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Lyn contributes to regulation of multiple Kit-dependent signaling pathways in murine bone marrow mast cells☆

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Abstract

SCF induces autophosphorylation of Kit and activates a variety of signaling components including Jnks, Erks, PI 3 Kinase, the JAK-Stat pathway and members of the Src family. Previously we showed that Lyn is activated at multiple points during SCF-induced cell cycle progression and contributes to SCF-mediated growth, chemotaxis and internalization of Kit. However, the Kit-dependent biochemical events that require Lyn are unknown. In this study, we used Lyn-deficient bone marrow mast cells (BMMC) to examine the contribution of this Src family member to tyrosine phosphorylation of Kit and SCF-induced activation of Jnks, Akt, Stat3 and Erks. Although surface expression of Kit was increased in Lyn-deficient BMMC, SCF-induced phosphorylation and growth was reduced compared to wild-type BMMC. Downstream of Kit, SCF-induced activation of Jnks was markedly reduced in Lyn-deficient BMMC. Further, Lyn was required for SCF-induced tyrosine phosphorylation of Stat3. Interestingly, Kit was constitutively associated with PI 3 Kinase in Lyn-deficient BMMC and this correlated with constitutive phosphorylation of Akt. This was in marked contrast to wild-type BMMC, where both these events were induced by SCF. These data indicate that in BMMC, Lyn contributes to SCF-induced phosphorylation of Kit, as well as phosphorylation of Jnks and Stat3. In contrast, Lyn may negatively regulate the PI 3 Kinase/Akt pathway. The opposing effects of Lyn on these signaling pathways may explain the pleiotropic effects ascribed to this Src family member in the literature.

Keywords: Kit; Stem cell factor; Lyn; Tyrosine kinases; Signal transduction; Hematopoiesis

1. Introduction

Signals initiated by receptors at the membrane are transmitted to downstream signaling molecules and then to final effectors. The biological responses mediated by the receptor tyrosine kinase Kit involve a multitude of downstream signaling molecules that regulate functions such as differentiation, migration and proliferation. Kit is expressed on the surface of hematopoietic progenitors including cells of myeloid and lymphoid origin at specific stages of differentiation, such as pro-B and pro-T cells stages. It is also expressed on the surface of certain differentiated cells such as mast cells as well as basophils, melanocytes, germinal cells and interstitial cells of cajal [1]. Interaction of Kit with its ligand SCF results in receptor dimerization, autophosphorylation and subsequent activation of downstream signals. SCF stimulation results in the association of Kit with signaling molecules such as p85 PI 3 Kinase, Src family kinases Lyn and Fyn and PLCγ. It also results in the activation of pathways such as the PI 3 Kinase pathway, the Jak/Stat pathway and the Ras/Raf/MAPK pathway [2–5].

Autophosphorylation of Kit on tyrosine 568 and 570 results in binding and activation of Src family members [6]. Moreover, Src family kinases phosphorylate Kit on tyrosine 900 which creates a docking site for CrkII [7]. Earlier work from our lab demonstrated that SCF activates Lyn and suggested that this contributes to SCF-induced proliferation [8]. These data were supported by results with Lyn-deficient progenitors and mast cells which showed decreased proliferation and chemotaxis [9]. Ueda et al. [10] have shown that autophosphorylation at tyrosine 567 and tyrosine 719 (the binding sites for Src family members and p85

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PI 3 Kinase) is critical for SCF-mediated Ca^{2+} influx and migration. Hence, Src family kinases, especially Lyn, play a critical role in SCF-mediated responses [8,9,11–13]. Interestingly, Lyn is a positive regulator in many responses mediated via the B-cell receptor and Fc∈RI in mast cells [14,15]. In contrast, B cells from Lyn-deficient mice are hyper responsive to anti-IgM-induced proliferation [16,17]. Lyn can also contribute to stress-induced cell cycle arrest [18–20]. Thus, Lyn both positively and negatively regulates growth.

Lyn-dependent signaling pathways activated by Kit have not been clearly defined. To address this question, we compared Kit stimulus–response coupling mechanisms in bone marrow mast cells (BMMC) derived from Lyn-deficient or wild-type mice. While surface expression of Kit is increased in Lyn-deficient BMMC, there is a reduction in the capacity of these cells to proliferate in response to SCF. This correlates with reductions in phosphorylation of Kit, Stat3 and activation of Jnks. In contrast, Kit is constitutively associated with p85 regulatory subunit of PI 3 Kinase in Lyn-deficient cells and Akt is constitutively phosphorylated. Thus, Lyn has diverse functions in regulation of signaling pathways activated by Kit.

2. Materials and methods

2.1. Animals

Wild-type and Lyn-deficient mice were obtained as a gift from Dr. Clifford Lowell (University of California at San Francisco). The mice were generated as described and maintained at NCI-FCRF in a pathogen-free environment [17].

2.2. Cells

Bone marrow-derived mast cells (BMMC) were generated as described [9,20]. In brief, bone marrow from wild-type and Lyn-deficient mouse was collected aseptically and the mononuclear cells were separated using LSM (Lymphocyte Separation Media). The cells were cultured for a week in IMDM supplemented with 100 ng/ml murine SCF and 30 ng/ml murine IL-3, 10% FCS and 1% Penicillin, streptomycin and glutamine. The cells were then grown in media with IL-3 alone for 4–5 weeks. All experiments were done with cells on the third day following feeding to ensure maximum expression of the receptor on cell surface. Flow cytometry was performed to confirm the presence and percentage of BMMC after 5 weeks of culture as previously described [21].

2.3. Cell proliferation assay

^{3}H-thymidine incorporation assays to analyze cell proliferation were done as previously described [22].

2.4. Cytokines and antibodies

IMDM was purchased from JRH Bioscience (Lenexa, KS). Fetal calf serum (FCS) was obtained from Hyclone (Salt Lake City, UT). Murine SCF and murine IL-3 were purchased from PeproTech (Rocky Hill, NJ). Antibodies for Kit, Erk and Stat3 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies for phospho-Kit pY719, PI 3 Kinase, Akt, phosphorylated Akt and phosphorylated Stat3 were obtained from Cell Signaling Technology™. The different phosphospecific Kit antibodies (pY823, pY703, pY730, pY568/570) were from Biosource International. GST-Jun fusion protein and purified MBP protein were obtained from Cell Signaling Technology™. GST protein was obtained from Santa Cruz. Anti-phosphotyrosine monoclonal antibody was from Upstate Biotechnologies (Lake Placid, NY). Biotinylated rabbit/mouse/goat IgG and horseradish peroxidase labeled Streptavidin were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protein assay reagents were obtained from Pierce (Rockford, IL). Enhanced Chemiluminescence (ECL) reagent was from NEN™ Life Science Products (Boston, MA). LSM was purchased from ICN Biomedicals (Aurora, OH).

2.5. Immunoprecipitation and immunobloting

Cells were washed two times with IMDM containing 1% Penicillin, streptomycin, glutamine (PSG) and then resuspended in the same media at 1×10^7 cells/ml. The cells were divided into two parts and one part was treated with 100 ng/ml murine SCF at room temperature for 10 min. The cells were then pelleted and lysed at 1×10^7 cells/ml in ice-cold lysis buffer (10 mM Tris base, 50 mM NaCl, 5 mM EDTA, 5 mM Tetra Sodium Pyrophosphate, 5 mM NaF, 5 mM Na_3VO_4, 1 mM PMSF, 1% Triton-X-100 and 0.1% BSA, pH 7.6). The lysates were incubated on ice for 20 min and then centrifuged at 14,000 rpm for 30 min at 4 °C. The protein concentration of the clarified lysates was determined using BCA protein assay (Pierce). Protein lysate (600 μg) was immunoprecipitated with 1 μg/ml of the indicated antibody as described by Chian et al. [22]. The immunoprecipitates were resolved by SDS-PAGE and transferred onto Immobilon-P followed by Western blotting using the indicated antibodies. Blots were either stripped and reprobed or identical blots were made and probed in parallel with different antibodies, as indicated in the figure legend.

2.6. Kinase assays

Jnk kinase activity was assessed with a "pull-down" assay using the substrate GST-Jun as bait and subjecting the "pull-down" product to an in vitro kinase assay using [γ^{32}P]ATP. The kinase assay product was resolved by SDS-PAGE and transferred onto immobilon-P and exposed to an X-ray film. The blots were then probed with anti-jun antibody.