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Syk and Lyn mediate distinct Syk phosphorylation events in FcεRI-signal transduction: Implications for regulation of IgE-mediated degranulation

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ABSTRACT

Spleen tyrosine kinase (Syk) is a key regulatory factor in the IgE-mediated allergic signal transduction pathway in mast cells and basophils. Syk is phosphorylated on a number of tyrosines following the binding of IgE/allergen complexes to FcεRI receptors leading to initiation of inflammatory signaling via downstream enzymes and scaffolding proteins. We examined the kinases responsible for the phosphorylation of key Syk tyrosines in rat RBL-2H3 basophilic cells and bone marrow-derived mast cells (BMMCs). The phosphorylation of Syk tyrosine 346 was completely blocked by the novel Src family kinase inhibitor BIRA766, suggesting this tyrosine is a pure substrate for Src family kinases. This was supported by the findings that kinase-dead (KD) Syk was efficiently phosphorylated on this tyrosine and that a specific Syk inhibitor BAY61-3606 was without effect. The phosphorylation of other Syk tyrosines 317, 342, 519 and 520 was reduced by Syk and Src family inhibitors, suggesting a role for auto- and trans-phosphorylation. Lyn was the predominant Src family kinase expressed and activated in RBL-2H3 cells, meanwhile Lyn knockdown with a specific siRNA interfered with the phosphorylation of all Syk tyrosines and the Syk substrates SLP-76 and LAT. Pharmacological inhibition of Syk completely blocked the degranulation of RBL-2H3 and BMMCs. However, Lyn knockdown sensitized RBL-2H3 cells to FcεRI-induced degranulation. We showed that whilst interference with Lyn expression disrupts FcεRI proximal signaling via Syk and its direct substrates including SLP-76 and LAT, distal activation of downstream proteins including Erk is enhanced. This study identifies the responsible kinases for the phosphorylation of key Syk tyrosines and the propagation of FcεRI receptor mediated signal transduction in allergic responses.

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1. Introduction

Tyrosine phosphorylation events downstream of the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils are crucial for allergic responses including degranulation and cytokine production (Rivera and Olivera, 2008). The FcεRI receptor is a tetrameric complex of subunits which binds IgE/allergen and initiates intracellular inflammatory signaling. The FcεRI receptor itself is without enzymatic activity and signaling is dependent on Spleen tyrosine kinase (Syk). Syk becomes phosphorylated following binding to the immunoreceptor tyrosine-based activation motifs (ITAMs) of the FcεRI β and γ chains. Phosphorylated Syk tyrosines then act as docking sites for adaptor molecules including LAT, SLP-76 and Vav, which are then phosphorylated by Syk

to propagate downstream signaling (Wong et al., 2004). The absolute requirement for Syk in FcεRI signaling has been ascertained in a number of complementary studies. For example, Syk-deficient mast cells fail to degranulate or produce inflammatory cytokines or leukotrienes in response to FcεRI crosslinking (Costello et al., 1996). These findings implicate Syk as a master regulatory kinase in the propagation of allergic signaling.

Specific phosphorylated Syk tyrosines mediate either positive or negative regulatory roles in FcεRI signaling. For example, Syk Y³¹⁷F expressing RBL-2H3 basophilic cells display increased degranulation and phosphorylation of PLCγ, suggesting a negative regulatory role for this tyrosine (Sada et al., 2000). In contrast, expression of Y³⁴²F or Y⁵¹⁹F/Y⁵²⁰F Syk leads to decreased degranulation (Zhang et al., 2002; Zhang et al., 1998). The identity of the responsible kinases for phosphorylation of each of these Syk tyrosines following FcεRI activation has not been extensively investigated. In one report it was shown that kinase-dead Syk displayed less than 10% phosphorylation of tyrosines 519 and 520 in comparison to WT Syk (Zhang et al., 2000). This suggests that these two tyrosines may be predominantly auto-phosphorylated. However, knockout of the Src family kinases, Lyn or Fyn, in mouse mast cells also decreases the

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total phosphorylation of Syk suggesting that other Syk tyrosines are substrates for trans-phosphorylation (Parravicini et al., 2002; Yu et al., 2006). The identity of the individual Src family kinases that trans-phosphorylate Syk during FcεRI signaling is also complicated by their differential expression and activation patterns in different sources of mast cells and basophils. For example, bone marrow-derived mast cells (BMMCs) display high expression of Lyn and Fyn, which become phosphorylated following FcεRI activation (Parravicini et al., 2002), however, the mouse PT18 mast cell line shows only expression and activation of a single Src family kinase Yes (Eiseman and Bolen, 1992). The rat basophilic RBL-2H3 cell line has previously been postulated to express only very low levels of Fyn (Blank and Rivera, 2004), meanwhile Fyn can be readily detected in human basophils (Gomez et al., 2007).

Considering the different functional roles of Syk tyrosines in the transmission of signals via the FcεRI receptor, we investigated the responsible kinases for phosphorylation of each residue. We show that the phosphorylation of Syk tyrosine 346 is entirely dependent on the activity of the Src family member Lyn. The phosphorylation of other Syk tyrosines 317, 342, 519 and 520 was regulated by both auto-phosphorylation and Lyn. Whilst Syk inhibition completely blocked all aspects of FcεRI receptor signaling and degranulation, interference with Lyn expression led to increased degranulation. This was accompanied by increased phosphorylation of Erk and other tyrosine phosphorylated proteins distal to the FcεRI receptor.

2. Materials and methods

2.1. Reagents

The Syk (SYK-01, 4D10 and N19), β-actin (C4), Fgr, Lyn, Fyn and Yes antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Phospho-Syk (Tyr352), Phospho-Syk (Tyr525/Tyr526), Src and Phospho-Erk (Thr202/Tyr204) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The SLP-76 (Tyr128), Phospho-Syk (Tyr348), High Affinity IgE Receptor (FcεRI) (BC4) and PE-labeled anti-CD117 antibodies were from BD Biosciences (San Jose, CA). The FITC-labeled FcεRIα antibody was obtained from eBioscience (Frankfurt, Germany). The Phospho-LAT (Tyr226), phosphotyrosine (4G10) and Phospho-Syk (Tyr323) antibodies were purchased from Millipore (Billerica, MA). The anti-DNP IgE mAb was produced from the TIB142 hybridoma cell line (ATCC) and the polyclonal rabbit anti-human IgE (A0094) was from DAKO. Recombinant IL-3 was from R&D Systems (Wiesbaden-Nordenstadt, Germany). Bovine Serum Albumin-2,4-dinitrophenyl (DNP-BSA) was purchased from Invitrogen (Karlsruhe, Germany). PP2 was purchased from Merck (Darmstadt, Germany). The siRNAs to rat Lyn (J-093726-07, GAGAUGCAACGUCCAAUAA) and GAPDH were purchased from Thermo Scientific (Waltham, MA).

2.2. Culture and treatment of RBL-2H3 cells with small molecule inhibitors

Wild-Type (WT) RBL-2H3 cells and the Syk-deficient RBL-2H3 subclone TB1A2 (Zhang et al., 1996) were cultivated in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS). For the treatment of cells with inhibitors, 5×10^5 cells were seeded into 24 well plates and cultured overnight. Cells were washed with PBS and treated with inhibitors for 30 min in medium without serum. Crosslinking of the FcεRI was performed by addition of the BC4 antibody (0.02 μg/ml). Following washing with ice-cold PBS, cells were lysed at 4 °C for 30 min in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1% NP40, 5 mM EDTA, 50 mM NaF, 20 μM Na₃VO₄) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL). Lysates were

then used directly for immunoprecipitation or added to NuPAGE® Sample Reducing Agent and LDS Sample Buffer (Invitrogen) for analysis by Western blotting. Immunoprecipitation of rodent Syk was complicated by the lack of commercially available antibodies suitable for this purpose. Therefore total phosphotyrosine proteins were immunoprecipitated followed by blotting with Syk antibodies. For immunoprecipitation of human Syk, the 4D10 antibody was used.

2.3. Cultivation and treatment of primary mast cells of mouse and human origin

Mouse bone marrow-derived mast cell (mBMMCs) preparation: Female mice (8–15 weeks old of mixed genetic background) were euthanized with an overdose of pentobarbital (Narkoren). Long bones were removed and flushed with PBS/2% FCS. Red blood cells were lysed using an ACK lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Cells were routinely seeded at 1×10^6 cells/ml in mast cell medium (RPMI1640 supplemented with 10% FCS, 55 μM 2-mercaptoethanol, Penicillin/Streptomycin 1% (v/v) and 5 ng/ml IL-3). Medium was replaced three times a week. Cells were used in assays after four weeks of culture. To confirm the mast cell phenotype, expression levels of FcεRIα and CD117 were examined by FACS and only cells >90% positive for both markers were used.

For Western analysis, mBMMCs were seeded at 1×10^6 cells/ml in mast cell medium and incubated with anti-DNP-IgE (1 μg/ml) overnight. After washing twice with mast cell medium, cells were incubated for 30 min with inhibitors at the desired concentration followed by stimulation with DNP-BSA (100 ng/ml). Cells were lysed directly in NuPAGE® LDS Sample Buffer.

Primary human mast cells were matured from human peripheral blood derived CD34⁺ cells (2G-101C; LONZA, Tokyo, Japan) as described (Saito et al., 2006). Cells were cultured in IMDM supplemented with 2% FCS, SCF (100 ng/ml), IL-6 (50 ng/ml), Albumin (0.1%), Insulin-Transferrin-Selenium supplement (1%, v/v), Penicillin/Streptomycin (1%, v/v) and 2-Mercaptoethanol (55 μM). After 84 days of culture, mast cell maturation was confirmed by FACS analysis of FcεRIα and CD117 expression.

2.4. Transfection of RBL-2H3 cells with siRNAs and plasmids

RBL-2H3 cells were transfected with siRNA duplexes using the Nucleofector L kit (Lonza Cologne AG, Germany). 1×10^6 cells were electroporated with 420 nM of siRNA against rat Lyn or GAPDH and then seeded into 6 well plates with MEM/10% FCS to give a final siRNA concentration of 20 nM. Cells were used in assays following 2 days of culture. Cell viability following siRNA transfection was established using a lactate dehydrogenase (LDH) release assay and was in all cases above 93% of untransfected control cells.

For plasmid transfections, 6 well plates were seeded with 5×10^5 RBL-2H3 cells. On the next day, cells were transfected with 4 μg of plasmid and 10 μl of Lipofectamine™ (Invitrogen) per well in 500 μl Opti-MEM® (Invitrogen) added to 2 ml MEM/10% FCS. Cells were then cultured for 24 h prior to application in assays.

2.5. Measurement of β-hexosaminidase and histamine release

RBL-2H3 cells were seeded at 1×10^6 cells per well in 6 well plates and incubated overnight with 1 μg/ml anti-DNP IgE. Cells were then cultured for 30 min with inhibitors prior to activation for 1 h with DNP-BSA (100 ng/ml). Following stimulation, supernatants were collected and cleared by centrifugation. Neat supernatants were used in the β-hexosaminidase enzyme assay. Cells were lysed on ice for 30 min in 1 ml of cold 1% Triton and lysates were cleared by centrifugation and diluted 1/10 before use in the assays. The

Table 1

Specificity and activity of small molecule kinase inhibitors. IC₅₀ (nM) activities of each compound towards recombinant human Syk and Src family kinases Lyn, Fyn, Src, Fgr and Yes were determined as described in the materials and methods section. The EC₅₀ (nM) values for inhibition of β -hexosaminidase (β -HX) release from RBH-2H3 cells ($N=4$) and histamine release from human ($N=2$) or mouse primary mast cells ($N=1$) for each compound is also presented.

	BAY61-3606	BIRA766	R406
Syk (nM)	14	>10,000	26
Lyn (nM)	1666	1	67
Fyn (nM)	>1000	2	606
Src (nM)	>1000	1	>10,000
Fgr (nM)	>1000	1	2340
Yes (nM)	>1000	2	>10,000
RBL-2H3 β -HX (nM)	26	262	17
hBMMC Histamine (nM)	30	94	42
mBMMC Histamine (nM)	108	360	413

release of β -hexosaminidase enzyme activity from RBL-2H3 cells was measured as described previously (Silverman et al., 2006). Results are presented as the percentage of β -hexosaminidase activity measured in supernatant samples compared to the total levels in the corresponding cell lysates.

Mast cells were sensitized overnight with 1 μ g/ml of IgE and then washed twice with HEPES buffer [HEPES (20 mM), NaCl (137 mM), D-Glucose (5 mM), KCl (2.7 mM), NaH₂PO₄ (0.4 mM), MgCl₂·6H₂O (0.5 mM), CaCl₂·2H₂O (2.4 mM), pH 7.4, supplemented with 0.07% Albumin]. After resuspension in HEPES buffer (5600 cells/ml), cells were incubated with inhibitors for 30 min prior to activation with an anti-IgE antibody (3 μ g/ml) for a further 30 min. Plates were then rapidly cooled in ice-cold water to stop cell degranulation and then centrifuged for 5 min (1000 rpm) at 4 °C. The amount of released histamine in supernatants was determined using a histamine EIA kit (Immunotech).

2.6. Kinase assays for Syk and Lyn

The kinase activities of GST-Lyn (human Lyn residues 217–512) and GST-Syk (human Syk residues 342–635) fusion proteins were determined using the Kinase-Glo® Plus Luminescent Kinase Assay (Promega) as described by the manufacturer. All other kinase activity measurements were performed externally using the Z'-LYTE™ Assay at Invitrogen.

3. Results

3.1. Characterization of small molecule inhibitors of Syk and Src family kinases

Small molecule inhibitors were used to investigate the role of Syk and Src family kinases in the phosphorylation of specific Syk tyrosines following Fc ϵ RI crosslinking. The specificity and potency of each inhibitor was first tested in recombinant Syk and Src family kinase assays as well as functional assays examining the degranulation of RBL-2H3 cells and primary MCs of human and mouse origin (Table 1). BAY61-3606 (Yamamoto et al., 2003) was highly specific towards Syk, whereas R406 (Brasemann et al., 2006) displayed only a two-fold selectivity for Syk versus Lyn. BIRA766 (Goldberg et al., 2003) potently inhibited the activity of Src family kinases and displayed no activity on Syk. This data identified BAY61-3606 as a tool compound for the specific inhibition of Syk activity and BIRA766 as a specific Src family inhibitor, which we then used for the characterization of the roles of each of these kinases in Syk phosphorylation and Fc ϵ RI-signal transduction in RBL-2H3 cells and BMMCs.

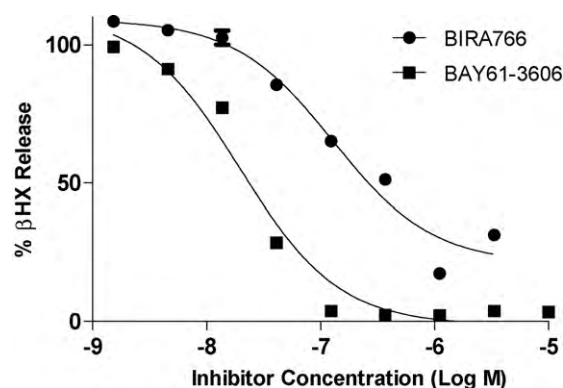


Fig. 1. Dose-response curves for the inhibition of RBH-2H3 degranulation by BAY61-3606 and BIRA766.

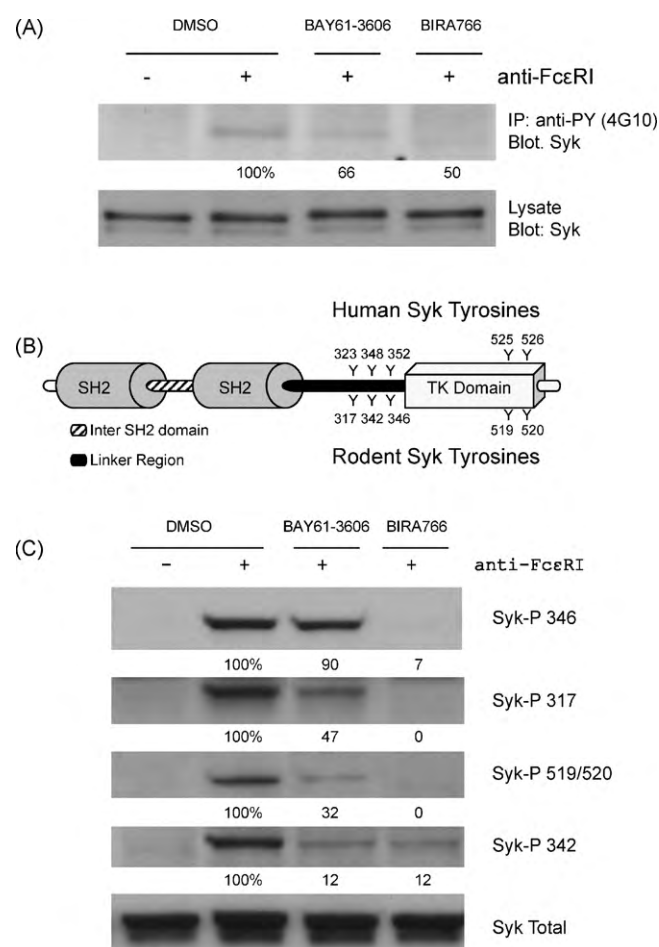


Fig. 2. Syk and Src family inhibitors reduce the Fc ϵ RI-stimulated tyrosine phosphorylation of Syk. (A) RBL-2H3 cells were treated with BAY61-3606 or BIRA766 (1 μ M) for 30 min in serum-free media and then stimulated with BC4 for 10 min. Tyrosine phosphorylated proteins were immunoprecipitated from cell lysates using the mouse 4G10 antibody and protein-G-sepharose beads. Solubilized immunoprecipitates were analyzed by Western blotting for levels of Syk using the rabbit N19 antibody. Total Syk levels in the lysates were also measured to control for protein loading using the SYK-01 anti-Syk antibody. The intensity of bands was quantitated by densitometry and levels of phosphorylated Syk expressed as a percentage of the signal observed in cells treated with BC4 and the vehicle DMSO. (B) Schematic representation of the position of analyzed tyrosines in rodent and human Syk. (C) RBL-2H3 cells were treated with BAY61-3606 or BIRA766 prior to activation of the Fc ϵ RI receptor. Lysates were analyzed for levels of specific Syk phosphotyrosines by Western blotting.

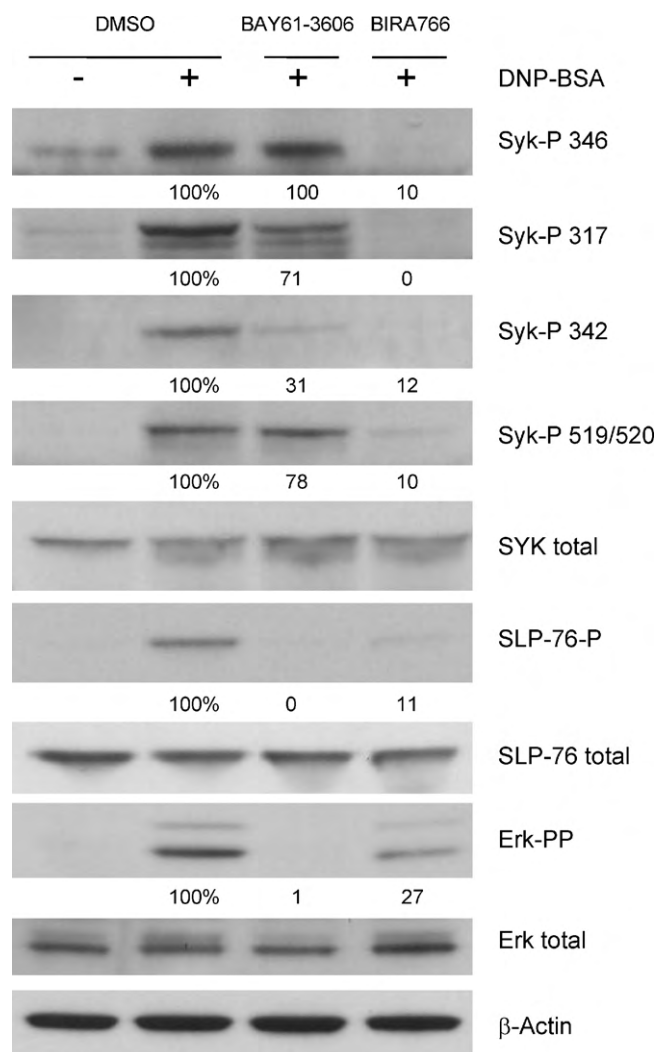


Fig. 3. Phosphorylation of Syk tyrosines and downstream proteins in IgE-stimulated mBMMCs. (A) Mouse BMMCs were sensitized with anti-DNP IgE overnight prior to incubation in BAY61-3606 or BIRA766 (1 μ M) for 30 min. Cells were then directly activated by the addition of DNP-BSA and cultivated for a further 10 min. Western blotting was used to identify phosphorylated Syk tyrosines 317, 342, 346 and 519/520 and the phosphorylation of SLP-76 and Erk. Total levels of Syk, SLP-76, Erk and β -actin were also analyzed. The intensity of bands was quantitated by densitometry and percentage levels of phosphorylated proteins are presented as performed above.

3.2. Both Syk and Src family inhibitors block Syk phosphorylation

The activity of Syk and Src family inhibitors on total Syk tyrosine phosphorylation was addressed in RBL-2H3 cells following Fc ϵ RI crosslinking. BAY61-3606 and BIRA766 were used on cells at concentrations of 1 μ M. At this concentration, maximal inhibition of RBL-2H3 cell degranulation was observed for each compound (Fig. 1), indicating that the cellular activities of either Syk or Src family kinases were completely inhibited. Meanwhile, based on the kinase data presented above, each inhibitor remained specific for either Syk or Src family kinases at this concentration. BAY61-3606 and BIRA766 both reduced total Syk tyrosine phosphorylation (Fig. 2A). Together, these results suggest that the phosphorylation of Syk following Fc ϵ RI crosslinking in RBL-2H3 cells is mediated by Syk autocatalytic activity and trans-phosphorylation by Src family kinases.

We next investigated the effects of Syk and Src family inhibitors on the phosphorylation of specific Syk tyrosines 317, 342, 346, 519 and 520. The positions of each tyrosine in rodent Syk and the cor-

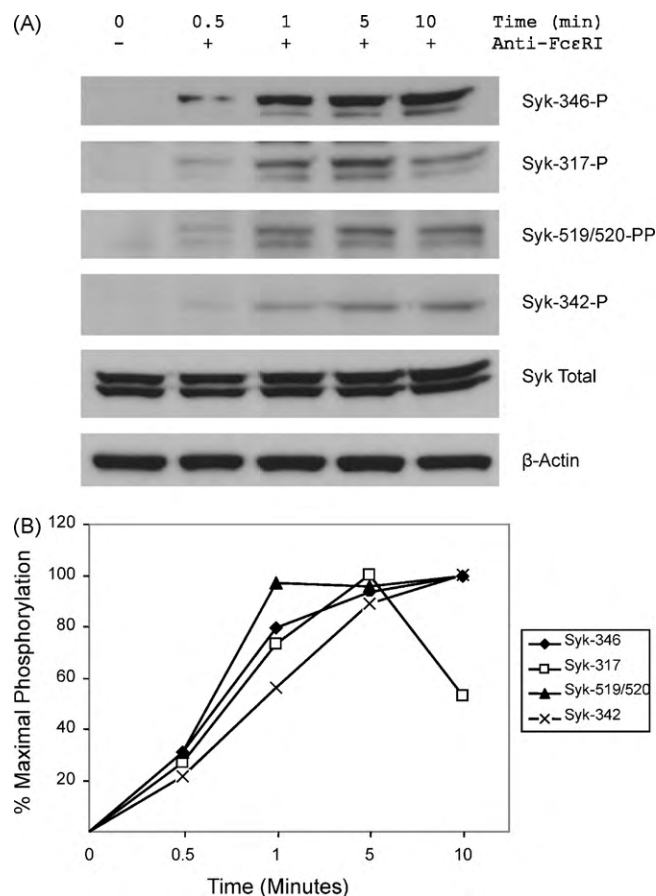


Fig. 4. Specific Syk tyrosines exhibit unique time-dependent phosphorylation patterns. (A) RBL-2H3 cells were treated for 0, 0.5, 1, 5 and 10 min with BC4 and cell lysates were analyzed for levels of phosphorylated Syk, total Syk and β -actin by Western blotting. (B) Intensities of phosphorylated Syk bands were determined by densitometry and are graphically presented as the percentage signal relative to the maximal phospho-Syk signal for each tyrosine.

responding human tyrosines are depicted in Fig. 2B. Tyrosine 346 phosphorylation was unaffected by the Syk inhibitor BAY61-3606 (Fig. 2C and also Fig. 5A). BIRA766 completely inhibited tyrosine 346 phosphorylation, suggesting that this residue is only trans-phosphorylated by Src family kinases. The phosphorylation of Syk tyrosines 317, 342 and 519/520 was inhibited by both BAY61-3606 and BIRA766, suggesting a role for both Syk and Src family kinases in the regulation of these phosphorylation events. In the case of tyrosines 317 and 519/520, phosphorylation was most potently inhibited by BIRA766, meanwhile tyrosine 342 phosphorylation was equally inhibited by BIRA766 and BAY61-3606.

3.3. The role of Syk and Src family kinases in Syk phosphorylation in activated mouse bone marrow-derived mast cells (mBMMCs)

Although different in several aspects of their biology, mast cells and basophils both undergo degranulation in response to Fc ϵ RI activation by IgE/allergen complexes (Warner and Kroegel, 1994). We used mBMMCs to investigate whether Syk undergoes analogous phosphorylation events to those we observed in the basophilic cell line RBL-2H3. Fc ϵ RI crosslinking led to the phosphorylation of Syk on all tyrosines examined and was accompanied by phosphorylation of the Syk substrate SLP-76 and downstream protein Erk (Fig. 3). Consistent with our results in RBL-2H3 cells, BIRA766 completely inhibited the phosphorylation of Syk tyrosine 346, whereas BAY61-3606 was without effect. Also analogous to the findings in RBL-2H3 cells were the observations that Syk tyro-

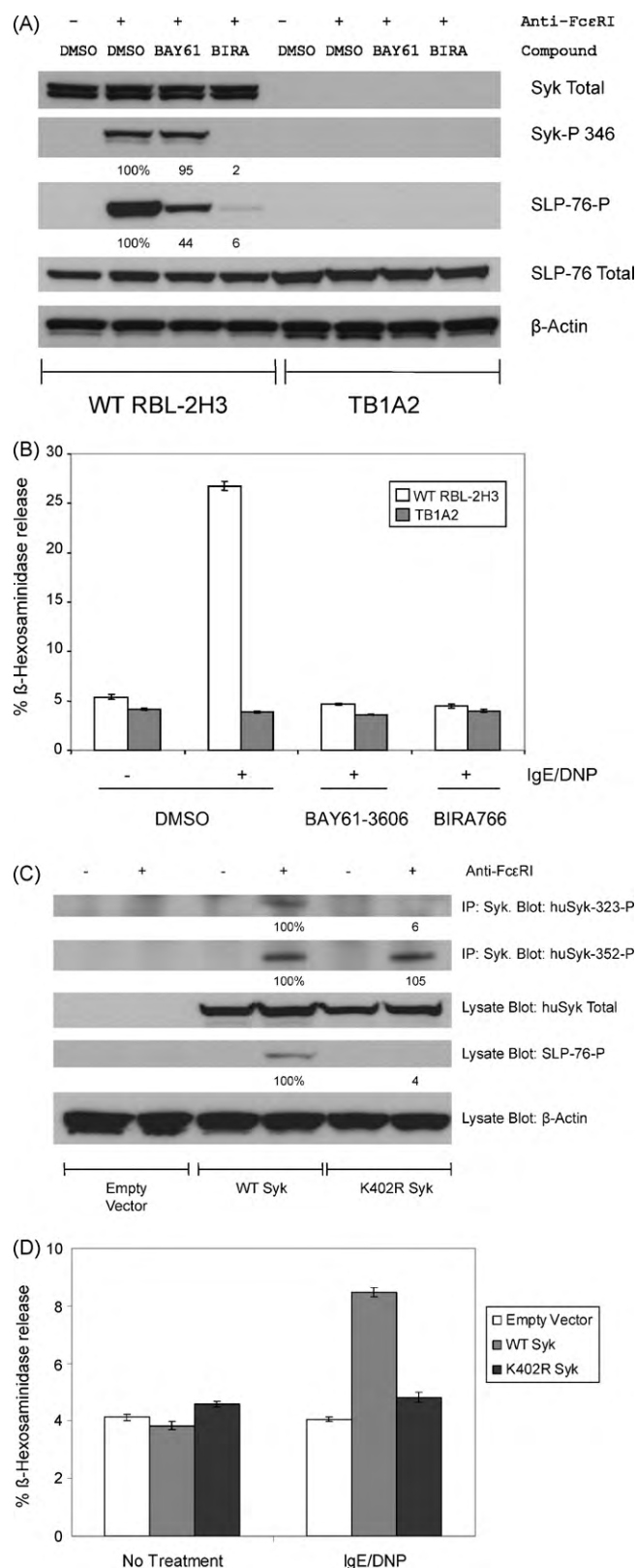


Fig. 5. Kinase-dead human Syk undergoes normal phosphorylation on tyrosine 352 (rodent Tyr 346), but tyrosine 323 (rodent Tyr 317) phosphorylation is defective. (A) RBL-2H3 and TB1A2 cells were incubated with BAY61-3606 or BIRA766 at 1 μ M and then stimulated with BC4. Levels of phosphorylated Syk (Tyr 352) and SLP-76 and Lyn as well as total Syk, SLP-76 and β -actin were analyzed by Western blotting. (B) RBL-2H3 and TB1A2 cells were loaded with anti-DNP IgE overnight and then incubated the next day with BAY61-3606 or BIRA766 at 1 μ M for 30 min. Cells were then activated by the addition of DNP-BSA for 1 h prior to the analysis of β -hexosaminidase release. Results are expressed as mean and standard

sine 342 phosphorylation was highly sensitive to both Syk and Src family inhibitors, meanwhile the phosphorylation of tyrosines 317 and 519/520 was more predominantly inhibited by BIRA766. These results point to a common regulatory mechanism of Syk phosphorylation in mast cells and basophilic cells. BAY61-3606 and BIRA766 also inhibited downstream FcεRI signaling via SLP-76 and Erk.

3.4. Time-course analysis of phosphorylation of Syk tyrosines

The alternate sensitivity of particular Syk tyrosines to Syk and Src inhibitors suggests that each phosphorylation event may be differentially regulated. We next investigated the time-course of phosphorylation of each Syk tyrosine. Peak phosphorylation of tyrosines 519/520 was observed 1 min after FcεRI crosslinking and was sustained until 10 min (Fig. 4A and B). Meanwhile, the phosphorylation signals for tyrosines 342 and 346 were found to progressively accumulate over the incubation period. In contrast, the tyrosine 317 signal peaked at 5 min and was then down-regulated by 10 min. These results indicate that the phosphorylation and down-regulation of specific Syk tyrosines are temporally regulated.

3.5. Kinase-dead human Syk displays defective phosphorylation of Syk tyrosine 323 but normal tyrosine 352 phosphorylation

As mentioned above, a kinase-dead ($K^{402}R$) Syk has previously been shown to undergo greatly reduced phosphorylation of tyrosines 519 and 520 when compared to WT Syk, indicating that autocatalytic activity is important for phosphorylation of these residues (Zhang et al., 2000). This is consistent with our results presented above which showed that Syk inhibitors reduced tyrosine 519/520 phosphorylation. We transfected a Syk-negative subclone of RBL-2H3 cells (TB1A2 cells) with expression constructs encoding either WT or $K^{402}R$ human Syk to investigate the role of Syk enzymatic activity on the phosphorylation of tyrosines 352 and 323. As depicted in above in Fig. 2B, human Syk tyrosines 352 and 323 are equivalent to rodent Syk tyrosines 346 and 317, respectively. WT RBL-2H3 and TB1A2 cells were first tested for their responsiveness to FcεRI crosslinking. The phosphorylation of the Syk substrate SLP-76 and degranulation of β -hexosaminidase were defective in TB1A2 cells confirming that TB1A2 cells were devoid of endogenous Syk activity (Fig. 5A and B).

$K^{402}R$ hSyk expressing TB1A2 cells displayed normal tyrosine 352 (rat tyrosine 346) phosphorylation (Fig. 5C). This supports the results shown above, which indicated that Syk inhibition with BAY61-3606 had no effect on tyrosine 346. Phosphorylation of hSyk tyrosine 323 (317 in rat Syk) was however reduced for $K^{402}R$ hSyk. This is also consistent with our findings that tyrosine 317 is sensitive to BAY61-3606. The reconstitution of Syk activity and functional FcεRI signaling in TB1A2 cells transfected with WT hSyk, but not $K^{402}R$ hSyk, was confirmed by examining SLP-76 phosphorylation and β -hexosaminidase release (Fig. 5C and D).

deviation values. A statistically significant difference between the release of β -hexosaminidase ($P < 0.0001$) by activated RBL-2H3 and TB1A2 cells was determined using a Student's *t*-test ($N = 3$). (C) TB1A2 cells were transiently transfected with expression plasmids for WT or kinase-dead ($K^{402}R$) human Syk. Following activation with BC4 for 10 min, human Syk was immunoprecipitated from cell lysates using the 4D10 antibody and protein-G-sepharose beads. Solubilized immunoprecipitates were analyzed by Western blotting for levels of phospho-Syk 323 and 352 using specific antibodies. Levels of total Syk and β -actin as well as phosphorylated SLP-76 in the corresponding cell lysates were also analyzed. (D) β -hexosaminidase release from TB1A2 cells transfected with WT or $K^{402}R$ human Syk. Results are expressed as mean and standard deviation values ($N = 3$). A statistically significant difference between the release of β -hexosaminidase ($P < 0.0001$) by activated TB1A2 cells expressing WT and $K^{402}R$ human Syk was determined as above.

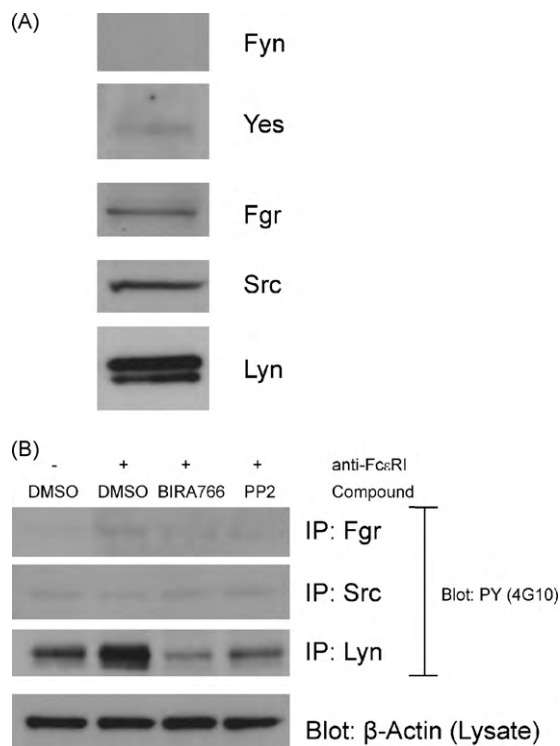


Fig. 6. Lyn is the predominant Src family kinase expressed and activated in RBL-2H3 cells. (A) Total cell lysates from RBL-2H3 cells were analyzed for levels of the Src kinases Fyn, Yes, Fgr, Src and Lyn by Western blotting. (B) RBL-2H3 cells were treated with BIRA766 (1 μ M) or PP2 (10 μ M) for 30 min and then activated by the addition of BC4 for 10 min. Fgr, Src and Lyn were immunoprecipitated from cell lysates using specific antibodies and protein-G-sepharose beads. Solubilized immunoprecipitates were analyzed for phosphotyrosine proteins by Western blotting using the 4G10 antibody. Levels of β -actin were analyzed in the corresponding cell lysates to control for protein loading.

3.6. Lyn displays the highest expression and phosphorylation amongst Src family tyrosine kinases in RBL-2H3 cells

The precise role of different Src kinases in FcεRI signaling may be cell type specific as different sources of mast cells or basophils display divergent expression levels for Src kinases (Parravicini et al., 2002; Blank and Rivera, 2004; Eiseman and Bolen, 1992; Gomez et al., 2007). We examined the expression and activation of Src kinases in RBL-2H3 cells in order to determine their involvement in FcεRI signaling and Syk phosphorylation. Lyn was detected at highest levels in RBL-2H3 cell extracts (Fig. 6A). Src and Fgr were only detected following prolonged Western blot exposures, meanwhile Fyn and Yes were virtually undetectable. These results were confirmed using different sources of antibodies against each Src family kinase (data not shown). Of the expressed Src family kinases, Lyn was also the only member to display a dramatic phosphorylation signal in response to FcεRI crosslinking, which was also inhibited by the Src family kinase inhibitors BIRA766 and PP2 (Fig. 6B). These data strongly implicate Lyn as the Src family kinase primarily activated via FcεRI signaling in RBL-2H3 cells.

3.7. Lyn knockdown reduces the phosphorylation of Syk and its direct substrates, whilst activating Erk phosphorylation and RBL-2H3 cell degranulation

Lyn expression was knocked down by 93% in RBL-2H3 cells using a specific siRNA (Fig. 7A). This decreased the phosphorylation of Syk at all analyzed tyrosines, confirming the fundamental role of this Src family kinase in Syk phosphorylation. Syk tyrosine 346 was most prominently inhibited in keeping with the notion that this

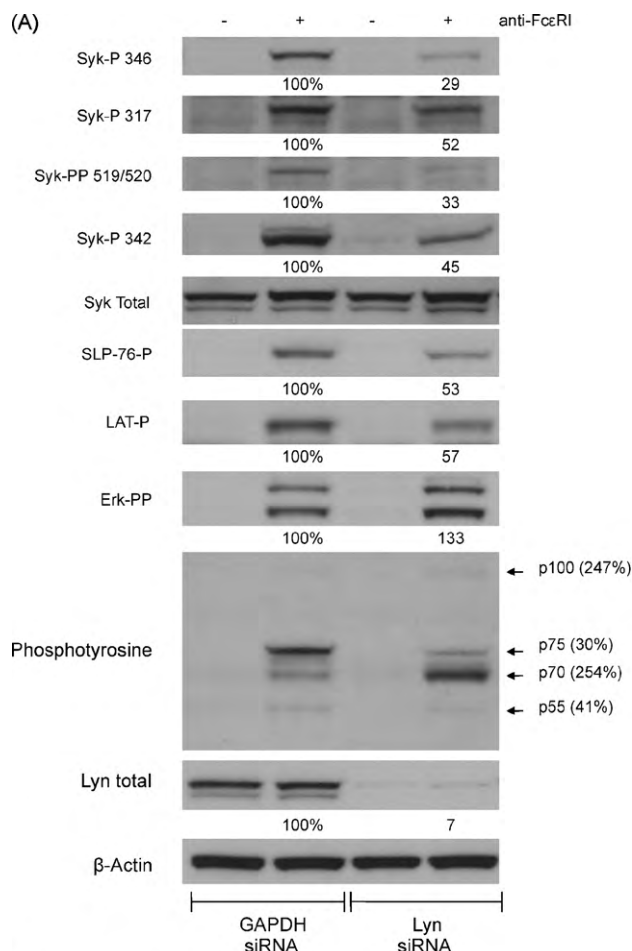


Fig. 7. Knockdown of Lyn reduces Syk phosphorylation meanwhile leading to hyper-degranulation of RBL-2H3 cells. (A) RBL-2H3 cells were transfected with siRNAs against rat Lyn or GAPDH. Two days later, cells were activated with BC4 for 10 min. Lysates were analyzed for levels of total Syk, Lyn and β -actin in addition to phosphorylated Syk, SLP-76, LAT, Erk and total cellular tyrosine phosphorylated proteins. (B) The release of β -hexosaminidase from RBL-2H3 cells transfected with GAPDH and Lyn siRNAs in response to anti-DNP IgE/DNP-BSA treatment was analyzed as described above. Results are expressed as mean and standard deviation values ($N=3$). A statistically significant difference between the release of β -hexosaminidase ($P<0.0001$) by activated RBL-2H3 cells transfected with GAPDH or Lyn siRNAs was determined as above.

site is a pure Lyn substrate. Lyn knockdown also led to the inhibition of phosphorylation of the Syk substrates SLP-76 and LAT, indicating that Lyn is required for activation of Syk enzymatic function. In contrast, the phosphorylation of the downstream protein Erk and of proteins in whole cell extracts with sizes of 100 and 70 kDa was increased by Lyn knockdown. Other cellular proteins at 75 and 50 kDa showed decreased phosphorylation. FcεRI-mediated degranulation of RBL-2H3 cells was enhanced following Lyn knockdown (Figure 7C). These results in RBL-2H3 cells are reminiscent of

those in Lyn-deficient BMMCs (Parravicini et al., 2002) and B cells (Gauld and Cambier, 2004), which are hyper-responsive to Fc ϵ RI and B cell receptor (BCR) activation, respectively, and suggest that interfering with Lyn expression concomitantly disrupts proximal Fc ϵ RI signaling via Syk, meanwhile activating distal signaling.

4. Discussion

Phosphorylation of Syk following Fc ϵ RI activation is an essential step in inflammatory signaling in mast cells and basophils (Sanderson et al., 2009). Phosphorylated Syk tyrosines 317, 342 and 346 in the linker region, which separates its two N-terminal SH2 domains from the C-terminal kinase domain, provide docking sites for interaction with Syk substrates including LAT, Vav and SLP-76. These proteins are then phosphorylated by Syk to initiate downstream signaling and effector functions (Siraganian et al., 2002; Latour et al., 1998). Two tyrosines (519 and 520) within the kinase domain are also phosphorylated following Fc ϵ RI activation (Zhang et al., 2000; Zhang et al., 1998) which also positively regulate Syk-mediated signaling (Siraganian et al., 2002; Zhang et al., 1998). The functions of specific Syk phosphotyrosines are therefore unique and crucial to the regulatory role of Syk in inflammatory signal transduction. The identity of the kinases responsible for the phosphorylation of each of these Syk tyrosines has not been characterized in depth. We aimed to identify the role of trans- and auto-phosphorylation events in the activation of specific Syk tyrosines and the degranulation of RBL-2H3 basophilic cells and BMMCs.

The phosphorylation of Syk tyrosine 346 was unique in that it was only sensitive to a Src family kinase inhibitor. The role of the Src kinase Lyn in tyrosine 346 phosphorylation was confirmed by Lyn knockdown and by the finding that kinase-dead (KD) Syk was efficiently phosphorylated on this residue. Phosphorylation of tyrosines 317 and 519/520 was completely blocked by the Src inhibitor BIRA766, but only partially blocked by the Syk inhibitor BAY61-3606. The predominant sensitivity of tyrosines 317 and 519/520 to BIRA766 does not fully correlate with our additional findings that KD-Syk did not undergo phosphorylation on tyrosine 317 and the findings of others that KD-Syk undergoes less than 10% phosphorylation of tyrosines 519/520 (Zhang et al., 2000), which are both suggestive of a role for auto-phosphorylation. It is possible that auto-phosphorylation of Syk tyrosines 317 and 519/520 occurs only after initial trans-phosphorylation of other Syk tyrosines by Lyn. Indeed, the phosphorylation of particular Syk tyrosines is crucial for enzyme activation. For example, Y³⁴²F and Y¹³⁰F Syk mutants display reduced kinase activity and phosphorylation of Syk substrates (Keshvara et al., 1997; Zhang et al., 2002; Zhang et al., 2008). Initial Lyn-mediated phosphorylation of Syk may switch the protein to an active state that then allows subsequent auto-phosphorylation of tyrosines including 317, 342 and 519/520. A recent interesting report indicated that three adjacent tyrosines (623, 624 and 625) in the COOH-terminal region of Syk are important for regulation of enzyme activity and cellular responses in RBL-2H3 cells (de Castro et al., 2010). The specific Syk and Lyn inhibitors used in our study may prove useful in establishing if tyrosines 623–625 are targets for auto-phosphorylation or trans-phosphorylation by Lyn.

Lyn knockdown in RBL-2H3 cells led to increased phosphorylation of Erk and other cellular proteins at 100 and 70 kDa. This may indicate an uncoupling of Fc ϵ RI signaling when Lyn expression is down-regulated and may explain the hyper-degranulation of these cells. Previous reports using Lyn-deficient BMMCs and B cells have also identified a hyper-responsive Fc signaling phenotype (Parravicini et al., 2002; Gauld and Cambier, 2004) and have led to Lyn being predominantly described as a negative regulator of leukocyte activation. However, these phenotypes identified

in Lyn knockout or knockdown cells may not reflect the function of Lyn in normal cells. Lyn knockout in mouse mast cells leads to the compensatory up-regulation of a second Src family kinase Fyn, which may uncouple downstream signaling and degranulation from the Fc ϵ RI receptor (Odom et al., 2004; Parravicini et al., 2002; Hernandez-Hansen et al., 2004). In our study, we treated RBL-2H3 cells and BMMCs with BIRA766 for a short-time period (30 min) in order to inhibit the catalytic activity of Lyn. This did not affect the expression of Lyn (data not shown). Under these conditions, all steps of Fc ϵ RI signaling including the phosphorylation of Syk and downstream substrates SLP-76, LAT and Erk and degranulation were inhibited. This is potentially a more meaningful approach to analyze Lyn function and may indicate that under normal conditions, Lyn in fact positively regulates Fc ϵ RI signaling via the control of Syk activation. Together, our results provide an insight into the kinases responsible for the phosphorylation of crucial Syk tyrosines and the transmission of signals via the Fc ϵ RI receptor.

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References

- Blank, U., Rivera, J., 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25, 266–273.
- Braselmann, S., Taylor, V., Zhao, H., Wang, S., Sylvain, C., Baluom, M., Qu, K., Herlaar, E., Lau, A., Young, C., Wong, B.R., Lovell, S., Sun, T., Park, G., Argade, A., Jurcevic, S., Pine, P., Singh, R., Grossbard, E.B., Payan, D.G., Masuda, E.S., 2006. R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-mediated inflammation. *J. Pharmacol. Exp. Ther.* 319, 998–1008.
- Costello, P.S., Turner, M., Walters, A.E., Cunningham, C.N., Bauer, P.H., Downward, J., Tybulewicz, V.L., 1996. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor for mast cells. *Oncogene* 13, 2595–2605.
- de Castro, R.O., Zhang, J., Jamur, M.C., Oliver, C., Siraganian, R.P., 2010. Tyrosines in the carboxy-terminus regulate Syk kinase activity and function. *J. Biol. Chem.* 285, 26674–26684.
- Eiseman, E., Bolen, J.B., 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355, 78–80.
- Gauld, S.B., Cambier, J.C., 2004. Src-family kinases in B-cell development and signaling. *Oncogene* 23, 8001–8006.
- Goldberg, D.R., Butz, T., Cardozo, M.G., Eckner, R.J., Hammach, A., Huang, J., Jakes, S., Kapadia, S., Kashem, M., Lukas, S., Morwick, T.M., Panzenbeck, M., Patel, U., Pav, S., Peet, G.W., Peterson, J.D., Prokopowicz III, A.S., Snow, R.J., Sellati, R., Takahashi, H., Tan, J., Tschantz, M.A., Wang, X.J., Wang, Y., Wolak, J., Xiong, P., Moss, N., 2003. Optimization of 2-phenylaminoimidazo[4,5-h]isoquinolin-9-ones: orally active inhibitors of Ick kinase. *J. Med. Chem.* 46, 1337–1349.
- Gomez, G., Schwartz, L., Kepley, C., 2007. Syk deficiency in human non-releaser lung mast cells. *Clin. Immunol.* 125, 112–115.
- Hernandez-Hansen, V., Smith, A.J., Surviladze, Z., Chigaev, A., Mazel, T., Kalesnikoff, J., Lowell, C.A., Krystal, G., Sklar, L.A., Wilson, B.S., Oliver, J.M., 2004. Dysregulated Fc ϵ RI signaling and altered Fyn and SHIP activities in Lyn-deficient mast cells. *J. Immunol.* 173, 100–112.
- Keshvara, L.M., Isaacson, C., Harrison, M.L., Geahlen, R.L., 1997. Syk activation and dissociation from the B-cell antigen receptor is mediated by phosphorylation of tyrosine 130. *J. Biol. Chem.* 272, 10377–10381.
- Latour, S., Zhang, J., Siraganian, R.P., Veillette, A., 1998. A unique insert in the linker domain of Syk is necessary for its function in immunoreceptor signalling. *EMBO J.* 17, 2584–2595.
- Odom, S., Gomez, G., Kovarova, M., Furumoto, Y., Ryan, J.J., Wright, H.V., Gonzalez-Espinosa, C., Hibbs, M.L., Harder, K.W., Rivera, J., 2004. Negative regulation of immunoglobulin E-dependent allergic responses by Lyn kinase. *J. Exp. Med.* 199, 1491–1502.
- Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L.E., O'Shea, J.J., Rivera, J., 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3, 741–748.

- Rivera, J., Olivera, A., 2008. A current understanding of Fc epsilon RI-dependent mast cell activation. *Curr. Allergy Asthma Rep.* 8, 14–20.
- Sada, K., Zhang, J., Siraganian, R.P., 2000. Point mutation of a tyrosine in the linker region of Syk results in a gain of function. *J. Immunol.* 164, 338–344.
- Saito, H., Kato, A., Matsumoto, K., Okayama, Y., 2006. Culture of human mast cells from peripheral blood progenitors. *Nat. Protoc.* 1, 2178–2183.
- Sanderson, M.P., Lau, C.W., Schnapp, A., Chow, C.W., 2009. Syk: a novel target for treatment of inflammation in lung disease. *Inflamm. Allergy Drug Targets* 8, 87–95.
- Silverman, M.A., Shoag, J., Wu, J., Koretzky, G.A., 2006. Disruption of SLP-76 interaction with Gads inhibits dynamic clustering of SLP-76 and FcepsilonRI signaling in mast cells. *Mol. Cell. Biol.* 26, 1826–1838.
- Siraganian, R.P., Zhang, J., Suzuki, K., Sada, K., 2002. Protein tyrosine kinase Syk in mast cell signaling. *Mol. Immunol.* 38, 1229–1233.
- Warner, J.A., Kroegel, C., 1994. Pulmonary immune cells in health and disease: mast cells and basophils. *Eur. Respir. J.* 7, 1326–1341.
- Wong, B.R., Grossbard, E.B., Payan, D.G., Masuda, E.S., 2004. Targeting Syk as a treatment for allergic and autoimmune disorders. *Expert. Opin. Invest. Drugs* 13, 743–762.
- Yamamoto, N., Takeshita, K., Shichijo, M., Kokubo, T., Sato, M., Nakashima, K., Ishimori, M., Nagai, H., Li, Y.F., Yura, T., Bacon, K.B., 2003. The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents. *J. Pharmacol. Exp. Ther.* 306, 1174–1181.
- Yu, M., Lowell, C.A., Neel, B.G., Gu, H., 2006. Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from FcepsilonRI. *J. Immunol.* 176, 2421–2429.
- Zhang, J., Berenstein, E., Siraganian, R.P., 2002. Phosphorylation of Tyr342 in the linker region of Syk is critical for Fc epsilon RI signaling in mast cells. *Mol. Cell. Biol.* 22, 8144–8154.
- Zhang, J., Berenstein, E.H., Evans, R.L., Siraganian, R.P., 1996. Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *J. Exp. Med.* 184, 71–79.
- Zhang, J., Billingsley, M.L., Kincaid, R.L., Siraganian, R.P., 2000. Phosphorylation of Syk activation loop tyrosines is essential for Syk function. An in vivo study using a specific anti-Syk activation loop phosphotyrosine antibody. *J. Biol. Chem.* 275, 35442–35447.
- Zhang, J., Kimura, T., Siraganian, R.P., 1998. Mutations in the activation loop tyrosines of protein tyrosine kinase Syk abrogate intracellular signaling but not kinase activity. *J. Immunol.* 161, 4366–4374.
- Zhang, Y., Oh, H., Burton, R.A., Burgner, J.W., Geahlen, R.L., Post, C.B., 2008. Tyr130 phosphorylation triggers Syk release from antigen receptor by long-distance conformational uncoupling. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11760–11765.