J. Biochem. **123**, 1199-1207 (1998)

Evidence for Involvement of Two Isoforms of Syk Protein-Tyrosine Kinase in Signal Transduction through the High Affinity IgE Receptor on Rat Basophilic Leukemia Cells

Toshiyuki Yamashita,[1] Liliana Kairiyama,[2] Mayu Araki, and Shigeharu Nagasawa

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-ku, Sapporo 060-0812

Received for publication, January 29, 1998

Recent evidence suggests a critical role for Syk in mast cell activation upon high affinity IgE receptor (FcεRI) aggregation. A rat basophilic leukemia cell line, RBL-2H3, expresses similar levels of two Syk isoforms that differ with respect to the presence of a 23-amino acid insert within the "linker" region located between the second Src homology 2 and the catalytic domain. Although they exhibit comparable intrinsic enzymatic activity, functional differences between the two isoforms are unknown. Here we report that the deleted Syk isoform can mediate signal transduction in RBL-2H3 cells. Aggregation of chimeric kinase, consisting of either form of Syk fused to the transmembrane and extracellular domains of guinea pig type II IgG Fc receptor, on RBL transfectants resulted in degranulation, release of leukotrienes, and enhanced gene expression of tumor necrosis factor-α. The chimeras as well as phospholipase C-γ1 and Vav became tyrosine-phosphorylated upon aggregation of chimeras. We also found that both Syk isoforms from transiently transfected COS-7 cells were capable of binding to phosphorylated FcεRI, and their kinase activities were similarly up-regulated in the presence of tyrosine-phosphorylated synthetic peptides based on the sequence of the γ subunit of FcεRI. Thus, these results establish that both isoforms of Syk can mediate signal transduction in mast cells and suggest that the 23-amino acid insert in the linker region of Syk may not be obligatory for FcεRI signaling.

Key words: FcεRI, linker region, RBL-2H3, signal transduction, Syk.

The aggregation of the high affinity receptor for IgE (FcεRI) on mast cells and basophils activates the multiple signaling pathways that lead to degranulation and the release of mediators of the allergic reaction (*1, 2*). FcεRI is a multisubunit receptor comprising an IgE-binding α chain, a β chain, and a homodimer of γ chains (*3*). Among the earliest biochemical events that follow FcεRI aggregation is the tyrosine phosphorylation of multiple signaling molecules including the β and γ subunits of FcεRI (*4*). The rapid tyrosine phosphorylation of FcεRI itself upon receptor aggregation suggests an aggregation-dependent activation of PTK that is closely associated with FcεRI. These FcεRI subunits lack intrinsic PTK activity; instead, Lyn kinase, a member of the Src family of PTK, is associated with FcεRI prior to receptor aggregation (*5-8*). Several lines of evidence suggest that Lyn, which is associated with the β chain of FcεRI and activated by receptor aggregation, is responsible for the tyrosine phosphorylation of FcεRI subunits (*7-9*).

The cytoplasmic domains of the β and γ chains each contain a sequence termed the immunoreceptor tyrosine-based activation motif (ITAM) that is also found in other multisubunit immune recognition receptors such as B-cell antigen receptor and T-cell antigen receptor (*10-13*). The phosphorylation of two conserved tyrosine residues within the ITAM consensus sequences plays a critical role in receptor-mediated signal transduction. Current evidence suggests that tyrosine-phosphorylated ITAM of β chain leads to reorientation of receptor-associated Lyn and also recruitment of additional Lyn due to SH2-mediated binding (*7, 8, 14, 15*). This binding is known to increase the specific activity of Lyn (*16, 17*). Thus, the FcεRI-associated kinase activity derived from Lyn is strongly enhanced by receptor aggregation.

On the other hand, tyrosine phosphorylation of ITAM of γ chain recruits another PTK, Syk, a member of the Syk/ZAP-70 family of PTK (*18*). In contrast to Lyn, Syk is not physically associated with FcεRI prior to receptor aggregation. Syk contains two tandem SH2 domains that mediate high affinity interaction with tyrosine-phosphorylated ITAM of γ chain (*7, 14*). This binding results in a confor-

[1] To whom correspondence should be addressed. Tel: +81-11-706-3245, Fax: +81-11-706-4989, E-mail: yamashit@pharm.hokudai.ac.jp

[2] L.K. is supported by the Hokkaido Overseas Association Inc. (Sapporo).

Abbreviations: Ab, antibody; Ag, antigen; DNP, 2,4-dinitrophenol; FcεRI, the high affinity receptor for IgE; FcγRIIB, the type IIB Fc receptor for IgG; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ITAM, immunoreceptor tyrosine-based activation motif; mAb, monoclonal antibody; PLC, phospholipase C; RBL, rat basophilic leukemia; RT-PCR, reverse transcription-polymerase chain reaction; SH2, Src homology 2; Syk(L), the longer isoform of Syk; Syk(S), the shorter isoform of Syk; TNF, tumor necrosis factor.

mational change of Syk and an increase in the specific activity (*19, 20*). The Syk thus recruited becomes tyrosine-phosphorylated by receptor-associated Lyn and/or autophosphorylation, leading to full activation of Syk and propagation of downstream signals. Recent studies have demonstrated that Syk plays a pivotal role in signal transduction through FcεRI (*9, 21-25*).

Besides the originally described form [termed Syk(L)], an additional Syk isoform [Syk(S)] was reported in a human basophilic leukemia cell line (KU812) (*26*). This isoform lacks a 23-amino acid insert located within the region that separates the two tandem SH2 domains from the catalytic domain. This insert is also deleted in ZAP-70, which is another member of Syk/ZAP-70 family and structurally related to Syk. Recently, a similar deleted form was found in a rat basophilic leukemia cell line (RBL-2H3) and a mouse IL-3-dependent mast cell line (PT18) (*27*). Syk genomic DNA analysis indicated that the sequence encoding the 23-amino acid insert comprises a distinct exon. The 23 amino acids within the insert, containing a potential tyrosine phosphorylation site, are highly conserved among species studied to date (*18, 27-29*), suggesting that this sequence may be structurally and/or functionally important. However, little is known regarding functional differences between the two Syk isoforms. Although intrinsic enzymatic activity of the two isoforms was shown to be comparable (*30*), whether Syk(S) becomes associated with FcεRI and activated upon aggregation of FcεRI has not been explored. In this study, we introduced chimeric Syk isoforms into RBL-2H3 cells and monitored the cell activation triggered by aggregation of chimeras. The results presented here suggest that both forms of Syk are capable of mediating signal transduction leading to mast cell activation.

MATERIALS AND METHODS

Cell Culture—RBL-2H3 cells and various RBL transfectants were grown in a humidified atmosphere containing 5% CO_2 in Eagle's MEM (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (ICN Biomedicals Inc., Irvine, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (Life Technologies, Grand Island, NY; 0.25 μg/ml). Cells were harvested following exposure to 0.05% trypsin/0.53 mM EDTA in Dulbecco's PBS and assayed in suspension. COS-7 cells were cultured in DMEM (Sigma) containing 5% FCS and antibiotics as described above.

Ab—Monoclonal anti-DNP IgE was prepared from the ascites of mice bearing the H1 DNP-ε-26.82 hybridoma (*31*). Rabbit polyclonal anti-rat Syk Ab was prepared as reported by Benhamou *et al.* (*18*). Briefly, a peptide, NH_2-EPTGGAWGPDRGLC-CO_2H, which corresponds to residues 318 to 330 of rat Syk with a cysteine residue at the carboxy terminus, was synthesized, conjugated with maleimide-activated keyhole limpet hemocyanin (Pierce Chemical, Rockford, IL), and used for immunizing rabbits. Total IgG fraction was obtained from antiserum by precipitation with ammonium sulfate followed by DEAE-cellulose column chromatography. Some of the IgG was affinity-purified using the immunizing peptide-bound FMP-activated Cellulofine (Seikagaku, Tokyo). The Fab′ fragment of a mAb directed against the guinea pig type IIB Fc receptor for IgG (FcγRIIB) was prepared as described (*32*). Anti-mouse IgE Ab was raised in guinea pigs, and the affinity-purified IgG2 fraction was used for immunoprecipitation of IgE-bound FcεRI. Goat $F(ab')_2$ fragment of anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA) and its FITC-conjugate was from TAGO (Burlingame, CA). The anti-phosphotyrosine mAb 4G10, rabbit polyclonal anti-Lyn serum, and mixed monoclonal anti-PLC-γ1 Ab were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-Vav Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugates of donkey $F(ab')_2$ anti-rabbit Ig, sheep $F(ab')_2$ anti-mouse Ig, and streptavidin were obtained from Amersham (UK). A biotinylated monoclonal anti-rabbit Ig, which reacts with an epitope sensitive to reduction (clone RG-16), was from Sigma.

Reagents—Dinitrophenyl (DNP)-conjugated BSA (11 mol of DNP/mol of protein) was prepared as described (*33*). Protein A- and Protein G-Sepharose 4 Fast Flow were purchased from Pharmacia Biotech AB (Uppsala, Sweden). The materials for electrophoresis were purchased from Novex (San Diego, CA) and Tefco (Tokyo). Nitrocellulose membranes (BA83; 0.2 μm pore size) were purchased from Schleicher & Schuell (Keene, NH). The enhanced chemiluminescence reagents were from Amersham.

cDNA and Generation of Syk Chimeras—The cDNAs encoding the two isoforms of rat Syk (*27*) were obtained by using total RNA from RBL-2H3 cells as the template and reverse transcription (RT)-PCR with RNA LA PCR Kit (Takara Shuzo, Otsu) with primers bearing appropriate restriction sites. The PCR products were cut with restriction enzymes and cloned into the pBluescript II SK^+ or KS^+ vector (Stratagene, La Jolla, CA). Several clones were sequenced and mutation-free coding sequences were inserted into pcDNA1 expression vector (Invitrogen, San Diego, CA). For the construction of the chimeric Syk, the entire coding region of rat Syk(L) or Syk(S) was attached to the sixth amino acid of the cytoplasmic domain of the guinea pig FcγRIIB (*34*) by PCR using primer 5′ AAG CAG CCT CCA GCC ATG GCG GGC AAT GCT 3′. Underlined nucleotides are from the 5′ end of rat Syk. After confirming their DNA sequences, the resulting constructs were subcloned into pcDNA3 expression vector (Invitrogen).

Transfections—COS-7 cells were transfected with pcDNA1-Syk(L) or -Syk(S) by the DEAE-dextran method as previously described (*34*). Two days after transfection, cells were harvested and lysed. RBL cells expressing chimeric Syk were produced according to protocols provided by the supplier of the LIPOFECT AMINE reagents (GIBCO BRL, Gaithersburg, MD). Briefly, 40 μg of the pcDNA3-Syk chimeras was transfected into RBL-2H3 cells (1×10^7), and stably transfected clones were selected with 600 μg/ml of G418 (GIBCO BRL). As a control, RBL-2H3 cells were also transfected with pcDNA3-guinea pig FcγRIIB1. Surface expression was analyzed on a FACSort (Becton Dickinson) using anti-guinea pig FcγRIIB mAb and FITC-goat anti-mouse IgG (*34*). Several monoclonal transfectants were obtained and cells stably expressing the highest levels of chimeras were used for experiments [RBL-C2.0, RBL-L9.0, and RBL-S6.5 express guinea pig FcγRIIB1, chimeric Syk(L), and chimeric Syk(S), respectively].

Analysis of Mediator Release—RBL transfectants were incubated with 10 μg/ml mouse anti-DNP IgE or 10 μg/ml Fab′ fragments of mouse monoclonal anti-guinea pig FcγRIIB for 1 h at 4°C. Cells were washed and resuspended in assay buffer containing 25 mM PIPES (pH 7.2), 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5.4 mM glucose, and 0.1% BSA at 1×10^7 cells/ml. Cells were stimulated with DNP-BSA (1 μg/ml) or $F(ab')_2$ fragments of goat anti-mouse IgG (10 μg/ml) for 40 min at 37°C. The culture supernatant was collected and the cells were lysed with assay buffer containing 0.2% Triton X-100. For determination of hexosaminidase release, 10 μl of supernatant or cell lysate was incubated with 3.3 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer, pH 4.5 for 30 min at 37°C. At the end of the incubation, 150 μl of 0.2 M glycine buffer, pH 10.4 was added and the absorbance at 405 nm was determined. Secreted leukotrienes were analyzed using the Leukotriene $C_4/D_4/E_4$ enzyme immunoassay system (Amersham).

Detection of Cytokine mRNA by RT-PCR—RBL transfectants were stimulated as in the case for analysis of mediator release. After 90 min of stimulation, total RNA was extracted from 1×10^6 cells by the acid guanidinium thiocyanate method (*35*). RT using RNA LA PCR kits (Takara Shuzo) synthesized first strand cDNA with oligo-(dT) as primer and 0.25 μg of total RNA. PCR amplification was performed using the same kits. The rat TNF-α and G3PDH primer sets were purchased from Clontech Laboratories (Palo Alto, CA). Thirty cycles were run for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. PCR products were analyzed by ethidium bromide staining in agarose gels.

Immunoprecipitation and Immunoblotting—Cells were solubilized for 30 min at 4°C in lysis buffer containing 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. The lysates were centrifuged for 5 min at $16,000\times g$ at 4°C to remove insoluble materials. The clarified supernatants were precleared with protein A- or protein G-Sepharose 4 Fast Flow for 1 h at 4°C, then immunoprecipitated with various Ab for 2 h at 4°C, followed by incubation with protein A- or protein G-Sepharose (35 μl of beads). After rotation for 1 h at 4°C, the beads were washed three times with ice-cold lysis buffer. SDS-PAGE sample buffer was added to a final concentration of 62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, and 50 mM DTT. All samples were boiled for 5 min before electrophoresis on an 8% SDS-PAGE gel. Unless otherwise indicated, 5×10^6 cell equivalents were loaded per lane. Proteins were transferred to nitrocellulose membranes for 1 h at 1.2 mA/cm^2 with a semi-dry transblotter (MilliBlot-Graphite Electroblotter System; Millipore, Bedford, MA). Nonspecific binding sites were blocked by incubating the nitrocellulose membranes for 1 h or overnight in blocking buffer (50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20, 4% protease-free BSA, 0.01% human IgG, 0.01% thimerosal). The blots were then incubated for 1 h with optimal concentrations of various primary Ab, washed three times with the blocking buffer, and incubated with horseradish peroxidase-conjugated secondary Ab or streptavidin-horseradish peroxidase complex (Amersham) for 1 h. After washing them again with the blocking buffer, the immunoblots were developed by enhanced chemiluminescence. For some experiments, membranes were stripped of primary Ab according to the protocol of the reagents and reprobed.

In Vitro Kinase Assay—Immunoprecipitates were washed twice with kinase buffer (20 mM Tris/HCl pH 7.5, 0.15 M NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4) and resuspended in 50 μl. Kinase reactions were initiated by the addition of 0.5 mM ATP, and the mixture was incubated at 30°C for indicated times with occasional mixing. For some experiments, washed immunoprecipitates were resuspended in 100 μl of kinase buffer containing 50 μM of doubly tyrosine-phosphorylated ITAM peptide [NH_2-DAV(pY)-TGLNTRNQET(pY)ETL-CO_2H], preincubated for 15 min at 4°C, and ATP was added to the samples. The mixture was incubated at 4°C for indicated times. Tyrosine-phosphorylated proteins were detected by immunoblotting with biotinylated 4G10 and streptavidin-horseradish peroxidase complexes as described above.

RESULTS

Two Isoforms of Syk Expressed in RBL-2H3 Cells—To investigate whether the short form of Syk [Syk(S)] can be activated by aggregation of FcεRI, we first examined the expression of Syk and its association with FcεRI in RBL-2H3 cells. We confirmed that the cells expressed two isoforms of Syk using RT-PCR with internal sense and antisense primers for the linker region (data not shown). Consistent with the previous reports (*27*), immunoblotting with anti-Syk Ab showed that unstimulated RBL-2H3 cells expressed two Syk isoforms (Fig. 1A). The amount of short isoform expressed in the cells was comparable to that of long form [Syk(L)]. We also overexpressed each Syk isoform in COS-7 cells, and the results of immunoblotting showed that only a single protein band corresponding to each isoform was observed (Fig. 1B). In the immunoblots of stimulated RBL-2H3 cells, an additional protein that migrated slightly slower was detected (Fig. 1A). Immunoprecipitation with anti-Syk Ab and immunoblotting with anti-phosphotyrosine Ab 4G10 revealed that this slower migrating protein was a tyrosine-phosphorylated Syk (Fig. 1C). It should be noted that those doublet or triplet bands were detected when the SDS-PAGE was run under reducing conditions; under non-reducing conditions, only a single band was observed in unstimulated cells and doublet bands in stimulated cells. Because of the low resolution of immunoblots, it is unclear whether Syk(S) is tyrosine-phosphorylated upon FcεRI aggregation. In addition, Syk was undetectable in the FcεRI immunoprecipitates from stimulated cells (data not shown). Thus, it remained unclear whether Syk(S) is capable of binding to the aggregated FcεRI in RBL-2H3 cells.

Introduction of Chimeric Syk into RBL-2H3 Cells—Kolanus *et al.* (*29*) reported that simple clustering of nonreceptor kinases such as Syk(L) was sufficient to initiate a cellular activation signal in T cells. Recently, Rivera and Brugge (*22*) also showed that aggregation of Syk(L), which had been introduced into RBL cells as part of a chimeric transmembrane protein containing the extracellular and transmembrane domains of CD16 and CD7, induced degranulation, leukotriene synthesis, and expression of cytokine genes. To determine functional differences between the two Syk isoforms, we used a similar experimental

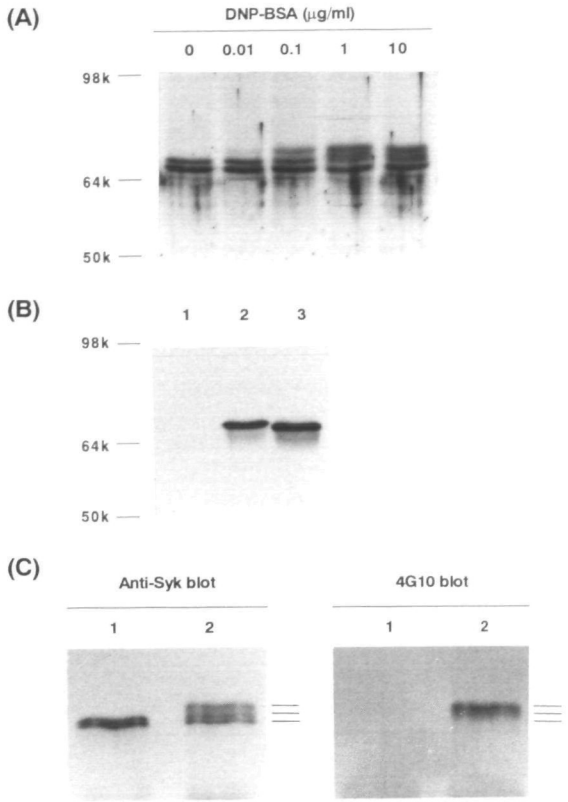

Fig. 1. **(A) Immunoblotting of Syk in lysates of RBL-2H3 cells.** Anti-DNP IgE-sensitized RBL-2H3 cells were stimulated with various amounts of DNP-BSA for 2 min and solubilized with 0.5% Triton-X100. The supernatants equivalent to 5×10^5 cells were subjected to immunoblotting with anti-Syk Ab. SDS-PAGE (8% gel) was performed under reducing conditions. **(B) Anti-Syk immunoblot of lysates of transfected COS-7 cells.** COS-7 cells were transiently transfected with vector (pcDNA1) alone (lane 1) or vector containing cDNA of rat Syk(L) (lane 2) or Syk(S) (lane 3) using the DEAE-dextran method. After 48 h, cells were harvested and solubilized with 0.5% Triton-X100. The lysates equivalent to 1×10^4 cells were subjected to immunoblotting with anti-Syk Ab. **(C) Tyrosine phosphorylation of Syk upon FcεRI aggregation.** Anti-Syk immunoprecipitates equivalent to 5×10^6 cells were prepared from unstimulated (lane 1) or stimulated cells (lane 2), and subjected to immunoblotting with anti-Syk Ab. After stripping of the Ab, the same blot was reprobed with anti-phosphotyrosine mAb 4G10.

system. We constructed gene fusions in which the extracellular and transmembrane domains consist of those of guinea pig type II FcγR, and the cytosolic domains consist of the entire coding region of either long or short form of rat Syk (Fig. 2A). The resulting chimeric cDNA constructs and cDNA encoding guinea pig type II FcγR (FcγRIIB1) were introduced into RBL-2H3 cells. Individual colonies were screened by flow cytometry using anti-FcγRIIB mAb. Several cloned transfectants expressing different levels of chimeric Syk were obtained, and cells stably expressing the highest levels of chimeras or guinea pig FcγRIIB1 were used for experiments [RBL-C2.0, RBL-L9.0, and RBL-S6.5 express guinea pig FcγRIIB1, chimeric Syk(L), and chimeric Syk(S), respectively] (Fig. 2B). Immunoprecipitation of the resulting Syk chimeras with anti-FcγRIIB Ab and *in vitro* kinase assay revealed the chimeras, both of which showed the expected molecular masses, had comparable autophosphorylation activities (Fig. 3). In addition, the chimeras were not tyrosine-phosphorylated in those transfectants.

Aggregation of the Short Syk Isoform Can Induce the Release of Inflammatory Mediators and Cytokine Synthesis—We next examined whether aggregation of the chimeric Syk(S) could induce mast cell activation. The RBL transfectants were sensitized with mouse anti-FcγRIIB mAb and stimulated with goat anti-mouse IgG Ab. The extent of degranulation was determined by measuring the intracellular and extracellular activities of β-hexosaminidase. Consistent with the previous report (*22*), aggregation of Syk(L) chimera on RBL-L9.0 cells resulted in degranulation (Fig. 4A). Similarly, aggregation of Syk(S) chimera on RBL-S6.5 cells induced the β-hexosaminidase release. The levels of degranulation were comparable to those when FcεRI were aggregated. RBL-C2.0 cells expressing guinea pig FcγRIIB did not release β-hexosaminidase after aggregation of FcγRIIB, but did degranulate after aggregation of FcεRI with IgE and antigen. This indicates that the effect of anti-FcγRIIB mAb is mediated by aggregating the Syk chimeras and not by aggregating other cell surface proteins.

We also determined whether aggregation of the Syk(S) chimera could induce leukotriene synthesis and release, since direct crosslinking of the Syk(L) chimera resulted in production of leukotrienes (*22*). As shown in Fig. 4B, aggregation of Syk(S) stimulated leukotriene synthesis and release, as was the case with degranulation.

In mast cells, aggregation of FcεRI induces expression of several cytokine genes including TNF-α (*36, 37*). To assess the ability of Syk(S) to induce cytokine gene expression, RNA levels of TNF-α were examined by RT-PCR analysis. RBL-L9.0 and RBL-S6.5 cells were sensitized with either IgE or anti-FcγRIIB mAb, and stimulated with either antigen or anti-mouse IgG Ab. After 90 min of stimulation, total RNA was isolated and subjected to RT-PCR analysis. As shown in Fig. 5, aggregation of Syk(S) as well as Syk(L) induced synthesis of TNF-α genes as potently as did aggregation of FcεRI. RT-PCR analysis using the primer pair specific to the G3PDH gene showed that equivalent levels of total RNA were analyzed. Thus, direct aggregation of Syk(S) results in the activation of signaling pathways leading to mast cell activation.

Aggregation of the Short Syk Isoform Induces the Tyrosine Phosphorylation of Syk Itself, PLC-γ1, and Vav—We next examined the tyrosine phosphorylation of Syk chimeras upon aggregation, since it is well known that aggregation of FcεRI induces the tyrosine phosphorylation of various signaling molecules including Syk itself. Syk chimeras as well as endogenous Syk were immunoprecipitated from unstimulated or stimulated cells and examined for their tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine Ab 4G10. As shown in Fig. 6, aggregation of Syk chimeras resulted in very weak but substantial tyrosine phosphorylation of the chimera itself [lane 6 for Syk(L), lane 9 for Syk(S)]. Aggregation of Syk chimera did not induce tyrosine phosphorylation of endogenous Syk. This indicates that mediator release and cytokine synthesis of cells induced by aggregation of Syk chimeras is not due to the activation of endogenous Syk. Interestingly, aggregation of FcεRI resulted in very strong tyrosine phosphorylation of chimeric Syk as well as endogenous Syk. This suggests that chimeric Syk as well as

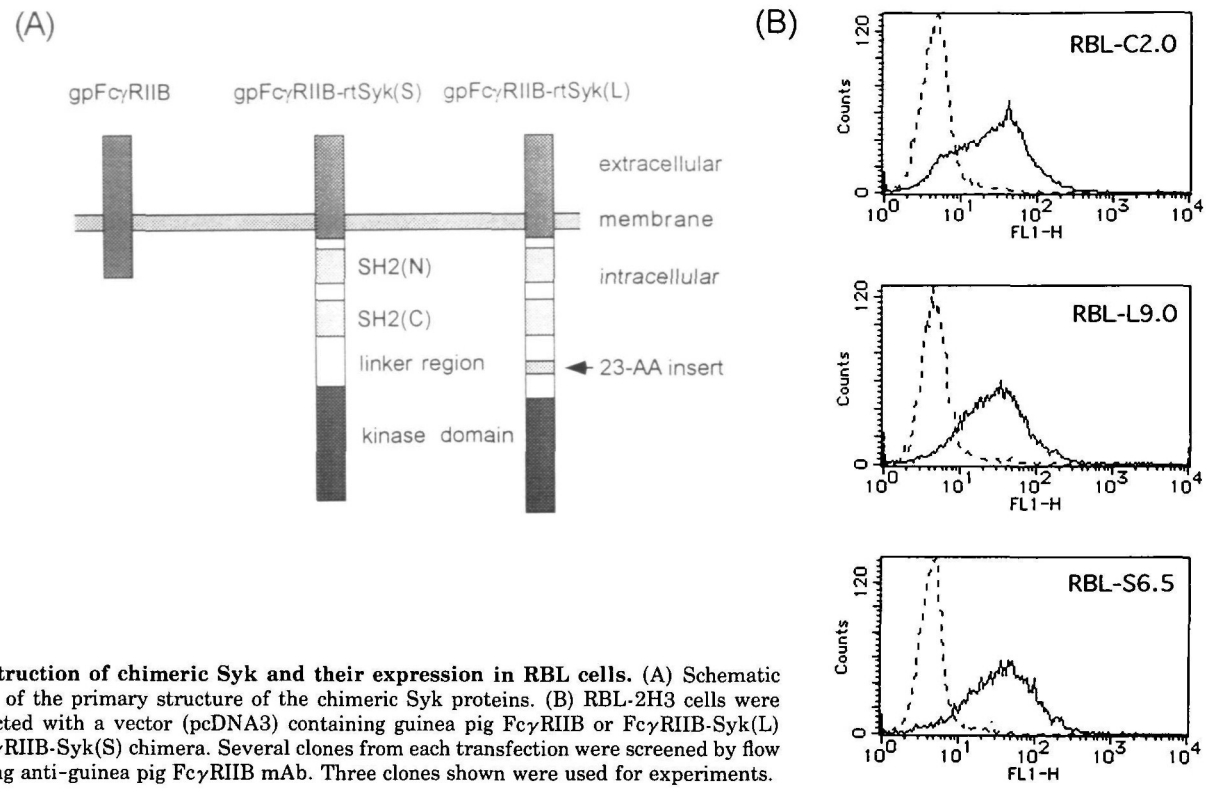

Fig. 2. **Construction of chimeric Syk and their expression in RBL cells.** (A) Schematic representation of the primary structure of the chimeric Syk proteins. (B) RBL-2H3 cells were stably transfected with a vector (pcDNA3) containing guinea pig FcγRIIB or FcγRIIB-Syk(L) chimera or FcγRIIB-Syk(S) chimera. Several clones from each transfection were screened by flow cytometry using anti-guinea pig FcγRIIB mAb. Three clones shown were used for experiments.

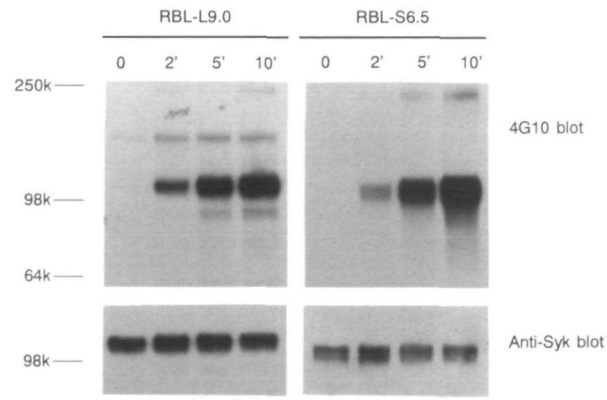

Fig. 3. **Autophosphorylation activity of chimeric Syk.** Chimeric Syk was immunoprecipitated using anti-guinea pig FcγRIIB mAb. Immunoprecipitates were subjected to *in vitro* kinase assay and analyzed with immunoblotting with anti-phosphotyrosine mAb 4G10. After stripping of the Ab, the same blot was reprobed with anti-Syk Ab.

endogenous Syk were recruited to the aggregated FcεRI and efficiently tyrosine-phosphorylated by autophosphorylation and/or FcεRI-associated kinase, probably Lyn.

It is well established that PLC-γ1 and protooncogene Vav are rapidly tyrosine-phosphorylated upon FcεRI aggregation (*38-40*). The phosphorylation of PLC-γ1 leads to its activation and results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol, which act as second messengers leading to a calcium flux and the activation of protein kinase C, respectively. The role of Vav in FcεRI signaling is less clear but Vav may link the mitogen-activated protein kinase (MAP kinase) pathways to FcεRI (*41*). To determine whether aggregation of Syk(S) induces the tyrosine phosphorylation of these signaling molecules, PLC-γ1 and Vav were immunoprecipitated from unstimulated or stimulated RBL transfectants. Antiphosphotyrosine blotting showed that PLC-γ1 became tyrosine-phosphorylated upon aggregation of chimeras on both RBL-L9.0 and RBL-S6.5 cells (Fig. 7A). Similarly, antiphosphotyrosine blotting of the immunoprecipitates with anti-Vav Ab showed that tyrosine phosphorylation of Vav was enhanced by aggregating chimeras as well as aggregating FcεRI (Fig. 7B).

Thus far, we have shown that Syk(S) is potentially capable of triggering activation of multiple signaling pathways that lead to release of allergic mediators by tyrosine phosphorylation of some signaling molecules that exist downstream of Syk.

The Short Form of Syk Binds to Aggregated FcεRI and Is Activated—It has been shown that Syk(L) binds to the aggregated FcεRI (*7, 14, 18*). This binding is mediated by an interaction between the two tandem SH2 domains of Syk and ITAM of FcεRI γ-chain phosphorylated by the Src-family kinase Lyn. Furthermore, this binding leads to Syk activation (*19, 20*). However, as described above, we could not demonstrate the Syk-FcεRI interaction in RBL-2H3 cells. As an alternative approach, we examined the ability of each Syk isoform to bind to aggregated FcεRI *in vitro*. Lysates prepared from COS cells overexpressing Syk(L) or Syk(S) were incubated with FcεRI immunoprecipitates from unstimulated or stimulated cells. Proteins bound to immunoprecipitates were eluted and subjected to immunoblotting analysis with anti-Syk Ab. As shown in Fig. 8,

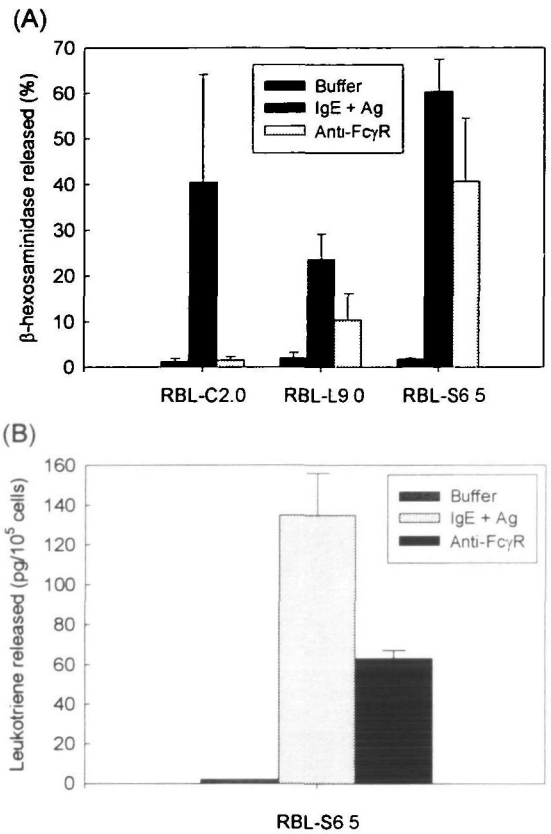

Fig. 4. **Aggregation of the short Syk isoform can induce the release of inflammatory mediators.** Cells were sensitized with saturating amounts of anti-DNP IgE or Fab' fragments of anti-guinea pig FcγRIIB mAb. The IgE-sensitized cells were incubated with buffer alone or 1 μg/ml of DNP-BSA. The mAb-sensitized cells were incubated with 10 μg/ml of goat $F(ab')_2$ anti-mouse IgG. After 40 min, β-hexosaminidase activity was measured from cell lysates and supernatants, and expressed as the percentage of the activity released (A). The same supernatants from cells expressing Syk(S) chimeras were analyzed for the presence of leukotrienes (B). The data shown represent the mean±standard error for at least three separate experiments.

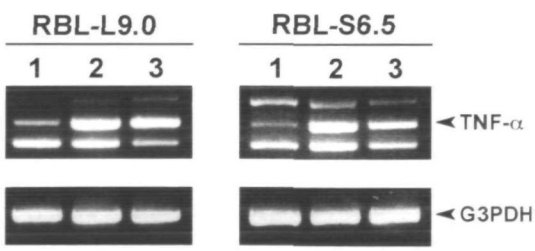

Fig. 5. **Aggregation of the short Syk isoform can induce cytokine synthesis.** RBL-L9.0 cells expressing Syk(L) chimeras or RBL-S6.5 cells expressing Syk(S) chimeras were unstimulated or stimulated as described in Fig. 4. After 90 min of stimulation, total RNAs were extracted and subjected to RT-PCR analysis using primer pairs specific to rat TNF-α or rat G3PDH.

Syk(S) as well as Syk(L) bound to stimulated FcεRI, suggesting that Syk(S) can be recruited to aggregated FcεRI, probably tyrosine-phosphorylated ITAM of γ-chain, in RBL-2H3 cells.

To determine whether the binding to FcεRI enhances the

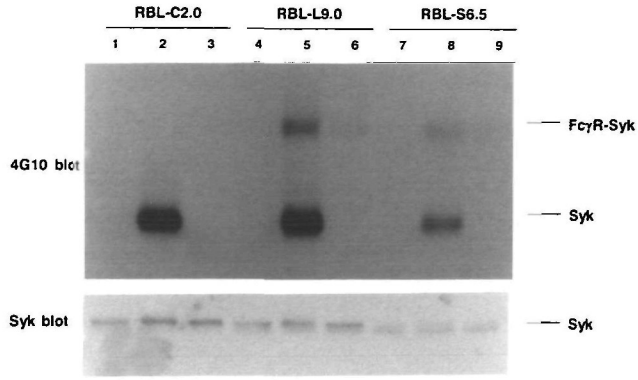

Fig. 6. **Aggregation of Syk induces tyrosine phosphorylation of Syk chimeras.** Cells were unstimulated (lanes 1, 4, 7) or stimulated with IgE and antigen (lanes 2, 5, 8) or anti-FcγRIIB and anti-mouse IgG (lanes 3, 6, 9) at 37°C for 5 min. Cells were then solubilized, immunoprecipitated with anti-Syk, and subjected to immunoblot with anti-phosphotyrosine 4G10. After stripping of the Ab, the same blot was reprobed with anti-Syk Ab.

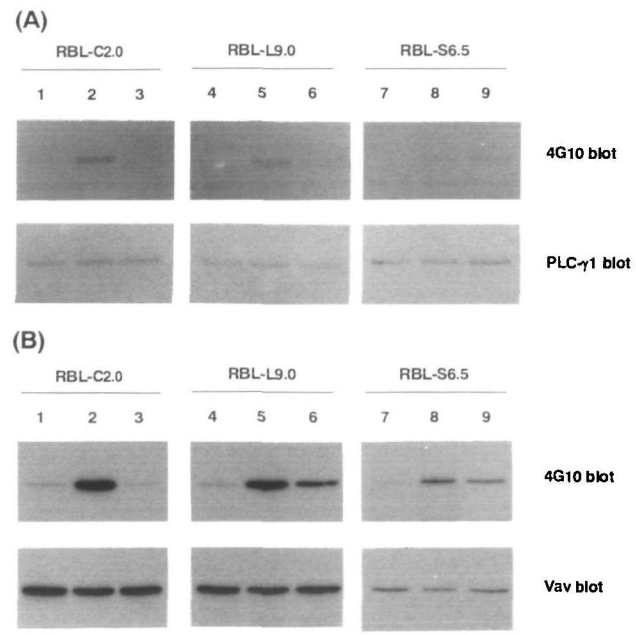

Fig. 7. **Aggregation of Syk induces tyrosine phosphorylation of PLC-γ1 and Vav.** Cells were unstimulated (lanes 1, 4, 7) or stimulated with IgE and antigen (lanes 2, 5, 8) or anti-FcγRIIB and anti-mouse IgG (lanes 3, 6, 9) at 37°C for 5 min. Cell were then solubilized, immunoprecipitated with anti-PLC-γ1 (A) or anti-Vav (B), and subjected to immunoblotting with anti-phosphotyrosine 4G10. The same blots were stripped and reprobed with anti-PLC-γ1 or anti-Vav Ab.

enzymatic activity of Syk(S), each isoform of Syk was immunoprecipitated from transfected COS-7 cells and subjected to *in vitro* kinase assay in the presence or absence of phosphorylated peptides containing the ITAM from the cytoplasmic domain of the γ chain of FcεRI. As a control, Syk isoforms were also immunoprecipitated from unstimulated RBL-2H3 cells. As shown in Fig. 9, the autophosphorylation activity of Syk from RBL-2H3 cells was enhanced by the phosphorylated γ ITAM peptides, which is

consistent with the previous reports (*19, 20*). Both Syk isoforms from transfected COS-7 cells were not tyrosine-phosphorylated before kinase assay, and the addition of the phosphorylated peptides enhanced the autophosphorylation of both Syk isoforms. These results indicate that Syk(S) can bind to FcεRI and this binding leads to its activation, as is the case with Syk(L).

DISCUSSION

In this report, we have shown that Syk(S) is capable of inducing mast cell activation by direct aggregation. Through *in vitro* analysis, it was also established that Syk(S) can bind to aggregated and tyrosine-phosphorylated FcεRI and this binding enhances its enzymatic activity. Taken together, these findings strongly suggest that Syk(S) as well as Syk(L) participates in the signal transduction through mast cell FcεRI.

Syk(S) is an alternative Syk isoform identified in the human basophilic leukemia cell line KU812 and T-cell leukemia cell line Jurkat (*26*). Thereafter a similar isoform was found in the rat basophilic leukemia cell line RBL-2H3 (*27*). In thymus, spleen, and a variety of hematopoietic cell lines, ~10% of *syk* RNA is of the Syk(S) configuration and expression of the Syk(S) protein in these cells is hardly observed. However, in mouse bone marrow cells, the short form represents ~50% of all *syk* transcripts and proteins (*30*). We and others (*27*) also found that RBL-2H3 cells expressed comparable amounts of Syk(S) to those of Syk(L). Therefore it seems likely that the short isoform may have a unique role in bone marrow cell and mast cell signaling.

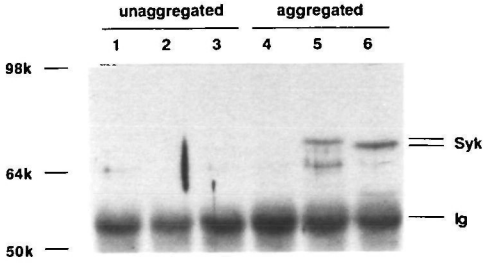

Fig. 8. ***In vitro* association of two Syk isoforms with aggregated FcεRI.** FcεRI immunoprecipitates were prepared from unstimulated (unaggregated) or IgE- and antigen-stimulated (aggregated) RBL-2H3 cells. Immunoprecipitates were incubated with lysates of COS-7 cells transfected with vector alone (lanes 1, 4) or Syk(L) (lanes 2, 5) or Syk(S) (lanes 3, 6) for 1 h at 4°C. The washed immunoprecipitates were subjected to immunoblotting with anti-Syk Ab.

To examine the role of each Syk isoform in mast cell signaling, we used chimeric transmembrane Syk, because the previous studies (*22, 29*) has shown the usefulness of this strategy. In those studies, a chimeric kinase consisting of Syk fused to the transmembrane domain of CD7 and the extracellular domain of CD16 was introduced in T-cell leukemia cell line Jurkat or RBL-2H3. By anti-CD16 Ab and secondary Ab, chimeric Syk was aggregated, then probably activated by autophosphorylation, and triggered the signaling pathways leading to mast cell activation. Thus, aggregating chimeric Syk mimics recruitment of endogenous Syk and its activation upon receptor aggregation. Based on the results in Fig. 6, aggregation of chimeric Syk does not seem to activate endogenous Syk, which allows us to examine the specific effects of Syk activation. One problem of those studies is that the chimeric Syk is tyrosine-phosphorylated before its aggregation, while the endogenous Syk in unstimulated cells is not. The study using Lyn-deficient B cells suggested that Lyn is required for full activation of Syk (*42*). Therefore, it had remained unexplored whether basal phosphorylation of Syk is mandatory for activating downstream signaling pathway in this experimental system. In contrast to those previous studies, our chimeric Syk, which contains the extracellular and transmembrane domains of guinea pig FcγRIIB, was not tyrosine-phosphorylated and became tyrosine-phosphorylated upon its aggregation (Fig. 6). Thus, it is likely that aggregating tyrosine-unphosphorylated Syk is sufficient to induce mast cell activation. The reason why our Syk chimeras are not tyrosine-phosphorylated before aggregation is unclear. It was reported that, when transiently expressed in COS cells, unlike CD8-Syk chimera, its kinase-negative version was not tyrosine-phosphorylated, suggesting that the tyrosine phosphorylation is due to the chimera's autophosphorylation activity (*43*). We reported that the mobility of guinea pig FcγRIIB on the cell surface was reduced by interaction between the extracellular domain of FcγRIIB and unknown membrane proteins (*44*). Therefore the possible reduced mobility of our chimeras may prevent their spontaneous aggregation and their tyrosine phosphorylation. However, when overexpressed in COS-7 cells, our chimeras were heavily tyrosine-phosphorylated (data not shown), suggesting that the extent of tyrosine phosphorylation of chimeras may be also depen-

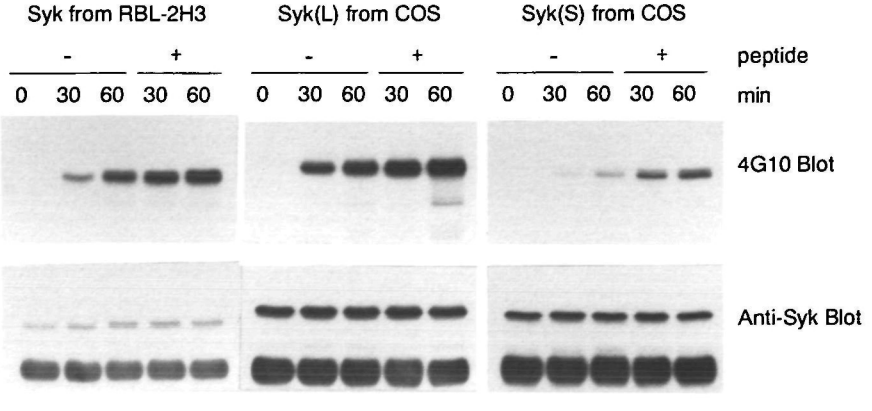

Fig. 9. **Effect of tyrosine-diphosphorylated γ ITAM peptides on autophosphorylation activity of two Syk isoforms.** Anti-Syk immunoprecipitates from RBL-2H3 cells and Syk(L)- or Syk(S)-transfected COS-7 cells were preincubated with the diphosphorylated γ ITAM peptides. Then the samples were subjected to *in vitro* kinase assay for the indicated times. The precipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10. After stripping of the Ab, the same blots were reprobed with anti-Syk Ab.

dent on their density on the cell surface.

Our analysis indicated that the long and short form of Syk exhibited comparable activities to induce mast cell activation, which implies that the 23-amino acid insert found in Syk(L) is not obligatory for mast cell signaling. Interestingly, this insert contains a potential tyrosine phosphorylation site highly conserved among species analyzed to date. In fact, the tyrosine in the insert (Y290 in rat) is one of the autophosphorylation sites (*45*). Furthermore, the sequence YSFP in the insert agrees with the YXXP motif that the SH2 domain of PLC-γ1 has been predicted to bind by using a phosphopeptide library (*46*). However, PLC-γ1 fails to bind to this site and instead binds to other sites in the linker region [Y342, Y346 in rat Syk(L)] (*43*). These tyrosine residues exist in Syk(S), and besides PLC-γ1, the SH2 domain of Vav has been shown to bind to these phosphorylated tyrosines (*47*). Thus, while the linker region of Syk may be involved in signaling, tyrosine phosphorylation of Y290, but not Y342 and Y346, is not mandatory. The 23-amino acid insert is also deleted in ZAP-70 kinase, another member of the Syk family. In B cells it was reported that ZAP-70 and Syk(L) are functionally homologous in antigen receptor signaling (*48*). This also suggests that the insert is not required for signaling.

So far the functional difference between the two isoforms of Syk is not clear. Both isoforms were reported to exhibit comparable enzymatic activity (*30*). Using the methods employed here, we were not able to observe any notable functional difference. However, although not required for mast cell signaling leading to mast cell secretion of allergy mediators, it is probable that the 23-amino acid insert may play an important role in other mast cell functions such as redistribution of actin filaments leading to changes in cell morphology. Because we used an artificial, membrane-localized chimeric Syk, we might have missed the possible functional difference. It was reported that activated and tyrosine-phosphorylated Syk was found predominantly in the soluble fraction and was not associated with components of the antigen receptor in B cells (*49*). Similarly, we were unable to demonstrate the association of Syk with FcεRI in RBL-2H3 cells, even though a large fraction of Syk (~40%) became tyrosine-phosphorylated upon FcεRI aggregation (Fig. 1A), suggesting that once recruited and tyrosine-phosphorylated, Syk is released from the aggregated FcεRI. In addition, it has been reported that activated and released Syk interacts with and phosphorylates α-tubulin (*49*). The deletion in the linker region may remove possible binding sites for such cellular proteins. On the other hand, the deletion may affect cellular localization of Syk in resting cells and, therefore, the extent of its recruitment to FcεRI upon receptor aggregation. These hypotheses are currently being tested. Obviously, it will be interesting to test whether transfection of Syk(S) in the Syk-deficient RBL cells (*25*) reconstitutes FcεRI-induced mast cell activation.

We gratefully acknowledge gifts of RBL-2H3 cells and H1 DNP-ε-26.82 hybridomas from Drs. Henry Metzger and Juan Rivera, NIAMS/NIH.

REFERENCES

1. Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.P., Pribluda, V., and Quarto, R. (1986) The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* **4**, 419-470
2. Ravetch, J.V. and Kinet, J.P. (1991) Fc receptors. *Annu. Rev. Immunol.* **9**, 457-492
3. Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J.P. (1989) Complete structure and expression in transfected cells of high affinity IgE receptor. *Nature* **337**, 187-189
4. Benhamou, M. and Siraganian, R.P. (1992) Protein-tyrosine phosphorylation: an essential component of FcεRI signaling. *Immunol. Today* **13**, 195-197
5. Eiseman, E. and Bolen, J.B. (1992) Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* **355**, 78-80
6. Hutchcroft, J.E., Geahlen, R.L., Deanin, G.G., and Oliver, J.M. (1992) FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. USA* **89**, 9107-9111
7. Jouvin, M.H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A., and Kinet, J.P. (1994) Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.* **269**, 5918-5925
8. Yamashita, T., Mao, S.Y., and Metzger, H. (1994) Aggregation of the high-affinity IgE receptor and enhanced activity of $p53/56^{lyn}$ protein-tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **91**, 11251-11255
9. Oliver, J.M., Burg, D.L., Wilson, B.S., McLaughlin, J.L., and Geahlen, R.L. (1994) Inhibition of mast cell FcεRI-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J. Biol. Chem.* **269**, 29697-29703
10. Reth, M. (1989) Antigen receptor tail clue. *Nature* **338**, 383-384
11. Weiss, A. (1993) T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73**, 209-212
12. Cambier, J.C., Pleiman, C.M., and Clark, M.R. (1994) Signal transduction by the B cell antigen receptor and its coreceptors. *Annu. Rev. Immunol.* **12**, 457-486
13. Cambier, J.C. (1995) New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL). *Immunol. Today* **16**, 110
14. Kihara, H. and Siraganian, R.P. (1994) Src homology 2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high affinity IgE receptor. *J. Biol. Chem.* **269**, 22427-22432
15. Vonakis, B.M., Chen, H., Haleem-Smith, H., and Metzger, H. (1997) The unique domain as the site on Lyn kinase for its constitutive association with the high affinity receptor for IgE. *J. Biol. Chem.* **272**, 24072-24080
16. Johnson, S.A., Pleiman, C.M., Pao, L., Schneringer, J., Hippen, K., and Cambier, J.C. (1995) Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J. Immunol.* **155**, 4596-4603
17. Clark, M.R., Johnson, S.A., and Cambier, J.C. (1994) Analysis of Ig-α-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-α stimulation of Fyn activity. *EMBO J.* **13**, 1911-1919
18. Benhamou, M., Ryba, N.J., Kihara, H., Nishikata, H., and Siraganian, R.P. (1993) Protein-tyrosine kinase $p72^{syk}$ in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor γ chain after receptor aggregation. *J. Biol. Chem.* **268**, 23318-23324
19. Shiue, L., Zoller, M.J., and Brugge, J.S. (1995) Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE. *J. Biol. Chem.* **270**, 10498-10502
20. Kimura, T., Sakamoto, H., Appella, E., and Siraganian, R.P. (1996) Conformational changes induced in the protein tyrosine kinase $p72^{syk}$ by tyrosine phosphorylation or by binding of phosphorylated immunoreceptor tyrosine-based activation motif peptides. *Mol. Cell. Biol.* **16**, 1471-1478
21. Scharenberg, A.M., Lin, S., Cuenod, B., Yamamura, H., and Kinet, J.P. (1995) Reconstitution of interactions between tyrosine kinases and the high affinity IgE receptor which are controlled by receptor clustering. *EMBO J.* **14**, 3385-3394

22. Rivera, V.M. and Brugge, J.S. (1995) Clustering of Syk is sufficient to induce tyrosine phosphorylation and release of allergic mediators from rat basophilic leukemia cells. *Mol. Cell. Biol.* **15**, 1582-1590
23. Taylor, J.A., Karas, J.L., Ram, M.K., Green, O.M., and Seidel-Dugan, C. (1995) Activation of the high-affinity immunoglobulin E receptor FcεRI in RBL-2H3 cells is inhibited by Syk SH2 domains. *Mol. Cell. Biol.* **15**, 4149-4157
24. Costello, P.S., Turner, M., Walters, A.E., Cunningham, C.N., Bauer, P.H., Downward, J., and Tybulewicz, V.L. (1996) Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene* **13**, 2595-2605
25. Zhang, J., Berenstein, E.H., Evans, R.L., and 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
26. Yagi, S., Suzuki, K., Hasegawa, A., Okumura, K., and Ra, C. (1994) Cloning of the cDNA for the deleted syk kinase homologous to ZAP-70 from human basophilic leukemia cell line (KU812). *Biochem. Biophys. Res. Commun.* **200**, 28-34
27. Rowley, R.B., Bolen, J.B., and Fargnoli, J. (1995) Molecular cloning of rodent $p72^{syk}$. Evidence of alternative mRNA splicing. *J. Biol. Chem.* **270**, 12659-12664
28. Taniguchi, T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamura, S., and Yamamura, H. (1991) Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. *J. Biol. Chem.* **266**, 15790-15796
29. Kolanus, W., Romeo, C., and Seed, B. (1993) T cell activation by clustered tyrosine kinases. *Cell* **74**, 171-183
30. Latour, S., Chow, L.M.L., and Veillette, A. (1996) Differential intrinsic enzymatic activity of Syk and Zap-70 protein-tyrosine kinases. *J. Biol. Chem.* **271**, 22782-22790
31. Liu, F.T., Bohn, J.W., Ferry, E.L., Yamamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R., and Katz, D.H. (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J. Immunol.* **124**, 2728-2737
32. Shimamura, T., Nakamura, T., and Koyama, J. (1986) Analysis of Fc receptors for IgG on guinea pig peritoneal macrophages by the use of a monoclonal antibody to one of the Fc receptors. *J. Biochem.* **99**, 227-235
33. Farah, F.S., Kern, M., and Eisen, H.N. (1960) The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group. *J. Exp. Med.* **112**, 1195-1210
34. Yamashita, T., Shinohara, K., and Yamashita, Y. (1993) Expression cloning of complementary DNA encoding three distinct isoforms of guinea pig Fc receptor for IgG1 and IgG2. *J. Immunol.* **151**, 2014-2023
35. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
36. Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P., and Paul, W.E. (1989) Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature* **339**, 64-67
37. Ohno, I., Tanno, Y., Yamauchi, K., and Takishima, T. (1990) Gene expression and production of tumour necrosis factor by a rat basophilic leukaemia cell line (RBL-2H3) with IgE receptor triggering. *Immunology* **70**, 88-93
38. Atkinson, T.P., Kaliner, M.A., and Hohman, R.J. (1992) Phospholipase C-γ1 is translocated to the membrane of rat basophilic leukemia cells in response to aggregation of IgE receptors. *J. Immunol.* **148**, 2194-2200
39. Li, W., Deanin, G.G., Margolis, B., Schlessinger, J., and Oliver, J.M. (1992) FcεRI-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor βγ2 complex, in RBL-2H3 rat basophilic leukemia cells. *Mol. Cell. Biol.* **12**, 3176-3182
40. Margolis, B., Hu, P., Katzav, S., Li, W., Oliver, J.M., Ullrich, A., Weiss, A., and Schlessinger, J. (1992) Tyrosine phosphorylation of vav proto-oncogene product containing SH2 domain and transcription factor motifs. *Nature* **356**, 71-74
41. Hirasawa, N., Scharenberg, A., Yamamura, H., Beaven, M.A., and Kinet, J.P. (1995) A requirement for Syk in the activation of the microtubule-associated protein kinase/phospholipase A2 pathway by FcεRI is not shared by a G protein-coupled receptor. *J. Biol. Chem.* **270**, 10960-10967
42. Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, T., and Yamamura, H. (1994) Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. *J. Exp. Med.* **179**, 1725-1729
43. Law, C.L., Chandran, K.A., Sidorenko, S.P., and Clark, E.A. (1996) Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Mol. Cell. Biol.* **16**, 1305-1315
44. Isashi, Y., Yamashita, T., Nagasawa, S., Tanaka, K., Murakami, M., and Uede, T. (1998) The mechanism by which proteolysis enhances the ligand binding activity of guinea pig type II Fc receptor for IgG (FcγRIIB). *J. Biochem.* **123**, 959-967
45. Furlong, M.T., Mahrenholz, A.M., Kim, K.H., Ashendel, C.L., Harrison, M.L., and Geahlen, R.L. (1997) Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk. *Biochim. Biophys. Acta* **1355**, 177-190
46. Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fafardo, J.E., Chou, M.M., Hanafusa, J., Schaffhausen, B., and Cantley, L.C. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-778
47. Deckert, M., Tartare-Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996) Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. *Immunity* **5**, 591-604
48. Kong, G.H., Bu, J.Y., Kurosaki, T., Shaw, A.S., and Chan, A.C. (1995) Reconstitution of Syk function by the ZAP-70 protein tyrosine kinase. *Immunity* **2**, 485-492
49. Peters, J.D., Furlong, M.T., Asai, D.J., Harrison, M.L., and Geahlen, R.L. (1996) Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates α-tubulin on tyrosine. *J. Biol. Chem.* **271**, 4755-4762