Brief Definitive Report

A Double-Edged Kinase Lyn: A Positive and Negative Regulator for Antigen Receptor-mediated Signals

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Summary

B cells from young lyn−/− mice are hyperresponsive to anti-IgM-induced proliferation, suggesting involvement of Lyn in negative regulation of B cell antigen receptor (BCR)-mediated signaling. Here we show that tyrosine phosphorylation of FcγRIIIB and CD22 coreceptors, which are important for feedback suppression of BCR-induced signaling, was severely impaired in lyn−/− B cells upon their coligation with the BCR. Hypophosphorylation on tyrosine residues of these molecules resulted in failure of recruiting the tyrosine phosphatase SHP-1 and inositol phosphatase SHIP, SH2-containing potent inhibitors of BCR-induced B cell activation, to the coreceptors. Consequently, lyn−/− B cells exhibited defects in suppressing BCR-induced Ca2+ influx and proliferation. Thus, Lyn is critically important in tyrosine phosphorylation of the coreceptors, which is required for feedback suppression of B cell activation.

The Src family protein-tyrosine kinase, Lyn, is highly expressed in hematopoietic cells. Lyn physically associates with the BCR in B cells and with FcγRI in mast cells, and is rapidly activated upon cross-linking of the antigen receptors (1–3). Lyn interacts with and phosphorylates a number of substrates, such as the Syk kinase (4, 5), H51 protein (6, 7), and Cbl protooncogene product (8). Taken together, Lyn is thought to play important roles in the antigen receptor-mediated positive signaling.

Recently, however, two groups reported that B cells from young lyn−/− mice were hyperresponsive to anti-IgM-induced proliferation due in part to the impairment of FcγRIIIB-mediated feedback suppression of the B cell antigen receptor (BCR) signaling (9, 10). Therefore, it is suggested that Lyn may play some roles in the antigen receptor-mediated negative signaling, too. In this study, using splenic B cells or bone marrow–derived mast cells (BMMCs) from lyn−/− mice, we addressed molecular mechanisms by which the Lyn kinase would act as a key regulator of antigen receptor signaling.

Materials and Methods

Cells and Cell Culture. All lyn−/− mice had been back-crossed at least six generations to C57BL/6J. Splenic B cells were isolated from 6- to 8-wk-old mice by T cell depletion with anti-Thy 1.2 mAb and with rabbit complement, followed by Percoll gradient purification (11). The resulting cells were >85% B220+ as determined by FACS™ (Becton Dickinson, Mountain View, CA) analysis. BMMCs were obtained from bone marrow cells cultured with IL-3 for at least 4 wk as described (12).

Proliferative Responses. For proliferation assay, B cells (105/well) were cultured in 96-well flat-bottomed tissue culture plates either alone or in the presence of goat F(ab')2 anti-IgM (Cappel, Durham, NC) or intact anti-IgM (Southern Biotechnology Associates, Birmingham, AL). Cultured cells were pulse-labeled and assayed as described (13). All assays were performed in triplicate with <20% variation among assays.

Qualification of Total Ig by ELISA. Amounts of each Ig isotype in sera and in culture supernatants were determined by ELISA with antibodies specific for each membrane-bound Ig (mIg) isotype (13).

Immunoprecipitation and Immunoblotting. For the activation of B cells, splenic B cells were treated with goat F(ab')2 anti-IgM (Cappel) or intact anti-IgM (Southern Biotechnology Associates, Inc.) for 2 min at 37°C. For the activation of mast cells, BMMCs were sensitized for 1 h with antidinitrophenyl (anti-DNP) monoclonal IgE (10 μg/ml; Sigma Chemical Co., St. Louis, MO) followed by stimulation for 2 min at 37°C with 30 ng/ml DNP-conjugated human serum albumin (DNP-HSA; resulting in FcεRI cross-linking; Sigma Chemical Co.), or DNP-HSA/rabbit anti-HSA IgG immune complexes (resulting in FcγRII coligation; FcγRII). The stimulated splenic B cells (105) or BMMCs (106) were lysed in TNE (1% [vol/vol] N onidet P-40, 50 mM Tris-HCl [pH 8], 20 mM EDTA, and 0.2 mM sodium orthovanadate with aprotinin at 10 μg/ml) buffer and subjected to immunoprecipitation/immunoblotting using SDS-7.5% PAGE as described (14). Antibodies used in these experiments were 2.4G2 mAb (PharMingen, San Diego, CA), anti-FcγRIIIB (α-mb1, gift from J.V. Ravetch, The Rockefeller University, New York), and anti-CD22.
anti-CD22 (gift from E.A. Clark, University of Washington, Seattle, WA), anti-SHP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-SHIP (gift from M.N. Lioubin, Fred Hutchinson Cancer Research Center, Seattle, WA). Biotinylated 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) was used to detect phosphorylated proteins in the immunoblotting experiments. The blots were treated with horseradish peroxidase (HRPO)-streptavidin, and then antibody-reacted bands were visualized by the use of enhanced chemiluminescence detection system (Amersham, Co., Arlington Heights, IL).

FcR Cross-linking and Degranulation Assay. FcεRI-triggered mast cell activation was induced by 1 h of sensitization with 10 μg/ml biotinylated mouse IgE, followed by cross-linking with 10 μg/ml streptavidin (0% of FcγRII coligation). FcγRII was coligated to FcεRI by adding biotinylated anti-FcγRII monoclonal antibody (biotin-2.4G2) at the sensitization step (100% of FcγRII coligation). A mixture of biotinylated/nonbiotinylated 2.4G2 (1/9 for 10% or 1/99 for 1% coligation) was used to vary the extent of FcγRII coligation. The degree of degranulation was determined by measuring the release of β-hexosaminidase as described (12).

Measurement of Internal Ca²⁺ Concentration. Splenic B cells and IgE-sensitized BMMCs from lyn−/− and lyn+/− mice were incubated with 3 μM Fura-2/acetoxymethyl (AM; Dojindo, Osaka, Japan) in PBS containing 20 mM Hepes (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM CaCl₂ at 37°C for 45 min. After the reaction, the cells were resuspended in 500 μl of the same buffer at 2 × 10⁶ cells/ml in a stirring cuvette. Then, by using CAF-110 fluorescence spectrophotometer (JASCO, Osaka, Japan), emission at 500 nm was monitored after excitation of the sample with two different wavelengths (340 and 380 nm).

Results and Discussion

lyn−/− mice, generated by gene targeting, exhibit splenomegaly as they age. The enlarged spleen accumulates un-
Figure 3. (A) Anti-IgM-induced tyrosine phosphorylation of FcγRIIB. Splenic B cells from wild-type (lanes 1-3), lyn\(^{-/-}\) (lanes 4-6), or lyn\(^{-/-}\) (lanes 7-9) mice were stimulated with F(ab\(^{b}\))\(_{2}\) anti-IgM (30 \(\mu\)g/ml; lanes 2, 5, and 8) or intact anti-IgM (50 \(\mu\)g/ml; lanes 2, 5, and 8) for 2 min. Proteins were immunoprecipitated with 2.4G2 anti-FcγRIIB monoclonal antibody from the cell lysates, and the precipitates probed with \(\alpha\)-PY (top) or anti-FcγRIIB antibody (\(\alpha\)-mβ1; bottom) by immunoblotting. (B) Recruitment of SHIP to FcγRIIB. Splenic B cells from wild-type (lanes 1-3) or lyn\(^{-/-}\) (lanes 4-6) mice were stimulated with F(ab\(^{b}\))\(_{2}\) anti-IgM (30 \(\mu\)g/ml; lanes 3 and 6) or intact anti-IgM (50 \(\mu\)g/ml; lanes 2 and 5) for 2 min. Anti-FcγRIIB (2.4G2) immunoprecipitates from the cell lysates were probed with anti-SHIP antibodies by immunoblotting. (C) Time course of Ca\(^{2+}\) flux in splenic B cells upon BCR coligation to FcγRIIB. Panels show real time Fura-2 ratios (340/380) for splenic B cells of wild-type mice (top) and lyn\(^{-/-}\) mice (bottom). Splenic B cells were stimulated with F(ab\(^{b}\))\(_{2}\) anti-IgM (5 \(\mu\)g/ml; line), intact anti-IgM (10 \(\mu\)g/ml; dotted line), or intact anti-IgM (10 \(\mu\)g/ml) in the presence of 1.5 mM EGTA (perforated line).
extremely impaired in lyn−/− B cells (lanes 5 and 6), as compared with that in wild-type B cells (lanes 2 and 3). A level of tyrosine phosphorylation of SHP-1 was also low, and association of CD22 with SHP-1 was poor in the absence of Lyn (Fig. 2 B and data not shown).

Next, we examined the level of tyrosine phosphorylation of anti-FcγRIIIB antibody (α-mβ1) (bottom) by immunoblotting. (B) Inhibitory effect of FcγRIIIB coligation to FcεRI in lyn−/− BMMCs. The mast cell was sensitized with biotinylated mouse IgE, followed by cross-linking with streptavidin (no FcγRIIIB coligation). FcγRIIIB was coligated to FcεRI by adding biotinylated anti-FcγRII monoclonal antibody (biotin-2.4G2) at the sensitization step (100% FcγRIIIB coligation). A mixture of biotinylated/nonbiotinylated 2.4G2 (1/9 for 10% or 1/99 for 1% coligation) was used to vary the extent of FcγRIIIB coligation. The degree of degranulation was determined by measuring the release of β-hexosaminidase as described (12). Closed columns, the results of wild-type mice; open columns, the results of lyn−/− mice. Standard errors of triplicate samples are indicated on each column.

Figure 4. (A) Tyrosine phosphorylation of FcγRIIIB upon FcεRI and FcγRIIIB coligation. BMMCs from wild-type (lanes 1–4), lyn−/− (lanes 5–8) mice were sensitized with anti-DNP monoclonal (10 μg/ml) followed by stimulation for 2 min at 37°C with 30 ng/ml DNP-HSA (FcεRI cross-linking; lanes 2 and 6), or DNP-HSA/rabbit anti-HSA IgG immune complexes (FcγRIIIB coligation to FcεRI; lanes 3 and 7). BMMCs were stimulated with DNP-HSA/rabbit anti-HSA IgG immune complexes without IgE sensitization (lanes 4 and 8). Anti-FcγRIIIB (2.4G2) immunoprecipitates from the cell lysates were probed with α-PY (top) or anti-FcγRIIIB antibody (α-mβ1) (bottom) by immunoblotting. The mast cell was sensitized with biotinylated mouse IgE, followed by cross-linking with streptavidin (no FcγRIIIB coligation). FcγRIIIB was coligated to FcεRI by adding biotinylated anti-FcγRII monoclonal antibody (biotin-2.4G2) at the sensitization step (100% FcγRIIIB coligation). A mixture of biotinylated/nonbiotinylated 2.4G2 (1/9 for 10% or 1/99 for 1% coligation) was used to vary the extent of FcγRIIIB coligation. The degree of degranulation was determined by measuring the release of β-hexosaminidase as described (12). Closed columns, the results of wild-type mice; open columns, the results of lyn−/− mice. Standard errors of triplicate samples are indicated on each column.

The similarities between the BCR- and FcεRI-mediated signaling prompted us to examine whether tyrosine phosphorylation of FcγRIIIB coligated with FcεRI was impaired in lyn−/− mast cells. As shown in Fig. 4 A, tyrosine phosphorylation of coligated FcγRIIIB was suppressed in the lyn−/− BMMCs. This likely results in the failure of recruitment of SHIP and/or SHP-1 to the ITIM of FcγRIIIB, leading to the lack of suppression of FcεRI signaling by the inhibitory coreceptor. However, in both wild-type and lyn−/− BMMCs, the calcium response after IgE cross-linking was similarly suppressed by coligation of FcγRIIIB with FcεRI-IgE complexes (data not shown). Furthermore, coligation of FcγRIIIB with FcεRI-IgE complexes with increasing amounts of the cross-linking antibody resulted in increasing inhibition of degranulation, as measured by β-hexosaminidase release, in both wild-type and lyn−/− BMMCs (Fig. 4 B). Coligation of FcγRIIIB with FcγRIIIB on both wild-type and lyn−/− BMMCs also inhibited receptor-triggered degranulation (the pair columns at the foremost right in Fig. 4 B). These results suggest that, unlike the case with the B cells, the negative signaling pathways appear to function similarly in wild-type and lyn−/− BMMCs.

The apparent contradiction between B cells and mast cells with regard to the FcγRIIIB feedback mechanism may be due to differences in their FcγRIIIB-mediated signalings. Such differences could produce the cell-type specific balance between the positive and negative signalings. Present data together with previous observations (9, 10, 12, 13, 15) indicate that positive signaling is less affected than negative signaling in lyn−/− B cells, and vice versa in lyn−/− mast cells. The balance of the positive and negative signalings in lyn−/− cells could well be influenced by such other Src-like kinases and Syk kinase expressed in the cells. In B cells, the imbalance induced by lyn deficiency seems to severely affect the immune responses. As a consequence, lyn−/− mice, which have lost the control of positive and negative signalings, exhibit abnormal phenotypes such as hyperactivation of B cells (9, 10) and elevated serum levels of IgM and IgA (13, 15), resulting in development of autoimmune disease (13, 15).

Our data show that Lyn is critically involved in tyrosine phosphorylation of particular proteins such as CD22 and FcγRIIIB that are involved in the feedback regulation in B cells and/or BMMCs (for review see references 33–35). Lyn is also important in tyrosine phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) family of proteins and the proteins involved in positive regulation of the BCR and FcεRI signaling events (4, 5, 12, 13, 15, 36). The other membrane molecules with the putative ITIMs...
have been described (37, 38). These include KIR on NK cells, glycoprotein 49B and MAFA-1 on mast cells, and CD23 and CD72 on B cells. To understand the molecular basis of the balance of the positive and negative signalings, it is important to address what kinases preferentially tyrosine phosphorylate and regulate these molecules. The candidates include the Src family kinases, JAK kinases, Tec family kinases, and Syk/ZAP-70 kinases. The balance, of course, could be affected by the absence of a critical kinase(s).

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