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Roles for SH2 and SH3 Domains in Lyn Kinase Association with Activated FcεRI in RBL Mast Cells Revealed by Patterned Surface Analysis

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Abstract

In mast cells, antigen-mediated cross-linking of IgE bound to its high affinity surface receptor, FcεRI, initiates a signaling cascade that culminates in degranulation and release of allergic mediators. Antigen-patterned surfaces, in which the antigen is deposited in micron-sized features on a silicon substrate, were used to examine the spatial relationship between clustered IgE-FcεRI complexes and Lyn, the signal-initiating tyrosine kinase. RBL mast cells expressing wild-type Lyn-EGFP showed co-redistribution of this protein with clustered IgE receptors on antigen-patterned surfaces, whereas Lyn-EGFP containing an inhibitory point mutation in its SH2 domain did not significantly accumulate with the patterned antigen, and Lyn-EGFP with an inhibitory point mutation in its SH3 domain exhibited reduced interactions. Our results using antigen-patterned surfaces and quantitative cross-correlation image analysis reveal that both the SH2 and SH3 domains contribute to interactions between Lyn kinase and cross-linked IgE receptors in stimulated mast cells.

Keywords

IgE receptors; cross-correlation analysis

Introduction

Mast cells are the primary cellular mediators of allergic reactions (Metcalf et al., 1997), and they play important roles in both innate (Marshall, 2004) and adaptive (Galli et al., 2005) immune responses. The high-affinity receptor for IgE, FcεRI, is expressed at the plasma membrane of mast cells where it binds IgE to sensitize the cells (Gould et al., 2003). Antigen-mediated cross-linking of IgE-FcεRI complexes results in receptor phosphorylation, an event that links receptor recognition of extracellular signal to the intracellular signaling cascade that culminates in degranulation and the release of allergic mediators, such as histamine (Metcalf et al., 1997; Turner and Kinet, 1999).

Intracellular signaling requires recruitment of cytosolic proteins to the plasma membrane and redistribution of membrane components relative to cross-linked IgE receptors. The first biochemically detectable step within this signaling cascade is phosphorylation of intracellular

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receptor segments by Lyn, a member of the Src kinase family (Sheets et al., 1999). Accumulating evidence supports the view that antigen-mediated cross-linking promotes stable association of clustered IgE receptors with ordered regions of the plasma membrane, often called lipid rafts, that are enriched in cholesterol, sphingomyelin, and phospholipids with saturated acyl chains (Dykstra et al., 2003; Holowka et al., 2005). Lyn is anchored to the membrane by saturated acyl chains, and active, phosphorylated Lyn is predominately associated with dynamic, ordered lipid domains in unstimulated cells. Net phosphorylation of monomeric FcεRI in unstimulated cells is prevented by transmembrane phosphatases that are more readily accommodated in regions of the membrane composed of lipids with unsaturated, disordered acyl chains. Co-localization of active Lyn with antigen-crosslinked IgE receptors in ordered lipid rafts results in FcεRI phosphorylation and consequent downstream signaling events (Young et al., 2005).

To initiate signaling, Lyn phosphorylates FcεRI β and γ subunits (Jouvin et al., 1994; Kihara and Siraganian, 1994), leading to recruitment and activation of Syk kinase (Kihara and Siraganian, 1994; Reth and Brummer, 2004; Sada et al., 2001; Shiue et al., 1995). Syk then phosphorylates several proteins, including the linker for the activation of T cells (LAT), which mediates recruitment of phospholipase C γ (PLCγ) to the plasma membrane (Saitoh et al., 2003). Upon tyrosine phosphorylation, PLCγ hydrolyzes phosphatidylinositol-4,5-bisphosphate to produce two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol (Gilfillan and Tkaczyk, 2006), leading to Ca²⁺ mobilization and protein kinase C activation, which are necessary for degranulation (Beaven and Metzger, 1993).

Src family kinases have common structure components, including an N-terminal unique domain, SH3 and SH2 domains, and a C-terminal kinase domain (Boggon and Eck, 2004; Ingley, 2008). At the N-terminus, all family members are myristoylated and most, including Lyn, are also palmitoylated (Koegl et al., 1994; Wolven et al., 1997) causing their association with the plasma membrane (Liang et al., 2001; Resh, 1996). The SH3 and SH2 domains mediate interactions with proline-rich motifs (Rickles et al., 1994; Yu et al., 1994) and with phosphotyrosine-containing sequences (Malek and Desiderio, 1993; Waksman et al., 1993), respectively. Phosphorylation of a C-terminal regulatory tyrosine by Csk maintains Src family kinases in an inactive conformation involving an intramolecular interaction between this phosphotyrosine residue and the SH2 domain. This “closed” conformation is further stabilized by an additional interaction between the SH3 domain and a proline-rich sequence between the SH2 and kinase domains (Panchamoorthy et al., 1994; Superti-Furga et al., 1993; Xu et al., 1999). Upon dephosphorylation of the regulatory C-terminal tyrosine, Src family kinases adopt an “open” conformation in which the activation loop tyrosine is phosphorylated and the kinase is fully activated (Ingley, 2008). Lyn activity is down-regulated by dephosphorylation of the active site tyrosine kinase by transmembrane phosphatases such as PTPα (Young et al., 2005).

Lyn SH2 and SH3 domains also mediate inter-protein interactions that influence signal transduction in mast cells. The SH2 domain interacts with phosphorylated FcεRI β to amplify the phosphotyrosine signaling cascade (Kihara and Siraganian, 1994), and its deletion results in inhibition of stimulated Syk phosphorylation and activation in an RBL cell reconstitution system (Honda et al., 2000). Studies of the SH3 domain showed that it also contributes to Lyn kinase activity (Abrams and Zhao, 1995). Peptides that bind competitively to this domain were found to inhibit stimulated IgE receptor phosphorylation and Ca²⁺ signaling (Stauffer et al., 1997). In contrast, deletion of this domain did not significantly alter Lyn-dependent signaling leading to Ca²⁺ mobilization (Honda et al., 2000).

Based on this previous work demonstrating functional roles for the SH2 and SH3 domains in Lyn kinase regulation and Lyn-dependent signaling, we investigated how specific mutations

of these domains affects physical association of Lyn with FcεRI during signaling. Co-localization of Lyn with cross-linked IgE receptors has been observed with electron microscopy (Wilson et al., 2000), fluorescence imaging of cells on patterned antigen surfaces (Torres et al., 2008b; Wu et al., 2004), fluorescence correlation spectroscopy (Larson et al., 2005), and cross-correlation fluorescence imaging (Das et al., 2008). To directly assess the respective roles of the SH2 and SH3 domains in mediating Lyn co-localization with FcεRI, we generated Lyn mutants in which the SH2 or SH3 domain is inactivated by a point mutation. We took advantage of antigen-patterned surfaces to cluster the IgE receptors in well defined features, which enables co-redistributing fluorescently labeled species to be reliably visualized (Torres et al., 2008a). In addition to visual inspection, we used cross-correlation analysis of Lyn co-localization with the antigen patterns to quantify the effects of these mutations on stimulated Lyn redistribution. We found that mutation of the SH2 domain blocks co-redistribution of Lyn with clustered IgE receptors, and mutation of the SH3 domain substantially reduces this antigen-dependent accumulation. Our results provide new information about binding interactions between Lyn kinase and FcεRI that relate to regulation of this receptor-mediated signaling.

Materials and Methods

Reagents

Mouse monoclonal IgE specific for 2,4-dinitrophenyl (DNP) was purified as described previously (Subramanian et al., 1996). This anti-DNP IgE in borate-buffered saline (BBS, 200 mM boric acid, 33 mM NaOH, and 160 mM NaCl, pH 8.5) was fluorescently labeled with Alexa488 as previously described (Gosse et al., 2005) and dialyzed extensively in PBS with EDTA (0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) at 4 °C. The fluorescently modified IgE had ~ 7-10 dye molecules per protein. BSA was conjugated with an average of 15 DNP groups per protein (Posner et al., 1992). DNP-BSA in BBS was fluorescently labeled with Alexa555 dye kit (Molecular Probes) at the recommended dye:protein ratio for 24 hr at room temperature (RT~ 22 °C) in the dark. Reaction mixtures were dialyzed extensively in PBS with EDTA at 4 °C. Fluorescently modified Alexa555-DNP-BSA had ~ 2 dye molecules per protein.

Lyn Constructs

The wt Lyn-EGFP construct was previously described (Hess et al., 2003). The Lyn constructs with single point mutations, Lyn-EGFP-SH2mut and Lyn-EGFP-SH3mut, were generated from the corresponding Lyn-mRFP (monomeric RFP) mutants by exchange of mRFP for EGFP at the BamHI and NotI restriction sites. Lyn-mRFP was generated by amplifying mRFP using the sense primer 5'-TTAAAGGATCCA ATGGCCTCCTCCGAGGACG-3' and the antisense primer 5'-GCGCAAACGGCCGCT TAGGCGCCGGTGGAGTGG-3' and then exchanging EGFP for the subcloned mRFP at the BamHI and NotI restriction sites in the Lyn-EGFP plasmid. The mRFP-pRSETb plasmid (Campbell et al., 2002) was a gift from Dr. Edward Cox, Princeton University. Lyn-mRFP-SH2mut was generated by the Arg→Ala mutation at position 135 using the sense primer 5'-GGGCTTTCCTGATCGCAGAAAGTGAACTTTAAAGG-3' and the antisense primer 5'-CCTTTAAAGTTTCACTTTCTGCGATCAGGAAAGCCC-3'. Lyn-mRFP-SH3mut was generated by the Trp→Ala mutation at position 78 using the sense primer 5'-GGAAGAGCACGGGGAAGCGTGGAAGCTAAGTCCC-3' and the antisense primer 5'-GGGACTTAGCTTTCCACGCTTCCCCGTGCTCTTCC-3'.

Clustering of IgE Receptors on Antigen-Patterned Surfaces

Preparation of parylene-patterned silicon wafers was described previously (Ilic and Craighead, 2000). Antigen was immobilized on these surfaces as previously described (Torres et al., 2008b). Briefly, the silicon surface, coated with parylene and patterned with the use of a

fabricated mask, was first derivatized with 2% v/v 3-(mercaptopropyl) trimethoxysilane (Sigma-Aldrich) in toluene for 20 min at RT. This surface was reacted with 2 mM N- γ -maleimidobutyryloxy succinimide ester (Sigma-Aldrich) in absolute ethanol for 1 hr at RT. Antigen was then immobilized on the surface by adding a 2:1 mixture of DNP-BSA:Alexa555-DNP-BSA (50 μ g/mL DNP-BSA:25 μ g/mL Alexa555-DNP-BSA) to the silicon chips for 2 hrs at RT. Chips were rinsed with PBS containing 2 mg/mL BSA before and after the patterned parylene layer was mechanically peeled away in solution.

Transient transfection was performed using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories). RBL cells were resuspended in cold electroporation buffer (137 mM NaCl, 2.7 mM KCl, 1 MgCl₂, 1 mg/mL glucose, 20 mM Hepes, pH 7.4) at 30×10^6 cells/mL. For each construct, ~ 10 μ g of wt Lyn-EGFP, Lyn-EGFP-SH2mut, or Lyn-EGFP-SH3mut cDNA was added to 0.3 mL of suspended RBL cells in a 4 mm cuvette, and cells were electroporated with 1 exponential pulse at 280 V and 950 μ F. Transfected cells were sensitized overnight with 0.5 μ g/mL anti-DNP IgE. Experiments were performed 24 hr after transfection.

Suspended cells at a concentration of $\sim 0.5-1 \times 10^6$ cells/mL were gently pipetted on top of the antigen-patterned silicon chip and incubated for 30 min at 37 °C. Then cells were fixed with 4% w/v paraformaldehyde for 10 min at RT and quenched with PBS containing 0.01% sodium azide and 10 mg/mL BSA for 10 min at RT. Confocal images were acquired on a Leica TCS SP2 laser scanning confocal system with a 63 $\times$, 0.9 NA, HCX APO L U-V-I water immersion objective. Images were collected sequentially to minimize bleedthrough by alternatively exciting and collecting emission from the green probe (EGFP: λ_{ex} = 488 nm, λ_{em} = 500-550 nm), and then exciting and collecting emission from the red probe (Alexa555-DNP-BSA: λ_{ex} = 543 nm, λ_{em} = 560-630 nm). These images were evaluated both by visual inspection and by cross-correlation analysis. For each of wt and mutant Lyn constructs, 150-180 transfected cells from 5-6 experiments were evaluated.

Cross-correlation analysis of Lyn construct co-localization with patterned antigen

Cross-correlation coefficients are calculated for individual cells as follows: First, the cell boundary is traced by hand to create the mask applied to paired images of cells and underlying patterned features. The mean pixel intensity is then subtracted from each masked image respectively. Auto-correlation and cross-correlation functions are defined as:

$$\text{autocorr}(\vec{r}) = \frac{\langle I(\vec{R} + \vec{r}) \cdot I(\vec{R}) \rangle}{\langle M(\vec{R} + \vec{r}) \cdot M(\vec{R}) \rangle}$$

$$\text{crosscorr}(\vec{r}) = \frac{\frac{1}{N} \langle I(\vec{R} + \vec{r}) \cdot J(\vec{R}) \rangle}{\langle M(\vec{R} + \vec{r}) \cdot M(\vec{R}) \rangle}$$

where $I(\vec{r})$ and $J(\vec{r})$ are the pixel values at position $\vec{r}$ of the masked images, $M(\vec{r})$ is the binary mask (ones on the cell and zeros off the cell), N is a normalization factor, and the averages are over all pixels (values of R). Correlation functions are divided by the autocorrelation of the mask to account for the finite nature of the image, because the autocorrelation of the mask represents that maximum autocorrelation values possible in the measurement. In practice, auto-correlation and cross-correlation values are evaluated using Fourier Transforms (FT),

$$\begin{aligned}\langle I(\vec{R} + \vec{r}) \cdot I(\vec{R}) \rangle &= FT^{-1}(|FT(I(\vec{r}))|^2) \\ \langle I(\vec{R} + \vec{r}) \cdot J(\vec{R}) \rangle &= \text{real} \{ FT^{-1}(FT(I(\vec{r})) \cdot FT(J(\vec{r}))^*) \}\end{aligned}$$

where FT^{-1} is the inverse Fourier Transform, $*$ denotes a complex conjugate, and M refers to the binary mask (on or off traced cell). Autocorrelations calculated using Fourier transforms are less computationally intensive and are mathematically equivalent to those calculated using brute force computations (Weisstein). The cross-correlation function normalization factor is defined as:

$$N = \sqrt{\langle I(\vec{R}) \cdot I(\vec{R}) \rangle \cdot \langle J(\vec{R}) \cdot J(\vec{R}) \rangle}$$

For acquired microscope images, the autocorrelation values at zero shift ($r=0$), which are used to calculate this normalization factor, contain contributions from camera or photomultiplier noise, as well as autocorrelations from the cell itself. To correct for noise, autocorrelation functions obtained from individual images are extrapolated to $r=0$. Cross-correlation coefficients are defined as the magnitude of the cross-correlation function at this zero shift. In this scheme, a cross-correlation value of 1 indicates perfect correlation of the paired images, whereas a value of -1 indicates perfect anti-correlation. Figure 1 illustrates the application of this method to a sample image.

For each of the three wt or mutant Lyn-EGFP constructs, cross-correlation coefficients were evaluated for 150-180 transfected cells. Each experimental condition resulted in a broad distribution of cross-correlation values, which are well fit by a Gaussian line-shape, centered around the most probable cross-correlation value.

Results

Lyn SH2 and SH3 domain point mutations

Our previous studies with sensitized RBL mast cells demonstrated that Lyn-EGFP co-redistributes with IgE receptors that cluster over patterns of specific antigen presented in micron-size features. To investigate the structural basis for this accumulation, we generated Lyn-EGFP constructs containing point mutations that target critical residues in Lyn's SH2 and SH3 domains. For Lyn-EGFP-SH2mut, we mutated a conserved arginine residue (R135 in LynB) to alanine within the phosphotyrosine-binding pocket (Ingley, 2008). The analogous mutation in Src (R175A in Src) eliminates phosphotyrosine binding to this domain (Shvartsman et al., 2007; Waksman et al., 1993). For Lyn-EGFP-SH3mut, we mutated a conserved tryptophan residue (W78 in LynB) to alanine in the ligand binding surface (Bauer et al., 2005; Kuga et al., 2008). The analogous mutation in Src (W118A in Src) blocks interaction between Src and PI-3-Kinase via this SH3 domain (Erpel et al., 1995; Shvartsman et al., 2007). As previously shown for Lyn-EGFP (Gosse et al., 2005), we determined that wt Lyn-EGFP, Lyn-EGFP-SH2mut, and Lyn-EGFP-SH3mut expressed in RBL cells show strong plasma membrane localization in cells on unpatterned surfaces (Figure 2).

Redistribution of Lyn on Antigen-Patterned Surfaces

Previously we used antigen presented in patterned lipid bilayers with feature dimensions of ~1-4 μm to study membrane trafficking and the reorganization of membrane associated

components that occurs at the cell-surface interface (Torres et al., 2008b; Wu et al., 2004; Wu et al., 2007). We demonstrated that for RBL cells sensitized with Alexa488-IgE, these labeled IgE receptors cluster specifically over antigen patterns on the time scale of several minutes as the cells contact the substrate, and no clustering occurs when antigen is omitted from the patterned features (Orth et al., 2003). Our subsequent experiments with transfected cells showed that micron-scale co-redistribution of Lyn-EGFP with IgE receptors occurs on patterned bilayer surfaces, becoming detectably clustered after 5-10 min at 37°C and maximally clustered after 15-20 min. No accumulation of Lyn-EGFP is detectable in the presence of cytochalasin D, which inhibits actin polymerization (Wu et al., 2004). Our present experiments used an alternative means of patterning the antigen with fluorescently labeled DNP-BSA immobilized on the silicon chips. With this method also, anti-DNP IgE bound to receptors on RBL cells accumulate over the patterned antigen, similar to that observed with patterned antigen presentation with lipid bilayers (Torres et al., 2008b). To evaluate the effects of functionally inhibitory point mutations in Lyn-EGFP on binding interactions, RBL cells transiently expressing Lyn-EGFP, Lyn-EGFP-SH2mut and Lyn-EGFP-SH3mut were incubated for 30 min with these antigen-patterned surfaces at 37°C. We found these conditions to be optimal for maximal Lyn-EGFP co-redistribution with patterned antigen, as represented in Figure 3, left panel. Visual inspection showed that Lyn-EGFP-SH3mut co-clusters to a reduced extent over antigen patterns (Figure 3, right panel), whereas Lyn-EGFP-SH2mut does not detectably co-cluster on these surfaces (Figure 3, middle panel).

As in our previous studies with patterned antigen, we assessed the tendency of the Lyn-EGFP constructs to co-redistribute with antigen-clustered receptors by scoring transfected cells that settled on surfaces and showed EGFP accumulation that matched the patterned features. By this visual assessment of 150-180 transfected cells of each type in 5-6 experiments, we found that 60% of Lyn-EGFP, 0% of Lyn-EGFP-SH2mut, and 20% of Lyn-EGFP-SH3mut clustered over antigen patterns (Figure 4A). The spatial definition of patterns provide reliability to this visual assessment, as described previously (Torres et al., 2008a). We also applied cross-correlation analysis to quantify the extent of co-clustering for the transfected cells in the same experimental samples. In this approach, a mask is used to trace the outline of adherent cells, and quantitative image analysis calculates the extent of overlapping fluorescent labels corresponding to the Lyn construct and the patterned antigen (Figure 1). A cross-correlation coefficient was determined for each transfected cell that had settled on the patterned surface. We found that each of the constructs displays a broad distribution of values corresponding to different cells, and these collectively are well fit by a Gaussian distribution with a peak (most probable) value (Figure 4B). Lyn-EGFP showed a wide range of cross-correlation coefficients, with a peak value of 0.37, compared to 1.0 for theoretically perfect overlap of the fluorescence labels. Values less than 1.0 and variation within a single population arises from fluorescence labeling of internal structures as well as typical cell-to-cell variation. Lyn-EGFP-SH2mut showed very low cross-correlation coefficients, with a peak value of 0.06. Lyn-EGFP-SH3mut showed cross-correlation coefficients intermediate to these two constructs, with a peak value of 0.14. Although the statistical basis is different for visual inspection and cross-correlation analysis, the trends in these results are consistent (Figure 4).

Discussion

Our study used surfaces that present patterned antigen to examine Lyn co-redistribution with IgE receptors (FcεRI) after these receptors are clustered to initiate transmembrane signaling. Previous studies showed that Lyn-mediated phosphorylation of FcεRI becomes maximal within 2-5 min of soluble antigen stimulation at 22°C, and this is followed by a reduction in Lyn-EGFP diffusion that temporally correlates with interactions between Lyn-EGFP and IgE receptors, as measured with fluorescence correlation spectroscopy (Larson et al., 2005). Additionally, micron-scale accumulation of Lyn-EGFP in cells stimulated on antigen-

patterned surfaces can be visualized about ten minutes after both IgE-receptor clustering and co-localized tyrosine phosphorylation is detected at earlier times in the same cells (Wu et al., 2004). Accumulation of Lyn-EGFP over antigen patterns is prevented by pretreatment with cytochalasin D, an inhibitor of actin polymerization. In contrast, this treatment enhances antigen-stimulated tyrosine phosphorylation of FcεRI (Holowka et al., 2000), further supporting the view that micron-scale accumulation of Lyn-EGFP with clustered IgE receptors can be uncoupled from its role in initial phosphorylation events. The stabilized, cytoskeleton-dependent interactions between Lyn-EGFP and IgE-receptors we observe with patterned antigen appear to be a consequence of the initial phosphorylation events that occur in ordered lipid domains in the plasma membrane (Holowka et al., 2005).

We evaluated antigen-patterned surfaces for co-redistribution of wt and mutant Lyn constructs with cross-linked IgE receptors; the same samples were assessed both by visual inspection and by cross-correlation analysis (Figure 4). The latter is a rigorous mathematical approach and yields a distribution of cross-correlation coefficients, which quantify the extent of overlapping fluorescent labels in imaged cells. The most probable cross-correlation coefficient, i.e., the peak of the distribution, can be determined for each construct. We observed ~ 60% of cells expressing wt Lyn-EGFP with visible concentration of this probe over the patterned antigen, and we determined a peak cross-correlation value of 0.37. Mutation of the SH2 domain (Lyn-EGFP-SH2mut) blocked this stimulated accumulation almost completely, such that negligible positive cells were scored, and correspondingly peak cross-correlation for Lyn-EGFP-SH2mut had a very low value of 0.06. These consistent results indicate that the SH2 domain participates in stabilized, F-actin-dependent, co-localization of Lyn with clustered IgE receptors. Mutation of the SH3 domain of Lyn substantially reduces, but does not eliminate, Lyn accumulation in the regions of patterned antigens. Only ~ 20% of cells expressing Lyn-EGFP-SH3mut exhibited co-clustering, and an intermediate peak cross-correlation value of 0.14 was determined. Evidently, the SH3 domain of Lyn contributes to, but is not essential for, its interaction with clustered IgE receptors.

Our results suggest that both the SH2 and SH3 domains play a positive role in IgE-mediated signaling by stabilizing interactions between Lyn, FcεRI, and focal adhesion proteins such as paxillin (Stauffer et al., 1997; Torres et al., 2008b). We previously observed that the saturated acyl anchor of Lyn (PM-EGFP) is sufficient to mediate its accumulation with patterned antigen in an IgE-dependent manner (Wu et al., 2004). These palmitate and myristoylate chains may facilitate Lyn's localization with cross-linked IgE receptors that preferentially partition into ordered membrane domains (Field et al., 1997), allowing the SH2 domain of recruited Lyn to interact with focal adhesion proteins in a dynamic complex. Indeed, our previous fluorescence photobleaching recovery measurements showed that, whereas clustered IgE receptors are immobilized, Lyn-EGFP and PM-EGFP show diffusive exchange, and the latter exhibits a larger mobile fraction than the former (Torres et al., 2008a; Wu et al., 2004). Lyn may mediate receptor-actin interactions by binding to the phosphorylated β subunit of FcεRI via its SH2 domain (Kihara and Siraganian, 1994) and from that position recruit other focal adhesion proteins, including paxillin (Minoguchi et al., 1994), to form an F-actin-stabilized network, similar to a focal adhesion complex.

These interactions may also negatively regulate IgE-mediated signaling, as implicated in gene knockout studies of bone marrow-derived mast cells from mice (Odom et al., 2004). Paxillin was shown participate in down-regulation of Src family kinase activity by recruiting the C-terminal Src kinase, Csk (Rathore et al., 2007). The knock-down of paxillin in RBL cells reveals evidence for both negative and positive effects of this protein: it enhances stimulated tyrosine phosphorylation of FcεRI β while reducing Ca²⁺ responses in these cells (Torres et al., 2008b). Thus, Lyn interactions with both phosphotyrosine and proline-rich sequences of adaptor proteins linked to the actin cytoskeleton may mediate both positive and negative

regulation of FcεRI signaling in these cells. Further studies will be necessary to distinguish the specific protein-protein interactions that participate in this process. In the current work, we over-expressed fluorescently labeled Lyn constructs, which may compete with endogenous Lyn for binding partners. Expression of these constructs in a Lyn-deficient background, generated by using siRNA that targets endogenous Lyn in RBL mast cells, may provide more insight into the role of these domains early in the signaling pathway.

A striking advantage of patterned substrates is their reliability for visual evaluation of co-localized fluorescent labels (Torres et al., 2008a). Our mathematical approach for quantifying the co-redistribution of Lyn constructs with patterned antigen, and thereby clustered IgE receptors, yields a distribution of cross-correlation coefficients for a large number of cells. These distributions reflect variations within the sample that likely arise from background fluorescence labeling as well as typical cell-to-cell variation. Nonetheless, significant differences in the peaks of these distributions demonstrate distinction in the capacities of these constructs to interact with clustered IgE receptors. Complementary and improving upon visual inspection, this cross-correlation approach provides an objective and quantitative method for evaluating co-localization in experiments utilizing patterned substrates.

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Figure 1. Illustration of cross-correlation methodology

A) Cell and B) pattern images are acquired in parallel. Pixel intensity, I , is displayed in the false color scale shown, where higher numbers correspond to brighter fluorescence. A mask is created from the cell image by tracing by hand the cell outline that defines a region of interest (white trace). Scale bar represents 5 μm . C) Cell and D) pattern images are prepared for further processing by applying the mask and by subtracting mean pixel values ($\langle I \rangle$) from each image. E) Radially averaged autocorrelation functions are calculated from each processed image as described in the Methods section. Autocorrelation values at $r=0$ contain additional contributions from photomultiplier (shot) noise; therefore the extrapolated values at zero shift ($r=0$, denoted with an 'x' symbols) are used to normalize cross-correlation values. F) The value

of the radially averaged cross-correlation function at zero shift ($r=0$; dotted line) is the cross-correlation coefficient. At long distances ($>1.5\mu\text{m}$), correlation function values can fall below zero, indicating that images become anti-correlated at large shifts. In these experiments, the decay of the correlation functions is a measure of patterned feature size.

Figure 2. Wild type Lyn-EGFP and constructs with point mutations in SH2 or SH3 domains localize to the plasma membranes of transfected RBL mast cells
Representative images of RBL cells expressing wt Lyn-EGFP, Lyn-EGFP-SH2mut, and Lyn-EGFP-SH3mut. Scale bar represents 5 μ m.

Figure 3. Distribution of Lyn-EGFP constructs in transfected cells adhering to surfaces with patterned antigen

Representative images of cells expressing Lyn-EGFP (left image, green), Lyn-EGFP-SH2mut (middle image, green), and Lyn-EGFP-SH3mut (right image, green) on surfaces patterned with immobilized antigen (red). Scale bars represent 5 μm.

Figure 4. Quantification of variant Lyn-EGFP distribution in transfected cells adhering to surfaces with patterned antigen

Approaches represented in (A) and (B) were carried out on the same samples: For each of wt Lyn-EGFP, Lyn-EGFP-SH2mut, and Lyn-EGFP-SH3mut, 150-180 cells from 5-6 independent experiments were evaluated. A) Visual inspection: Bar graph shows average percent of transfected cells on patterned antigen surfaces exhibiting Lyn-EGFP co-localization with patterned features. Error bars indicate the standard error for independent experiments. B) Cross-correlation analysis: histogram shows the distribution of calculated cross-correlation coefficients that quantify overlap of Lyn-EGFP constructs and patterned fluorescent antigen. The fitted Gaussian line-shapes are centered around the value corresponding to the most probable (peak) cross-correlation coefficient value for each construct. Standard errors are included to demonstrate the fit to the mean.