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Regulation of Rat Basophilic Leukemia-2H3 Mast Cell Secretion by a Constitutive Lyn Kinase Interaction with the High Affinity IgE Receptor (FceRI) 1

Becky M. Vonakis, 2 Scott P. Gibbons, Jr., Masashi J. Rotté, Elizabeth A. Brothers, Seok C. Kim, Kristin Chichester, and Susan M. MacDonald

Signaling through the high affinity IgE receptor is initiated by noncovalently associated Lyn kinase, resulting in the secretion of inflammatory mediators from mast cells. A fraction of the total cellular Lyn is associated via its N-terminal unique domain with the cytoplasmic domain of the FceRI β subunit before receptor aggregation. In the current study, we stably transfected the unique domain of Lyn into rat basophilic leukemia-2H3 mast cells and examined the consequences on FceRI-induced signal transduction and mediator secretion to further define the role of the unique domain of Lyn in mast cell secretion. Tyrosine phosphorylation of FceRI β and γ subunits was partially inhibited in the Lyn unique domain transfectants after Ag stimulation. Ag stimulation of Lyn unique domain transfectants was accompanied by enhanced phosphorylation of MEK and ERK-2, which are required for leukotriene C4 (LTC4) release, and production of LTC4 was increased 3- to 5-fold, compared with cells transfected with vector alone. Conversely, tyrosine phosphorylation of the adaptor protein Gab2, which is essential for mast cell degranulation, was inhibited after Ag stimulation of Lyn unique domain transfectants, and Ag-induced release of histamine was inhibited up to 48%.  

In rat basophilic leukemia-2H3 cells, Lyn thus plays a dual role by positively regulating FceRI phosphorylation and degranulation while negatively regulating LTC4 production. This study provides further evidence that the constitutive interaction between the unique domain of Lyn and the FceRI β subunit is a crucial step in the initiation of FceRI signaling and that Lyn is limiting for FceRI-induced secretion of inflammatory mediators.  


The FcRs (for IgE, IgM, IgA, and IgG) and TCRs and BCRs belong to the multichain immune recognition receptor (MIRR) family of immunoreceptors (1, 2). The MIRR family receptors initiate signaling by tyrosine phosphorylation of their ITAMs by noncovalently associated Src family tyrosine kinases (3–6). A common feature of the kinase-receptor interaction is a constitutive association between a small fraction of the total kinase with resting, i.e., unphosphorylated receptors, mediated by the unique domain of each kinase (7, 8). After aggregation of the MIRR family receptors, the Src family kinases reorient and bind to the phosphorylated receptor ITAMs via their Src homology 2 (SH2) domains (9).

The unique domain of Src family kinases appears to function as both a membrane-targeting domain (the SH4 region) and a receptor association domain essential for the initiation of receptor signal transduction (Fig. 1A) (10, 11). Two forms of Lyn are expressed in mast cells. The larger A form of Lyn kinase (56 kDa) contains a 21-aa insert in its unique domain that is lacking in the smaller (53-kDa) Lyn B isoform (Fig. 1A). Previous studies have shown that transfection of Lyn unique domain A into Chinese hamster ovary (CHO) fibroblasts containing high affinity receptors for IgE (FceRI) led to an inhibition of receptor tyrosine phosphorylation, compared with cells containing FceRI alone (8). Transfection of equivalent amounts of catalytically inactive Lyn B kinase into FceRI-transfected CHO cells resulted in a similar degree of inhibition of receptor tyrosine phosphorylation (8). Furthermore, transfection of increasing amounts of active Lyn B kinase into FceRI-containing CHO cells gave a linear increase in receptor phosphorylation. A mathematical analysis of data from the Lyn-FceRI transfectants indicated a single Lyn molecule was sufficient to phosphorylate a dimerized receptor (12). Yeast two-hybrid studies (8) indicated that the unique domain of both Lyn A and Lyn B could bind directly to the unphosphorylated C-terminal cytoplasmic domain of the FceRI β-chain, while peptide-binding studies (9, 13) indicated that the SH2 domain of Lyn bound to phosphorylated FceRI β ITAMs. Furthermore, chemical cross-linking studies demonstrated that 5% of total cellular Lyn in rat basophilic leukemia (RBL)-2H3 cells was bound to resting, unphosphorylated receptors (7). The Lyn unique domain appears to be the sole site of interaction with resting IgE receptors because an equivalent strength of association, as measured by production of β-galactosidase, was present between the unique domain and full-length Lyn protein in yeast two-hybrid studies (8). Taken together, the data implicate the constitutive interaction of the Lyn unique domain and the FceRI β as a crucial positive regulatory point in initiating FceRI signal transduction, which ultimately results in the release of allergic mediators.

Emerging data also strongly point to a role for Lyn kinase in negatively regulating signal transduction in bone marrow-derived mast cells. While Lyn positively regulates FceRI signaling, it negatively regulates LTC4 production. This study provides further evidence that the constitutive interaction between the unique domain of Lyn and the FceRI β subunit is a crucial step in the initiation of FceRI signaling and that Lyn is limiting for FceRI-induced secretion of inflammatory mediators. The Journal of Immunology, 2005, 175: 4543–4554.

1 Abbreviations used in this paper: MIRR, multichain immune recognition receptor; BMMC, bone marrow-derived mast cell; CHO, Chinese hamster ovary; CM, calcium measurement; DSP, dithiothreitol (succinimidylpropionate); HSA, human serum albumin; IP, immunoprecipitate; LTC4, leukotriene C4; PCA, passive cutaneous anaphylaxis; PY, phosphotyrosine; RBL, rat basophilic leukemia; SH, Src homology; PAgG, PIPES-bovine albumin-glucose.
The phenotype of Lyn knockout mice (Lyn⁻/⁻) indicates that Lyn is important in FcεRI signaling and establishing B cell tolerance. Lyn⁻/⁻ mice (on a C57BL/6 genetic background), as studied by Hibbs et al. (14), fail to undergo passively cutaneous anaphylaxis (PCA), are IgM hypergammaglobulinemic, and develop an autoimmune disease similar to human lupus. Recently, the lack of a PCA in these mice was correlated with a failure to sensitize mast cells with the injected Ag-specific IgE, due to a lack of unoccupied FcεRI (15). However, a second group prepared Lyn⁻/⁻ mice on the same genetic background and found them capable of a normal PCA reaction (16). Lyn⁻/⁻ mice can signal positively through the BCR, but activated B cells accumulate, resulting in splenomegaly, indicating an indispensable role for Lyn in negatively regulating B cell signaling (16). Several groups have examined the consequences of Lyn deficiency on signaling in BMMCs. In common with Lyn⁻/⁻ B cells stimulated through the BCR, Ag stimulation of Lyn⁻/⁻ BMMCs induces prolonged activation of ERKs, and both cytokine and histamine secretion are up-regulated (16–19). Lyn was found to negatively regulate phosphorylation of Gab2 (20). Degranulation was partially reduced in Btk-deficient BMMCs (18). However, degranulation was severely reduced in Lyn and Btk doubly deficient BMMCs, pointing to a positive role for Lyn in Ag-stimulated mediator secretion (18).

Furthermore, a complementary role for the Src family kinase Lyn in FcεRI-induced signaling to degranulation was indicated based on the dramatic reduction in FcεRI-induced histamine release detected in Lyn⁻/⁻ BMMCs (20). In BMMCs, Lyn was found to associate with the FcεRI β-chain and to positively regulate phosphorylation of the adaptor protein Gab2. Lyn deficiency results in a reduction in ERK phosphorylation in both Ag-treated BMMCs and CD2-stimulated T cells (20, 21). The specific roles of Lyn and Fyn in regulating FcεRI signaling and mediator secretion to further define the role of the unique domain of Lyn in mast cell secretion.

Materials and Methods
Reagents and cells
Anti-DNP-specific mouse IgE (clone SPE-7) was obtained from Sigma-Aldrich or was a gift from H. Metzger (National Institutes of Health, Bethesda, MD) (clone ε-26-82) (23), while high valency DNP₃-BSA or DNP₄₋₅ human serum albumin (HSA) Ags were purchased from Molecular Probes or Sigma-Aldrich, respectively. Polyclonal Abs to Lyn, FcεRI y, and Gab2, as well as a mAb to phosphotyrosine (PY; 4G10-biotin or 4G10-HRP) were purchased from Upstate Biotechnology; polyclonal Abs to ERK1 and to Gab2 were obtained from Santa Cruz Biotechnology; an Ab to tubulin (clone DM1A) was from Neomarkers; while phospho-ERK (Thr202, Tyr204)-specific, and phospho-MEK (Ser217, Ser221)-specific Abs were obtained from Cell Signaling Technology. Monoclonal and polyclonal anti-Syk Abs were a gift from P. Draber (Institute of Genetic Sciences, Prague, Czech Republic) and U. Blank (Institute Pasteur, Paris, France), respectively. The DNP₃-BSA used in this study was a gift from H. Metzger. HRP-conjugated anti-rabbit and anti-mouse secondary Abs were obtained from Amersham Biosciences. Avidin-coupled HRP was purchased from Sigma-Aldrich, and HRP-conjugated anti-goat Abs were obtained from Roche Diagnostics. Fura 2-AM and pluronic 27 were purchased from Molecular Probes or Sigma-Aldrich, respectively. Polyclonal Abs to Lyn, FcεRI, and Fyn in regulating FcεRI signaling to degranulation was indicated based on the dramatic reduction in FcεRI-induced histamine release detected in Lyn⁻/⁻ BMMCs (20).

Lyn- AND FcεRI-MEDIATED MAST CELL SECRETION
Low and moderate valency Ag preparation
Lower valency Ags (DNP₂-BSA, DNP₃₋₅-BSA) were prepared by reacting DNP-succinimidyl ester (Molecular Probes) with fatty acid-free BSA (Sigma-Aldrich) at various molar ratios for 1 h at room temperature. The reaction was stopped by adding 1.5 M hydroxyamine (pH 8.5), and the conjugate separated from unreacted dye by chromatography on a Sephadex G-25 column from Amersham Biosciences. The protein-containing fractions (by absorbance at 280 nm) were pooled and dialyzed against borate-buffered saline (0.2 M boric acid, 0.16 M NaCl; pH 8).

Ag valency determination
The Ag valency (degree of labeling (DOL)) was calculated for all Ags used in the study using the formula: DOL = Aₘₐₓ × MW/[protein] × εᵥₑᵥₑ, where Aₘₐₓ = absorbance at 349 nm (Aₘₐₓ for DNP), MW = molecular mass of BSA (66,000 kDa), [protein] = protein concentration in mol/L (M) as determined by absorbance at 280 nm, corrected for the contribution of DNP to the absorbance at 280 nm (λᵥₑᵥₑ = 0.18, and εᵥₑᵥₑ = the extinction coefficient of DNP (18,000 cm⁻¹ mol⁻¹⁻¹).

Preparation of stable Lyn A unique domain-RBL transfectants
The cDNA for rat Lyn A unique domain was subcloned into the vector pSVL and linearized by digestion with PstI. The construct (10 µg) was then cotransfected into RBL-2H3 cells along with pZeoSV2⁺ (1 µg, encoding resistance to the antibiotic zeocin) as a selectable marker by electroporation (0.4-cm gap, 700 V, 25 µF). After 48 h, the cells were passaged into growth medium supplemented with 250 µg/ml zeocin. Colonies were picked after 7 days using sterile cloning cylinders and expanded for screening of Lyn expression. Whole cell SDS lysates were Western blotted with polyclonal anti-Lyn. Control transfectants were similarly generated by cotransfection of PstI-linearized pSVL and pZeoSV2⁺. Several clones of each type of transfectant were expanded for further testing.

FcεRI determination
Expression of FcεRI was quantified in a binding assay with ¹²⁵I-labeled mouse IgE (8).

Determination of ratio of endogenous Lyn to Lyn unique domain
To determine the ratio of Lyn unique domain to endogenous Lyn, various amounts of SDS whole cell lysates (equivalent to 10,000–60,000 cell equivalents) of the transfectants were Western blotted using a polyclonal anti-Lyn Ab, and the blots were developed using Supersignal chemiluminescent substrate.

Subcellular fractionation
The subcellular location of transfected Lyn unique domain protein was measured by centrifuging the postnuclear supernatant from sonicated clone UC3 cells at 140,000 × g to generate a high-speed pellet and supernatant. After detergent extraction (0.5% Triton X-100) of the fractions, an aliquot was Western blotted with anti-Lyn. Co- sedimentation of ¹²⁵I-labeled IgE-sensitized FcεRI served as a membrane marker for proteins recovered from the detergent-extracted pellet, whereas recovery of tubulin from the supernatant fraction (as detected by Western blotting) served as a cytosolic marker.

Histamine release
After sensitization with anti-DNP-specific IgE overnight, control and Lyn unique domain-transfected RBL cells (150,000 cells per condition) were seeded into 96-well plates, washed with HBSS (Sigma-Aldrich), and stimulated with various doses of DNP₃-BSA or DNP₃₋₅-BSA Ag in PIPES-bovine albumin-glucose (PABG) buffer (25 mM PIPES, 5 mM KCl, 110 mM NaCl, 0.1% BSA, and 0.1% dextrose) supplemented with 1 mM CaCl₂ for 45 min. Supernatants were collected for quantification of histamine by automated fluorometry (24). Spontaneous release was assessed by incubation in PA₄₅-G-calcium buffer, while total cellular histamine was determined by lysis in 1.6% HClO₄ acid. The results are expressed as the percentage of total histamine release after subtraction of spontaneous release.

Cell-associated and secreted TNF-α
To compare TNF-α secretion between transfectants, cells were seeded into six-well plates (2.4 × 10⁶ cells per condition) in growth medium without antibiotics, sensitized with anti-DNP-specific IgE overnight, and washed.
twice with HBSS. The cell monolayers were then stimulated for 2.5 h with DNP-BSA or DNP-BSA Ag in growth medium without antibiotics. The supernatants were collected, clarified by centrifugation to remove any detached cells, and TNF-α quantified by specific ELISA (BioSource International), according to the manufacturer’s instructions. The percentage of cell-associated TNF-α was determined by washing the cell monolayer with HBSS and freeze thawing the cells recovered from the wells by trypsination in HBSS-containing protease inhibitors three times (25).

**Leukotriene C4 (LTC4) release**

RBL transfectants or parental RBL-2H3 cells (150,000 cells per condition) were sensitized with anti-DNP IgE, washed, and plated as for histogram release. The cells were then stimulated with DNP-pHSA or DNP-pBSA Ag for 15–45 min, cell supernatants were collected, and leukotriene C4 (LTC4) release was quantified by specific RIA (26).

**Quantification of Lyn and Lyn unique domain associated with FcεRI**

RBL Lyn unique domain UC3 transfectants were sensitized with 5 μg/ml anti-DNP-specific IgE for 1 h, room temperature, and washed three times with PAAG buffer. Aliquots of cells (10^8) were then incubated with 2 mM DSP for 1 h on ice in XL Assay Buffer (2.5 mM HEPES buffer supplemented with 11.9 mM NaCl, 0.5 mM Na3VO4, 5 mM Mn2+PO4, 0.5 mM CaCl2, 1 mM MgCl2, 5 mM EDTA, 1 mM Na3VO4, 5 mM Na3PO4, 0.5 μM EDTA, 1 mM MgCl2, 0.5 mM dextrose, 0.5 mM EDTA, 1 mM Na3VO4, 5 mM Mn2+PO4, and 1.5X protease inhibitors). The reaction was quenched with an excess of amine groups in the form of 10 mM glycine for 20 min on ice, and then the cross-linked receptors were extracted with 0.5% Triton X-100 in XL of amine groups in the form of 10 mM glycine for 20 min on ice, and then 1 mM Na2EDTA, 1 mM MgCl2, 5 mM EDTA, 1 mM Na3VO4, 5 mM Na3PO4, and 1.5X protease inhibitors. The reaction was quenched with an excess of amine groups in the form of 10 mM glycine for 20 min on ice, and then the cross-linked receptors were extracted with 0.5% Triton X-100 in XL of amine groups in the form of 10 mM glycine for 20 min on ice, and then the cross-linked receptors were extracted with 0.5% Triton X-100 containing buffer, and Lyse immunoprecipitated with goat anti-mouse IgE (Cappel-ICN) and protein A-Sepharose (Amersham Biosciences) overnight. The control immunoprecipitate (IP) consisted of chemically cross-linked lysate from UC3 cells incubated with protein A-Sepharose alone. The IPs were washed three times with lysis buffer to remove any noncovalently associated proteins, boiled in SD-sample buffer reduced with 1 mM DTT, and FcεRI-associated Lyn quantified by anti-Lyn Western blotting.

**FcεRI tyrosine phosphorylation**

Lyn unique domain and control transfectants were sensitized with anti-DNP-specific mouse IgE (trace labeled with 32P-labeled IgE) and stimulated with 250 ng/ml DNP3,BSA Ag for various times. The cells were lysed in 0.5% Triton X-100, 25 mM PIPES, 5 mM KCl, 110 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 5 mM Na3PO4, and 1X protease inhibitors. The FcεRI were immunoprecipitated with goat anti-mouse IgE, and equal amounts of recovered cpm were loaded on 12% Tris-glycine gels. The transferred blots were probed with anti-PY (4G10) and then monoclonal anti-Syk. The blots were scanned, and the anti-PY densitometric value was divided by the anti-Syk reprobe value to give a normalized Syk PY value. The scanned value of a single tyrosine-phosphorylated band in the Western blot standard was used to normalize the phosphoprotein values for samples run on different gels. The same procedure was used to quantify FceRI PY, Gab2 PY, and phosphorylation of ERK-2 and MEK.

**Cytosolic-free calcium measurements**

Stable rat Lyn A unique domain transfectants were sensitized with 5 μg/ml DNP-specific IgE for 2 h at room temperature and washed three times in calcium measurement (CM) buffer consisting of 10 mM HEPES buffer containing 135 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.25 mM probenecid, and 0.1% BSA, pH 7.4. The cells were then incubated with 2 μM fura-2-AM in CM buffer supplemented with 0.02% pluronic 27 for 45 min, 37°C. After three washes in CM buffer, the cells were placed into the prewarmed 37°C chamber of a QM-8 spectrofluorometer (Photon Technology International). After establishing a baseline, separate samples were stimulated with 20 ng/ml DNP-pBSA Ag in the presence or absence of 2 mM EGTA to chelate extracellular calcium. The change in cytosolic-free calcium concentration is expressed as the ratio of emitted fluorescence (at 510 nm) excited at 340 nm over that excited at 380 nm.

**Statistical analysis**

Statistical analysis software (Statview SE + Graphics, version 1.03; Abacus Concepts) was used to compare the FcεRI-induced mediator release from Lyn unique domain transfectants to control transfectants by means of a paired (two-tailed) t test. For Western blot data, the scanned normalized phosphoprotein or Lyn value was calculated and statistical differences between transfectants were compared by a paired (two-tailed) t test.

**Results**

**Expression of endogenous Lyn A and Lyn B, Lyn A unique domain, and FcεRI in RBL transfectants**

Stable rat Lyn A unique domain transfectants were prepared in RBL-2H3 cells, a widely studied model of mucosal mast cells, to examine the consequences on FcεRI-induced signaling and mediator secretion. To determine the ratio of Lyn unique domain to endogenous Lyn, various amounts of whole cell lysates (equivalent to 10,000–60,000 cell equivalents) of the transfectants were Western blotted using a polyclonal anti-Lyn Ab. As shown in Fig. 1B, a band of apparent molecular mass of 53/56 kDa was observed corresponding to endogenous Lyn A/B in all transfectants. The Lyn A unique domain contains 66 aa and has an expected molecular mass of 7 kDa (Fig. 1A). A single immunoreactive band of 7 kDa was observed only in our Lyn A unique domain transfectants. Quantification of the amounts of these three Lyn species by use of a digital camera and densitometry software from six separate experiments indicated that clone UC3 expressed, on average, 3.8-fold more Lyn A unique domain than endogenous Lyn A/B, while clone UAI expressed 2.4-fold more transfected Lyn A unique protein than endogenous Lyn A/B (Table I). By densitometry of six total blots, we observed that transfection did not alter the amount of endogenous Lyn expressed, when compared with the same amount of RBL-2H3 lysate run as a standard on each gel (average ± SEM, 1.04 ± 0.3, n = 6 control transfectants; 1.08 ± 0.3, n = 6 Lyn unique transfectants).
Membrane localization of transfected Lyn unique domain and endogenous Lyn

The subcellular location of transfected Lyn unique domain protein was measured by centrifuging the postnuclear supernatant from sonicated clone UC3 cells to generate a high-speed pellet and supernatant. After detergent extraction of the fractions, an aliquot was Western blotted with anti-Lyn. As shown in Fig. 1C, >95% of transfected Lyn unique domain was detected in the total membrane fraction, while endogenous p56/p53 Lyn was found exclusively in the membrane fraction, as expected. Furthermore, the Lyn unique domain protein in our RBL transfectant UC3 was found to partition equally into both raft and nonraft membranes by sucrose gradient fractionation (M. Rotté and B. Vonakis, unpublished results). These data indicate that transfected Lyn unique domain is membrane localized, in which it can compete with endogenous Lyn for association with FcεRI.

The parental RBL-2H3 cells used for transfection were found to express 350,000 FcεRI per cell in binding studies with 125I-labeled mouse IgE, while receptor expression on our RBL transfectants ranged from 270,000 to 530,000 (Table I), in accord with the heterogeneity previously reported for this cell line (27). At least two different control transfectants were tested in each of the assays described subsequently.

Association of transfected Lyn unique domain with unaggregated FcεRI in RBL cells

RBL Lyn unique domain UC3-transfected cells were sensitized with anti-DNP-specific IgE. The samples were then incubated with 2 mM DSP, a membrane-permeable, thiol-cleavable, homobifunctional, and amine-reactive chemical cross-linking agent. The FcεRI were immunoprecipitated with goat anti-mouse IgE and protein A-Sepharose. The control IP consisted of chemically cross-linked lysate from UC3 cells incubated with protein A-Sepharose alone. The IPs were reduced with 1 mM DTT and subjected to anti-Lyn Western blot. The results in Fig. 2 demonstrate association of both p7 Lyn A unique domain and p53/p56 Lyn A and Lyn B in the FcεRI IPs from UC3 cells (one experiment representative of three conducted). The control IP does not contain Lyn-reactive bands, indicating that the Lyn and Lyn unique domain recovered in the anti-IgE IP are receptor associated and not due to nonspecific binding of Lyn or Lyn unique domain to the protein A beads. These results provide direct proof of our hypothesis concerning a constitutive FcεRI β-Lyn kinase unique domain association.

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**Table I. Expression of endogenous Lyn, Lyn unique domain, and FcεRI in RBL transfectants**

<table>
<thead>
<tr>
<th>Cotransfected cDNA</th>
<th>Clone</th>
<th>FcεRI (×10^3)</th>
<th>Lyn Unique per Endogenous Lyn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyn Unique</td>
<td>UC3</td>
<td>350</td>
<td>3.8</td>
</tr>
<tr>
<td>Lyn Unique</td>
<td>UA1</td>
<td>340</td>
<td>2.4</td>
</tr>
<tr>
<td>pSVL</td>
<td>PB2</td>
<td>380</td>
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</tr>
<tr>
<td>pSVL</td>
<td>PB4</td>
<td>270</td>
<td>NA</td>
</tr>
<tr>
<td>pSVL</td>
<td>PC5</td>
<td>530</td>
<td>NA</td>
</tr>
<tr>
<td>pSVL</td>
<td>PC6</td>
<td>440</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* Stable transfectants were prepared by electroporation of the Lyn A unique domain cDNA (in the vector pSVL) along with pZeoSV2 (as a selectable marker) into RBL-2H3 cells, followed by selection of stable clones with zeocin. Control transfectants were prepared by cotransfection of pSVL and pZeoSV2.

*b* Expression of FceRI was determined using 125I-labeled IgE in duplicate in two separate experiments.

*c* The ratio of transfected Lyn unique domain to endogenous LynA/LynB was determined by anti-Lyn Western blotting (n = 6).

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**FIGURE 1.** Domain structure, expression, and membrane localization of endogenous Lyn and transfected Lyn unique domain in RBL Lyn unique domain and vector control transfectants. *A*, Domain structure of Lyn A and Lyn B kinase. *B*, Whole cell lysates were Western blotted with a polyclonal anti-Lyn Ab (one experiment representative of six conducted). On the blot represented in *B*, three doses of a lysate from clones UC3, UA1, and PC6 were run on the same gel. Only the highest dose of lysate run for each clone is shown. *C*, Subcellular fractionation of Lyn unique domain-transfected RBL cells (clone UC3). Fractions were Western blotted with anti-Lyn (one experiment representative of three conducted).

**FIGURE 2.** Constitutive association of transfected Lyn A unique domain in RBL-2H3 cells. Cellular proteins from IgE-sensitized UC3 cells were cross-linked with DSP and lysates immunoprecipitated with anti-IgE and protein A-Sepharose beads (alμE) or beads alone (control). IPs were Western blotted with polyclonal anti-Lyn. One experiment representative of three conducted is shown.
Stimulation of transfected cells for mediator production and signaling

As carefully studied by Gonzalez-Espinosa et al. (28), release of granule mediators and lymphokines by BMMC is differentially sensitive to FcεRI occupancy and dose of Ag, with many lymphokines being preferentially released upon stimulation with low doses of Ag or partial receptor occupancy. LTC₄ production was not examined in their study. In addition, we have shown that under conditions in which cells expressing different ratios of Lyn to FcεRI are compared, a weak stimulus such as a dimer of covalently cross-linked IgE molecules or low valency Ag can detect signaling differences between cell types (8). For example, Ag-stimulated RBL-2H3 cells (high Lyn:FcεRI ratio) and FcεRI-transfected CHO cells (low Lyn:FcεRI ratio) have similar FcεRI tyrosine phosphorylation, but weak IgE dimer stimulation induces FcεRI tyrosine stimulation in RBL-2H3, but not FcεRI-transfected CHO cells (8). Therefore, stimulation experiments to compare mediator release (histamine, LTC₄, and TNF-α) between transfectants were conducted with high valency Ag (DNP₂₅BSA, DNP_{35}HSA) as well as lower valency Ags (DNP₁₂BSA, DNP_{10}BSA, DNP₂BSA).

Histamine release stimulated by FcεRI aggregation in Lyn unique domain RBL transfectants

Control and Lyn unique domain-transfected RBL cells (with similar FcεRI expression; see Table I) were sensitized with anti-DNP-specific IgE and stimulated with various doses of high valency DNP₂₅BSA Ag. Supernatants were collected for quantification of histamine by automated fluorometry. When the kinetics of histamine release were compared between control clone PB2 and Lyn unique clone UC3, 40% inhibition was observed in the Lyn unique-containing cells at time points between 1 and 45 min after stimulation with 10 ng/ml DNP₂₅BSA Ag (Fig. 3A; n = 2). Rel-

![Graph A](image)

**FIGURE 3.** Histamine release induced by FcεRI aggregation and calcium ionophore in Lyn unique domain-transfected RBL cells. Cells were sensitized with anti-DNP IgE and stimulated with Ag for 45 min. A, Kinetics of FcεRI-induced histamine release upon stimulation with 10 ng/ml DNP₂₅BSA Ag; n = 2. B, DNP₂₅BSA Ag dose response; n = 3–10. C, DNP₁₂BSA Ag dose response; n = 4–7. D, A23187 (2 μM)-induced histamine release; n = 3. Control clone PC5; Lyn unique clone UC3. Data expressed as the mean ± SEM; *, p < 0.05; **, p < 0.005 (t test).
ative to the four different control transfectants tested (PB2, PB4, PC5, PC6), Lyn unique domain clone UC3 demonstrated a consistent 35% inhibition of histamine release when stimulated with 5–50 ng/ml DNP35BSA Ag for 45 min (Fig. 3B; *, p < 0.05; **, p < 0.005; t test, n = 3–10). However, Lyn unique domain clone UA1, which expresses 37% less Lyn unique domain as clone UC3, did not give statistically different histamine release than the control transfectants when stimulated with DNP35BSA Ag (Fig. 3B).

When Lyn unique domain clones UC3 and UA1 were compared with control transfectant PC5 for histamine release induced by moderate valency DNP12-BSA Ag, 43–46% inhibition was observed at 50 or 100 ng/ml Ag doses, respectively (Fig. 3C; *, p < 0.05, either UC3 or UA1 vs PC5, n = 4–7). These results are consistent with our model that disruption of the constitutive protein-protein interaction between Lyn kinase and the FcRI can partially prevent release of a preformed mediator. In contrast, stimulation of either Lyn unique transfectant UC3 or control clone PC5 with 2 μM A23187 calcium ionophore gave statistically equivalent histamine release, indicating the secretory machinery downstream of calcium release is unaffected by transfection (Fig. 3D; n = 3).

Cell-associated and FcεRI-induced TNF-α production in Lyn unique domain RBL transfectants

The cytokine TNF-α is important in host defense against bacterial infection, and has been reported to be both stored in granules in RBL mast cells and newly synthesized upon IgE receptor cross-linking (25). In two preliminary experiments, we compared TNF-α secretion between control PC6 and Lyn unique domain UC3 transfectants after the cells were stimulated for 2.5 h with moderate valency DNP10BSA Ag. The supernatants were collected, and TNF-α was quantified by specific ELISA. No difference in TNF-α secretion was noted between the two cell types (e.g., PC6, 124.3 pg; UC3, 127 pg after 30 ng/ml DNP10BSA stimulation). We next stimulated control PC6 cells with 10–1200 ng/ml low valency DNP2-BSA Ag. TNF-α secretion reached a maximum at 800 ng/ml DNP2-BSA Ag, and therefore, we compared UC3, UA1, and PC6 at that dose. As shown in Fig. 4, secretion of TNF-α was decreased, on average, 32.9–37.6% in Lyn unique domain clones UC3 and UA1, respectively, compared with control transfectant PC6 (n = 4; p value NS). The percentage of cell-associated TNF-α was found to decrease with increasing time of stimulation and increasing concentrations of Ag. Under the conditions reported in Fig. 4 (800 ng/ml, 2.5 h), 84% of the total TNF-α was secreted.

**LTC4 production stimulated by FcεRI aggregation in Lyn unique domain RBL transfectants**

As before, RBL transfectants were sensitized with anti-DNP IgE and stimulated with high valency DNP10-HSA Ag for 45 min; cell supernatants were collected; and LTC4 release was quantified by specific RIA. As shown in Fig. 5A, transfection of Lyn unique domain up-regulated production of this eicosanoid 3- to 5-fold at doses of Ag between 5 and 100 ng/ml (n = 6–7; *, p < 0.05) in a clone (UC3) in which the ratio of Lyn unique domain to active Lyn is 3.8:1. The weaker expressing Lyn unique domain clone (UA1, 2.4:1 ratio) also showed a small, but statistically significant

**FIGURE 4.** FcεRI-induced TNF-α secretion by Lyn unique domain RBL transfectants. Cells were sensitized with anti-DNP IgE and stimulated for 2.5 h with low valency DNP2-BSA Ag. The cell supernatants were collected, and TNF-α was quantified by specific ELISA (mean ± SEM, n = 4, NS).

**FIGURE 5.** LTC4 secretion induced by Ag stimulation in Lyn unique domain transfectants. RBL transfectants were sensitized with anti-DNP IgE and stimulated with Ag; cell supernatants were collected; and LTC4 release was quantified by specific RIA. Stimulation with DNP10-HSA Ag for 45 min (A, n = 6–7) or DNP12-BSA Ag for 15 min (B, n = 3). Mean ± SEM; *, p < 0.05 (t test).
increase in LTC₄ production upon stimulation with the highest dose of DNP₃₅-HSA Ag tested (100 ng/ml). To explore this issue further, we sensitized Lyn unique clone UA1 and control cells PC5 or PC6 and stimulated with moderate valency Ag DNP₁₂-BSA to amplify any differences in Fc/H₉₂₈₀ RI-Lyn association. As represented in Fig. 5, under these conditions, Fc/H₉₂₈₀ RI-induced LTC₄ secretion was up-regulated 2- to 18-fold (n=11005). These data suggest that Fc/H₉₂₈₀ RI-associated Lyn has a negative regulatory role in signaling to eicosanoid production, which was explored in subsequent experiments.

For the mediators examined, the lower valency Ags required a higher dose of Ag to reach the same maximum release in control transfectants (Figs. 3–5). The EC₅₀ for histamine release by control transfectants was 4.7 ng/ml for DNP₃₅-BSA Ag and 37.3 ng/ml for DNP₁₂-BSA Ag, although cells stimulated with either Ag reached a similar maximum percentage of release (Fig. 3, and data not shown). The EC₅₀ for LTC₄ release by control transfectants was 22.4 ng/ml for DNP₃₅-HSA Ag and 45.2 ng/ml for DNP₁₂-BSA Ag, although cells stimulated with either Ag reached a similar maximum LTC₄ release (Fig. 5). For TNF-α secretion by control transfectant PC6, the EC₅₀ for DNP₂-BSA Ag was 501.7 ng/ml. For our subsequent experiments, we focused on signaling components that might explain the opposing effects of Lyn unique domain transfection on RBL cells, namely reduced histamine release, but increased LTC₄ production. We used DNP₁₀-BSA, DNP₁₁-BSA, or DNP₁₃-BSA Ag for these experiments because stimulation of Lyn unique domain transfectants with these Ags produced statistically significant differences in secretion of both mediators, compared with control transfectants. The concentration of Ag for the phosphorylation or calcium experiments was chosen based on Ag dose-response assays to determine the minimum Ag dose required for maximal stimulation (results not shown).

**FcεRI tyrosine phosphorylation of Lyn unique domain-transfected RBL cells**

We compared the aggregation-induced FcεRI tyrosine phosphorylation between our Lyn unique domain and control transfectants by sensitizing them with anti-DNP-specific mouse IgE and stimulating them with 250 ng/ml DNP₃₅-BSA Ag for various times. The FcεRI were immunoprecipitated with goat anti-mouse IgE,
probed with anti-PY, and then reprobed with anti-FcεRI γ. The FcεRI phosphorylation is more transient in Lyn unique domain clone UC3 than control clone PB4 (Fig. 6A, upper panel). The kinetics of FcεRI tyrosine phosphorylation for Lyn unique domain clone UC3 and control clone PB4 are summarized for five experiments in Fig. 6B, and indicate a 40–60% reduction in the phosphorylation of FcεRI β and γ subunits from the Lyn unique domain transfectant compared with the control transfectant between 1 and 5 min (*, p < 0.05; **, p < 0.005). The data from Figs. 2 and 6 are consistent with disruption of the interaction between catalytically active Lyn kinase and FcεRI β subunits by inactive Lyn unique domain, resulting in reduced FcεRI tyrosine phosphorylation.

**Kinetics of Syk tyrosine phosphorylation induced by FcεRI aggregation in Lyn unique domain RBL transfectants**

We next examined the consequences of Lyn unique domain transfection on IgE receptor-mediated tyrosine phosphorylation of Syk kinase, which is recruited to phosphorylated FcεRI γ ITAMs via its tandem SH2 domains after FcεRI aggregation (9, 13). Control and Lyn unique domain transfectants were sensitized as before with DNP-specific IgE and stimulated with 50 ng/ml DNP$_2$-BSA Ag, and the Syk protein immunoprecipitated with polyclonal anti-Syk. The anti-Syk IPs were sequentially probed with anti-PY and then monoclonal anti-Syk, as depicted in Fig. 7A. As depicted in Fig. 7B, after normalizing for recovered Syk protein, the two Lyn unique domain clones demonstrated slightly enhanced Syk activation after 1–9 min of stimulation compared with control transfectants (n = 5, NS).

**Kinetics of MAPK activation stimulated by FcεRI aggregation in Lyn unique domain RBL transfectants**

In RBL cells, secretion of newly synthesized LTC$_4$ and TNF-α, but not histamine release, requires activation of the MAPK pathway, specifically MEK and ERK-2 (29). We compared FcεRI-stimulated phosphorylation of MEK and ERK-2 between our two Lyn unique domain transfectants and a control transfectant. Cells were sensitized as before and stimulated with 20 ng/ml DNP$_1$-BSA Ag for 0–30 min. Triton X-100 lysates were Western blotted sequentially with anti-phospho-MEK (Ser217, Ser221), anti-phospho-ERK (Thr202, Tyr204), and anti-ERK-1 (which cross-reacts with anti-ERK-2), as shown in Fig. 8A. In the case of MEK, signaling initiated earlier in Lyn unique domain transfectant UC3 and, on average, a 4-fold increase in MEK activation were observed after 2 min of stimulation (Fig. 8B; *, p < 0.05). For ERK-2, an 11-fold increase in phosphorylation was noted, on average, after 2 min of stimulation in our Lyn unique domain UC3 transfectant, and ERK-2 activation was increased at all time points tested up to 15 min (Fig. 8C; *, p < 0.05). Furthermore, as shown in Fig. 8D, in addition to increased ERK-2 phosphorylation in transfectant UC3, ERK-2 phosphorylation was also increased in Lyn unique domain transfectant UA1 compared with control PC6 transfectant after 3 min of 20 ng/ml DNP$_1$BSA Ag stimulation.
may result from the observed change in MAPK pathway kinetics from a 5-min peak to a 2-min peak and the increased amplitude of ERK-2 activation. The increased ERK-2 activation would be expected to result in increased PLA2 activation and arachidonic acid release.

Kinetics of Gab2 tyrosine phosphorylation stimulated by FceRI aggregation in Lyn unique domain RBL transfectants

Tyrosine phosphorylation of the adaptor protein Gab2 appears crucial to the positive regulation of mast cell degranulation based on studies using Gab2-deficient BMMCs and Gab2-overexpressing RBL transfectants (30, 31). We explored the effect of Lyn unique domain transfection on Gab2 phosphorylation by comparing the kinetics of Ag-induced phosphorylation between Lyn unique domain clone UC3 and control transfectant PB4. Cells were sensitized with DNP-specific IgE as before and stimulated with 150 ng/ml DNP35-BSA Ag for 1–27 min, and Gab2 IP were immunoblotted with anti-PY. As shown in Fig. 9A, tyrosine phosphorylation of Gab2 was observed in unstimulated cells, peaked at 1 min, and had declined substantially by 9 min. Both the constitutive phosphorylation as well as the stimulated phosphorylation at 1 and 3 min were sharply reduced in the Lyn unique domain transfectant compared with control transfectants tested (n = 5; *p < 0.05). In a single experiment, Gab2 phosphorylation in Lyn unique clone UA1 was nearly identical with that of control clone PC6 (relative phospho-Gab2 for PC6, 2.08, and for UA1, 2.31 after 1-min stimulation). In Ag-treated Gab2−/−BMMCs, activation of PI3K is reduced and degranulation is nearly abrogated, consistent with a positive role for Gab2 in degranulation (30). Decreased phosphorylation of Gab2 is consistent with the decreased histamine release observed in DNP35BSA Ag-stimulated Lyn unique domain UC3 transfectant (Lyn unique:Lyn ratio 3.8:1). The inability to reduce Gab2 phosphorylation would predict the lack of inhibition of DNP35BSA Ag-induced histamine release observed in Lyn unique domain clone UA1 (Lyn unique:Lyn ratio 2.4:1).

Calcium release upon FceRI aggregation in Lyn unique domain-transfected RBL cells

Control (PC6, PB4) or Lyn unique domain transfectants (UA1, UC3) were sensitized with DNP-specific IgE, as before, and then loaded with the calcium-sensitive fluorescent dye fura 2. After establishing a baseline, separate samples were stimulated with 20 ng/ml DNP35-BSA Ag in the presence or absence of 2 mM EGTA to chelate 1 mM extracellular calcium. As depicted in Fig. 10A,
control transfectants treated with Ag produced an increase in cytosolic-free calcium that was sustained for at least 3.5 min, similar to that reported for untransfected RBL-2H3 cells (32). Pretreatment with EGTA indicated that the sustained phase requires an influx of extracellular calcium (Fig. 10B). By comparison, Lyn unique domain-transfected UC3 cells displayed elevated baseline calcium, and an accelerated initial phase of calcium release, but reached the same plateau of sustained influx. EGTA chelation produced a transient, but elevated increase in calcium release in these Lyn unique domain transfectants. Lower expressing Lyn unique domain transfectant UA1 gave results similar to the control PC6 cells. Therefore, we have observed an increase in the rate and amount of calcium release from the endoplasmic reticulum, but no difference in the sustained calcium channel opening phase of calcium secretion compared in Lyn unique domain UC3 vs control or UA1 transfectants.

Discussion

The integration of signals culminating in mast cell secretion subsequent to FceRI aggregation appears to be regulated both spatially and temporally (33, 34). Upon aggregation by Ag, FceRI translocate into specialized areas of the plasma membrane, so-called membrane rafts, which allows coupling with essential components of the secretory machinery and the cytoskeleton (35, 36). Investigation of the membrane topography of FceRI and its essential signaling components by electron microscopy of immunogold-labeled membrane sheets prepared from RBL-2H3 cells has demonstrated a colocalization of Lyn with 25% of the FceRI in unstimulated cells (37). We have observed the association of both endogenous Lyn and transfected Lyn unique domain with FceRI in our RBL-2H3 transfectants by chemical cross-linking (Fig. 2). Transfection of the unique domain of Lyn kinase, the domain responsible for association of the kinase with unaggregated FceRI, has now been shown to partially reduce tyrosine phosphorylation of transfected FceRI on CHO fibroblasts as well as endogenous FceRI on RBL-2H3 cells (8) (Fig. 6). The partial inhibition of FceRI tyrosine phosphorylation upon Lyn unique domain transfection thus most likely reflects a reduction in the amount of Lyn kinase constitutively associated with FceRI β due to competition with excess Lyn unique domain.

Current information indicates a dual role for Lyn in regulating activation of the tyrosine kinase Syk in mast cells and B cells, and may explain the similar Syk tyrosine phosphorylation observed in both our control and Lyn unique domain transfectants. Mutation of the negative regulatory tyrosine 317 of Syk in B cells resulted in an increase in both the amplitude and duration of the BCR-mediated increase in intracellular calcium (38). In RBL-2H3 cells, the same mutation enhanced Ag-induced histamine release (39). In contrast, the reduction in FceRI γ subunit phosphorylation upon Lyn unique domain transfection would be expected to reduce the amount of activated Syk available for signaling to secretion.

Because Lyn appears to be responsible for phosphorylating SHIP-1 and allowing it to associate with the plasma membrane and degrade phosphatidylinositol-3,4,5-trisphosphate, a reduction in activated Lyn in Lyn unique domain transfecants would predict increased phospholipase C_{γ2} activation and subsequent IP₃-mediated calcium flux (Fig. 10) (40, 41). However, the same decrease in activated Lyn has apparently led to a sharp reduction in activated Gab2, which is required for degranulation in BMMCs (Fig. 9) (30). It appears the balance of these two opposing effects has led to a partial, albeit statistically significant, reduction in histamine release in our Lyn unique domain transfecants.

More recently, a complementary role for the Src family kinase Fyn in FceRI-induced signaling to degranulation was elucidated based on the dramatic reduction in FceRI-induced histamine release detected in Fyn⁻/⁻ BMMCs (20). In BMMC, Fyn was found to associate with the FceRI β-chain and to positively regulate phosphorylation of the adaptor protein Gab2. Lyn was found to negatively regulate phosphorylation of Gab2. In contrast, our data are most consistent with a reduction in activated Lyn upon FceRI aggregation in RBL cells, resulting in...
a reduction in histamine release and, thus, consistent with a positive role for Lyn kinase in RBL mast cell degranulation. A protein-protein BLAST analysis indicated no significant homology between the 85-aa unique domain of Fyn and the 66-aa unique domain of the Lyn A kinase expressed in these studies. Lyn’s unique domain binds directly to the FceRI β subunit, while Fyn’s unique domain may associate directly or indirectly with the FceRI β-chain (8, 20). Lyn phosphorylates the FceRI β and γ ITAMs, while Fyn does not (41). These data point to distinct mechanisms of association and function in FceRI signaling for Lyn and Fyn. Also, protein levels of Fyn are 3-fold greater in BMMC than RBLs, while both cell types contain similar levels of Lyn (20, 41). These data suggest that the same Src family kinase may play a different role in a given mast cell type (BMMC or RBL), based on its level of expression, activation state, and competition with other similar kinases. Alternatively, the difference may lie in the complete absence of Lyn or Fyn in knockout BMMCs vs a reduction in Lyn kinase available to be activated in Lyn unique domain-transfected RBL cells.

Signals downstream of aggregated FceRI appear to occur as a complex network sensitive to both the presence and the amount of an activated protein or generated lipid, as well as kinetic proof-reading (42, 43). Signal transduction in the RBL-2H3 cell is in part regulated by the shuttling of limited amounts of the Lyn kinase that initiates a cascade of phosphorylation events (44). The dose response of histamine release or LTC4 secretion in Lyn unique domain RBL transfectants is similar to that of control transfectants in terms of the dose required for maximum secretion. However, the amount of histamine released at the optimal Ag dose is reduced in Lyn unique domain transfectants, which contain reduced amounts of activated Lyn and Gab2. Also, the amount of LTC4 secreted at the optimal Ag dose is heightened in Lyn unique domain transfectants, which contain reduced amounts of activated Lyn, elevated basal intracellular calcium, and heightened ERK-2 phosphorylation upon Ag stimulation. Our study therefore implicates Lyn as a negative regulator of LTC4 secretion in mast cells, impacting FceRI signaling at the level of ERK-2 activation or an earlier step. Thus, it appears that there is an insufficient amount of activated Lyn in our Lyn unique domain transfectants at optimal Ag doses to permit maximal histamine secretion or to limit LTC4 production. These studies further demonstrate that for secretion of inflammatory mediators, Lyn is limiting in FceRI signaling in RBL-2H3 mast cells.

Disclosures

The authors have no financial conflict of interest.

References