3.4. In Vitro Kinase Assays

Two representative methods will be described here: one is an assay on Btk, in which reaction products are analyzed by SDS-PAGE and followed by blotting, and the other is an assay on Akt, in which reaction products are blotted onto phosphocellulose filters followed by counting the radioactivity. Both methods can be adapted for other PTKs and PS/TKs using proper substrates and kinase reaction conditions (*see Note 6*). These methods are also easy to be adapted when different sources of enzyme, for example, purified or recombinant kinases, can be used in place of cell lysates.

3.4.1. Cell Lysis

1. Add 200 μ L of lysis buffer (+ inhibitors) to the tube containing 2×10^7 cells (*see Note 7*).

2. Incubate on ice with occasional vortexing for 10 min.
3. Spin down the cells at 4°C for 12 min at 15,000g and save sup.

3.4.2. Measurement of Protein Concentration

1. Measure the protein concentration of cleared cell lysate using a Bio-Rad Dc Protein Assay kit following the manufacturer's protocol.
2. Calculate the protein concentration.
3. Make 0.2–1 mg (depends on the protein you want to immunoprecipitate) aliquots at 200–500 μ L (make up with lysis buffer as needed).

3.4.3. Btk Kinase Assay

3.4.3.1. IMMUNOPRECIPITATION

1. To 1 mg of the lysate, add 4 μ g of anti-Btk (M138) antibody.
2. Let sit on ice for 1.5 h.
3. Add 20 μ L of Pansorbin to each tube and vortex at 4°C for 30 min.
4. Wash Pansorbin 4X with 1 mL of lysis buffer and 1X with 1 mL of Btk kinase buffer (–ATP).
5. Remove all sup using Pipetman.

3.4.3.2. KINASE REACTION

1. Add 20 μ L of kinase buffer (–ATP) to the Pansorbin, mix well.
2. Let sit on ice.
3. Add 20 μ L of kinase buffer (+ATP) and mix well by tapping.
4. Incubate at 30°C for 10 min.
5. Stop reaction by adding 10 μ L of 5X SDS/DTT Sample buffer.

3.4.3.3. SDS-PAGE AND BLOTTING

1. Boil sample and load onto 8% SDS–polyacrylamide gel.
2. Run the gel overnight at approx 40 V.
3. Transfer the proteins to PVDF membrane (Millipore), dry the membrane, and then detect the kinase activity by autoradiography.

3.4.4. Akt Kinase Assay

3.4.4.1. IMMUNOPRECIPITATION

1. To 0.5 mg of the lysate, add 2 μ g of anti Akt1 (C-20).
2. Let sit on ice for 1.5 h.
3. Add 20 μ L of Protein G PLUS agarose and shake at 4°C for 30 min.
4. Wash the agarose 4X with 1 mL lysis buffer and 1X with 1 mL Akt kinase buffer (–ATP).
5. Remove the buffer exhaustively using 1-mL syringe with 30-gauge needle.

3.4.4.2. KINASE REACTION (SEE NOTE 8)

1. Add 15 μ L of kinase buffer (–ATP) to the agarose, carefully mix by swirling the tube using tip of Pipetman (see NOTE 9).
2. Let sit on ice.

3. Add 10 μ L of kinase buffer (+ATP).
4. Incubate at 30°C for 10 min.
5. Stop reaction by adding 45 μ L of ice-cold 10% trichloroacetic acid.
6. Spin at 8000g for 2 min.
7. Spot one third of the volume of the total reaction (23.3 μ L) of sup to P81 phosphocellulose filter squares (Upstate cell signaling solutions; 11.6 μ L to each square, and use 2 squares/rxn).
8. Wash filters with 100 mL of cold 0.5% phosphoric acid for 5 min.
9. Repeat washes four times.
10. Wash once with 100 mL of acetone at room temperature.
11. Dry the filters at room temperature.
12. Put filters in a scintillation vial and measure Cerenkov counts.

3.5. Co-Immunoprecipitation

Protein–protein interactions can be studied by a variety of methods: the yeast two hybrid experiment has been used to identify a novel binding partner for many years. With the advent of proteomics techniques, a more recently invented method termed tandem affinity purification is used more often. Structural requirements for interactions between a given protein and its partner have been studied using “pull-down” methods with a precipitable affinity ligand incubated with cell lysates that express the binding partner or a purified, recombinant, or in vitro translated binding protein. A wide selection of affinity ligands, such as glutathione *S*-transferase (GST), hexahistidine, and maltose-binding protein, are available. However, interactions between endogenous cellular proteins are usually confirmed by co-immunoprecipitation of interacting proteins: a protein will be immunoprecipitated by a specific antibody, and immune complexes are analyzed by SDS-PAGE and followed by immunoblotting with an antibody to the interacting partner (see NOTE 10). What follows is the protocol used in our previous study (13).

3.5.1. Co-immunoprecipitation of Btk and PKC β I

1. The cells are sensitized and stimulated as shown in Subheading 3.3.
2. Add 500 μ L of 1% NP-40 lysis buffer containing inhibitors to 4–5 $\times$ 10⁷ cells. Incubate on ice with occasional vortexing for 10 min, centrifuge at 15,000g for 12 min, and save the sup.
3. Measure the protein concentration using Bio-Rad Dc Protein Assay kit following the manufacturer's protocol.
4. Preclear the lysate with Pansorbin. Add 20 μ L of Pansorbin/1 mg of lysate and incubate at 4°C for 30 min on the shaker.
5. Spin down at 15,000g for 5 min and save the sup.
6. Add 5 μ g of anti-Btk (raised in rabbit against mouse Btk carboxyl terminal peptide) or 5 μ g of normal rabbit IgG to 2 mg of the precleared lysate, let sit on ice for 1.5 h. Use 5 μ g of anti-PKC β I (C-16) for the protein kinase C (PKC) immunoprecipitation.

7. Add 25 μ L of bovine serum albumin (BSA)-coated Pansorbin and shake at 4°C for 30 min (see **Note 11**).
8. Wash once with 1% NP-40 lysis buffer, once with high salt wash buffer, then twice with 1% NP-40 lysis buffer.
9. Remove all the washing buffer using Pipetman, then add 40 μ L of SDS/DTT sample buffer.
10. Boil at 95°C for 3 min and load onto 8% polyacrylamide/Tris-SDS gel.
11. Transfer the protein onto PVDF membrane and blot against anti-PKC (C-16) or anti-Btk (C-20).

4. Notes

1. The number of BMMC obtained by our protocol widely varies from 5×10^6 to 4×10^8 cells per mouse, mainly as the result of the mouse strain: C57BL/6 and BALB/c mice tend to give lower numbers of BMMC whereas 129/SvJ mice give larger numbers. Mice with a mixed background of C57BL/6 and 129/SvJ give the greatest numbers of BMMC in our experience. Knockout of some genes might also affect mast cell generation. Although we use mouse IL-3 gene-transfected fibroblasts (D11) as a source of IL-3 for an economic reason, one can use recombinant IL-3 instead. Some laboratories use WEHI-3 culture supernatants as a source of IL-3. Previous optimization for IL-3 sources is required by culturing bone marrow cells. Similarly, a large amount of fetal bovine serum should be tested using bone marrow cells or IL-3-dependent mouse mast cell lines such as PT-18 or MC/9. The heterogeneity of mast cells generated in vitro as well as those in tissues has been well known (14). BMMC generated in our standard protocol are “immature” mast cells with similarities to mucosal mast cells. When bone marrow cells or BMMC that have been generated in IL-3 are exposed to SCF for some time, the resultant mast cells become more “mature,” with similarities to connective-tissue mast cells. Therefore, it will not be so surprising to note differences in phenotypes between BMMC and sBMMC. One should carefully assess whether an observed difference is the result of an effect of differentiation or genetic difference or simply a result of c-Kit (SCF receptor) signaling. One should also be cautious in interpreting data using RBL-2H3 rat mast cells because these cells have an activating mutation in c-Kit. *D11 is culture supernatant of IL-3 gene-transfected cells S15 72F-D11 (7). Optimal amounts of D11 supernatant must be titrated by culturing IL-3-dependent mast cells, such as BMMC, PT-18, or MC/9.
2. NP-40 is not commercially available. Igepal CA-630 is recommended as the substitute for NP-40 by Sigma, and we have been using this without any problem.
3. The optimum cell concentration is approx 3×10^5 cells/mL.
4. Retroviral transduction of a transgene results in its integration into the host (sBMMC) genome. Although the integration event is thought to be a nonsequence-specific phenomenon, the randomness of integration may not be absolute. Regardless of the extent of randomness, the positional effect associated with transgene insertion is an inherent problem with retroviral transduction (be reminded of the recent leukemia/lymphoma complications in patients who received a gene

therapy). One way to avoid this problem is to use other vectors that do not involve transgene integration. Unfortunately, no systematic application of such vectors has been attempted with mast cells. However, a practical solution is to study mass populations of retrovirally transduced cells. Even using such populations of mast cells, one has to keep it in mind that the resultant cells might be skewed to have a growth advantage in SCF-containing media. We usually perform three or more transduction experiments before we draw a conclusion on the effect of gene knockout on a mast cell phenotype.

5. This sensitization protocol is different from the one described next. Because sBMMC begin to die from apoptosis 10–24 h after SCF depletion, one needs to shorten the sensitization time.
6. Most PTKs have an autophosphorylating capacity. Autophosphorylating activity is usually more robust in in vitro kinase assays than the activity to phosphorylate an exogenous substrate. It is generally assumed that autophosphorylating activity is correlated with endogenous substrate-phosphorylating activity. Traditionally, acid-denatured enolase has been used as an exogenous substrate for Src family PTKs. Enolase is also a substrate for Btk. Although several other substrates such as poly-(Glu-Tyr) and band III have been used for the same purpose, it is not known whether phosphorylation of these substrates in vitro faithfully reflects the activity of the tested kinase *in situ*. This question can be addressed by immunoblotting cell lysates by a phospho-specific antibody that detects an activated kinase (or an antibody that detects an inactivated kinase) and by confocal microscopic analysis of cells stained by a phospho-specific antibody. Synthetic peptides have been extensively used as a substrate for a variety of kinases, particularly PS/TKs. Phosphorylation of synthetic peptides can be quantified by SDS-PAGE and autoradiography or by counting the radioactivity of filter papers that are spotted with reaction mixtures. Unlike the latter method, SDS-PAGE analysis gives a higher level of confidence for results because the detected radioactive band can make sure the identity of the substrate. However, short peptides (<10 residues) cannot be analyzed by this method. Another potential problem is that some peptides contain more than one phosphorylatable residue. This could be particularly problematic when an enzyme source comes from a complex mixture of proteins such as cell lysates. Fortuitous co-precipitation of an unintended kinase might phosphorylate the peptide at an unintended residue.
7. 2×10^7 cells make approx 1 mg of lysate when lysed in 1% NP-40 lysis buffer.
8. Make samples in triplicate. To reduce the background, perform kinase assays without crosstide, and subtract the count from the one with crosstide.
9. Do not vortex or tap hard. The agarose beads will jump up and stick on the wall.
10. To perform a proper negative control precipitation is critical, given that non-specific bands are usually seen by immunoblotting total cell lysates even with preimmune sera. Our experience also indicates that antibody-precipitating media, such as Pansorbin, protein A agarose, and protein G agarose, precipitate from cell lysates numerous proteins that be detected by staining SDS-PAGE gels. Therefore, it is imperative to show negative control data. We believe such data are essential even when time course studies are carried out. Remember that some

proteins, such as PKC, are very sticky. How can you reduce the chance of fortuitous precipitations of your protein? Because of its unpredictable nature, there is no fixed way to solve this problem. Once you are faced with it, you can try several tricks: (1) change the precipitating media; (2) coat the precipitating media with BSA or gelatin; (3) change the lysis buffer from a mild detergent to a harsher detergent; (4) wash the precipitate with high salt buffer; or (5) a combination of these changes. These changes were all tried before we could generate the clean co-immunoprecipitation data on Btk–PKC interactions (**13**).

11. Pansorbin usually is incubated with 1 mg/mL BSA during the preparation, but to reduce the nonspecific PKC binding, one needs to incubate Pansorbin with 10 mg/mL BSA in PBS for 30 min right before use, and wash 3X with PBS followed by washing with 1% NP40 lysis buffer.

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