Antibodies. Anti-FcεRIβ subunit mAb and anti-IgE mAb B1E3 were originally provided by Drs. Juan Rivera, NIH, and Daniel H. Conrad, Virginia Commonwealth University, respectively. Rabbit polyclonal antibody to the cytoplasmic domain of human Cbp/PAG (used for probing immunoblots) that cross-reacts with the murine homolog was previously described [28]. Anti-NTAL/LAB mAb used for immunoprecipitation and probing was previously described [29]. Anti-Fyn polyclonal antibody was described previously [30]. Commercial sources of other antibodies are as follows: anti-phosphotyrosine mAb 4G10 and anti-FcεRIγ subunit polyclonal antibody from Upstate Cell Signaling Solutions, Charlottesville, VA; anti-Cbp/PAG (P-18 for immunoprecipitation), anti-Btk, anti-RhoA, anti-Lyn, anti-Hck (M-28), anti-Syk, anti-p38, and anti-Akt from Santa Cruz Biotechnology, Santa Cruz, CA; anti-ERK from Invitrogen, Carlsbad, CA; anti-phospho-Btk (Tyr223), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-phospho-Akt (Ser473), anti-phospho-Gab2 (Tyr452), anti-phospho-Src (Tyr416), anti-phopho-$p56^{Dok-2}$ (Tyr351), and anti-phospho-SHIP1 (Tyr1020) from Cell Signaling; anti-α-tubulin mAb from Sigma-Aldrich, St Louis, MO; Alexa 488-conjugated anti-mouse IgG from Molecular Probes, Eugene, OR.

Proliferation assays. BMMCs were incubated with the indicated concentrations of mouse recombinant IL-3 or mouse recombinant SCF (both kindly donated by Kirin Brewery Co., Tokyo) for 24 h. [^{3}H]Thymidine was added to the cultures for the last 8 h. Acid-insoluble tritium counts were measured in a scintillation counter.

Apoptosis assays. BMMCs cultured in IL-3-free medium were assessed by staining with annexin V and 7-amino-actinomycin D. Stained cells were analyzed using a FACSCalibur.

GST-fusion proteins. N-terminal regions of mouse Lyn (residues 1-283) and mouse Hck (residues 1-236) were PCR-amplified using cDNA clones as templates and appropriate primers. PCR products were cloned into the pGEX-3T vector. pGEX-2T-mouse fyn (coding for residues 7-84) plasmid was provided by Alexander Y. Tsygankov. GST fusion proteins were expressed and purified with glutathione agarose beads (Sigma-Aldrich). Concentrations of fusion proteins were determined using SDS-PAGE and staining with Coomassie Brilliant Blue compared to known amounts of BSA.

Confocal microscopy. IgE-sensitized mast cells were stimulated with 100 ng/ml DNP23 -HSA for 5 min at 37°C, and fixed with 3.7% paraformaldehyde for 10 min at 37°C. Cells washed twice with PBS were attached to glass slides using a Cytospin (Thermo Shandon) at 600 rpm for 6 min. Primary and secondary stainings were performed on glass slides with anti-α-tubulin at a dilution of 1:50, Alexa 488-conjugated anti-mouse IgG at 1:50, and phalloidin-rhodamine (Molecular Probes, Inc.) at 1:150. Staining was performed for 45 min in the dark at RT; the slides were then washed with PBS and coverslips were mounted with anti-fade mounting solution (Molecular Probes, Inc). Confocal microscopy was performed using a Marianas microscope (Intelligent Imaging Innovation Inc., Santa Monica, CA).

Polymeric tubulin assay. IgE-sensitized mast cells were stimulated with 100 ng/ml DNP23 -HSA for the indicated periods. Cells were then suspended in extraction buffer (0.1 M Pipes, pH 7.1, 1 mM MgSO4, 1 mM EGTA, 2 M glycerol, 0.1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, and 0.5 mM phenylmethysulfonyl fluoride). After incubation on ice for 15 min, cell lysates were centrifuged at 15,000 rpm for 15 min and the supernatants were transferred to a new tube. The remaining pellets were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 0.4 M NaCl, and 0.5% SDS) and boiled for 10 min at 95 °C. The samples were centrifuged at 15,000 rpm for 5 min, and polymeric tubulin-containing supernatants were collected and placed gently into another tube. The 0.1% Triton-insoluble solutions were subjected to SDS-PAGE, and polymeric tubulin was detected by immunoblotting with anti-αtubulin antibody. The 0.1% Triton-soluble proteins were subjected to SDS-PAGE and the gels were stained with Coomassie Brilliant Blue G.

In vitro kinase assay using FcεRIβ ITAM peptides as substrates. Mast cells were lysed in NP-40 lysis buffer. 0.2 mg of lysates were immunoprecipitated with anti-Lyn antibody and 25fold more (5 mg) lysates were precipitated with anti-Hck antibody. Washed immunoprecipitates were incubated at 25°C for 15 min with FcεRIβ ITAM peptides (YYY, KRKKKVPDDRL<u>Y</u>EELNV<u>Y</u>SPI<u>Y</u>SA; mutant peptides have F in place of Y) in kinase buffer containing [γ-^{32}P]ATP. Reaction mixtures were spotted onto phosphocellulose paper discs. The paper discs were washed in phosphoric acid and acetone-dried. The radioactivity of the discs was measured in a scintillation counter. The autophosphorylating activity was also measured by SDSPAGE followed by blotting and autoradiography.