

Chapter 13

Basic Techniques to Study FcεRI Signaling in Mast Cells

Yuko Kawakami and Toshiaki Kawakami

Abstract

Mast cells are the crucial effector cells for allergic reactions. They are activated through the aggregation of the high-affinity 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 (PTKs) and serine/threonine kinases (PS/TKs) such as Akt (protein kinase B), protein kinase C (PKC), 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

In spite of a recent flurry of papers indicating roles of mast cells in innate immunity, autoimmune disease models, angiogenesis, chronic cardiac failure, etc., immediate hypersensitivity and allergic diseases remain the most important focus in mast cell research [1]. As allergic reactions in these diseases are largely dependent on allergen and allergen-specific 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]. Upon receptor aggregation, receptor-bound Src family PTKs, such as Lyn [3], Fyn [4], Hck [5], and Fgr [6] 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, etc.), transcription factors, and cytoskeletal proteins. These phosphorylations eventually lead to the activation of several signaling pathways such as phospholipase C/ Ca^{2+} , Ras/MAP kinase, NF- κ B, AP-1, and NFAT. Finally, coordinate activation of these pathways results in degranulation, synthesis and release of lipid mediators, and synthesis and secretion of cytokines and chemokines [7, 8].

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 [9]. 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 cDNA. The current standard method of gene transduction in mast cells is retroviral transduction [10], although a recent study used lentiviral transduction in human mast cells [11]. 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, i.e., 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 Reagents for BMMC Generation

1. Bone marrow-derived mast cell (BMMC) medium: 10 % *not* heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 50 $\mu\text{g}/\text{mL}$ gentamicin sulfate, 50 μM 2-mercaptoethanol (2-ME) in RPMI 1640 supplement with ~ 7 % D11 (IL-3)-conditioned media (or 10 ng/mL recombinant mIL-3).
2. To make 600 mL BMMC media: To 500 mL RPMI 1640, add 60 mL FCS, 40 mL D11 (*see* **Note 1**), 6 mL L-glutamine (200 mM stock), 6 mL NEAA (10 mM stock), 0.6 mL gentamicin sulfate (50 $\mu\text{g}/\text{mL}$ stock), and 0.6 mL 2-ME (50 μM stock).

2.2 Reagents for Retroviral Transduction

1. SCF-derived bone marrow-derived mast cell (sBMMC) medium: 15 % FCS, 2 mM L-glutamine, 0.1 mM NEAA, 50 $\mu\text{g}/\text{mL}$ gentamicin sulfate, 50 μM 2-ME in RPMI 1640

supplemented with 10 % D11 (IL-3)-conditioned media (or 10 ng/mL recombinant mIL-3), and 50 ng/mL recombinant mouse stem cell factor (rmSCF).

2. To make 600 mL sBMMC media: To 438 mL RPMI 1640, add 90 mL FCS, 60 mL D11 (*see Note 1*), 6 mL L-glutamine (200 mM stock), 6 mL NEAA (10 mM stock), 0.6 mL gentamicin sulfate (50 µg/mL stock), and 0.6 mL 2-ME (50 µM stock). Supplement with 50 ng/mL rmSCF.
3. Plat-E medium: 10 % *heat-inactivated* FCS (HI-FCS), 2 mM L-glutamine, 0.1 mM NEAA, 50 µM 2-ME in Dulbecco's Modified Eagle's Medium (DMEM).
4. To make 500 mL Plat-E medium: To 439.5 mL DMEM, add 50 mL heat-inactivated FCS, 5 mL L-glutamine (200 mM stock), 5 mL NEAA (10 mM stock), and 0.5 mL 2-ME (50 mM stock).
5. TransIT-LT1 transfection reagent (Mirus, Madison, WI).
6. Polybrene stock: 8 mg/mL.

2.3 Reagents for FcεRI Stimulation

1. Tyrode's buffer: 112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.6 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7.94, 0.05 % gelatin, 0.1 % glucose.
2. Anti-DNP IgE [12].
3. DNP₂₃-HSA (Biosearch Technologies, Inc.)

2.4 Reagents for Kinase Assays

1. Lysis buffer: 1 % NP40 or 1 % Igepal CA-630 (*see Note 2*), 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1 % NaN₃.
2. Add protease and phosphatase inhibitors to lysis buffer right before use: 1 mM Na₃VO₄, 1 mM PMSF, 1 mg/mL aprotinin, 1 µg/mL leupeptin, 1 µM pepstatin, 25 µM *p*-nitrophenyl *p*'-guanidinobenzoate, 2 mM NaF.
3. 5× SDS/DTT sample buffer: 0.5 M DTT, 10 % SDS, 0.4 M Tris-HCl (pH 6.8), 50 % glycerol.
4. PTK (protein-tyrosine kinase) assay buffer (without ATP): 20 mM HEPES (pH 7.5), 10 mM MgCl₂ 10 mM MnCl₂.
5. Autophosphorylation PTK assay buffer: 20 mM HEPES (pH 7.5), 10 mM MgCl₂ 10 mM MnCl₂ with 0.1 mM "cold" ATP with 10 µCi [γ-³²P] ATP per reaction.
6. Substrate PTK assay buffer: 20 mM HEPES (pH 7.5), 10 mM MgCl₂ 10 mM MnCl₂ with 0.1 mM "cold" ATP with 10 µCi [γ-³²P] ATP and 2 µg enolase per reaction.
7. Preparation of acid-denatured enolase.
 - (a) Thaw a 100 µL aliquot of frozen enolase (2 mg/mL in water kept at -20 °C).
 - (b) Add 100 µL of 50 mM acetic acid.

- (c) Incubate at room temperature (RT) for 5 min.
- (d) Add 50 μ L of 1 M HEPES (pH 7.0).
- (e) Keep at RT until used in assay.

2.5 Reagents for Immunoprecipitation

1. Antibodies (clone name): Anti-Lyn (44), anti-Btk (M138), anti-PKC α (C-20), anti-PKC β I (C-16), anti-PKC β II (C-18). All antibodies are from Santa Cruz Biotechnology.
2. Protein G PLUS-agarose (Santa Cruz).
3. High salt wash buffer for co-immunoprecipitation: 1 M NaCl, 1 % Triton X-100, 10 mM Tris-HCl (pH 7.2).

2.6 Equipment

1. Culture flasks and tubes.
2. Dissection kit: Forceps, scissors.
3. 10 cc syringes and 26 G needles.
4. SDS-PAGE apparatus.
5. Gel transfer apparatus.

3 Methods

The methods described below will outline (Subheadings 3.1–3.2) the generation and activation of mouse bone marrow-derived mast cells (hereafter abbreviated as BMMC), (Subheading 3.3) the genetic manipulation of BMMC by retroviral transduction, (Subheading 3.4) the measurement of PTKs and PS/TKs, and (Subheading 3.5) a detailed procedure to detect protein-protein interactions by co-immunoprecipitation.

3.1 Generation of BMMC

The following are the description of our standard procedures for BMMC generation that usually take 5–6 weeks. After this period, mast cells constitute more than 95 % of live cells, as assessed by flow cytometry for positive expression of Fc ϵ RI 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, etc.), genetic (e.g., cDNA transfection, siRNA, etc.), and pharmacological (e.g., inhibitors and activators) experimentations.

Day 1

1. Euthanize a mouse by CO₂ asphyxiation followed by cervical dislocation (or other approved humane method of euthanasia).
2. Drench the mouse in 70 % alcohol to sterilize it.
3. Strip off the skin from one leg.
4. Holding the femur with the forceps, cut away as much of the quadriceps muscle as possible. Then cut the muscles behind the knee joint.

5. Bend the knee joint in the “unnatural” or “incorrect” direction to dislocate it. Cut off the lower leg at the broken knee joint.
6. Snip off a small amount of bone from each end to expose the bone marrow.
7. While holding the femur above the open small flasks (25 cm²), load a syringe with 10 mL BMMC media, install a 26 G needle, and insert the tip of the needle into the exposed bone marrow.
8. 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.
9. Repeat **steps 3–7** with the other leg.
10. Store the flasks in a 37 °C, 5 % CO₂ incubator for 2 days.

Day 3

11. Transfer each 10 mL culture from the small flask to a medium flask (75 cm²) containing 40 mL of BMMC media.

Day 8

12. Transfer the cultures to 50 mL tubes.
13. Centrifuge the cells at 300×*g* for 5 min and aspirate supernatant.
14. Resuspend the cells in an optimum volume of BMMC medium and transfer to a new flask (*see Note 3*).
15. Return the cultures to the incubator and maintain for 1 week.

Day 15

16. Change the media and flasks again.
17. Maintain the cultures in the incubator for 1 week.

Day 22 and 29

18. Change the media and flasks again as on Day 15.
19. Maintain the cultures in the incubator for 1 week.

Day 35

20. Change the media and flasks again.
21. The cells are now ready to use. At this point, BMMC cells can be maintained in culture with weekly changes to the media for about 1–2 more weeks (*see Note 4*).

3.2 FcεRI Stimulation of BMMC

Sensitization

1. Enumerate mast cells in culture manually using a hemocytometer.
2. Transfer BMMCs to 200 or 50 mL conical centrifuge tubes.

3. Spin down the cells in at $300\times g$ for 5 min.
4. Resuspend the cells in BMMC medium to a density of 2×10^6 cells/mL.
5. Add IgE to a final concentration of $0.5\text{ }\mu\text{g/mL}$.
6. Incubate overnight (37°C , 5 % CO_2).

Stimulation

7. Spin down the cells in a 50 mL tube for 5 min at $300\times g$.
8. Wash once in Tyrode's buffer.
9. Resuspend the cells in Tyrode's buffer to 2×10^7 cells/mL.
10. Make 1 mL aliquots.
11. Add antigen (DNP₂₃-HSA) to a final concentration of 1–1,000 ng/mL.
12. Incubate at 37°C for predetermined periods (depending on the purpose of the experiment).
13. Spin down the cells and aspirate supernatant. In some experiments (e.g., degranulation assay or isolation of exosomes), one may wish to keep the supernatants for analysis.
14. If you continue the experiment, add an appropriate buffer to the pellet. Otherwise, freeze down the cells in dry ice and keep frozen at -80°C until use.

3.3 Retroviral Transduction of BMMC

This section can be divided into Subheadings “the Construction of Retroviral Vector, the Generation of Recombinant Retrovirus, and the Infection of Mast Cells and Selection of Transgene-Expressing Cells.”

Construction of Retroviral Vector

A Moloney murine leukemia virus-based vector, pMX-puro [13, 14], 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 (e.g., a green fluorescent protein (GFP) 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 can accommodate a gene or cDNA in the region between the *Bam* *HI* (nucleotide 1884) and *Not* *I* (nucleotide 3161) sites. Standard molecular biological techniques are used to construct recombinant vectors.

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: e.g., BOSC23 [15], Phoenix [16], and Plat-E [17]. Our experience indicates that all of these packaging cell lines yield titers of viruses high enough to

produce transfected BMMC in a scale of $5\text{--}20 \times 10^7$ 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 Plat-E cells.

*Infection of Mast Cells
and Selection
of Transgene-
Expressing Cells*

Retroviral genomes can integrate into a host genome only when host cells are replicating. While IL-3 is usually 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 stem cell factor (SCF) to generate BMMC that are ready for retroviral infection (hereafter abbreviated as sBMMC) (*see* **Notes 5 and 6**).

Ten days prior to transfection

1. Reconstitute a vial of frozen Plat-E cells in Plat-E medium.
2. After the cells become confluent, passage every 3 or 4 days by three- to fivefold dilution.
3. Aspirate medium very carefully with Pasteur pipette and wash once with 5 mL PBS.
4. Add 1 mL of trypsin-EDTA and incubate at RT for 1–2 min.
5. Add 3 mL of DMEM and pipette well to separate individual cells. It is very important to disperse cell clumps.
6. Centrifuge the cells for 5 min at $300 \times g$.
7. Suspend in 8 mL of Plat-E medium per 10 cm dish.
8. Plate Plat-E cells homogenously. Pipette the cells well in order to prevent them from making clumps.

One day prior to transfection

9. Plate 5×10^6 Plat-E (I) cells in 10 mL Plat-E medium in 10 cm dish for Day 1 transfection.
10. Plate 4×10^6 Plat-E (II) cells for Day 2 transfection.

Day 1: Transfect Plat-E (I) cells

11. Add 45 μ L TransIT-LT1 to 1.5 mL DMEM containing 20 mM HEPES (pH 7.4) (without FCS), vortex briefly, let it sit at room temperature (RT) for 5 min.
12. Add 10–15 μ g plasmid, vortex briefly, and then incubate at RT for 15 min.
13. Add the above plasmid mixture dropwise to Plat-E (I) cells.
14. Put the Plat-E cells back into the incubator (37°C , 5 % CO_2).

Day 2: Change Plat-E (I) medium and transfect Plat-E (II) cells

15. Change medium of Plat-E (I). Aspirate old medium, add 10 mL of Plat-E medium.

16. Transfect Plat-E (II) cells with plasmid as described on Day 1, **steps 1–4**.
17. Harvest BMMC as described in Subheading 3.1. Resuspend the cells to 1×10^6 cells/mL in sBMMC medium. Seed 5 mL of BMMC in a 6-well tissue culture plate.

Day 3: Infect BMMC culture

18. Harvest virus supernatant from Plat-E (I), centrifuge at $50 \times g$ for 5 min at RT.
19. Make 10 mL of virus cocktail (sufficient for 2 BMMC transfection wells (**step 21**):
 - (a) 8 mL Plat-E culture supernatant.
 - (b) 1 mL D11-conditioned media (IL-3).
 - (c) 1 mL FCS.
 - (d) 5 μ L SCF (100 μ g/mL stock).
 - (e) 7 μ L Polybrene (8 mg/mL stock).
20. Remove 2.5 mL sBMMC medium from each well of the 6-well plate.
21. Add 5 mL virus cocktail to each well of sBMMC.
22. Centrifuge the whole 6-well plate at $800 \times g$ for 1 h at 32 °C.
23. Incubate the cells in the incubator set at 32 °C with 5 % CO₂ for 4 h (*see Note 7*).
24. Transfer the cells to 37 °C incubator with 5 % CO₂.

Day 4: Infect BMMC culture

25. Make 10 mL of virus cocktail from Plat-E (II) cell supernatant as in **step 19** of Day 3.
26. Remove 3 mL of medium from each well of the sBMMC.
27. Repeat **steps 21–23** of Day 3.

Day 5: Change sBMMC medium

28. Change medium containing virus to sBMMC medium.
29. Incubate the cells at 37 °C with 5 % CO₂.

Day 8 (48 h after the second infection): Begin drug selection

30. Add selection reagent (such as puromycin) or sort the cells.
31. Maintain the cultures with sBMMC medium as described in the Subheading 3.2.

Experimentation with infected sBMMCs

32. Two days prior to experimentation, wash the cells twice with sBMMC medium to remove the selection reagent.
33. Resuspend the cells in sBMMC medium and maintain in culture for 48 h.

34. Wash the cells twice with BMMC medium to remove SCF from medium.
35. Incubate the cells in BMMC medium with or without 0.5 µg/mL of IgE for 6–8 h (*see Note 8*).
36. Stimulate the cells with antigen as described in Subheading 3.2, steps 7–14.

3.4 In Vitro Kinase Assay

Although anti-phospho-specific antibodies are widely used for the evaluation of activities of many kinases, in vitro kinase assay is still the most reliable, direct assay to measure the activity of kinases. A representative method will be described here for Btk, in which reaction products are analyzed by SDS-PAGE and followed by blotting. The method can be adapted for other PTKs or protein serine/threonine kinases P(S/T)Ks using proper substrates and kinase reaction conditions. These methods are also easy to be adapted when different sources of enzyme, e.g., purified or recombinant kinases, are used in place of cell lysates (*see Notes 9 and 10*).

Cell lysis

1. Add 200 µL of lysis buffer (+inhibitors) to the tube containing 2×10^7 cells (*see Note 11*).
2. Incubate on ice with occasional vortexing for 10 min.
3. Spin down the cells at 4 °C for 12 min at $13,000 \times g$ and save supernatant.

Measurement of protein concentration

4. Measure the protein concentration of cleared cell lysate using a Bio-Rad (detergent compatible (DC)) Protein Assay Kit following the manufacturer's protocol.
5. Calculate the protein concentration.
6. Make 0.5–1 mg (depends on the protein you want to IP) aliquots at 200–500 µL (make up with lysis buffer as needed).

Immunoprecipitation for Btk kinase assay

7. To 1 mg of the lysate, add 4 µg of anti-Btk (M138) antibody.
8. Let it sit on ice for 1.5 h.
9. Add 20 µL of Protein G PLUS beads to each tube and vortex at 4 °C for 30 min.
10. Wash Protein G PLUS beads 4× with 1 mL lysis buffer.
11. Wash beads 1× with 1 mL PTK assay buffer (–ATP).
12. Remove all supernatant using a 1 cc syringe with 30 G needle.

Kinase assay reaction

13. Add 20 µL of PTK assay buffer (–ATP) to the Protein G PLUS-agarose, carefully mix by swirling the tube using plastic pipette tip (200 µL) (*see Note 12*).

14. Let it sit on ice.
15. Add 20 μL of PTK assay buffer (+ATP) and mix well by tapping.
16. Incubate at 30 °C for 10 min.
17. Stop reaction by adding 10 μL of 5 $\times$ SDS/DTT sample buffer.

SDS-PAGE and blotting

18. Boil the samples and load onto 8 % SDS-polyacrylamide gel.
19. Run the gel overnight at ~40 V.
20. Transfer the proteins to PVDF membrane (Millipore) and then dry the membrane.
21. Detect the kinase activity by autoradiography.

3.5 Co-immunoprecipitation (co-IP)

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 (TAP) [18] is more often used. 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 or recombinant binding protein. There is a wide selection of affinity ligands such as GST (glutathione S-transferase), hexahistidine, maltose-binding protein, etc. However, interactions between endogenous cellular proteins are usually confirmed by co-IP 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 13*).

Co-IP of Btk and PKC β I

1. The cells are sensitized and stimulated as shown in Subheading 3.3.
2. Add 500 μL of 1 % NP40 lysis buffer containing inhibitors to $4\text{--}5 \times 10^7$ cells. Incubate on ice with occasional vortexing for 10 min, centrifuge at $13,000 \times g$ for 12 min, and save the supernatant.
3. Measure the protein concentration using Bio-Rad detergent-compatible (DC) Protein Assay Kit following the manufacturer’s protocol.
4. Preclear the lysate with Protein G PLUS-agarose. Add 30 μL of Protein G PLUS-agarose per 1 mg of lysate, incubate at 4 °C for 30 min on the shaker.
5. Spin down at $13,000 \times g$ for 5 min and save the supernatant.
6. Add 5 μg of anti-Btk (raised in rabbit against mouse Btk carboxyl terminal peptide) or 5 μg of normal rabbit IgG to 2 mg of the precleared lysate, let it sit on ice for 1.5 h.

7. Use 5 µg of anti-PKCβI (C-16) for the PKC IP.
8. Add 25 µL of Protein G PLUS-agarose and shake at 4 °C for 30 min.
9. Wash once with 1 % NP40 lysis buffer, once with high salt wash buffer, then twice with 1 % NP40 lysis buffer.
10. Remove the washing buffer using a 1 cc syringe with 30 G needle, then add 40 µL of SDS/DTT sample buffer.
11. Boil at 95 °C for 3 min and load onto 8 % Tris-SDS acrylamide gel.
12. Transfer the protein onto PVDF membrane and blot against anti-PKCβI (C-16) or anti-Btk (C-20).

4 Notes

1. D11 is a culture supernatant of IL-3 gene-transfected cells S15 72 F-D11 [19].
2. NP40 is not commercially available. Igepal CA-630 is recommended as the substitute for NP40 by Sigma, and we have been using this agent without any problem.
3. The optimum cell concentration is about $3\text{--}5 \times 10^5$ cells/mL.
4. The number of BMMC obtained by our protocol widely varies from 5×10^7 to 4×10^8 cells per mouse, mainly due to mouse strain: BALB/c mice tend to give lower numbers of BMMC while C57BL/6 mice give larger numbers. 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 because of economy, one can use recombinant IL-3 instead. Some laboratories use WEHI-3 culture supernatants as a source of IL-3. Prior optimization for IL-3 sources is required by culturing bone marrow cells as described above. Similarly, lots of fetal bovine serum should be tested using bone marrow cells.
5. Heterogeneity of mast cells generated in vitro as well as those in tissues has been well known [20]. 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 [21]. Therefore, it will not be so surprising to see differences in phenotypes between BMMC and sBMMC. One should carefully assess whether an observed difference is due to an effect of differentiation or genetic difference or simply due to c-Kit (SCF receptor) signaling.

6. Retroviral transduction of a transgene results in its integration into the host (sBMMC) genome. Although the integration event is thought to be a sequence-nonspecific 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 complications in patients who received a gene therapy [22]). 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.
7. The optimum temperature for virus replication is generally lower than that for cells. Keep the plates at 32 °C for the first 4 h to obtain the best efficiency of retrovirus infection.
8. This sensitization protocol is different from the one described in Subheading 3.3. Because sBMMC start to die from apoptosis 10–24 h after SCF depletion, one needs to shorten the sensitization time.
9. 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.
10. 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 of the identity of the substrate. However, short peptides (<10 residues) cannot be analyzed by the former 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.

11. 2×10^7 cells makes ~1 mg of lysate when lysed in 1 % NP40 lysis buffer.
12. Do not vortex or tap hard. The agarose beads will jump up and stick on the wall.
13. To perform a proper negative control precipitation is critical, given that nonspecific 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 of numerous proteins that can 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 of precipitating media, (2) coating precipitating media with BSA or gelatin, (3) change of lysis buffer from a mild detergent to a harsher detergent, (4) wash the precipitate with high salt buffer, and (5) a combination of these changes. These changes were all tried before we could generate the clean co-IP data on Btk-PKC interactions [23].

References

1. Galli SJ, Lantz CS (1999) *Fundamental immunology*, 4th edn. Lippincott-Raven Publishers, Philadelphia, PA
2. Kinet JP (1999) The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu Rev Immunol* 17:931–972
3. Eiseman E, Bolen JB (1992) Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355:78–80
4. Parravicini V, Gadina M, Kovarova M, Odom S, Gonzalez-Espinosa C, Furumoto Y, Saitoh S, Samelson LE, O'Shea JJ, Rivera J (2002) Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat Immunol* 3:741–748
5. Hong H, Kitauro J, Xiao W, Horejsi V, Ra C, Lowell CA, Kawakami Y, Kawakami T (2007) The Src family kinase Hck regulates mast cell activation by suppressing an inhibitory Src family kinase Lyn. *Blood* 110:2511–2519
6. Lee JH, Kim JW, Kim do K, Kim HS, Park HJ, Park DK, Kim AR, Kim B, Beaven MA, Park KL,

- Kim YM, Choi WS (2011) The Src family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice. *J Immunol* 187:1807–1815
7. Turner H, Kinet JP (1999) Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 402:B24–B30
8. Kawakami T, Galli SJ (2002) Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2:773–786
9. Kawakami T, Inagaki N, Takei M, Fukamachi H, Coggeshall KM, Ishizaka K, Ishizaka T (1992) Tyrosine phosphorylation is required for mast cell activation by Fc epsilon RI cross-linking. *J Immunol* 148:3513–3519
10. Hata D, Kawakami Y, Inagaki N, Lantz CS, Kitamura T, Khan WN, Maeda-Yamamoto M, Miura T, Han W, Hartman SE, Yao L, Nagai H, Goldfeld AE, Alt FW, Galli SJ, Witte ON, Kawakami T (1998) Involvement of Bruton's tyrosine kinase in FcepsilonRI-dependent mast cell degranulation and cytokine production. *J Exp Med* 187:1235–1247
11. Furumoto Y, Brooks S, Olivera A, Takagi Y, Miyagishi M, Taira K, Casellas R, Beaven MA, Gilfillan AM, Rivera J (2006) Cutting Edge: lentiviral short hairpin RNA silencing of PTEN in human mast cells reveals constitutive signals that promote cytokine secretion and cell survival. *J Immunol* 176:5167–5171
12. Liu FT, Bohn JW, Ferry EL, Yamamoto H, Molinaro CA, Sherman LA, Klinman NR, Katz DH (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J Immunol* 124:2728–2737
13. Onishi M, Kinoshita S, Morikawa Y, Shibuya A, Phillips J, Lanier LL, Gorman DM, Nolan GP, Miyajima A, Kitamura T (1996) Applications of retrovirus-mediated expression cloning. *Exp Hematol* 24:324–329
14. Kawakami Y, Miura T, Bissonnette R, Hata D, Khan WN, Kitamura T, Maeda-Yamamoto M, Hartman SE, Yao L, Alt FW, Kawakami T (1997) Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. *Proc Natl Acad Sci U S A* 94:3938–3942
15. Pear WS, Nolan GP, Scott ML, Baltimore D (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A* 90:8392–8396
16. Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Lanfrancone L, Peschle C, Nolan GP, Pelicci PG (1998) High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res* 58:14–19
17. Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 7:1063–1066
18. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17:1030–1032
19. Yokota T, Lee F, Arai N, Rennick D, Zlotnick A, Mosmann T, Miyajima A, Takebe Y, Kastelein R, Zurawski G, Arai K (1986) Strategies for cloning mouse and human lymphokine genes using mammalian cDNA expression vector. *Lymphokines* 13:1–19
20. Wong GW, Friend DS, Stevens RL (1998) Mouse and rat models of mast cell development. In: Razin E, Rivera J (eds) *Signal transduction in mast cells and basophils*. Springer, New York, pp 39–53
21. Galli SJ, Tsai M, Lantz CS (1999) The regulation of mast cell and basophil development by the Kit ligand, SCF, and IL-3. In: Razin E, Rivera J (eds) *Signal transduction in mast cells and basophils*. Springer, New York, pp 11–30
22. Staal FJ, Pike-Overzet K, Ng YY, van Dongen JJ (2008) Sola dosis facit venenum. *Leukemia in gene therapy trials: a question of vectors, inserts and dosage?* *Leukemia* 22:1849–1852
23. Yao L, Kawakami Y, Kawakami T (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc Natl Acad Sci U S A* 91:9175–9179