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Functional Analysis of Lyn Kinase A and B Isoforms Reveals Redundant and Distinct Roles in FcεRI-Dependent Mast Cell Activation

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Engagement of FcεRI causes its phosphorylation by Lyn kinase. Two alternatively spliced variants, Lyn A and B, are expressed in mast cells, and both isoforms interact with FcεRI. Unlike Lyn A, Lyn B lacks a 21-aa region in the N-terminal unique domain. In this study, we investigated the role of Lyn A and B isoforms in mast cell signaling and responses. Lyn B was found to be a poor inducer of mast cell degranulation and was less potent in both inositol 1,4,5-triphosphate production and calcium responses. Expression of Lyn B alone showed reduced phosphorylation of both phospholipase Cγ-1 and -2 and decreased interaction of phospholipase Cγ-1 with the phosphorylated linker for activation of T cells. Lyn B also showed increased binding of tyrosine-phosphorylated proteins, which included the negative regulatory lipid phosphatase SHIP-1. In contrast, both Lyn A and B caused similar total cellular tyrosine phosphorylation and FcεRI phosphorylation and neither Lyn A nor Lyn B alone could completely restore mast cell degranulation or dampen the excessive cytokine production seen in the absence of Lyn. However, expression of both isoforms showed complementation and normalized responses. These findings demonstrate that Lyn B differs from Lyn A in its association with SHIP-1 and in the regulation of calcium responses. However, complementation of both isoforms is required in mast cell activation. *The Journal of Immunology*, 2010, 184: 5000–5008.

Mast cells are important innate immune cells that can amplify the adaptive immune response (1). They are also known as the central effector cell in IgE-mediated allergic and inflammatory disorders. In an allergic reaction, mast cell activation is initiated through the recognition of an Ag by Ag-specific IgE bound to the α subunit of FcεRI, which is expressed on the cell surface. The Src family protein tyrosine kinase Lyn provides the key recognition signal that interprets receptor engagement into intracellular events by transphosphorylating the FcεRI β and γ subunits (2). Efficient phosphorylation of the FcεRI requires specialized regions of the cell membrane that are enriched in cholesterol and sphingolipids (commonly termed lipid rafts) as both Lyn and FcεRI can be concentrated in these domains upon receptor engagement (3). Phosphorylation occurs within the cytoplasmic tails of the β and γ subunits in a domain that encodes the ITAM, which is characterized by a YXXL-X₇-YXXL amino acid sequence (4). Once phosphorylated, phospho-ITAMs constitute a novel docking site for the binding and subsequent

activation of Src homology 2 (SH2)-domain containing molecules, such as Syk, a tyrosine kinase that is crucial for mast cell activation (5). The activation of Syk results in the phosphorylation of multiple substrates, among which the membrane-localized linker for activation of T cells (LAT) coordinates the assembly of a molecular complex that includes proteins like phospholipase C (PLC)γ. PLCγ catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol 1,4,5-triphosphate (IP₃) and diacylglycerol. IP₃ binds to its receptors on the endoplasmic reticulum, promoting calcium release from the intracellular stores, which, upon emptying, trigger calcium influx from the extracellular environment via store-operated calcium channels like Orai1/CRACM (6, 7). Diacylglycerol binds the C1 domain of a number of proteins (like protein kinase C) promoting their membrane localization and activity. Both the calcium influx and protein kinase C activation are essential for the release of pre-formed granule-stored allergic mediators and the de novo synthesis of cytokines and eicosanoids from mast cells (8, 9).

As the key initiating kinase, the therapeutic targeting of Lyn is of interest, because intervening at this step should presumably abrogate mast cell activation. However, recent studies suggest that Lyn has both positive and negative regulatory roles (10–12) and, in the context of a particular genetic background, Lyn deficiency could result in either reduced or increased mast cell degranulation and anaphylactic responses (13, 14). In mast cells as well as in other cell types (15), Lyn kinase exists as two isoforms, Lyn A and Lyn B of 56 and 53 kDa, respectively. These isoforms are generated by alternative splicing and differ by a 21-aa insert found in the N-terminal unique domain of Lyn A (Fig. 1A) (16). Prior studies have shown that both Lyn A and Lyn B coimmunoprecipitate with FcεRI (17). In addition, both isoforms can be found in lipid rafts (18). In mast cells derived from a mouse model of Smith-Lemli-Opitz Syndrome (an inborn error of cholesterol metabolism leading to loss of cholesterol from lipid rafts) both isoforms of Lyn are lost from lipid rafts, and these mast cells

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Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; IP₃, inositol 1,4,5-triphosphate; LAT, linker for activation of T cells; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; SH2, Src homology 2; SHP-1, Src homology region 2 domain-containing phosphatase 1; WCL, whole cell lysate; WT, wild-type.

showed a hyperresponsive phenotype (19). Although distinguishing the individual roles of Lyn A and Lyn B (or whether one isoform has a more dominant negative function) is of considerable interest, this was not feasible by means of silencing RNA or genetic deletion strategies, as both isoforms arise from a single gene and differ in a relatively short stretch of nucleotide sequence.

In this study, we investigate the roles of the two Lyn isoforms by expressing each individually or together in mast cells derived from *lyn*^{-/-} mice. Our results show unique properties as well as redundant roles for the Lyn A and B isoforms. Lyn B was found to dominantly associate with the negative regulatory lipid phosphatase SHIP-1 and was less effective in eliciting calcium responses and mast cell degranulation. However, Lyn B was equivalent to Lyn A in total cellular tyrosine phosphorylation and FcεRI phosphorylation. Lyn A showed stronger calcium responses and degranulation than Lyn B and was better in promoting the interaction of PLCγ with phospho-LAT. Importantly, expression of each individual isoform did not fully restore calcium fluxes or degranulation or controlled hypercytokine production, but expression of both isoforms fully normalized these responses. The findings demonstrate differences in the role of these two Lyn kinase isoforms in mast cell signaling and responses. However, they also demonstrate a requirement for both Lyn A and Lyn B in generating normal mast cell responses.

Materials and Methods

Animals

Mice used in this study were wild-type (WT) and *lyn*^{-/-} (129/Sv, or 129/Sv × C57BL/6 [N8]), which were bred in-house. Animals were maintained and used according to National Institutes of Health guidelines and a National Institute of Arthritis and Musculoskeletal and Skin Diseases-approved animal study proposal.

Abs

Mouse anti-DNP mAb was purified from culture supernatants of the H1-DNP-ε-26.82 hybridoma (20). Mouse mAb to FcεRIβ was previously described (21). Rabbit polyclonal Ab to GST or Lyn were purchased from Invitrogen (Carlsbad, CA) or Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-GST, anti-SHIP-1, anti-PLCγ1 and PLCγ2, anti-phospho-PLCγ1 (Y783), anti-phospho-Syk (Y352), and anti-phospho-LAT (Y191) Abs were purchased from Cell Signaling Technology (Boston, MA). Anti-phosphotyrosine 4G10 mAb was from Upstate Biotechnology (Lake Placid, NY). Anti-mouse Ig linked to infrared fluorescent dye IR800 (Rockland Immunochemicals, Philadelphia, PA) and anti-rabbit Ig linked to Alexa Fluor 680 (Invitrogen) were used as secondary Abs for western blotting.

Cell cultures

Total bone marrow from the femurs of *lyn*^{-/-} and WT 6-wk-old female mice was extracted and used to obtain cultures of bone marrow-derived mast cells (BMMCs). Cells were cultured for 4 wk in the presence of IL-3 and stem cell factor (PeproTech, Rocky Hill, NJ). Cultures were checked periodically for mast cell differentiation and purity by FcεRI and c-Kit expression. Cells were used when cultures achieved ≥95% of the population expressing both markers.

Lentiviral expression constructs

To generate the cDNA construct expressing Lyn A, murine cDNA was amplified using the specific primers LynA sense: 5'-ATG GGA TGT ATT AAA TCA AAA AGG AAA G-3'; and Lyn A antisense: 5'-CTA CGG TTG CAG CTATAC TG-3'. Lyn B was subsequently generated by deleting the 63 nucleotides encoding the 21-aa insert using the primers Lyn B sense: 5'-AAG ACT CAA CCA GTA CCT GAA TTT CAT CTT-3'; and Lyn B antisense: 5'-AAG ATG AAA TTC AGG TAC TGG TTG AGT CTT-3'. Both Lyn A and Lyn B were then cloned into pLenti6/V5-TOPO vector using the Gateway technology kit following manufacturer's instructions (Invitrogen).

Lentiviral gene transduction

Supernatants containing virus were harvested after a 48 h transfection of 293FT cells with Lyn A or B constructs (Cell Biolabs, San Diego, CA) as previously described (22). Viral particles were concentrated 25× by cen-

trifugation for 2 h at 20,000 × g. BMMCs (10 × 10⁶) were incubated with the viral preparations, medium was changed after 24 h, and selection was started 48 h later with 8 μg/ml blasticidin (Invitrogen). Expression of Lyn A or Lyn B was analyzed by Western blot 2 wk postselection.

Ag stimulation, immunoprecipitation, and Western blotting

For Ag stimulation, BMMCs were sensitized at 37°C for 3 h with 1 μg/ml IgE in RPMI 1640 without cytokines. Cells were washed and stimulated at 37°C with 100 ng/ml Ag (DNP-BSA) or with the indicated concentration in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 5.6 mM glucose, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.05% BSA [pH 7.4]). Stimulation time varied with the type of experiment as indicated. Cell lysates were prepared by incubation of 20 × 10⁶ cells in 1 ml BSS buffer containing 0.5% Triton X-100 and 20 mM octyl β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) on ice for 15 min. Lysates were clarified by centrifugation for 15 min at maximum speed at 4°C and supernatants collected. Immunoprecipitation was performed with protein A (GE Healthcare, Piscataway, NJ) and the indicated Abs by incubation of cell lysates for 3 h at 4°C in rotation. Proteins were resolved in 10% NuPAGE Bis-Tris gels and transferred to nitrocellulose membranes (Invitrogen). For Western blotting, the membranes were blocked for 1 h at room temperature with Odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE) diluted two times in PBS. Membranes were then incubated with the indicated primary Abs, followed by secondary Abs linked to infrared fluorescence dyes. After washings with PBS-0.1% Tween-20 (Sigma-Aldrich), blots were analyzed in the Odyssey Infrared Imaging system (Li-COR).

Measurement of IP₃ production and calcium fluxes

Intracellular IP₃ was extracted with 20% cold perchloric acid from IgE-sensitized cells (10 × 10⁶/sample) challenged with 25 ng/ml Ag. The acidified samples were centrifuged at 2000 × g for 15 min at 4°C, and the supernatants were neutralized with a 1:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane:trioctylamine. The concentration of IP₃ in each sample was measured with a competitive binding assay (IP₃-[3H] Biotrak Assay System, GE Healthcare).

To measure calcium mobilization, two methods were used. In some experiments, cells were sensitized overnight and loaded with 3 μM fura 2-AM for 20 min, washed, and aliquoted in 96-well plates (9 × 10⁴ cells/well). Cells were then challenged with Ag (25–50 ng/ml), and changes in intracellular calcium were monitored with a microplate fluorescence reader (Opti-Fluor; BMG Labtech, Cary, NC) as previously described (23). In some experiments, single-cell calcium imaging was performed using a Zeiss LSM510 META confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY). The procedure was essentially described (24). Briefly, 5 × 10⁶ BMMCs were sensitized with 0.5 μg/ml IgE for 2 h at 37°C in HEPES-Tyrode's buffer. Cells were loaded with 1.5 μM Fluo 4-AM and 5 μM fura Red-AM (Invitrogen), plated on coverslips, and incubated for 30 min at 37°C in the dark. Ag was added after capture of the first 10 images. A total of 75 images at 5-s intervals were captured. The mean of the fluorescent signal of all cells from a field was obtained with the Zeiss LSM510 META software (Zeiss, Oberkochen, Germany). Data were plotted as a Fluo 4/fura Red ratio.

β-Hexosaminidase release assays

Mast cell degranulation was measured by the release of the enzyme β-hexosaminidase as described elsewhere (25). Briefly, cells were sensitized for 2 h with 1 μg/ml anti-DNP IgE in medium without growth cytokines. Cells were stimulated for 30 min with varying concentrations of Ag (ranging from 0.1 to 1000 ng/ml) in Tyrode's buffer followed by supernatant collection. β-Hexosaminidase released into supernatants was measured by incubation with substrate and quantified colorimetrically as described by Blank and Rivera (25).

Cytokine production

For cytokine secretion analysis, 1 × 10⁶ BMMCs were incubated with anti-DNP IgE as described above and stimulated with 100 ng/ml DNP for 4 h. Supernatants were collected, and the amounts of IL-6, IL-13, and TNF-α were measured by Millipore Cytokine Multiplex Panel (Millipore, Billerica, MA).

Statistical analysis

Statistical significance of observed differences (*p* value) was assessed by two-tailed Student *t* test using the Prism software (GraphPad, San Diego, CA).

Results

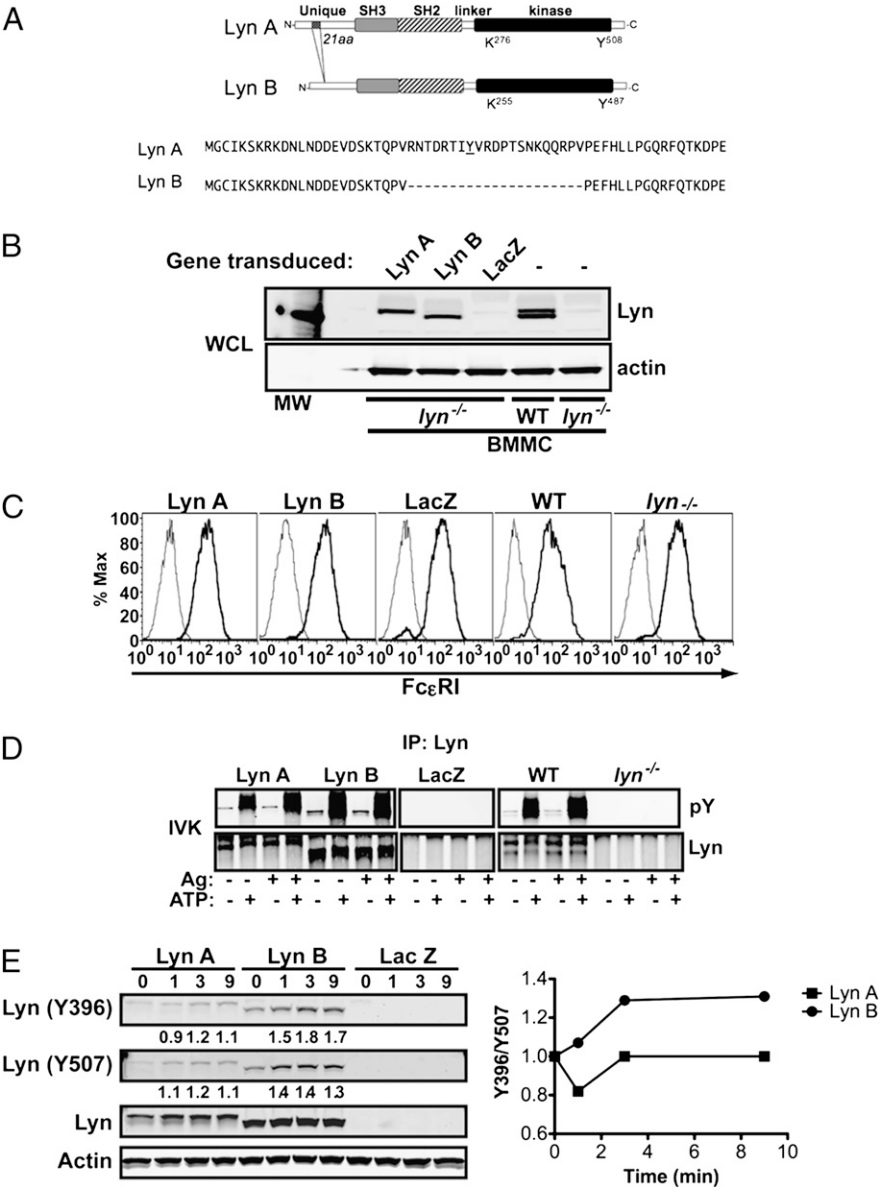
Expression of the individual isoforms, Lyn A or Lyn B, does not alter Lyn-dependent phosphorylation of FcεRI or Syk activation

Lyn A and Lyn B isoforms (Fig. 1A) were expressed in BMMCs derived from *lyn*^{-/-} mice. Stable expression was achieved after 2 wk of blasticidin selection, as described in *Materials and Methods*, with the protein levels being comparable to those found in WT mast cells (Fig. 1B). Expression of Lyn A or Lyn B alone caused no significant change in mast cell differentiation and FcεRI expression (Fig. 1C). Given the extensive regulatory cross talk among Src protein tyrosine kinases (26), we verified that each individual isoform was active when expressed individually. The kinase activity, after immunoprecipitation of each isoform from cells expressing them individually, was determined in an in vitro kinase assay and compared with the activity in WT cells. As shown in Fig. 1D, each individual isoform was active only in the presence of ATP. Ag stimulation caused only a minimal increase in Lyn activity for both isoforms as measured in vitro, consistent with previously published reports (13), suggesting that large

fractions of both Lyn A and B can be readily activated by the presence of ATP in the absence of FcεRI engagement. No kinase activity was found in the immunoprecipitates from Lyn-null mast cells whether transfected to express LacZ or not (Fig. 1D). Thus, expression of the individual Lyn isoforms did not appear to alter their intrinsic kinase activity. To assess the relative activation of Lyn A and B in cells, we measured the phosphorylation of the activation loop Y396 (as recognized by an Ab to Y416 in human Lyn) versus the inhibitory Y507 prior to and after FcεRI stimulation. Fig. 1E shows that, when normalized for the amount of protein expressed, the FcεRI-dependent phosphorylation of Lyn B at Y396 is more prominent than that of Lyn A. Thus, the ratio of Y396/Y507 increased after FcεRI stimulation, whereas the fraction of Lyn A phosphorylated at Y396 showed an initial decrease and a subsequent return to baseline levels. This suggested that Lyn B may be more active in mast cells, but the fraction of Lyn A and Lyn B that is involved in FcεRI-stimulated mast cell responses is unknown, and this would likely determine their relative activities in vivo.

As shown in the control LacZ-transduced *lyn*^{-/-} BMMCs (Fig. 2A), and also as previously reported (10), the absence of Lyn kinase caused a marked reduction in the total cellular tyrosine

FIGURE 1. Lyn A and Lyn B isoforms and their expression in Lyn-null mast cells. *A*, Schematic representation of Lyn isoforms with the sequence of the 21-aa insert found in Lyn A, but not in Lyn B, indicated. *B*, Protein expression of Lyn A and Lyn B relative to WT mast cells. *C*, Analysis of FcεRI expression on cells transduced with lentiviral constructs of Lyn A and Lyn B after blasticidin selection and cell differentiation (5 wk) in IL-3 and stem cell factor. *D*, Activity of Lyn A and Lyn B as measured by in vitro kinase assays (autophosphorylation). The activity of Lyn A and Lyn B isoforms is not impaired by their individual expression and is regulated by ATP. *E*, Immunoblot of the phosphorylation status of the activation loop Y396 (using an Ab to human Lyn Y416) and of inhibitory Y507 on Lyn A and Lyn B isoforms, upon FcεRI stimulation. Graph shows the ratio of Y396/Y507 for both isoforms. *B–E*, A representative experiment is shown.



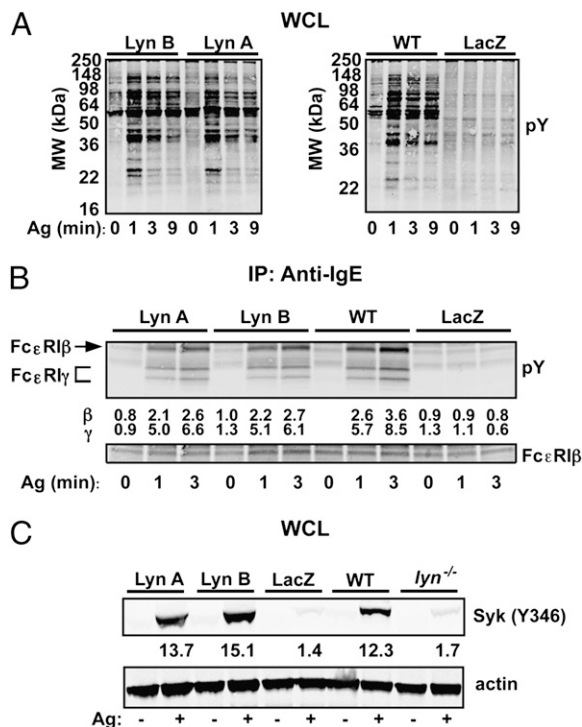


FIGURE 2. Protein tyrosine, FcεRI, and Syk (Y346) phosphorylation is similar in Lyn A- and Lyn B-expressing cells. *A*, Total protein tyrosine phosphorylation (pY) in whole cell lysates (WCLs) of cells expressing Lyn A, Lyn B, and LacZ (negative control) and from WT cells. Kinetic analysis following FcεRI stimulation for the indicated times. *B*, Tyrosine phosphorylation (pY) kinetics of stimulated (for the indicated time) FcεRI immunoprecipitated from cells expressing Lyn A or Lyn B and compared with WT or LacZ controls. Fold induction (for β and γ) normalized to the 0 min time point for WT cells (= 1.0) is shown. *C*, Phosphorylation of Syk at Y346 prior to or after FcεRI stimulation (3 min) in cells expressing Lyn A, Lyn B, and LacZ as compared with Lyn-null (*lyn*^{-/-}) or WT cells. An Ab that detects phosphorylation of the Y352 of human Syk was used to determine the phosphorylation status of the equivalent mouse residue (Y346). Quantitation of all experiments was by densitometry, and data are shown in *C*. One representative of a minimum of four experiments (*A–C*) is shown.

phosphorylation in resting and FcεRI-stimulated mast cells. This demonstrates that the detectable tyrosine phosphorylation seen in mast cells is highly dependent on Lyn activity. Therefore, it was of interest to determine if the profile of tyrosine phosphorylated proteins might differ considerably in cells individually expressing Lyn A or Lyn B. As shown in Fig. 2*A*, the total cellular tyrosine phosphorylation did not differ markedly between the two isoforms, and the gross pattern of phosphorylation observed was similar (albeit not identical) to that of WT mast cells. Because Lyn is the kinase responsible for ITAM phosphorylation of β- and γ-chains of FcεRI, we evaluated the relative contribution of the Lyn isoforms to FcεRI tyrosine phosphorylation following the stimulation of this receptor. As shown in Fig. 2*B*, both Lyn A and Lyn B were equally effective in the phosphorylation of the FcεRI β- and γ-chains, albeit at levels that appeared to be slightly less than that of WT mast cells. Quantitation of the phosphorylation of the FcεRI β- and γ-chains for all experiments showed that at 3 min poststimulation, the β-chain in WT mast cells had a significantly higher phosphorylation (30%) than in Lyn A- or Lyn B-expressing cells. However, although a trend for increased phosphorylation of the FcεRI γ-chain at 3 min was observed, no significant difference was found for the phosphorylation of the FcεRI γ-chain, and the kinetics of FcεRI phosphorylation were unaltered

upon expression of either isoform when compared with WT cells. Thus, Lyn A and Lyn B were equally efficient in the phosphorylation of FcεRI and showed similar ability in inducing total cellular tyrosine phosphorylation.

Given that FcεRI phosphorylation is essential for the activation of Syk kinase (5) and that phosphorylation of mouse Syk on Y346 (which is recognized by an Ab to human Syk Y352, the equivalent site on human Syk) requires Lyn kinase (13), we investigated the effect of Lyn A and Lyn B on phosphorylation of Syk. As shown in Fig. 2*C*, phosphorylation of Syk at 3 min poststimulation occurred efficiently in cells expressing Lyn A or Lyn B and appeared to be comparable to that seen in WT cells. Varying doses of Ag did not reveal any difference in Syk Y346 phosphorylation between Lyn A- and Lyn B-expressing cells (data not shown). Thus, it is clear that the extent of Syk phosphorylation at Y346 did not differ significantly between Lyn A and Lyn B (Fig. 2*C*), arguing that both isoforms can promote Lyn-dependent phosphorylation of this site. This was verified, because cells expressing LacZ or Lyn-null cells showed minimal phosphorylation of Y346, consistent with our previous results (13). Thus, both Lyn A and Lyn B are capable of causing Syk phosphorylation at the Y346 site in a manner similar to that of WT cells, consistent with the ability of each isoform to similarly elicit FcεRI phosphorylation.

Lyn A and Lyn B differ in promoting mast cell degranulation but not cytokine production

We assayed cells expressing the individual Lyn isoforms for their ability to promote FcεRI-mediated degranulation, as measured by release of granule-localized enzyme β-hexosaminidase. Our past studies (11, 13) demonstrated that Lyn-null mast cells derived from 129/Sv mice or from early crosses to C57BL/6 mice showed hyperresponsive degranulation. In contrast, mast cells from Lyn-null C57BL/6 mice or mice backcrossed extensively (N8) into this background showed hyporesponsive degranulation when compared with WT mice. This difference was due, in part, to the relative levels of Fyn expression, as C57BL/6 mice showed lower levels of Fyn kinase, and hyperdegranulation was restored by overexpression of this kinase (13).

At the onset of these studies, we decided to use *lyn*^{-/-} BMMCs derived from mice backcrossed extensively (N8) into a C57BL/6 background. These cells showed a marked loss of FcεRI-induced degranulation (Fig. 3*A*), thus allowing us to determine if Lyn A or Lyn B or both isoforms were needed to promote degranulation. Cells expressing Lyn A or Lyn B showed reduced degranulation response when compared with WT cells (Fig. 3*A*), albeit the impairment observed in cells expressing Lyn B was more marked. Although Lyn A-expressing cells also showed a reduction in their degranulation response, in some experiments, these cells approached the degranulation response of WT cells. Moreover, Lyn A-expressing cells consistently showed a degranulation response that was considerably higher than Lyn B-expressing cells. The impaired degranulation of Lyn B-expressing cells was not due to a deficiency in granule content or to a developmental defect in the granule fusion apparatus because PMA and calcium ionophore (A23187) elicited a similar and potent degranulation from all cells, regardless of whether they expressed the individual Lyn isoforms or none at all (Fig. 3*A*).

Previous work (11) demonstrated that the loss of Lyn kinase led to increased cytokine responses in mast cells and that this phenotype was independent of the genetic background of the mice from which the cells were derived (13). Thus, we explored if the different isoforms of Lyn had differing roles in suppressing cytokine responses. In these experiments, we used BMMCs derived from *lyn*^{-/-} 129/Sv mice because of their potent cytokine responses relative to BMMCs

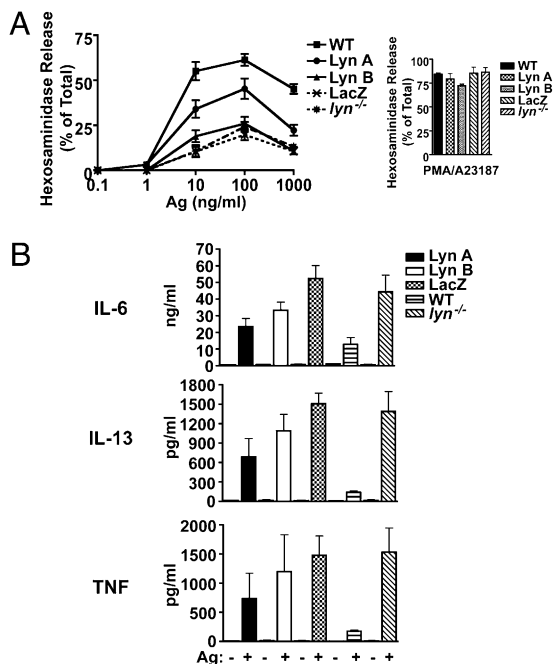


FIGURE 3. Lyn A- and Lyn B-expressing mast cells differ in the extent of degranulation, and each individual isoform fails to dampen cytokine production. *A*, Mast cell degranulation (as measured by release of hexosaminidase) is impaired in both Lyn A- and Lyn B-expressing cells relative to WT cells. However, Lyn A elicits a more robust degranulation response at all Ag concentrations tested than Lyn B. PMA and calcium ionophore (A23187) elicit degranulation of similar magnitude from the indicated genotypes. Data shown (mean $\pm$ SE) are a compilation of four experiments from individual cultures. More than 10 experiments were performed. *B*, Cytokine production (as measured by secretion into the medium) is hyperresponsive to Fc ϵ RI stimulation (4 h) in all genotypes (Lyn A, Lyn B, LacZ, and *lyn*^{-/-}) relative to WT cells. The amount produced is normalized to 10^6 cells. Data shown (mean $\pm$ SD) are compiled from three individual experiments.

from *lyn*^{-/-} C57BL/6 mice. We reasoned that a more potent cytokine response might allow us to more easily ascertain the relative contributions of Lyn A and Lyn B to this response. As shown in Fig. 3*B*, both Lyn A and Lyn B showed a similar loss in the ability to suppress the hyperproduction of IL-6, IL-13, or TNF, similar to Lyn-null mast cells but markedly differing from WT cells. Neither isoform alone was able to completely restore the negative control of cytokine production to the levels seen in WT mast cells. However, cells expressing Lyn A showed a trend (albeit not significant) toward dampening of the hypercytokine response than those expressing Lyn B. Thus, the findings show that Lyn A more effectively promotes mast cell degranulation and is seemingly better in dampening cytokine responses.

Lyn B is less effective than Lyn A in evoking Ca²⁺ responses and optimal IP₃ production following Fc ϵ RI engagement

To explore the underlying mechanism for the observed difference between Lyn A and Lyn B in inducing mast cell degranulation, we investigated whether the two isoforms were similarly able to induce intracellular Ca²⁺ mobilization upon Fc ϵ RI stimulation. A rise in the intracellular Ca²⁺ concentration that is coupled to the influx of Ca²⁺ from the extracellular medium is essential for the degranulation response (9). As shown in Fig. 4*A*, both Lyn A and Lyn B showed altered Ca²⁺ fluxes when compared with WT cells. For both isoforms, the onset and extent of the calcium response was impaired. Lyn B-expressing cells showed the most marked delay in Ca²⁺ fluxes relative to Lyn A-expressing or WT cells. Nonetheless, in many experiments, the Ca²⁺ flux of Lyn A-expressing cells was also de-

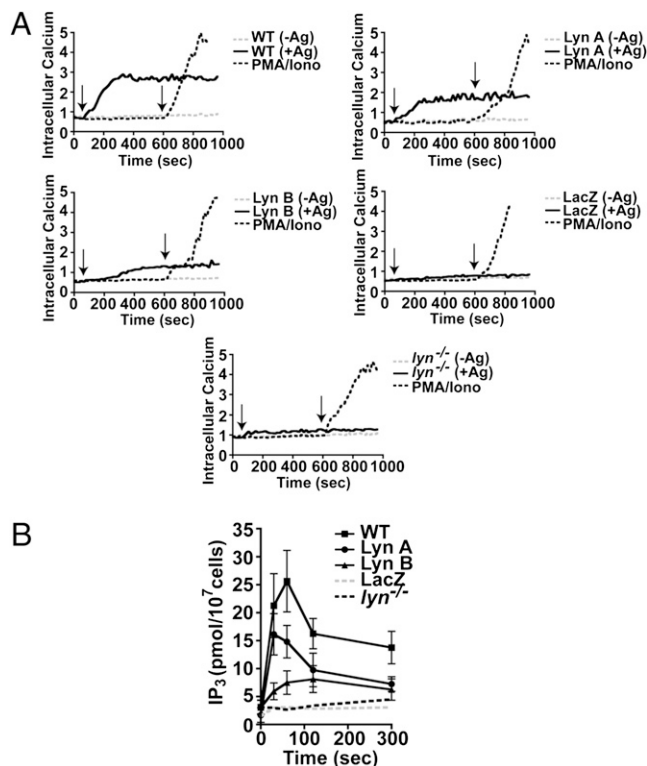


FIGURE 4. Lyn A and Lyn B differ in generating IP₃ and inducing Ca²⁺ fluxes following Fc ϵ RI engagement. *A*, Relative induction (340:380 ratio) of intracellular Ca²⁺ fluxes in cells expressing Lyn A, Lyn B, and LacZ as well as WT and Lyn-null (*lyn*^{-/-}) mast cells stimulated (+) or not (-) with Ag. Time of addition of Ag is indicated by the left arrow, whereas the time of stimulation with PMA and calcium ionophore (A23187) is indicated by the right arrow. One representative of seven experiments from four individual cultures is shown. *B*, Measurement of IP₃ production after Fc ϵ RI stimulation in cells expressing Lyn A, Lyn B, and LacZ compared with WT or *lyn*^{-/-} mast cells. Data are from three experiments using individual cell cultures.

layed when compared with WT cells, and the extent of the response appeared to be diminished for both Lyn A- and Lyn B-expressing cells. Relative to the Ca²⁺ flux of LacZ or Lyn-null mast cells, both Lyn A and B elicited a significant rise in intracellular Ca²⁺ concentration, and all the analyzed transductants showed a marked increase in intracellular Ca²⁺ concentrations when stimulated by PMA and the calcium ionophore A23187 (Fig. 4*A*).

Because the production of IP₃ is key for the mobilization of Ca²⁺ from intracellular stores, which subsequently leads to extracellular Ca²⁺ influx, the role of Lyn A and Lyn B in IP₃ production was also explored. These experiments showed that both Lyn A or Lyn B alone were unable to generate IP₃ at levels comparable to that of WT cells, following Fc ϵ RI stimulation (Fig. 4*B*). For Lyn B, there was a marked impairment in the onset and extent of IP₃ production relative to WT cells. In contrast, it was found that WT and Lyn A-expressing cells showed a similar early rise in IP₃ production, although the extent of the response appeared to be diminished in Lyn A-expressing cells. Thus, the findings demonstrate that Lyn A and Lyn B differ in their ability to induce IP₃ production and Ca²⁺ fluxes, consistent with their observed ability to elicit mast cell degranulation.

Lyn A and Lyn B differ in their ability to associate with phosphoproteins and LAT

The difference of Lyn A and Lyn B in promoting IP₃ production and Ca²⁺ fluxes suggested that these isoforms might differ in protein-protein or protein-lipid interactions required for these events. To explore such possibilities, we first assessed whether the two

isoforms could coimmunoprecipitate similar profiles of tyrosine-phosphorylated proteins. Fig. 5A shows that GST-fusion proteins of Lyn A and Lyn B differed in their binding to tyrosine-phosphorylated proteins from FcεRI-stimulated cell lysates. Relative to Lyn A, Lyn B showed enhanced binding to tyrosine-phosphorylated proteins, including a prominent band with the molecular mass of LAT. This was further demonstrated by Far Western. Cells were stimulated with pervanadate (to maximize the abundance of phosphorylated proteins), and the total cellular proteins were transferred to nitrocellulose and probed with GST-fusion proteins encoding Lyn A and Lyn B without their catalytic domains. As shown in Fig. 5B, GST-Lyn B bound more effectively than Lyn A to tyrosine-phosphorylated proteins in cell lysates from both nonstimulated and stimulated cells. Collectively, the results show that Lyn B forms stronger or more abundant interactions with tyrosine-phosphorylated proteins than Lyn A. To further explore whether these differences might be seen in cells, we immunoprecipitated Lyn A or Lyn B or both from cells expressing them individually or from WT cells, respectively. Fig. 5C shows that a dominant band identified as LAT was found to coimmunoprecipitate with Lyn B and not Lyn A (albeit weak interactions with Lyn A could be seen in some experiments). LAT was also found to coimmunoprecipitate with Lyn kinase from WT cells and was present in resting cells, although at reduced levels when all experiments were analyzed.

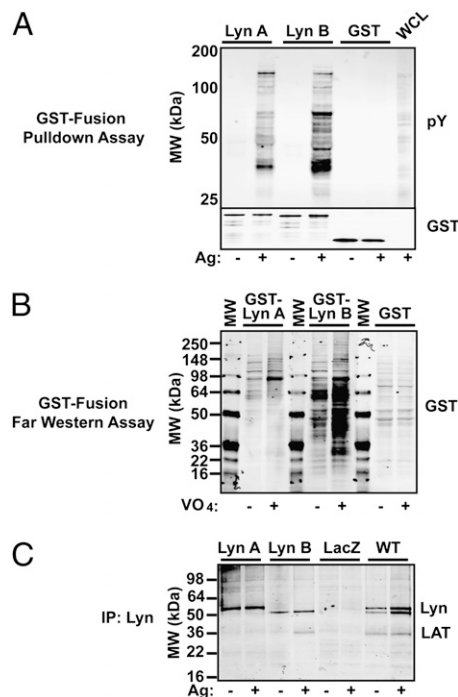


FIGURE 5. Lyn B shows increased binding of phosphoproteins, like LAT, when compared with Lyn A. *A*, GST-fusion proteins (10 μ g) encoding Lyn A and Lyn B (without the catalytic domain) were used in pulldown assays from lysates derived from mast cells stimulated (+) or not (–) with Ag. Interacting phosphoproteins were resolved on SDS-PAGE and immunoblotted with an Ab to phosphotyrosine (pY). GST alone was used as a control, and a sample of WCL from stimulated cells was included as a control for testing anti-phosphotyrosine Ab recognition. *B*, Proteins in the cell lysates (75 μ g/lane) from cells stimulated (+) or not (–) with pervanadate (VO₄) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. GST-Lyn A, GST-Lyn B, or GST was used to probe the membranes (Far Western), and binding was detected by use of an Ab to GST. *C*, Immunoprecipitation of Lyn isoforms from Ag stimulated (+) or resting (–) mast cells expressing Lyn A, Lyn B, or LacZ and from WT cells. Bands corresponding to the Lyn isoforms and LAT were detected with the respective Abs.

Lyn B forms a stable complex with SHIP-1 and decreases the interaction of PLC γ with phosphorylated LAT

Among the many proteins described to interact with Lyn kinase, the lipid phosphatase SHIP-1 is key in controlling the levels of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) following FcεRI stimulation (27). SHIP-1 has been shown to interact with PLC γ 1 and modulate its activation (28). Lyn is also required for the phosphorylation of the Tec family kinase BTK (29), which was shown to be required for full phosphorylation of PLC γ and increased activity (29, 30). Because both BTK and PLC γ require PIP₃ for their membrane localization and activation, we explored if the Lyn isoforms showed differences in coimmunoprecipitation of SHIP-1, which might explain differences in promoting calcium responses. Fig. 6A shows that Lyn B binds SHIP-1 more efficiently than Lyn A following FcεRI stimulation of mast cells expressing the respective kinases. Tyrosine phosphorylation of SHIP-1 could be seen in the Lyn B coimmunoprecipitates, whereas it was difficult to detect in Lyn A coimmunoprecipitates (data not shown). This interaction was confirmed in pulldown assays in which a GST-Lyn B fusion protein showed the increased presence of SHIP-1 relative to a GST-Lyn A fusion protein (Fig. 6B). Moreover, a GST-Lyn SH2 domain construct showed similar efficiency in its interaction with SHIP-1 (data not shown), demonstrating that this domain is key in promoting this interaction. Because of these findings and the observation that Lyn B associates more prominently with LAT than Lyn A, we explored if LAT was required for the interaction of Lyn B with SHIP-1. Fig. 6B shows that the absence of LAT had only a minor effect on Lyn B–SHIP-1 interactions. A more marked effect was seen for Lyn A, particularly after FcεRI stimulation. Moreover, a phosphoprotein of the molecular mass of NTAL/LAB/LAT2 (the Ab recognizing Y191 of LAT also recognizes an equivalent site on LAT2) was also more prominently associated with Lyn B. Regardless, the association of Lyn B with SHIP-1 was not significantly altered by the absence of LAT, showing that this interaction was not LAT-dependent.

Because Lyn B was less effective than Lyn A in inducing Ca²⁺ fluxes, we further explored if Lyn B-expressing cells might be less efficient in causing PLC γ phosphorylation, which would be consistent with the marked reduction in IP₃ production (Fig. 4B). As shown in Fig. 6C, tyrosine phosphorylation of PLC γ 1 and PLC γ 2 was markedly diminished (albeit not completely absent) in Lyn B-expressing cells when compared with Lyn A or WT cells. In general, cells expressing Lyn A were also slightly impaired in PLC γ 1 phosphorylation, although this was not apparent in all experiments. Strikingly, the amount of phosphorylated LAT coimmunoprecipitating with PLC γ 1 was reduced in cells expressing Lyn B relative to WT cells (Fig. 6C). Lyn A-expressing cells also showed less binding of phospho-LAT to PLC γ , but this interaction was less affected relative to Lyn B-expressing cells. Thus, the findings demonstrate that Lyn B-expressing cells have an impaired phosphorylation of PLC γ 1 and 2 and a reduced association with phosphorylated LAT relative to Lyn A or WT cells. Because Lyn kinase has been reported to phosphorylate the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1), we explored the possibility of differential control of this phosphatase by Lyn A and Lyn B, which might explain the differences in the level of LAT and PLC γ phosphorylation. Coimmunoprecipitation experiments revealed no significant association of SHP-1 with Lyn A or B, under conditions where SHIP-1 was detected, and no difference in the phosphorylation of Y536 on SHP-1 (the site phosphorylated by Lyn) was observed (data not shown).

This demonstrates that the mechanism underlying the ability of the two Lyn isoforms to evoke a Ca²⁺ response lies in their relative

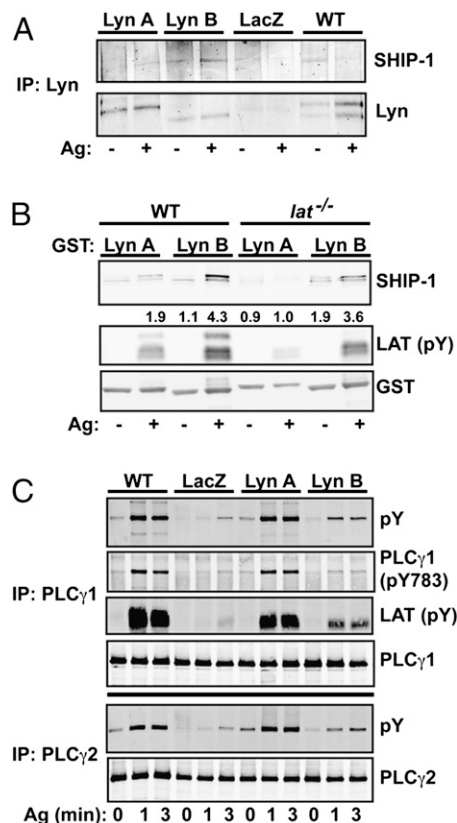


FIGURE 6. SHIP-1 association with Lyn B is prominent, and PLCγ phosphorylation and its association with LAT1 is impaired in Lyn B-expressing mast cells. *A*, Immunoprecipitation of Lyn from mast cells, stimulated (+) or not (-) with Ag, that were expressing Lyn A, Lyn B, or LacZ in comparison with WT cells. Resolved proteins were immunoblotted with Abs to SHIP-1 or Lyn. One of three representative experiments is shown. *B*, GST-fusion proteins encoding Lyn A and Lyn B (without the catalytic domain) were used in pull-down experiments to detect interacting proteins derived from lysates of Ag stimulated (+) or not (-) WT or *lat^{-/-}* mast cells. SHIP-1 was recognized by Ab to this protein. Phospho-LAT1 Ab (Y191) and LAT1 deficiency was used to recognize LAT1. This Ab also reacts with phospho-LAT2 (NTAL/LAB), which is the remaining band in the immunoblot from *lat^{-/-}* mast cell lysates. Ab to GST was used as a control for the amount of GST-fusion proteins used in this assay. One representative of four experiments is shown. Fold increase of SHIP-1 interaction with Lyn A or Lyn B is shown and is normalized to the interaction of SHIP-1 with Lyn A from non-stimulated cells. *C*, Immunoprecipitation of PLCγ1 and 2 from mast cells expressing Lyn A, Lyn B, and LacZ in comparison with WT cells. Phosphorylation of PLCγ1 and 2 was assessed with an Ab to phosphotyrosine (pY) and also (for PLCγ1) by phosphorylation at Y783 (whose phosphorylation is required for PLCγ activity) of this protein. Association of phosphorylated LAT with PLCγ1 was determined by the Ab to LAT (Y191). One representative of five experiments from three individual cultures is shown.

capacity to activate PLCγ and IP₃ production, and this appears to be independent of a role in SHP-1 phosphorylation.

Complementation of Lyn A and Lyn B activity is required for normal mast cell activation and responses

The presence of both Lyn A and Lyn B isoforms in mast cells, and their association with FcεRI, suggests that both isoforms are likely to complement for maximal FcεRI-induced mast cell activation. As noted in the above experiments, expression of Lyn A or Lyn B alone did not fully restore many of the cellular responses to those seen in WT cells. In these experiments, we set out to investigate

whether expression of both Lyn isoforms in *lyn^{-/-}* BMMCs complemented to normalize some of the responses, such as calcium mobilization, degranulation, and cytokine production, to the levels observed in WT cells. As shown in Fig. 7*A*, left panel, the ectopic expression of both Lyn A and Lyn B together was similar to the levels of these two isoforms in WT cells, and FcεRI stimulation did not alter this expression. Analysis of the effect on Ca²⁺ responses when both isoforms are restored showed that the isoforms complemented in restoring this response to WT levels (Fig. 7*A*, right panel). Interestingly, in most experiments, the rise in intracellular Ca²⁺ was still slightly delayed in cells expressing both Lyn A and Lyn B, suggesting that the rate may be determined by factors independent of Lyn or that minor differences in the expression levels of each isoform may affect the rate of this response. As shown in Fig. 7*B*, the degranulation of cells reconstituted with both Lyn A and Lyn B isoforms was now similar to that of WT cells, and consistently, the response was greater than that of cells expressing Lyn A or Lyn B alone. Thus, the presence of both isoforms caused a full degranulation response in an apparently additive manner (Fig. 7*B*). Finally, analysis of whether expression of both Lyn A and B would restore control (dampen) on cytokine responses revealed a marked inhibition of IL-6 production (which was similar to the levels of IL-6 production in WT cells) when both isoforms were expressed (Fig. 7*C*). Collectively, the findings demonstrate that, although Lyn A and Lyn B differ in the efficacy of PLCγ activation and Ca²⁺ fluxes following FcεRI stimulation, both

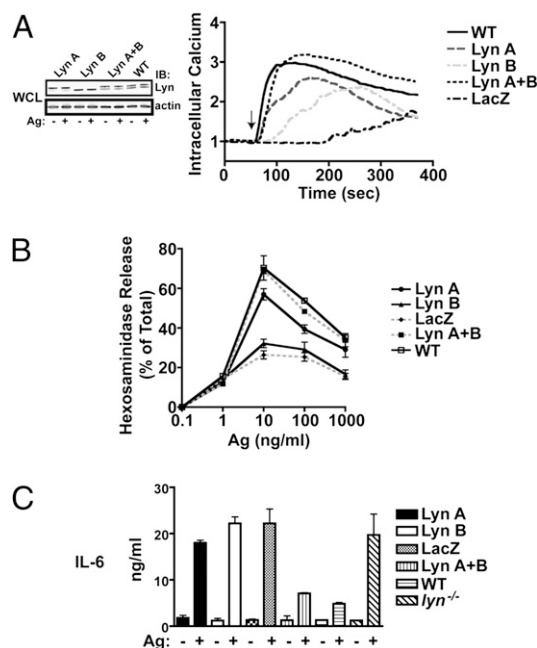


FIGURE 7. Lyn A and Lyn B complement to elicit full Ca²⁺ mobilization and mast cell degranulation and to suppress cytokine production. *A*, Mast cells expressing Lyn A or Lyn B individually or jointly were analyzed for Ca²⁺ responses and compared with WT or control LacZ-expressing cells. Lyn expression levels were determined from WCLs of each genotype pre- (-) or post- (+) stimulation of cells with Ag. Ab to actin was used to assess similar protein loading. Relative induction (340:380 ratio) of intracellular Ca²⁺ fluxes was measured by single-cell analysis (see Materials and Methods). One representative of six experiments is shown. *B*, Degranulation as measured by the release of hexosaminidase from Ag-stimulated cells (at the indicated concentrations) expressing the individual or both Lyn isoforms in comparison with WT or LacZ-expressing cells. One representative of six experiments is shown. *C*, Cytokine secretion by mast cells expressing the indicated Lyn isoforms and appropriate controls. IL-6 was measured using an ELISA assay. One representative of three experiments is shown.

isoforms complement to achieve a full Ca^{2+} response, normal degranulation, and to dampen cytokine responses.

Discussion

The presence of two Lyn kinase isoforms in hematopoietic cells where this kinase is expressed has long been recognized (16). However, how these two isoforms function and whether both isoforms are required for normal cellular responses has long remained a mystery. In the current study, we explored this unknown by reconstituting Lyn A and Lyn B isoforms individually or together in Lyn-null mast cells. This investigation was not amenable to RNA silencing strategies or gene deletion because the two Lyn isoforms are alternatively spliced products from a single gene and differ only in a 21-aa N-terminal insert found in Lyn A and not in Lyn B (16). Moreover, the requirements for the alternative splicing that generates the Lyn B isoform are not understood, hampering a genetic knock-in approach. How the 21-aa difference in Lyn A and B can cause the marked changes in functional responses analyzed in this study is not yet completely clear. However, some clues may be derived from their relative ability to coimmunoprecipitate tyrosine-phosphorylated proteins. The 21-aa insert contains a tyrosine (Y32) residue (Fig. 1) that could potentially be phosphorylated. As identified from Prosite database analysis, the amino acid sequence surrounding Y32 has some structural homology with the Y43 site on enolase, which is a protein readily phosphorylated by tyrosine kinases (31). We explored this possibility and found no evidence for phosphorylation of Y32 nor did a Y32F mutant of Lyn A show altered Ca^{2+} responses or degranulation (data not shown). In contrast, the marked difference of Lyn A and B isoforms in interacting with phosphoproteins suggests that the 21-aa insert functions to control Lyn A interactions presumably by creating a conformation that limits access to its protein interaction domains (SH2 and/or SH3). How this may occur is undetermined; however, given that GST fusion proteins of Lyn A and Lyn B (which are not tyrosine phosphorylated) maintained their differing ability to bind phosphoproteins, one can conclude that this behavior is not likely to be regulated by tyrosine phosphorylation of the 21-aa insert but most likely results from differences in protein folding/conformation.

Unlike Lyn A, Lyn B poorly induced Fc ϵ RI-mediated Ca^{2+} mobilization and mast cell degranulation. The underlying mechanism for the effect on Ca^{2+} mobilization and mast cell degranulation was the inability of the Lyn B isoform to effectively promote the phosphorylation of PLC γ and the production of IP_3 , crucial steps for these responses (9). Phosphorylated LAT1 was coimmunoprecipitated with PLC γ from Lyn A-expressing cells in greater amounts than from cells expressing Lyn B. This suggests that Lyn B fails to promote and/or impairs this interaction. This finding is consistent with the previous observation that loss of LAT1, or mutation of the PLC γ binding site on LAT1, causes loss of PLC γ phosphorylation, impaired Ca^{2+} responses, and defective mast cell degranulation (32, 33).

The ability of Lyn B, and not Lyn A, to efficiently associate with SHIP-1 may also be the key factor in the impaired PLC γ activity and reduced Ca^{2+} response. It has been shown that SHIP-1 coimmunoprecipitates with Lyn kinase from monocytes (34) and from mast cells (35). Moreover, SHIP-1 association with Fc γ RIIB is a key step for the inhibitory properties of this receptor on Fc ϵ RI-mediated Ca^{2+} responses, following coligation of these receptors (36). Recently, the negative regulatory role for SHIP-1 has been extended to its association with LAT1 in both mast cells as well as in T cells. In T cells, LAT1 organizes an inhibitory complex that includes the adapter Dok-2 and SHIP-1, which serves to dampen the extent of early activation upon TCR engagement (37). In mast cells, LAT1 can also exert an inhibitory role under certain circumstances.

Analysis of LAT1 mutants showed the recruitment of SHIP-1 into an Fc ϵ RI-induced signaling complex (38). SHIP-1 was found to be recruited to LAT1 in a cooperative manner, in which binding of Grb-2 to tyrosine Y195 potentiates the binding of SHIP-1 to tyrosine Y235. However, our findings suggest that LAT1 is not required for the association of Lyn B with SHIP-1. Deficiency in LAT1 had minimal effect on this interaction, suggesting that the interaction of SHIP-1 with Lyn B is not mediated through this adapter protein. Instead, Lyn B may bring SHIP-1 to LAT1, where it might exert its control by destabilizing PLC γ interaction with the plasma membrane (and thus with LAT1), possibly by reducing the levels of PIP_3 , a lipid required for PLC γ translocation to the plasma membrane. It should be noted that other modes of inhibition by SHIP-1 have been described. Dok-1, an adapter that binds SHIP-1, can associate with and negatively regulate Fc ϵ RI signaling without the involvement of inhibitory receptors (39, 40). In mast cells, Dok-1 was found to associate with the Fc ϵ RI β -chain (40), and its phosphorylation was demonstrated to be Lyn-dependent (41). Thus, it is possible that Lyn B interactions with SHIP-1 might be mediated through the adapter Dok-1. Nonetheless, our analysis of Lyn B immunoprecipitates for the presence of Dok-1 did not reveal detectable levels of this adapter protein (data not shown). Yet, mutation of the noncanonical tyrosine residue that lies in the middle of the Fc ϵ RI β -chain caused increased cytokine production in mast cells and led to a modest loss of SHIP-1 phosphorylation, suggesting that this tyrosine residue might interact with this negative regulator (42).

In this study, we also investigated whether the previously described negative regulatory role of Lyn kinase on cytokine production (11, 13) could be ascribed to Lyn A or Lyn B. Although Lyn A consistently showed a moderate dampening of cytokine responses, when compared with Lyn B-expressing cells, this effect was incomplete. That Lyn A showed a greater dampening effect than Lyn B was unexpected, given that the association of SHIP-1 is more prominent with Lyn B and thus one might expect that Lyn B would have a greater dampening effect. Our findings show that expression of both Lyn isoforms is required to restore the control on cytokine production. It is unlikely that the suppression of cytokine responses is related to dampening of Ca^{2+} mobilization, because expression of both Lyn A and Lyn B increased Ca^{2+} fluxes following Fc ϵ RI stimulation. A plausible explanation could derive from our previous finding that mutation of the Fc ϵ RI β -chain ITAM tyrosines, which causes the loss of Lyn association with this receptor, induced increased I κ B α degradation and enhanced nuclear NF- κ B activity following Fc ϵ RI stimulation (42). It is well established that in mast cells, TNF and IL-6 production are highly dependent on NF- κ B activity (43, 44). Thus, both Lyn A and Lyn B may be required to prevent the excessive degradation of I κ B α and thus dampen nuclear NF- κ B activity. However, a test of this hypothesis showed the inverse, as Fc ϵ RI-stimulated cells expressing the individual Lyn A or Lyn B isoforms were found to have higher amounts of I κ B α protein remaining when compared with cells expressing both isoforms, which had a similar content of I κ B α protein to stimulated WT cells (data not shown). Thus, it is unlikely that increased NF- κ B activity could account for increased cytokine response of Lyn A- or Lyn B-expressing cells, and how both Lyn kinase isoforms complement to dampen cytokine production remains to be determined.

We now appreciate that the 21-aa N-terminal insert found in Lyn A, and not in Lyn B, determines the efficacy of each isoform in activating PLC γ and generating IP_3 , and thus in promoting Ca^{2+} fluxes. Additionally, key differences were seen in the ability of each isoform to associate with phosphoproteins like LAT and SHIP-1, suggesting that the 21-aa insert governs protein interactions. Most importantly, our findings demonstrate that the Lyn A and Lyn B isoforms have

distinct roles with regards to mast cell signal transduction. Nonetheless, the two isoforms complement in inducing both positive and negative regulation of mast cell effector functions. These findings provide an explanation for the presence of both Lyn A and Lyn B isoforms in mast cells and likely for other cell types as well.

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Disclosures

The authors have no financial conflicts of interest.

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