Inhibition of Lyn Function in Mast Cell Activation by SH3 Domain Binding Peptides†

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ABSTRACT: While Lyn tyrosine kinase has been shown to be necessary for IgE-receptor (FcεRI)-mediated mast cell activation, the mechanism of Lyn activation is not yet understood. Using a micro-electroporation technique to quantitatively introduce peptides into the cytosol of tumor mast cells, we show that proline-rich peptides that preferentially bind Src family SH3 domains block receptor-induced repetitive calcium spikes in a concentration dependent manner. The Src family member Lyn was the likely target, since a series of phage displaying derived peptides with increased Lyn SH3 domain binding specificity inhibited FcεRI-mediated calcium signaling at concentrations consistent with binding to Lyn rather than other Src-type kinases. Furthermore, SH3 binding peptides prevented the plasma membrane translocation of a fluorescently labeled Syk tandem SH2 domain, which binds to phosphorylated FcεRI, suggesting that the peptides specifically block the Lyn-mediated step by which FcεRI cross-linking leads to receptor phosphorylation. Our study suggests that the binding of proline-rich peptides, or corresponding cellular interaction partners, to Lyn SH3 domain suppresses the Lyn-mediated phosphorylatation of FcεRI and calcium signaling.

Mast cells play a key role in type I hypersensitivity by releasing mediators (e.g., histamine, prostaglandins, cytokines) that trigger an inflammatory response. The release of these agents is initiated by cross-linking of FcεRI and mediated by the activation of the tyrosine kinase Lyn. Lyn plays an indispensable role in the FcεRI-mediated allergic reaction as shown in Lyn-deficient mice, which failed to respond to antigen (Hibbs et al., 1995; Nishizumi & Yamamoto 1997). The function of Lyn in mast cell activation is to activate downstream targets by tyrosine phosphorylation of substrates and possibly by binding of its SH3[1] domain to target proteins. Tyrosine phosphorylation of the γ subunit of the FcεRI by Lyn is required for binding and activation of the tyrosine kinase Syk (Scharenberg et al., 1995). An additional tyrosine phosphorylation of the β subunit of FcεRI potentiates the activation of Syk (Lin et al., 1996). Syk activation, in turn, induces calcium signals either by inositol 1,4,5-trisphosphate (InsP3) dependent (Choi et al., 1993) or possibly by InsP3-independent mechanisms (Choi et al., 1996). Calcium signals, together with the diacylglycerol-mediated activation of protein kinase C, have been shown to be important regulators for the secretion of histamine as well as for the synthesis of prostaglandins, leukotrienes, and cytokines (Jouvin et al., 1995).

We used rat basophilic leukemia (RBL) cells, a tumor mast cell line, as a model system to understand the signaling function of Lyn SH3 domain in living cells. In other signaling molecules, SH3 domains have been proposed to be involved in the localization of proteins, the self-regulation of enzyme activity, and the formation of binding interactions between signaling molecules and effectors (Cohen et al., 1995; Pawson, 1995). Experiments with Lyn itself have shown that deletion of its SH3 domain decreases kinase activity *in vitro* (Abrams & Zhao, 1995). This result was unexpected, since mutations of the SH3 domain of the related Src tyrosine kinase were shown to increase Src kinase activity, a result that lead to the hypothesis that the Src SH3 domain, together with the Src SH2 domain, is essential for autoinhibition of Src kinase activity (Superti-Furga, 1995). If this Src model also applied for Lyn and if, in the cellular context, Lyn SH3 domain is indeed important for autoinhibition of kinase activity, the binding of proline-rich peptides to Lyn should potentiate FcεRI-mediated calcium signaling. In addition to their role in autoregulation, Lyn SH3 domain may have alternative functions which will only become evident through their study in an *in vivo* environment.

Here, we investigate the role of Lyn SH3 domain in intact cells by electroporating Lyn-specific SH3 domain binding peptides into adherent RBL cells. Lyn SH3 domain binding peptides completely blocked FcεRI-mediated receptor phosphorylation and calcium signaling, a result that was not consistent with a significant autoinhibitory role for Lyn SH3 domain. Our studies suggest that the binding of cellular interaction partners to Lyn SH3 domain, mimicked in our experiments by proline-rich peptides, suppresses Lyn kinase activity and reduces FcεRI cross-linking-induced receptor phosphorylation and calcium signaling.

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[1] Abbreviations: GST, glutathione *S*-transferase; ITAM, immunoreceptor tyrosine-based activation motif; RBL, rat basophilic leukemia cell; SH2, Src homology domain 2; SH3, Src homology domain 3.

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EXPERIMENTAL PROCEDURES

Chemicals. Fluo-3 hepta-potassium and Cy 3.5 were purchased from Molecular Probes (Eugene, OR) and Amersham (Arlington Heights, IL), respectively. Peptides Lyn 1−5 and Src 1 (Table 1) as well as the control peptide (RSAYNRLYLGAK) were synthesized and HPLC-purified by D. Klapper, University of North Carolina, Chapel Hill. Peptide Src 2 (Table 1) was synthesized and purified by Bio-Synthesis (Lewisville, TX). Peptides were biotinylated at the N-terminal end to facilitate the ELISA binding measurements.

Fluorescent Labeling. The peptide Lyn 4 was labeled with FITC (Sigma, St. Louis, MO), 10 kDa dextran (Molecular Probes) was labeled with Cy 3.5, and tandem Syk SH2 domain was labeled with Cy 2. The reactions were performed at room temperature in 80 mM NaCl, 100 mM HEPES, pH 9.3, with a 2-fold molar excess of fluorescein isothiocyanate or Cy 3.5 to peptide, protein, or dextran, respectively. The labeled compounds were separated from the unbound dye by gel filtration chromatography.

In Vitro Peptide Binding Assay. PCR fragments encoding SH3 domians of c-Src (amino acids 87−143) and Lyn (amino acids 61−126) were cloned into the *Bam*HI site of pGex-2T. pGex-derived constructs expressing GST fusion proteins containing SH3 domains of Hck, Lck, Fyn, Yes, Abl, and PLC-γ were obtained from M. Sudol (Rockefeller University) and A. M. Pendergast (Duke University). The GST−SH3 domain fusion proteins were expressed in BL21 cells and purified using a glutathione−Sepharose column. The integrity and purity of the proteins were confirmed by SDS−PAGE. Protein concentrations were determined by a BioRad protein assay (BioRad, Hercules, CA). The *in vitro* peptide binding assay (ELISA) was performed as described in Sparks et al. (1994, 1995). Briefly, 1 μg of biotinylated peptide was applied to microtiter wells coated with the different GST−SH3 domains or GST (5−20 μg). After washing with 50 mM NaH_2PO_4, pH 8.0, 340 mM NaCl, 0.1% Tween 20, and 0.1% bovine serum albumin, the bound peptides were detected by streptavidin−alkaline phosphatase based ELISA (Sigma).

Cell Culturing and Electroporation. Rat basophilic leukemia cells (2H3 type, a tumor mast cell line) were grown in Dulbecco's minimum essential medium (DMEM) with 20% fetal bovine serum (Life Technologies, Gaithersburg, MD), 1 mM L-glutamine, 200 units of penicillin, and 0.2 mg/mL streptomycin added (Sigma) and maintained in a humidified chamber at 37 °C and 5% CO_2. Cells were plated on glass coverslips at least 12 h before experiments and sensitized by exposure to 10 ng/mL DNP-specific IgE. Adherent cells were washed 2 times with an extracellular buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4, 1.5 mM $CaCl_2$, and 1.5 mM $MgCl_2$), followed by a wash with the same buffer without calcium and magnesium (electroporation buffer). A small volume field electroporation device was used for cell loading (Teruel & Meyer, 1977). Electroporation was typically of 325 V/cm applied for a 40 ms period and repeated 3 times at 40 s intervals. The applied field transiently permeabilized the cells, leading to the exchange of a small fraction of the cytosol with the applied sample volumes. The percent uptake was determined by quantitative coelectroporation of fluorescent markers. After loading, cells were washed 5 times with extracellular buffer containing 250 μM sulfinpyrizone and left for at least 1 min at 37 °C to recover. The IgE receptor (FcϵRI)-mediated pathway in RBL cells was activated by addition of 10 ng/mL DNP-BSA.

Single Cell Calcium Measurement. The calcium indicator fluo-3 was excited at 488 nm using an inverted Nikon Diaphot microscope and an Odyssey laser scanning confocal imaging system (Noran Inc., Middleton, WI). Fluorescence intensity traces from individual cells were obtained by sampling the average overall intensity of each cell at 0.5 s intervals using Image-1 software (Universal Imaging Corp., Westchester, PA). Cell viability and maximal Ca^{2+} response were determined by adding 1 μM ionomycin at the end of each recording.

Diffusion Measurement of FITC-Labeled Peptides. Fluorescein-labeled Lyn 4 peptide was introduced into cells by electroporation, and images were recorded at 16.7 ms intervals using Eye Image Calculator hardware and software (IO Industries, London, Ontario, Canada). The fluorophores were locally photobleached with a second laser (488 nm; Enterprise, Coherent Inc., Palo Alto, CA), focused to an average beam diameter of approximately 2 μm. The laser was coupled to the optical axis through the epi-fluorescence port using a dichroic mirror. Shutter-controlled pulses of 5−8 ms were used to locally bleach the fluorophore. The diffusion analysis was based on the observation that the initial increase in peak fluorescence induced by a laser pulse could fit by the two-dimensional Gaussian distribution: $F_o(x, y) = 1 - C^*\exp[-(x - x_o)^2 + (y - y_o)^2]/a_o^2]$, with x and y as the pixel values and F as the local fluorescence intensity. Sequential images recorded after the UV pulse were normalized to an averaged image that was recorded before the pulse (Figure 5A). The decreased amplitude and the increased radius (a_n) of the bleached zone in each image were fit by two-dimensional Gauss functions: $F_n(x, y) = 1 - C^*(a_o^2/a_n^2)^*\exp[-[(x - x_o)^2 + (y - y_o)^2]/a_n^2]$. A least-squares fit routine was used to determine the radius of each Gaussian peak, a_n. The diffusion coefficient was then determined from the slope of a graph of the square of the radius, a_n^2, versus time ($dy/dt = 4^*D$; with D as the diffusion coefficient; Yokoe & Meyer, 1996).

Single Cell FcϵRI Tyrosine Phosphorylation Assay Using Syk Tandem SH2 Domains. Adherent RBL cells were electroporated with Cy 2-labeled Syk tandem SH2 domains together with or without the Src 1 peptide and activated by addition of DNP-BSA. The activation was stopped after 3 min by fixation with 4% paraformaldehyde for 15 min followed by a 15 min incubation with 0.1% Triton X-100. After washing with phosphate-buffered saline, the coverslips were mounted on slides and examined by confocal laser scanning microscopy (Zeiss LSM).

RESULTS

Electroporation of SH3 Binding Peptides. The role of the Lyn SH3 domain in FcϵRI-mediated calcium signaling was investigated by the electroporation of proline-rich SH3 domain binding peptides into RBL cells. The uptake of peptides by individual cells was determined by measuring the fluorescence intensity of the coelectroporated calcium indicator fluo-3 at basal calcium levels. In dual wavelength control measurements using fluo-3- and Cy 3.5-labeled

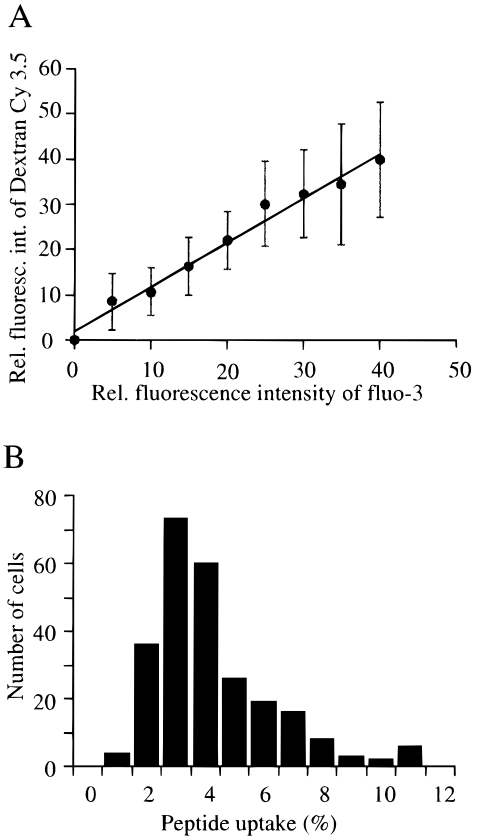

FIGURE 1: Quantification of peptide uptake by RBL-2H3 cells. (A) Control measurements show that fluo-3 fluorescence intensities at basal calcium concentration can be used to calibrate the amount of electroporated peptide. In each cell, the relative fluorescence intensity of fluo-3 (530 nm) was compared to that of the coelectroporated 10 kDa Cy 3.5 dextran (596 nm). The fitted line shows that the two fluorescence intensities increase nearly linearly with respect to each other. (B) Variability of peptide uptake by different cells. The number of cells at a given fluorescence intensity are shown in a bar diagram (loaded with fluorescein-labeled Lyn 4). The percent of extracellular added peptide that was electroporated into individual cells was determined in a confocal microscope by comparing the fluorescence intensity of a cell section to the intensity of a serial dilution of the peptide between two coverslips.

dextran, the relative fluorescence intensity of the fluo-3 signal increased linearly to that of Cy 3.5, indicating that the fluo-3 fluorescence intensity at basal calcium concentration can be used as an approximate determination for peptide uptake (Figure 1A).

The electroporation-mediated uptake of fluorescein labeled peptide (Lyn 4) was variable between cells. Figure 1B shows a histogram of the number of cells versus the cellular fluorescent intensity. The percentage of uptake was calculated by comparing the fluorescence intensity measured confocally inside individual cells to that of a dilution series of the same fluorescein-labeled peptide between two coverslips. The medium intracellular concentration of peptide in individual cells was 3%. Thus, cells electroporated with extracellular peptide concentrations of 1.5−50 mM yielded typical intracellular peptide concentrations of 50 μM to 1.5 mM.

Src Family Specific SH3 Binding Peptides Inhibit FcεRI-Mediated Calcium Signaling. Src family specific SH3 domain binding peptides with highest affinity for these SH3 domains were synthesized according to sequences previously derived from phage display analysis. These peptides were chosen to test the function of Lyn SH3 domain in intact cells, since they showed higher relative specificity between Lyn and other Src-type kinases than peptides modeled after existing proline-rich sequences. The peptide Src 1 (SGSGVLKRPLPILPVTR) was synthesized using a sequence by Rickles et al. (1995) and the peptide Src 2 (RWLSSRPLPPLPLPPRT) using a sequence by Sparks et al. (1994). The core Src binding motif which mediates high-affinity binding is underlined in Table 1. We determined the relative specificity of the two peptides for the SH3 domain of different Src-type kinases (Lyn, Hck, Fyn, Lck, Yes, and Src) as well as for two SH3 domains of molecules present in RBL-cells, e.g., PLC-γ and Abl (Figure 2A,B). Both peptides were quite selective for Src family SH3 domains and showed minimal binding interactions with Abl and PLC-γ SH3 domains. The peptides did not markedly distinguish between different Src-type SH3 domains. The observed residual binding of Src 1 and Src 2 peptides to Abl and PLC-γ was not distinguishable from background GST binding.

Table 1: Table of the Relative Peptide Concentrations Needed for Half-Maximal Inhibition of Calcium Signaling and Binding to Lyn and Src SH3 Domains

Peptide	Sequence [a]	*Rel. in vivo* I_{50} [b]	*Rel. in vitro* affinity Lyn [c]	*Rel. in vitro* affinity Src
Src 1	SGSGVLK<u>RPLPILP</u>VTR	1.00	1.0	1.0
Src 2	RWLSS<u>RPLPPLP</u>LPPRT	0.83	N.D.	N.D.
Lyn 1	GSGSYDTLALPSLPLHPMSS	0.22	~0.2	<0.01
Lyn 2	GSGSYDTRALPSLPLHPMSS	0.28	~0.2	~0.07
Lyn 3	GSGSSGVTMYPKLPPHWSMA	0.27	~0.2	<0.01
Lyn 4	GSGSSGVRMYPKLPPHWSMA	0.19	~0.2	<0.01
Lyn 5	GSGSSSMRMVPTIPGSAKHG	0.20	< 0.2	< 0.1

[a] Sequences were derived by screening phage display libraries. Peptides Src 1 and Src 2 had a high binding affinity for Src-type tyrosine kinases (Figure 2). Peptides Lyn 1 to Lyn 5 were selected for their binding specificity for Lyn over other Src-type tyrosine kinases (Figure 4A). [b] Peptide concentrations for half-maximal inhibition of calcium signaling were determined for all peptides from the inhibition curves shown in Figures 3B and 4B. The relative potency for the *in vivo* inhibition of calcium signaling by each of the peptides was normalized to Src 1 peptide which inhibited calcium signaling at the lowest concentration (~200 μM). [c] The relative *in vitro* binding affinity of the peptides for Lyn and Src SH3 domains was estimated from an ELISA binding assay using recombinant Lyn SH3 domain (the relative values for Src binding were derived from the data in Figure 4A). The approximate binding affinity of Src 1 peptide to Lyn and Src SH3 domains was 2−5 μM as estimated from this assay. While the relative potency of the peptides is similar *in vivo* and *in vitro*, peptide inhibition of calcium signaling required significantly higher peptide concentrations than SH3 domain binding *in vitro*.

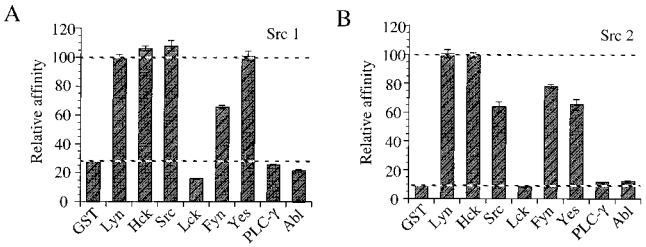

FIGURE 2: Characterization of the specificity of Src family SH3 binding peptides for different SH3 domains. (A) The relative binding affinities of biotinylated Src 1 were compared for different Src family kinase SH3 domains (Lyn, Hck, Src, Lck, Fyn, and Yes) and for two control SH3 domains (PLC-γ and Abl). Binding measurements were performed using an ELISA assay with immobilized recombinant GST−SH3 domains. (B) The same relative binding affinity measurements were performed for biotinylated Src 2 peptide.

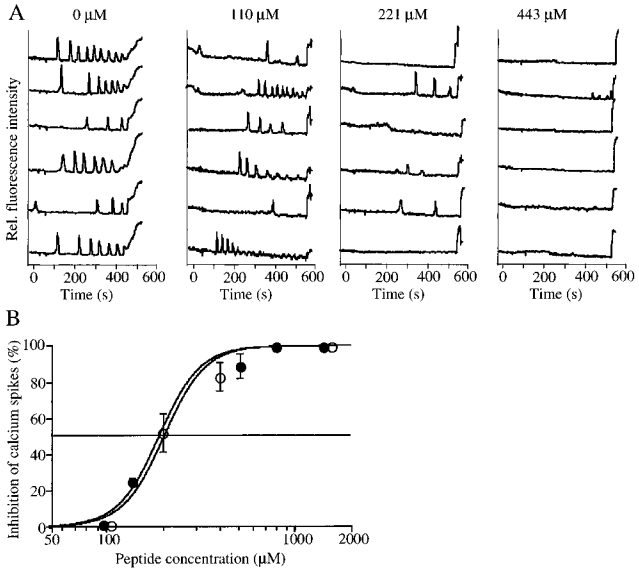

FIGURE 3: Effect of Src family specific SH3 binding peptides on FcεRI-mediated calcium signaling. RBL cells were coelectroporated with fluo-3 and peptides Src 1 or Src 2. Intracellular calcium spikes were recorded as a function of time after cross-linking of FcεRI by DNP-BSA. (A) Time course of calcium responses after FcεRI activation in the presence of increasing concentrations of Src 2 peptide inside the cell. Inhibition of calcium activation by FcεRI could be observed above an intracellular concentration of Src 2 of 100 μM. (B) Concentration-dependent inhibition of calcium spiking by Src 1 and Src 2 peptides. Cells were counted as responders when one or more calcium spikes were triggered by the antigen. Suppression of calcium signaling in a particular cell was defined when the relative increase in fluorescence intensity was smaller than 30% over base line. At least 3 experiments with 3 to 12 cells each were performed to calculate the degree of inhibition at a given range of intracellular peptide concentrations.

We determined whether the presence of Src 1 peptide potentiates or suppresses FcεRI-mediated calcium signaling. In the absence of peptide, low doses of antigen lead to the triggering of repetitive calcium transients in these cells (calcium spikes) (Figure 3A, 0 μM panel). In the presence of 110, 221, and 443 μM Src 1 peptide, antigen-mediated calcium spikes became progressively smaller in number and were nearly absent at peptide concentrations above 400 μM. A control peptide with a random sequence (RSAYNRLYLGAK) did not inhibit calcium signaling even at a concentration of 2 mM (data not shown).

In order to quantify the inhibition of calcium signaling, the percentage of cells that triggered calcium spikes was determined at different concentrations of Src 1 and Src 2. In this analysis, cells were counted as positive for calcium activity when one or more individual calcium spikes showed a relative fluorescence increase above baseline of >30%. The results of at least 3 experiments with 3−12 cells each were used to calculate the degree of inhibition at a given intracellular peptide concentration. Half-maximal block of calcium signaling was observed at ~200 μM for both Src 1 and Src 2 peptide (Figure 3B).

The relative *in vitro* specificity of the Src 1 and Src 2 peptides for Src family SH3 domains suggests that a Src-type kinase such as Lyn and not PLC-γ or other SH3 containing proteins is affected by the peptides. It should also be noted that Lyn and Src are the only Src-type family tyrosine kinases present in RBL cells whereas the latter is only present in small amounts (Eiseman & Bolen, 1992). Furthermore, Lyn and PLC-γ are thought to be the only known enzymes in the pathway between receptor activation and calcium signaling that contain SH3 domains.

SH3 Binding Peptides with Higher Lyn Specificity. To more conclusively identify the functional targets of the proline-rich peptides Src 1 and Src 2, peptides that were selective for Lyn SH3 domain were synthesized. The sequences for these peptides were obtained in the phage display approach using XΨPXXPXΨ as the core sequence and the Lyn SH3 domain for affinity selection. Hydrophobic amino acids are denoted with Ψ. The majority of the isolated phage expressed the motif RPLPPLP in the displayed peptide similar to Src 1 and Src 2. However, a small number of recovered Lyn SH3 binding phage (i.e., ~5%) had variations from this general Src binding motif and showed more restricted Lyn SH3 domain binding. Particularly, the peptides used in this study had the sequences YDTLALPSLPLHPMSS (Lyn 1), YDTLARPSLPLHPMSS (Lyn 2), SGVTMPKLPPHWSMA (Lyn 3), SGVTRPKLPPHWSMA (Lyn 4), and SSMRMVPTIPGSAKHG (Lyn 5). A linker sequence was added at the N-terminus of each of the peptides (GSGS) for biotinylation. Lyn 2 only differed from Lyn 1 by a Leu to Arg replacement, restoring a crucial amino acid for Src kinase SH3 domain recognition. The Lyn specificity of Lyn 5 was minimal although the peptide significantly deviated from the core Src binding sequence.

Figure 4A shows the relative specificities of these five peptides (Lyn 1 to Lyn 5) for SH3 domains from Lyn and other Src-type kinases as well as for PLC-γ and Abl. A markedly higher specificity was observed for the binding of Lyn 1 to Lyn 4 peptide to SH3 domains from Lyn and its closest sequence homologue Hck. No measurable binding of the peptides was observed for the SH3 domain of Abl, PLC-γ, Src, and other Src-type tyrosine kinases beside Hck and Lyn. The background binding to these SH3 domains was equal to that to the fusion tag GST itself. While these variant peptides showed a high relative specificity for Lyn over other Src family SH3 domains, their binding affinity for Lyn was lower than that of the Src peptides (Table 1). Nevertheless, the high specificity of these peptides for Lyn SH3 domains can be used to show more conclusively whether the previously observed inhibition of calcium signaling by the Src-type peptides is indeed mediated by their binding to Lyn.

Specificity of Inhibition of Calcium Signaling. We evaluated the relative specificity by which Lyn 1 to Lyn 5 inhibit calcium signaling by using the same analysis described above for the Src family binding peptides Src 1 and Src 2. All five Lyn SH3 binding peptides were similarly effective in inhibiting FcεRI-mediated calcium increases (Figure 4B) with an IC_{50} for inhibition that ranged from about 700 to 1000 μM. The relative half-maximal concentrations for inhibition of each of the peptides are listed in Table 1.

Interestingly, the relative concentration of peptide needed for half-maximal inhibition of calcium signaling closely matched that of their binding to Lyn SH3 domains but was not consistent with binding to the SH3 domain from Src (Table 1), to SH3 domains from other Src-type tyrosine kinases, or to SH3 domains from PLC-γ and Abl (see Figure 4A). However, we cannot completely exclude the binding of the peptides to Hck and other SH3 domains not tested in our *in vitro* assay. Since Hck is not present in RBL cells (Eiseman & Bolen, 1992), this suggests that the *in vivo* target of the peptides is Lyn and not another type of SH3 domain.

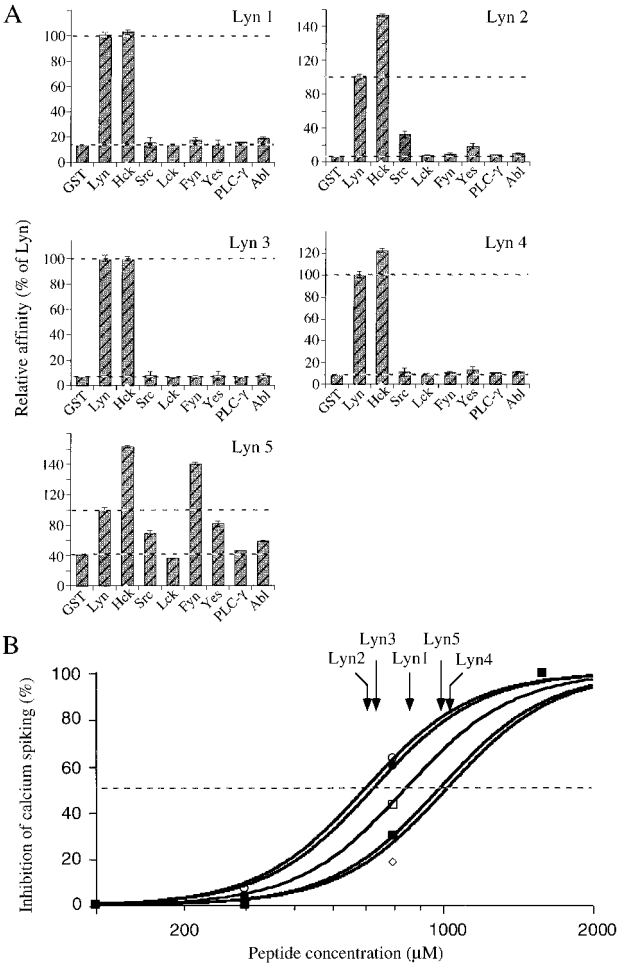

FIGURE 4: Identification of Lyn as the peptide target by using Lyn SH3 binding peptides with different specificities. (A) The relative binding affinities of Lyn 1 to Lyn 5 were compared for different Src family kinase SH3 domains (Lyn, Hck, Src, Lck, Fyn, and Yes) and for two control SH3 domains (PLC-γ and Abl). Binding measurements were performed using an ELISA assay. Lyn 1 to Lyn 4 showed significant binding to Lyn and Hck and almost no binding to SH3 domains from other Src-type kinases or PLC-γ or Abl. (B) Effectiveness of Lyn 1 to Lyn 5 peptides in blocking FcϵRI-mediated calcium signaling. (●) Lyn 1; (○) Lyn 2; (◇) Lyn 3; (□) Lyn 4; (▲) Lyn 5; (■). Approximate half-maximal inhibition was at 850 μM (Lyn 1), 700 μM (Lyn 2), 730 μM (Lyn 3), 1030 μM (Lyn 4), and 980 μM (Lyn 5).

It should be noted that the inhibition of calcium signaling by Lyn binding peptides required significantly higher concentrations (200 μM−1 mM) than binding to Lyn SH3 domains (3−20 μM). A first reason for this discrepancy is that a large fraction of Lyn may need to bind inhibitory peptide in order to fully suppress FcϵRI-induced calcium signaling. A second reason will be investigated below by using a diffusion analysis to determine whether the peptides are freely diffusible or are nonspecifically sequestered in the cytosol.

Mobility and Lifetime of SH3 Binding Peptide in the Cytosol. Since proline-rich sequences could exhibit significant unspecific binding interactions, the actual free peptide concentration in the cytosol may actually be lower than the total peptide concentration. We measured the cytosolic mobility of Lyn 4 as an indirect means to assess nonspecific binding. A relative mobility can be determined from the measurement of the peptide's mobility coefficient through the fluorescence recovery method. Figure 5A shows an

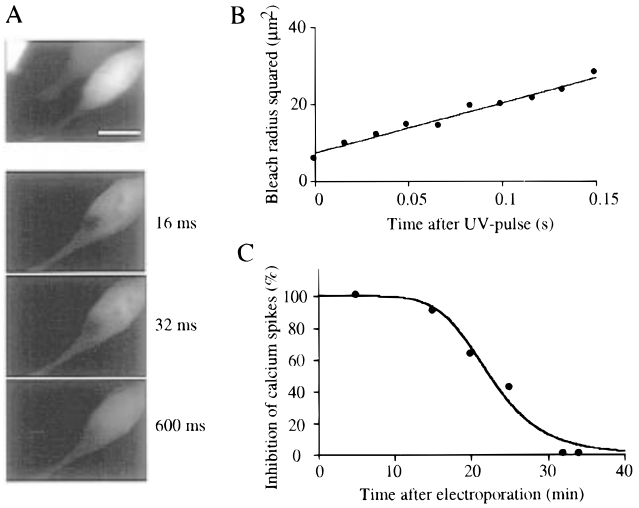

FIGURE 5: Cytosolic mobility and degradation of SH3 binding peptides. Measurement of the diffusion coefficient of FITC-labeled Lyn 4. The fluorophore coupled to peptide Src 1 was locally photobleached using a 5−8 ms pulse of a focused laser (488 nm). The average beam diameter in the image section was ~2 μm. (A) Image of RBL cell loaded with FITC-labeled Src 1 domain before photobleaching (top panel). Sequential images (ratioed to an averaged image before the laser pulse) are shown at different time points after the pulse (lower panels). The calibration bar corresponds to 10 μm. (B) Measurement of the diffusion coefficients of SH3 binding peptide. The profile of the bleached area was fit by a two-dimensional Gaussian distribution in each image. The square of the peak radius was graphed as a function of time. The slope in the graph is proportional to the diffusion coefficient ($dy/dt = 4D$). (C) Degradation time of Src 1 peptide in RBL cells. Inhibitory peptide Src 2 was introduced into RBL cells by electroporation (internal concentration ~1.5 mM). The FcϵRI-mediated pathway was activated at different time points after electroporation, and the percentage inhibition was measured as a function of the delay time between electroporation and antigen addition. Inhibition of the calcium pathway was transient, suggesting that this peptide is degraded on a time scale faster than 35 min.

example of such peptide diffusion measurements. In our analysis, the recovery from photobleaching was analyzed by a fit of a Gaussian intensity profile through each of a series of sequential images (Yokoe & Meyer, 1996). The diffusion coefficient (D) was then determined in a plot of the square of the peak radius as a function of time (Figure 5B). D for the SH3 binding peptide Lyn 4 was 20 $\mu m^2/s$, approximately 4 times slower than expected if a molecule of that size would freely diffuse in the cytosol (Yokoe & Meyer, 1996). This suggests that the actual free concentration of peptide in the cytosol is markedly lower than the total peptide concentration. This could contribute to the observation that higher SH3 binding peptide concentrations are needed for blocking signaling functions *in vivo*.

We also observed that the ability of the SH3 binding peptide to inhibit calcium signaling was lost as a function of time. To measure the time course of the presumed peptide degradation, the FcϵRI-mediated pathway was activated at different time points after electroporation of inhibitory peptide (Src 1). While near-maximal inhibition was observed for the first 10 min, cells showed normal calcium signaling 35 min after the introduction of Src 1 (Figure 5C). Half-maximal inhibition was observed at 25 min. It should be noted that the electroporation itself did not affect the signaling pathway since FcϵRI-induced calcium signaling could be measured immediately after electroporation in the presence of high concentrations of a control peptide (RSAYN-

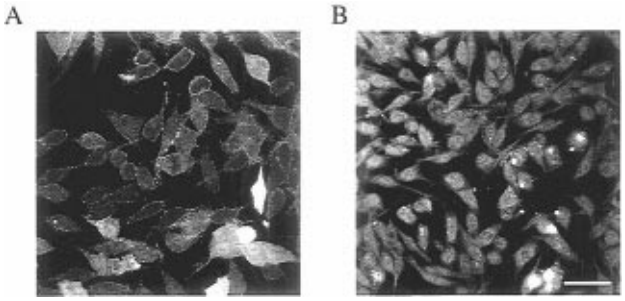

FIGURE 6: Lyn SH3 binding peptides prevent the phosphorylation of FcεRI receptors on ITAM motifs. Phosphorylation of FcεRI on ITAM motifs was measured indirectly by monitoring the plasma membrane translocation of fluorescently labeled Syk tandem SH2 domains. (A) RBL cells 3 min after FcεRI activation with BSA-DNP. FcεRI activation lead to the translocation of the Syk tandem SH2 domain to the plasma membrane. Cells were fixed 3 min after activation, and images were recorded by confocal microscopy. The calibration bar is 10 μm. (B) RBL cells loaded with Src 1 peptide 3 min after FcεRI activation with BSA-DNP. The presence of the peptide prevented the plasma membrane translocation of the Syk tandem SH2 domain.

RLYLGAK). Thus, the best explanation for the loss of inhibition is that the peptides are rapidly degraded within 35 min. These measurements may also be relevant for peptide studies in other cell systems since they are to our knowledge the first measurement of peptide stability and degradation in intact mammalian cells.

Plasma Membrane Translocation of a Fluorescent-Labeled Syk Tandem SH2 Domain Is Inhibited in the Presence of the SH3 Binding Peptides. We determined more directly whether the Lyn SH3 domain binding peptides prevent Lyn-mediated tyrosine phosphorylation of the FcεRI. Lyn is known to phosphorylate the β and γ subunits of the FcεRI at ITAM motifs. The tyrosine-phosphorylated ITAM motifs in the γ subunits are specifically recognized by the tandem Syk SH2 domain (Cambier, 1995; Jouvin et al., 1995). We found that this ITAM binding interaction can be observed in single cells by monitoring the translocation of a fluorescently labeled tandem Syk SH2 domain to the phosphorylated FcεRI receptor after antigen activation (T. Stauffer unpublished results). This translocation event was used to determine the phosphorylation state of the ITAM's in individual cells.

Fluorescently labeled tandem Syk SH2 domains were introduced into RBL cells by electroporation. Activation of the cells resulted in a markedly increased fluorescent staining of the plasma membrane, presumably due to the binding of the labeled SH2 domains to the tyrosine-phosphorylated ITAM motif of the FcεRI (Figure 6A). Strikingly, the introduction of the Lyn SH3 binding peptide Src 1, but not a control peptide (see Chemicals under Experimental Procedures), abolished the membrane localization of the tandem Syk SH2 domain (Figure 6B). The smaller surface area of the cells in the presence of Lyn SH3 domain binding peptide (Figure 6B) is likely a result of inhibition of FcεRI-mediated cell spreading (Pfeiffer et al., 1985). These results suggest that Lyn SH3 binding peptides block calcium signaling by suppressing the Lyn-mediated phosphorylation of FcεRI on ITAM motifs. Furthermore, these measurements strengthen the confidence that Lyn is indeed the functional target of the Lyn SH3 domain binding peptides.

DISCUSSION

This study has explored the *in vivo* function of the Lyn SH3 domain in antigen-mediated mast cell activation using a novel approach based on quantitative intracellular titration of Src and Lyn SH3 domain binding peptides. Rather than potentiating the FcεRI-mediated calcium response, which was expected for an autoinhibitory role of the Lyn SH3 domain, the introduction of Lyn SH3 domain binding peptides suppressed Lyn-mediated calcium signaling. The specificity of the peptides for Lyn SH3 domain was evaluated by comparing the *in vitro* specificity of a series of SH3 binding peptides to the relative concentration needed for *in vivo* functional inhibition. Further evidence for the specificity for Lyn was obtained by the finding that these peptides prevent the phosphorylation of FcεRI, a signaling step that is thought to be mediated by Lyn directly.

The *in vivo* properties of the peptides were analyzed by diffusion and degradation measurements. The diffusion coefficient of 20 $\mu m^2/s$ suggests that as much as 25% of the peptide is free in solution, and a lifetime of approximately 35 min for the peptide guarantees that measurements can be performed before a significant amount of peptide is degraded. The nearly 50-fold difference in the half-maximal concentration required for *in vitro* binding and functional inhibition can be attributed in part to the nonspecific sequestration of peptides. Since we count cells that trigger only a single calcium spike as positive, it is likely that also a significant fraction of Lyn has to be bound for complete inhibition of calcium signaling. This further increases the required peptide concentration in the cytosol necessary for functional inhibition. Our results suggest that micro-electroporation of combinatorial peptides is a generally useful method to understand the *in vivo* function of signaling domains for selected signal transduction pathways. The suppression or activation of targeted pathways by peptide binding to specific domains can also provide valuable information to identify target domains for the rational design of drugs.

The Lyn SH3 domains can bind several signaling molecules including PI3-kinase, Shc, K-protein, PLC-γ2, GAP, and others (Pleiman et al., 1993; Weng et al., 1994). Indeed, these targets contain proline-rich sequences which show some similarity to the sequences of the peptides obtained by phage display analysis (Sparks et al., 1996). Nevertheless, no evidence for an increased sequence similarity of these potential targets was observed when compared either to the sequences of highly selective Lyn SH3 binding peptides (Lyn 1 to 5) or to less selective peptides (Src 1 and 2). This may be due to the promiscuity of some of the proline-rich binding regions and SH3 domains which may mediate binding interactions between a variety of signaling molecules. Our study focused on the subset of phage display derived SH3 domain binding peptides with high specificity for Lyn SH3 domains. Although the sequence of these Lyn-specific peptides could not be used to identify potential Lyn SH3 binding partners, they allowed us to study the function of the Lyn SH3 domain more specifically in its native environment.

SH3 domains have three proposed functions: (1) the autoregulation of enzyme activity; (2) the localization of proteins; and (3) the formation of binding interactions between signaling molecules and effectors (Cohen et al., 1995; Pawson, 1995). What insights can be gained from

these measurements about the role of Lyn SH3 domains in FcϵRI-receptor activation? The mechanism of activation of Lyn by receptor cross-linking is not yet understood. It is conceivable that activation may occur similar to Src. Dephosphorylation of residue Tyr 507 of Lyn may prevent the autoinhibitory binding to the Lyn SH2 domain and then release the interaction of a linker region between the SH2 domain catalytic region with the SH3 domain (Xu et al., 1997; Sicheri et al., 1997). Since no potentiation of calcium signaling was observed, a role for Lyn SH3 domain in this process is not likely. Additionally, immunocytochemistry studies showed that Lyn remained plasma membrane localized in the presence of a high concentration of Src 1 peptide (T. Stauffer, unpublished results). A role for the SH3 domain in localizing Lyn to the plasma membrane is therefore also not supported by our data.

Overall, our result is consistent with the hypothesis that the Lyn SH3 domain provides a binding interaction that associates Lyn with the β subunit of the FcϵRI. Such a direct binding of Lyn to the β subunit of the FcϵRI has been shown previously (Yamashita et al., 1994; Pribluda et al., 1994) and may result from an interaction between a proline-rich sequence that is present in the N-terminus of the β subunit and the Lyn SH3 domain. In a second step, the localization of Lyn to FcϵRI and the Lyn-mediated phosphorylation of FcϵRI could be blocked by the binding of the Lyn SH3 domain to other signaling molecules (PI 3-kinase and others) with proline-rich sequences. Thus, the inhibitory effect of Lyn SH3 domain binding peptides observed in our studies may mimic the potential down-regulation of the Lyn-mediated phosphorylation of FcϵRI and calcium signaling by an interaction between the Lyn SH3 domain and alternative signaling partners. In antigen-stimulated mast cells, high-amplitude calcium spikes are usually only observed for a time period of minutes (Millard et al., 1988) which might suggest that binding of interaction partners to the Lyn SH3 domain is a potential mechanism for down-regulation of tyrosine kinase activity. Thus, Lyn SH3 domains may have two potential roles: (1) to colocalize Lyn with the FcϵRI receptor to facilitate FcϵRI phosphorylation; and (2) to down-regulate the calcium signaling pathway by mediating a delayed inhibition of Lyn-mediated FcϵRI phosphorylation.

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