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

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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)

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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 Journal of Immunology, 2005, 175: 4543–4554.

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 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.
mast cells (BMMC). 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 passive cutaneous anaphylaxis (PCA), are IgM hyperglobulinemic, 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 Fyn in FcεRI-induced signaling to degranulation was indicated based on the dramatic reduction in FcεRI-induced histamine release detected in Fyn<−<−> BMMCs (20). In BMMCs, Lyn was found to associate with the FcεRI β-chain and to positively regulate phosphorylation of the adaptor protein Gab2. Fyn deficiency results in a reduction in ERK phosphorylation in both Ag-stimulated BMMC and CD2-stimulated T cells (20, 21). The specific roles of Lyn and Fyn in regulating FcεRI-induced mediator secretion in the RBL-2H3 cell line are still being elucidated. A recent study by Choi et al. (22) determined that the Src family kinases Fyn and Fgr positively regulate degranulation in RBL-2H3 cells, at least in part, by activating phospholipase D2. In the current study, we stably transfected the unique domain of Lyn into RBL-2H3 mast cells and examined the consequences on FcεRI-induced signal transduction 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 DNP<sub>2</sub>-BSA or DNP<sub>12</sub>-BSA, human serum albumin (HSA) Ags were purchased from Molecular Probes or Sigma-Aldrich, respectively. Polyclonal Abs to Lyn, FceRIγ, 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 (Thr<sup>202</sup>, Tyr<sup>204</sup>)-specific, and phospho-MEK (Ser<sup>217</sup>, Ser<sup>221</sup>)-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 DNP<sub>2</sub>-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, while probenecid was obtained from Sigma-Aldrich. Dithiobis(succinimidylpropionate) (DSP) and Supersignal West Pico chemiluminescent substrate were ordered from Pierce. Tris-glycine-buffered polyacrylamide gels were ordered from NOVEX/Invitrogen Life Technologies, Triton X-100 (Ultrapure grade) was obtained from Sigma-Aldrich, and a 50X protease inhibitor mixture was purchased from BD Pharmingen. The 2H3 subline of RBL cells was cultured, as described before (6), except that the culture medium was supplemented with 13.6 µg/ml gentamicin (Invitrogen Life Technologies).

Low and moderate valency Ag preparation
Lower valency Ags (DNP<sub>2</sub>-BSA, DNP<sub>12</sub>-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] × ε<sub>PY</sub>, where AMAX = absorbance at 349 nm (λ<sub>MAX</sub> 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 (AMAX<sub>PY</sub> = 0.18), and ε<sub>PY</sub> = the extinction coefficient of DNP (18,000 cm<sup>-1</sup>, M<sup>-1</sup>).

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<sup>+</sup> (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<sup>+</sup>. Several clones of each type of transfectant were expanded for further testing.

FceRI determination
Expression of FceRI was quantified in a binding assay with <sup>125</sup>I-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. Cosedimentation of <sup>125</sup>I-labeled IgE-sensitized FceRI 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 DNP<sub>2</sub>-BSA or DNP<sub>12</sub>-BSA Ag in PIPES-buffered albumin-glucose (PA BG) buffer (25 mM PIPES, 5 mM KCl, 110 mM NaCl, 0.1% BSA, and 0.1% dextrose) supplemented with 1 mM CaCl<sub>2</sub> for 45 min. Supernatants were collected for quantification of histamine by automated fluorometry (24). Spontaneous release was assessed by incubation in PA<sub>G</sub> buffer, while total cellular histamine was determined by lysis in 1.6% HClO<sub>4</sub> 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 transfected cells, cells were seeded into six-well plates (2.4 × 10<sup>6</sup> 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_{5BSA} or DNP_{10BSA} 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 trypsinization 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 histamine release. The cells were then stimulated with DNP_{5BSA} or DNP_{10BSA} 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 PA_{5G} buffer. Aliquots of cells (10 * 10⁶) 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 KCl, 0.56 mM dextrose, 0.5 mM EDTA, 1 mM Na₃VO₄, 5 mM Na₂P₂O₇, and 1.5× 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

Mammalian Two-hybrid System (Promega). The linear range of anti-Lyn Ab staining fell within the linear range of Ab staining. The same procedure was followed for all Abs used for Western blotting. We previously published a validation of this approach and have shown we can discriminate 2-fold changes in protein expression (12). For quantification of phosphorylated proteins, a fixed amount of lysate from a single preparation of Ag-stimulated RBL cells was run on each gel as a Western blot standard. For example, to quantify Syk PY, the blot of Syk IPs was probed sequentially 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 FcεRI PY, Gab2 PY, and phosphorylation of ERK-2 and MEK.

Cytosolic-free calcium measurements

Control or Lyn 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 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_{10BSA} 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 transfected samples 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 UA1 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).

Quantification of Western blot data

Quantification of anti-Lyn Western blot data was accomplished by use of a digital camera and densitometry software (Kodak Electrophoresis Documentation and Analysis System). The linear range of anti-Lyn Ab staining was determined by Western blotting 2-fold dilutions of a lysate of RBL-2H3 cells. Film exposures were then chosen such that the values used in the determination fell within the linear range of Ab staining. The same procedure was followed for all Abs used for Western blotting. We previously published a validation of this approach and have shown we can discriminate 2-fold changes in protein expression (12). For quantification of phosphorylated proteins, a fixed amount of lysate from a single preparation of Ag-stimulated RBL cells was run on each gel as a Western blot standard. For example, to quantify Syk PY, the blot of Syk IPs was probed sequentially 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 FcεRI PY, Gab2 PY, and phosphorylation of ERK-2 and MEK.

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 transfected samples were compared by a paired (two-tailed) t test.
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. Rotte 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.
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 FceRI 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 (DNP35BSA, DNP35HSA) as well as lower valency Ags (DNP12BSA, DNP10BSA, 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 DNP35BSA 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 DNP35BSA Ag (Fig. 3A; n = 2). Rel-
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; \( p < 0.05 \); 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 \( \mu M \) 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-\( \alpha \) production in Lyn unique domain RBL transfectants

The cytokine TNF-\( \alpha \) 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-\( \alpha \) 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-\( \alpha \) was quantified by specific ELISA. No difference in TNF-\( \alpha \) 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-\( \alpha \) secretion reached a maximum at 800 ng/ml DNP2BSA Ag, and therefore, we compared UC3, UA1, and PC6 at that dose. As shown in Fig. 4, secretion of TNF-\( \alpha \) 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-\( \alpha \) 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-\( \alpha \) 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 DNP35-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

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**FIGURE 4.** FcεRI-induced TNF-\( \alpha \) 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-\( \alpha \) 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 DNP35-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 FcεRI-Lyn association. As represented in Fig. 5B, under these conditions, FcεRI-induced LTC4 secretion was up-regulated 2- to 18-fold (n = 3). These data suggest that FcεRI-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 DNP35BSA Ag and 37.3 ng/ml for DNP12BSA 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 DNP12BSA 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 DNP2BSA 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 DNP10BSA, DNP11BSA, or DNP13BSA 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.

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**FIGURE 6.** Tyrosine phosphorylation (PY) of FcεRI β and γ subunits after 250 ng/ml DNP35BSA Ag stimulation in RBL Lyn unique domain transfectants. A, Anti-PY blot of FcεRI IPs with anti-FcεRI γ reprobe. B, Normalized FcεRI PY (value from FcεRI PY blot divided by value from FcεRI γ blot, mean ± SEM, n = 5); *, p < 0.05; **, p < 0.005 (t test).

**FIGURE 7.** Tyrosine phosphorylation of Syk induced by Ag in RBL Lyn unique domain transfectants. Transfectants were sensitized as before with DNP-specific IgE and stimulated with 50 ng/ml DNP13-BSA Ag. A, Anti-PY blot of Syk IPs and anti-Syk reprobe. B, Normalized Syk PY (value from anti-PY blot divided by value from anti-syk reprobe, mean ± SEM, n = 5, NS).
FIGURE 8. Ag-induced phosphorylation kinetics for MEK and ERK-2. Cells were sensitized as before and stimulated with 20 ng/ml DNP₁₂-BSA Ag. A. Representative anti-phospho-MEK, anti-phospho-ERK, and anti-ERK-1/2 Western blots of lysates (in duplicate) from Lyn unique domain (labeled L, clone UC3) and control (labeled C, clone PC6) RBL transfectants. B. Relative MEK phosphorylation (value from anti-phospho-MEK blot divided by value for anti-ERK-2 blot); *, p < 0.05. C. Relative ERK-2 phosphorylation (value from anti-phospho-ERK blot divided by value for anti-ERK-1/2 blot); *, p < 0.05. B and C, The values are the average ± SEM from 0 to 30 min for the three experiments conducted. S, Western blot standard. D. Representative (n = 3) anti-phospho-ERK and anti-ERK-1/2 Western blots of lysates from two Lyn unique domain clones (UC3, UA1) and control clone PC6 after 3 min of 20 ng/ml DNP₁₂-BSA Ag stimulation.

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

In RBL cells, secretion of newly synthesized LTC₄ 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 DNP₁₂-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 Ag stimulation. In three experiments, ERK-2 phosphorylation was increased 1.3 ± 0.3-fold (average ± SEM) in Lyn unique UA1, and 3.2 ± 0.3-fold in Lyn unique UC3, consistent with the increased LTC₄ secretion noted in both Lyn unique domain clones. The increase in FcεRI-induced LTC₄ release upon Lyn unique domain transfection...
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 FcεRI 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 BMMC 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 reduced on average by 74.1 ± 0.02% in the Lyn unique domain transfectant compared with the two 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<sup>−/−</sup>/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 DNP<sub>35</sub>BSA 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 DNP<sub>35</sub>BSA Ag-induced histamine release observed in Lyn unique domain clone UA1 (Lyn unique:Lyn ratio 2.4:1).

**Calcium release upon FcεRI 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 DNP<sub>35</sub>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 FcεRI aggregation appears to be regulated both spatially and temporally (33, 34). Upon aggregation by Ag, FcεRI 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 FcεRI 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 FcεRI in unstimulated cells (37). We have observed the association of both endogenous Lyn and transfected Lyn unique domain with FcεRI 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 FcεRI, has now been shown to partially reduce tyrosine phosphorylation of transfected FcεRI on CHO fibroblasts as well as endogenous FcεRI on RBL-2H3 cells (8) (Fig. 6). The partial inhibition of FcεRI tyrosine phosphorylation upon Lyn unique domain transfection thus most likely reflects a reduction in the amount of Lyn kinase constitutively associated with FcεRI 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 FcεRI 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 transfectants would predict increased phospholipase Cγ2 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 BMNCs (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 transfectants.

More recently, a complementary role for the Src family kinase Fyn in FcεRI-induced signaling to degranulation was elucidated based on the dramatic reduction in FcεRI-induced histamine release detected in Fyn−/− BMNCs (20). In BMMC, Fyn was found to associate with the FcεRI 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 FcεRI aggregation in RBL cells, resulting in

FIGURE 10. Change in cytosolic-free calcium in Ag-stimulated Lyn unique domain-transfected RBL cells. A, Calcium release upon 20 ng/ml DNPε10BSA Ag addition from control PC6 and Lyn unique domain UC3 and UA1 transfectants. B, Same cell lines pretreated with 2 mM EGTA and then Ag stimulated. One experiment representative of three conducted with clones PB4, PC6, UC3, and UA1.
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 Lyn and the 66-aa unique domain of the Lyn A kinase expressed in these studies. Lyn’s unique domain binds directly to the FcεRI β subunit, while Lyn’s unique domain may associate directly or indirectly with the FcεRI β-chain (8, 20). Lyn phosphorylates the FcεRI β and γ ITAMs, while Lyn does not (41). These data point to distinct mechanisms of association and function in FcεRI signaling for Lyn and Fyn. Also, protein levels of Lyn 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 FcεRI 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, elevated basal intracellular calcium, and heightened ERK-2 phosphorylation (45). The dose response of histamine release or LTC4 secretion in Lyn unique domain-transfected RBL cells and indication of autoimmune disease in FcεRI-deficient mice. Immunity 3: 549–560.

References