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Clustering of Syk Is Sufficient To Induce Tyrosine Phosphorylation and Release of Allergic Mediators from Rat Basophilic Leukemia Cells

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In mast cells, antigen-mediated aggregation of the high-affinity receptor for immunoglobulin E, FcεRI, stimulates tyrosine phosphorylation and activation of multiple signaling pathways leading to the release of several classes of mediators of the allergic response. Early events induced upon cross-linking of FcεRI include tyrosine phosphorylation of FcεRI subunits and activation of the tyrosine kinase p72^{syk} (Syk), which binds to tyrosine-phosphorylated FcεRI. Clustering of Syk, as a result of its interaction with aggregated FcεRI, may play a role in activating one or more of the signaling pathways leading to mediator release. To test this possibility, Syk was introduced into a model mast cell line (rat basophilic leukemia cells) as part of a chimeric transmembrane protein containing the extracellular and transmembrane domains of CD16 and CD7, respectively. Clustering of the Syk chimera, using antibodies against CD16, was found to be sufficient to stimulate early and late events normally induced by clustering of FcεRI. Specifically, aggregation of Syk induced degranulation, leukotriene synthesis, and expression of cytokine genes. Induction of mediator release was dependent on the kinase activity of Syk. Consistent with this finding, clustering of Syk also induced the tyrosine phosphorylation of a profile of proteins, including phospholipase C-γ1 and mitogen-activated protein kinase, similar to that induced upon clustering of FcεRI. These results strongly suggest that Syk is an early and critical mediator of multiple signaling pathways that emanate from the FcεRI receptor and give rise to the allergic response.

Antigen-induced aggregation of the high-affinity receptor for immunoglobulin E (IgE), FcεRI, induces mast cells to release several classes of mediators that activate both early and late phases of the allergic response (37, 49). Mediators released upon mast cell activation fall into three classes which are represented by (i) histamines, which are preformed and secreted from granules within minutes of activation; (ii) leukotrienes and prostaglandins, which are newly generated from lipid precursors and are also rapidly released upon activation; and (iii) cytokines, which are newly expressed and released within the first few hours after activation.

Activation of the multiple signaling pathways that lead to the synthesis and release of these diverse mediators is preceded by aggregation-induced tyrosine phosphorylation of two subunits of FcεRI (33, 40). Subsequent events include (i) the increased tyrosine phosphorylation and activation of a number of signaling molecules, including Syk (7, 23), phospholipase C-γ1 (PLC-γ1) (2, 19, 41), Vav (36), and mitogen-activated protein (MAP) kinase (46); (ii) activation of protein kinase C (55, 56) and phospholipase A_2 (20); and (iii) elevation of intracellular calcium levels (6). Tyrosine phosphorylation appears to play a critical role in propagating the signal leading to these events since mediator release is blocked by tyrosine kinase inhibitors (27, 57).

FcεRI is a multisubunit receptor composed of an α chain, which binds the Fc portion of IgE, a β chain, and a homodimer of disulfide-linked γ chains (8). The cytoplasmic domains of the β and γ chains contain a sequence known as the immunoreceptor tyrosine activation motif (ITAM; also referred to as the antigen response activation motif or antigen receptor homology 1 motif) which is also found in the signal transducing components of other multisubunit receptors, including T- and B-cell receptors (12, 44, 54). Upon activation, ITAMs are phosphorylated on two tyrosine residues within the consensus D/ExxYxxL/I$(X)_{6-8}$YxxL/I (42, 45). The critical role of ITAMs in transducing signals was revealed by analyzing the effects of aggregating chimeric proteins containing an intracellular ITAM-containing polypeptide fused to the transmembrane and extracellular domains of an unrelated protein (54). Aggregation of such ITAM-containing proteins was sufficient to induce both early and late signaling events normally observed upon clustering of the intact oligomeric receptors in a manner that was dependent on the two tyrosines within the motif. In mast cells, clustering of a chimeric protein containing the cytoplasmic domain of the FcεRI γ chain is sufficient to induce tyrosine phosphorylation, elevation of intracellular calcium levels, and degranulation (26, 32).

The rapid tyrosine phosphorylation of FcεRI in the absence of any intrinsic kinase activity of the receptor suggests the presence of a closely associated tyrosine kinase. Members of the Src family of tyrosine kinases are associated with FcεRI as well as the T- and B-cell receptors prior to receptor cross-linking (12, 18, 54), and evidence suggests that they are responsible for phosphorylating the ITAMs upon receptor cross-linking (14, 22, 25). In mast cells, this role is likely to be fulfilled by Lyn, which is basally associated with the β chain and becomes tyrosine phosphorylated and activated upon clustering of FcεRI (18, 23, 26).

The aggregation-induced phosphorylation of the two tyrosines of the ITAMs allows members of the Syk/ZAP-70 family of tyrosine kinases to bind. In contrast to Src family kinases, Syk and ZAP-70 lack both an amino-terminal myristoylation site and a carboxy-terminal negative regulatory site and contain two Src homology 2 (SH2) domains (14, 51). These tandem SH2 domains mediate high-affinity interactions with doubly phosphorylated ITAMs (25, 28, 48, 53). While ZAP-70, and

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to a lesser degree Syk, is expressed in T cells, only Syk is expressed in mast cells and B cells (12, 15). In mast cells, Syk associates specifically with the doubly phosphorylated γ chain, allowing its recruitment to the membrane (7, 28, 48). The association of Syk and ZAP-70 with clustered ITAM-containing receptors also correlates with increased tyrosine phosphorylation and activation of these kinases (7, 13, 23, 24, 38).

A critical role for these kinases in T-cell activation was recently demonstrated by analyzing the effects of cross-linking chimeric transmembrane proteins bearing Syk or Src family kinase intracellular domains and unrelated transmembrane and extracellular domains. In this system, early and late signaling events normally induced by T-cell receptor clustering, including tyrosine phosphorylation, calcium mobilization and cytolytic effector function, could be mimicked by the clustering of Syk or the coclustering of ZAP-70 and a Src family kinase (30).

In mast cells, aggregation of Syk through its interaction with phosphorylated and aggregated FcεRI γ chain may play a critical role in triggering one or more of the signaling pathways leading to the release of mediators. To test this possibility, Syk was introduced into rat basophilic leukemia 2H3 (RBL) cells, a model mucosal mast cell line, as part of a chimeric transmembrane protein. Clustering of the Syk chimera was found to be sufficient to induce downstream responses normally induced upon antigen-mediated clustering of the entire FcεRI. These responses include the induction of tyrosine phosphorylation and the synthesis and release of all three classes of allergic mediators. Moreover, induction of these events required the kinase activity of Syk. Thus, Syk is likely to be a critical mediator of the signals induced by cross-linking of FcεRI.

MATERIALS AND METHODS

Cell culture. RBL cells were grown in minimal essential medium (GIBCO-BRL) supplemented with 15% fetal bovine serum (GIBCO-BRL), 100 U of penicillin and 100 μg of streptomycin (GIBCO-BRL) per ml, and 2 mM glutamine (GIBCO-BRL) at 37°C in 5% CO_2. At least 1 h prior to stimulation with 2,4-dinitrophenol (DNP)–bovine serum albumin (BSA) (Zymed), FcεRI was loaded with mouse monoclonal anti-DNP IgE by addition of a 1:100 dilution of conditioned medium from the hybridoma cell line TIB-142 (American Type Culture Collection).

Generation and analysis of cell lines expressing a chimeric Syk protein. To express in RBL cells a chimeric kinase consisting of human Syk fused to the transmembrane domain of CD7 and the extracellular domain of CD16, the DNA fragment encoding this tripartite gene fusion (16:7:Syk) (30) was cloned into the pRc/CMV expression vector (Invitrogen). Ten micrograms of the expression vector alone or of the vector expressing 16:7:Syk was electroporated into RBL cells, and stably transfected clones were selected with 600 μg of G418 (GIBCO-BRL) per ml. Several hundred clones from each electroporation were pooled. Surface expression was analyzed on a FACSort (Becton Dickinson) after staining with fluorescein isothiocyanate-labeled anti-CD16 antibodies (3G8-FITC; Medarex), and cells expressing the highest levels of 16:7:Syk chimera on the surface were selected. For analysis, 2.5×10^5 cells were plated in each well of a 24-well dish (in triplicate) or 7.5×10^6 cells were plated in a 100-mm-diameter dish 4 to 6 h prior to stimulation. It was noted that optimal levels of mediator release induced by clustering of Syk were obtained by plating cells 4 to 6 h, rather that 18 to 24 h, prior to stimulation due to increased surface expression of the chimera (data not shown).

Infection of RBL cells with vaccinia virus recombinants. Vaccinia virus recombinants expressing the chimeric proteins containing wild-type porcine Syk (16:7:Syk), human Syk in which the lysine in the ATP binding site was mutated to glycine (amino acid 397 according to numbering in reference 30) (16:7:Syk k-), and 16:7 alone were a generous gift (B. Seed and W. Kolanus, Harvard Medical School) and were generated as described previously (30). Viral stocks were prepared and viral titer was determined according to standard procedures (3). To infect RBL cells with recombinant virus, 2×10^5 cells were plated overnight in each well of a 24-well dish. Cells were mock infected or infected with recombinant vaccinia virus, in triplicate, at a multiplicity of infection of 20 for 1 h in serum-free minimal essential medium and further incubated for 4 h in minimal essential medium containing 2.5% fetal bovine serum. To obtain enough lysate for analysis of tyrosine-phosphorylated proteins, 6×10^6 cells plated in a 100-mm-diameter dish were infected. Under these conditions of infection, chimeric proteins were expressed efficiently and negative effects of vaccinia virus infection on mediator release were minimized.

Analysis of mediator release. To assay mediator release, stable cell lines or cells infected with vaccinia virus recombinants were plated in 24-well dishes and loaded with anti-DNP IgE. Cells were washed twice in assay buffer [25 mM disodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Na_2PIPES; pH 7.1), 100 mM NaCl, 5 mm KCl, 5 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 1% BSA] and incubated in 200 μl of assay buffer either alone or containing DNP-BSA (0.1 μg/ml) or a combination of mouse monoclonal anti-CD16 antibodies (3G8; 0.5 μg/ml; Medarex) and goat anti-mouse IgG1 antibodies (2.5 μg/ml; Southern Biotechnology) that had been premixed (this treatment was as effective as first incubating cells with anti-CD16 and then adding anti-IgG1 antibodies). Following stimulation for various amounts of time, the culture supernatant was collected and the cells were lysed by addition of 200 μl of assay buffer containing 0.2% Triton X-100.

To assay β-hexosaminidase activity, aliquots (10 μl) of culture supernatant and cell lysate obtained after 40 min of stimulation were incubated with 50 μl of 4 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M citrate phosphate buffer (pH 4.5) at 37°C for 30 min. At the end of the incubation, 150 μl of 0.2 M glycine (pH 10.7) was added. The A_{405} was measured. Each value (mean ± standard error) was expressed as the percentage of total β-hexosaminidase that was released into the supernatant.

To detect secreted leukotrienes, aliquots (50 μl) of culture supernatant collected after 40 min of stimulation were analyzed using the leukotriene $C_4/D_4/E_4$ enzyme immunoassay system (Amersham) according to the manufacturer's instructions.

To detect secreted tumor necrosis factor alpha (TNF-α), cells were stimulated for 3 h as described above but in complete medium instead of assay buffer. Aliquots (50 μl) of culture supernatant were analyzed by using a mouse TNF-α enzyme-linked immunosorbent assay kit (Genzyme). This assay utilizes hamster and goat anti-mouse TNF-α antibodies that cross-react with rat TNF-α. Quantitation of rat TNF-α secreted from RBL cells was based on a standard curve obtained with recombinant mouse TNF-α.

Northern (RNA) analysis. Total cytoplasmic RNA was isolated from 100-mm-diameter dishes by lysis in Nonidet P-40 (NP-40) (3), and 20-μg samples were fractionated by electrophoresis through 0.2 M formaldehyde–1% agarose gels. The RNA was transferred to Hybond-N+ (Amersham) and cross-linked to the nylon by UV irradiation. Following hybridization under conditions recommended by the manufacturer, membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 30 min at 42°C and then for 30 min at 68°C. ^{32}P-labeled probes were prepared by using a Prime-it (Stratagene) random primer labeling kit, using gel-purified cDNA fragments from the murine interleukin-3 (IL-3; American Type Culture Collection) gene and the murine c-*fos* and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes (gifts from M. Greenberg, Harvard Medical School). After autoradiography, membranes were stripped of radioactivity by boiling in 0.5% SDS before reprobing.

Immunoprecipitation and immunoblotting. Cell lines or infected cells stimulated in 100-mm-diameter dishes as described above were rinsed in cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM $NaHPO_4$, 1.5 mM KH_2PO_4 [pH 7.2]) and lysed in one of the following buffers: NP-40 lysis buffer (1% NP-40, 100 mM NaCl, 20 mM Tris [pH 7.5], 10% glycerol), radioimmunoprecipitation assay (RIPA) buffer (1.6 mM NaCl, 10 mM Tris [pH 7.2], 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100), or SDS lysis buffer (0.5% SDS, 10 mM Tris [pH 7.5]). In addition, each buffer contained 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, and 10 U of aprotinin per ml. Cells lysed in SDS buffer were boiled for 5 min and then diluted by the addition of 4 volumes of 1.25× RIPA buffer without SDS. Lysates were clarified by centrifugation at 10,000 × *g* for 30 min. Protein concentration was determined by using a Lowry protein assay (Bio-Rad).

Immunoprecipitations were performed by incubating 500 to 1,000 μg of total cell protein with the following antibodies for 2 h: rabbit polyclonal anti-β, -γ, and -Syk (all gifts from J. Bolen, Bristol-Myers Squibb) and mixed monoclonal anti-PLC-γ1 antibodies (Upstate Biotechnology Inc.). Antibodies were collected by the addition of Pansorbin (Calbiochem) for 40 min, with or without prebound rabbit anti-mouse antibody, and washed three times with 500 μl of NP-40 or RIPA buffer.

Immunoblotting was performed by separating immunoprecipitates or 50 μg of total cell protein by SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to polyvinylidene difluoride membranes (0.2-μm pore size; Bio-Rad), and the membranes were incubated for 1 h in blocking buffer (5% BSA, 150 mM NaCl, 10 mM Tris [pH 8.0], 0.1% Tween 20). Membranes were subsequently probed with antibodies at the following concentrations: anti-β, -γ, and -Syk, 1:500 dilution; anti-MAP kinase (Upstate Biotechnology Inc.), 1 μg/ml; and antiphosphotyrosine antibody 4G10 (a gift from T. Roberts, Dana Farber Cancer Institute, and B. Druker, Oregon Health Sciences University), 0.2 μg/ml. Secondary antibody (Bio-Rad) was used at a 1:20,000 dilution, and reactive proteins were visualized by enhanced chemiluminescence (Amersham).

RESULTS

Introduction of a chimeric transmembrane Syk protein into RBL cells. To analyze the role of Syk in mast cell activation events which are normally induced by antigen-mediated cross-linking of FcεRI, we examined the events induced by activating Syk independently of FcεRI. This was accomplished by clustering an artificial chimeric kinase consisting of Syk fused to the transmembrane domain of CD7 and the extracellular domain of CD16 (16:7:Syk) (30). A vector directing the expression of 16:7:Syk was stably introduced into RBL cells, and pools of clones expressing the highest levels of the chimera on the surface were sorted by using antibodies against CD16. Staining with anti-CD16 antibodies revealed the efficient surface expression of the 16:7:Syk chimera in the selected clones and the lack of anti-CD16 reactivity in the clones transfected with the expression vector alone (Fig. 1A). Immunoblot analysis using anti-Syk antibodies demonstrated that the level of expression of the Syk chimera is similar to that of the endogenous Syk (data not shown). In these cells, intracellular Syk, tethered to the plasma membrane, can be clustered by using antibodies against the extracellular CD16 portion of the chimera and the appropriate anti-IgG secondary antibody. In the same cells, FcεRI can be clustered by the addition of antigen, DNP coupled to BSA, following pretreatment with IgE specific for DNP.

Clustering of Syk is sufficient to induce degranulation. Aggregation of FcεRI leads to the rapid exocytotic release of preformed mediators stored in secretory granules. The extent of RBL cell degranulation can be determined by monitoring the intracellular and extracellular activity of β-hexosaminidase, an acid hydrolase that is stored in the secretory granules and coreleased with other mediators such as histamine and serotonin. Clustering of FcεRI with DNP led to the secretion of approximately 50% of the preformed β-hexosaminidase from both control cells and cells stably transfected with the 16:7:Syk chimera (Fig. 1B). Strikingly, clustering of the Syk chimera with anti-CD16 antibodies induced RBL cells to degranulate to an extent comparable to that induced by clustering of FcεRI. Addition of anti-CD16 antibodies to control cells transfected with the expression vector alone had no effect on β-hexosaminidase release (Fig. 1B), indicating that the effects of anti-CD16 antibodies are mediated by clustering the 16:7:Syk chimeric protein and not by clustering of an endogenous RBL cell surface protein.

Clustering of Syk is sufficient to induce leukotriene synthesis and release. A second signaling pathway activated by clustering of FcεRI leads to the rapid generation of arachidonic acid from phospholipids and its subsequent metabolism to form leukotrienes and prostaglandins which are then released from the cell. To assess activation of this signaling pathway, the amount of leukotrienes released following clustering of FcεRI or Syk was determined. As was the case with β-hexosaminidase release, clustering of Syk stimulated leukotriene synthesis and release as potently as did clustering of FcεRI (Fig. 1C). These results indicate that the pathways leading to degranulation and production of leukotrienes diverge downstream of Syk activation.

Clustering of Syk is sufficient to induce cytokine synthesis and release. A third signaling pathway activated by clustering of FcεRI leads to increased gene expression and the release of cytokines. Studies in a variety of mast cell lines have shown that genes whose expression is induced include several immediate-early genes as well as a number of proinflammatory and growth factor-type cytokines, including IL-1, -3, -4, -5, and -6 and TNF-α (37). However, any given mast cell type does not necessarily express all of these cytokines (11). In RBL cells, FcεRI activation has been shown to the induce rapid expression of two immediate-early genes, c-*fos* and c-*jun*, and expression and secretion of TNF-α (4, 39, 43). The products of the c-*fos* and c-*jun* genes are major components of the transcription factor AP-1 and are likely to play a role in the regulation of downstream genes such as cytokines.

To assess the ability of Syk aggregation to induce new gene

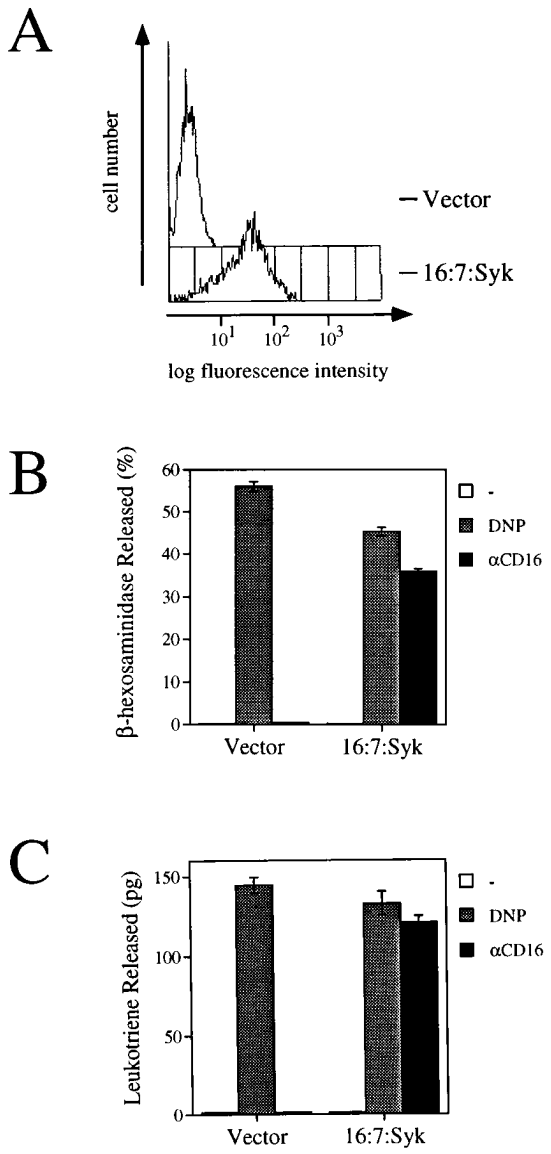

FIG. 1. Clustering of Syk induces degranulation and leukotriene release. RBL cells stably transfected with a vector expressing the 16:7:Syk chimera or the expression vector alone were analyzed as follows. (A) Flow cytometric analysis using fluorescein isothiocyanate-labeled anti-CD16 antibodies. (B and C) Cells that had been presensitized with anti-DNP IgE were incubated with buffer alone (−) or buffer containing DNP-BSA (0.1 μg/ml) (DNP) or a combination of anti-CD16 (0.5 μg/ml) and anti-IgG (2.5 μg/ml) (αCD16) for 40 min. (B) β-Hexosaminidase activity was measured from cell lysates and culture supernatants in triplicate and expressed as the mean percentage of β-hexosaminidase released ± standard error. The results shown are representative of more than 10 separate experiments. (C) The same culture supernatants assayed in panel B were analyzed for the presence of leukotrienes and expressed as mean picograms of leukotrienes released ± standard error. The results shown are representative of four separate experiments.

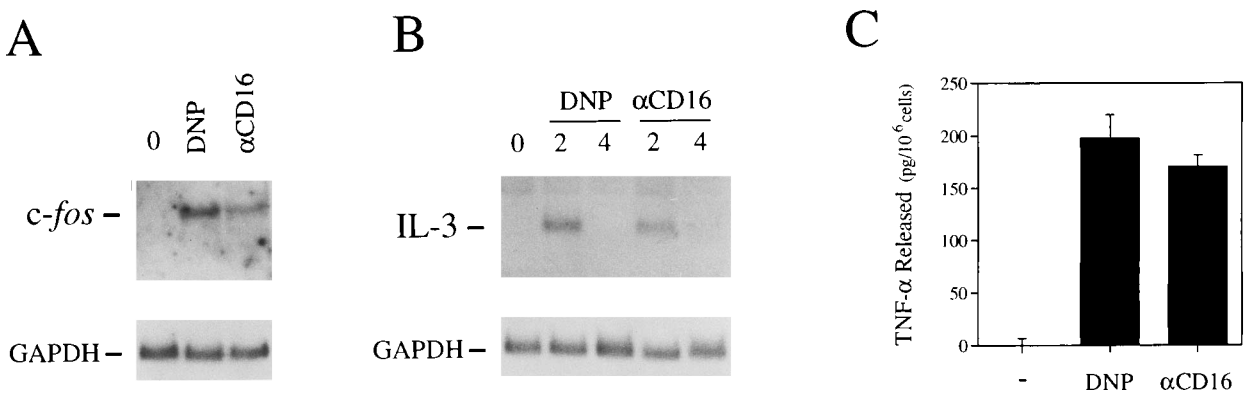

FIG. 2. Clustering of Syk induces synthesis of c-*fos* and cytokine genes. RBL cells stably transfected with a vector expressing the 16:7:Syk chimera were presensitized with anti-DNP IgE and incubated with medium alone (○) or medium containing DNP-BSA (0.1 μg/ml) (DNP) or a combination of anti-CD16 (0.5 μg/ml) and anti-IgG (2.5 μg/ml) (αCD16). (A and B) RNA was isolated from cell lysates following stimulation for 1 h (A) or 2 to 4 h (B), and Northern analysis was performed with ^{32}P-labeled probes from the c-*fos* (A) or IL-3 (B) genes. Blots were then stripped and rehybridized with a probe from the GAPDH gene (bottom panels). (C) TNF-α present in culture supernatants after 3 h of stimulation was assayed. Standard deviations of results obtained in triplicate are shown. The results shown are representative of two separate experiments.

expression, c-*fos* and IL-3 RNA levels from unstimulated cells or cells activated for various amounts of time were examined by Northern analysis. Antigen-mediated clustering of FcεRI induced the transient expression of c-*fos* (Fig. 2A [4]) and IL-3 RNA (Fig. 2B). Furthermore, cross-linking of Syk was also sufficient to induce these responses (Fig. 2A and B). Rehybridization of each blot with a probe to the GAPDH gene demonstrated that equivalent levels of total RNA were analyzed.

The synthesis of another cytokine, TNF-α, was analyzed by using antibodies to detect secreted TNF-α protein. As shown in Fig. 2C, clustering of Syk mimicked the induction of TNF-α secretion observed upon aggregation of FcεRI. In both cases, secretion of TNF-α was completely blocked when cells were stimulated in the presence of an inhibitor of RNA polymerase (data not shown), indicating the TNF-α was secreted as a result of increased transcription of the gene.

Thus far, we have shown that clustering of Syk is sufficient to activate several distinct signaling pathways which lead to the release of three different classes of mediators of the allergic response. We next used this system to investigate the mechanism by which clustering of Syk leads to the activation of these signaling pathways.

Mediator release induced by clustering of Syk is dependent on its kinase activity. The tyrosine kinase activity of Syk is activated upon aggregation of FcεRI (7, 23), suggesting that Syk's ability to induce release of mediators when clustered may depend on its kinase activity. To test this, a vaccinia virus expression system was used (30) to transiently introduce into RBL cells chimeric proteins containing wild-type Syk (16:7:Syk) or Syk containing a mutation in the ATP binding site (16:7:Syk k-) which results in the loss of its kinase activity (see below). To control for the effects of vaccinia virus infection on mediator release, cells were also infected with recombinant virus containing only the transmembrane domain of CD7 fused to the extracellular domain of CD16 (16:7). Staining with anti-CD16 antibodies demonstrated the efficient surface expression of each chimeric protein in RBL cells infected with the corresponding recombinant vaccinia virus (Fig. 3A).

Initial experiments revealed that infection of RBL cells with control virus had an inhibitory effect on FcεRI-mediated signaling, as evidenced by the finding that both β-hexosaminidase and leukotriene release were severely reduced (data not shown). However, by varying the length of infection and the amount of virus used, we found conditions that minimized the inhibitory effect of vaccinia virus infection on mediator release while still providing sufficient levels of surface expression of the chimeras to allow effects of clustering of Syk to be assessed (see Materials and Methods). Under these conditions, as was the case in the stably transfected cell lines, clustering of wild-type Syk induced degranulation and leukotriene release to levels comparable to those induced by FcεRI clustering in infected cells (Fig. 3B and C). However, clustering of kinase-inactive Syk was not sufficient to induce release of either mediator (Fig. 3B and C). These results indicate that the kinase activity of Syk is required to activate signaling pathways leading to mediator release. Since vaccinia virus infection blocks new gene expression (10), the effects that clustering of wild-type and kinase-inactive Syk had on cytokine expression could not be evaluated in this system.

Clustering of Syk induces tyrosine phosphorylation. It is well established that clustering of FcεRI leads to the tyrosine phosphorylation of numerous proteins, including the β and γ chains of FcεRI, and that increased tyrosine phosphorylation is critical for mediator release (5). To examine the role of Syk in the activation of tyrosine phosphorylation, we assessed the ability of the chimeric Syk to induce tyrosine phosphorylation when clustered. First, to examine whether the effects observed upon aggregation of the chimeric Syk were due to activation of endogenous FcεRI, the phosphorylation state of the FcεRI β and γ chains was examined. To detect tyrosine phosphorylated β and γ, antiphosphotyrosine blotting was performed on β and γ immunoprecipitated from RBL cells stably transfected with 16:7:Syk. While cross-linking of FcεRI induced the rapid tyrosine phosphorylation of β and γ, cross-linking of Syk had no effect (Fig. 4A and B). Blotting with anti-β and -γ antibodies confirmed that equal amounts of both proteins were immunoprecipitated. Similarly, aggregation of FcεRI induced the rapid tyrosine phosphorylation of the endogenous 72-kDa Syk, whereas clustering of the Syk chimera did not (Fig. 4C). These results indicate that the signals induced by clustering of the Syk chimera, which lead to mediator release, emanate from the chimeric Syk protein and not from the endogenous FcεRI. Furthermore, the failure of Syk aggregation to induce tyrosine phosphorylation of β and γ supports the notion that another

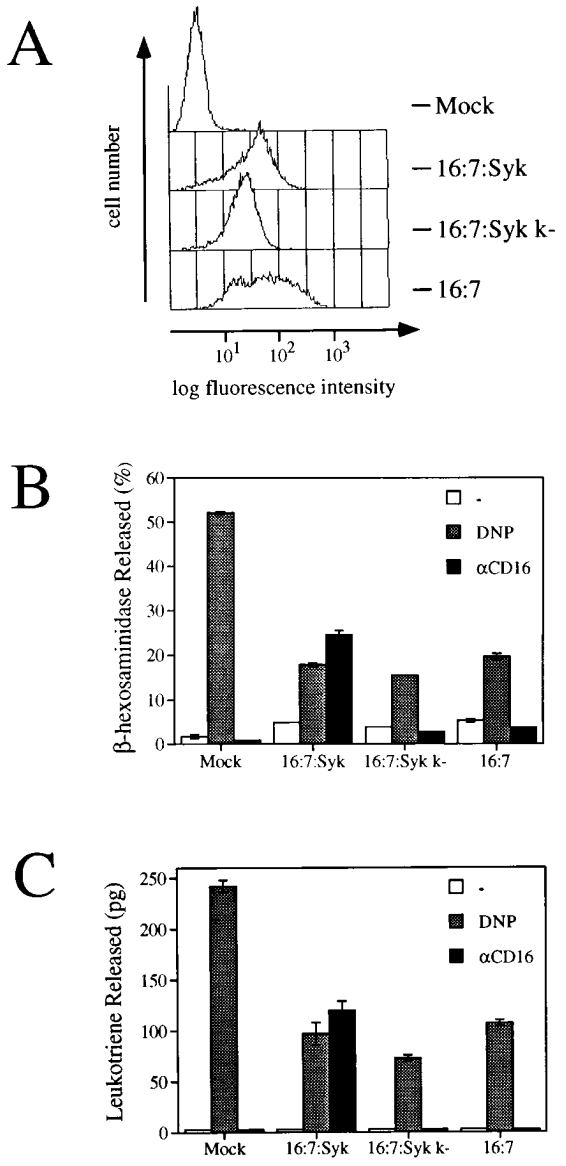

FIG. 3. The kinase domain of Syk is required for degranulation and leukotriene release. RBL cells that were mock infected or infected with vaccinia virus recombinants expressing the wild-type 16:7:Syk chimera (16:7:Syk), the kinase-inactive 16:7:Syk k-chimera, or 16:7 alone were analyzed to assess surface expression of the chimeras (A), β-hexosaminidase release (B), and leukotriene release (C) as described in the legend to Fig. 1.

tyrosine kinase, and not Syk, phosphorylates these chains upon activation.

To examine the role of Syk in the activation of potential downstream tyrosine phosphorylation events, the profile of tyrosine-phosphorylated proteins induced by clustering of Syk was compared with that induced by clustering of FcεRI. Control RBL cells and RBL cells stably transfected with 16:7:Syk were stimulated with antigen or anti-CD16 antibodies, and total cell lysates were prepared. Tyrosine-phosphorylated proteins were then detected by immunoblotting with antiphosphotyrosine antibodies. To enhance detection of a large number of proteins, lysates were electrophoresed on both high- and low-percentage acrylamide gels. With the exception of β, γ, and endogenous Syk, clustering of the Syk chimera was found to be sufficient to induce tyrosine phosphorylation of essentially all detectable proteins whose phosphorylation was induced by FcεRI clustering (Fig. 5A and B). Experiments in which tyrosine-phosphorylated proteins were first enriched by immunoprecipitation with antiphosphotyrosine antibodies revealed several additional proteins whose phosphorylation was also induced by both FcεRI and Syk cross-linking (data not shown). Interestingly, the findings that clustering of the Syk chimera induced tyrosine phosphorylation of several proteins in the 70- to 75-kDa size range (Fig. 5), without inducing phosphorylation of the endogenous 72-kDa Syk (Fig. 4C), are consistent with the previous observation that Syk is only one of several proteins in this size range that become tyrosine phosphorylated upon FcεRI activation (38) and further indicate that these other 70- to 75-kDa proteins become tyrosine phosphorylated as a result of Syk activation. Importantly, clustering of Syk did not lead to tyrosine phosphorylation of any proteins whose phosphorylation was not also induced by FcεRI clustering. This finding strongly suggests that clustering of the chimeric Syk protein induces mediator release by activating signaling pathways normally activated upon FcεRI activation.

Similar results were obtained when the vaccinia virus expression system was used to introduce the wild-type Syk chimera into RBL cells (Fig. 5C). In contrast, clustering of Syk containing a mutation in the kinase domain was not sufficient to induce tyrosine phosphorylation (Fig. 5C). This result demonstrates that tyrosine phosphorylation induced by clustering of Syk is likely to be effected by the kinase domain of Syk itself and not by the activation of an associated kinase which becomes coclustered with Syk. In addition, the correlation between the ability of Syk to induce mediator release and tyrosine phosphorylation when clustered strongly suggests that Syk-induced tyrosine phosphorylation initiates signals leading to mediator release. Also visible in Fig. 5C is a 125-kDa tyrosine-phosphorylated protein present only in cells infected with virus expressing the wild-type Syk chimera. Immunoblot analysis with anti-Syk antibodies confirms that this protein is the 16:7:Syk chimera itself (data not shown). Since the 16:7:Syk chimera was not expressed as highly in the stably transfected cells lines (data not shown), it was not easily detected by this analysis (Fig. 5A and B).

Finally, we examined the effect that aggregation of Syk had on specific proteins whose phosphorylation was known to be induced by FcεRI aggregation. PLC-γ1 becomes tyrosine phosphorylated within minutes of FcεRI clustering (2, 19, 41) and induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol, two important signal mediators. To test whether Syk clustering also induces the tyrosine phosphorylation of PLC-γ1, the 145-kDa PLC-γ1 protein was immunoprecipitated from RBL cells infected with the vaccinia virus vector expressing the Syk chimera. Antiphosphotyrosine blotting showed a low basal level of tyrosine phosphorylation of PLC-γ1 which was rapidly induced by either FcεRI or Syk cross-linking (Fig. 6A). Blotting of the immunoprecipitates with anti-PLC-γ1 antibodies showed that equal amounts of PLC-γ1 were immunoprecipitated.

The 42-kDa MAP kinase ($p42^{mapk}$) is another important signaling protein which becomes tyrosine phosphorylated upon FcεRI aggregation (46). The tyrosine phosphorylation of $p42^{mapk}$ results in a characteristic decrease in its mobility which can be detected by blotting with anti-$p42^{mapk}$ antibodies. As shown in Fig. 6B, clustering of Syk results in the rapid and transient phosphorylation of $p42^{mapk}$ on tyrosine with kinetics that match those seen upon clustering of FcεRI.

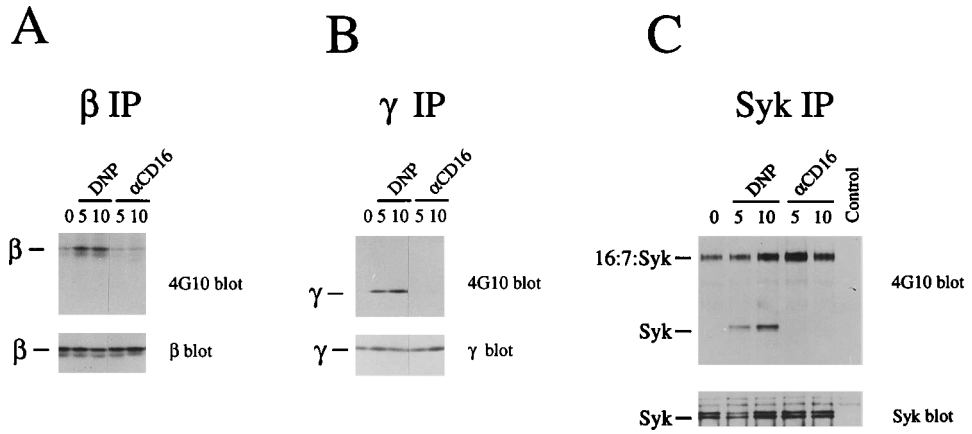

FIG. 4. Clustering of Syk does not induce tyrosine phosphorylation of endogenous β, γ, or Syk. RBL cells stably transfected with a vector expressing the 16:7:Syk chimera were presensitized with anti-DNP IgE and incubated with medium alone (○) or medium containing DNP-BSA (0.1 μg/ml) (DNP) or a combination of anti-CD16 (0.5 μg/ml) and anti-IgG (2.5 μg/ml) (αCD16) for the indicated amount of time (minutes). (A and B) Cells were lysed in RIPA buffer, and immunoprecipitations (IPs) performed with anti-β (A) or anti-γ (B) antibodies. (C) Cells were lysed in SDS lysis buffer, and immunoprecipitations performed with anti-Syk or preimmune (control) antibodies. Immunoprecipitates were then immunoblotted with antiphosphotyrosine antibody 4G10 (top panels) or the antibody used for the immunoprecipitation (bottom panels).

DISCUSSION

The studies in this report examined the role of Syk in triggering events induced by antigen-mediated clustering of the multisubunit FcεRI on mast cells. Syk-mediated events were examined by analyzing the effects of clustering Syk which was expressed on the inner face of the plasma membrane as part of a chimeric transmembrane protein. Using this system, we show that cross-linking of Syk is sufficient to activate the program of events normally induced upon cross-linking of FcεRI. Specifically, aggregation of the Syk chimera induced the tyrosine phosphorylation of essentially the same profile of proteins induced upon aggregation of FcεRI and induced the synthesis and release of all three classes of allergic mediators. These include preformed mediators released by degranulation of secretory vesicles, newly synthesized mediators generated from lipid precursors, and newly transcribed cytokines. In each case, the magnitude and kinetics of activation induced by cross-linking of Syk closely paralleled that seen after FcεRI cross-linking. These results indicate that Syk plays a critical role in mediating the effects of FcεRI clustering by triggering activation of multiple signaling pathways that lead to release of allergic mediators.

The use of this system to study the specific effects of Syk activation was validated by two findings. First, clustering of Syk did not induce tyrosine phosphorylation of the β or γ chain of FcεRI or of the endogenous Syk, indicating that the signal emanating from the Syk chimera did not feed back to activate the FcεRI receptor or endogenous Syk. Second, with the ex-

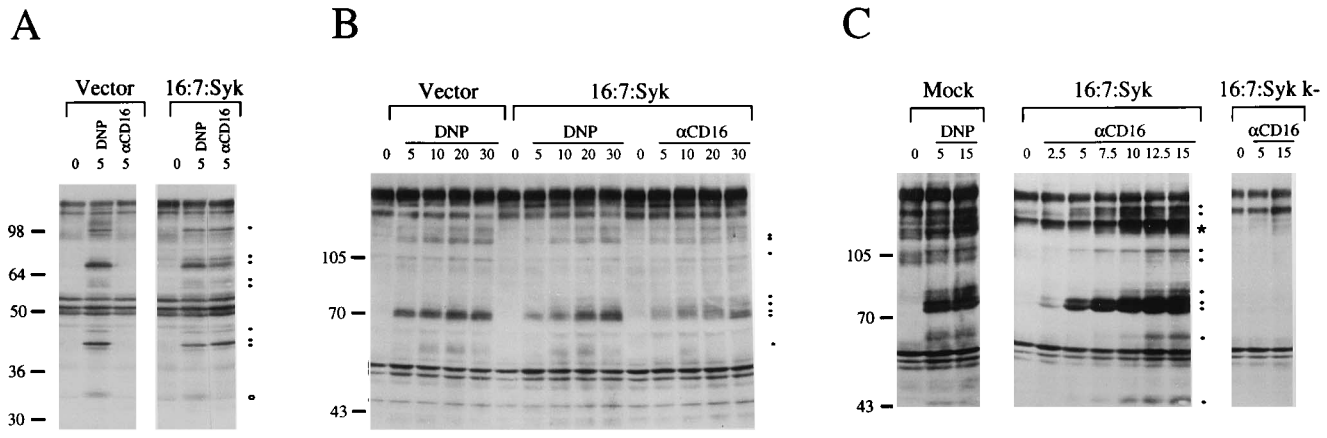

FIG. 5. Clustering of Syk induces tyrosine phosphorylation. (A and B) RBL cells stably transfected with a vector expressing the 16:7:Syk chimera or the expression vector alone were presensitized with anti-DNP IgE and incubated with buffer alone (○) or buffer containing DNP-BSA (0.1 μg/ml) (DNP) or a combination of anti-CD16 (0.5 μg/ml) and anti-IgG (2.5 μg/ml) (αCD16) for the indicated amount of time (minutes). Following stimulation, cells were lysed in RIPA buffer and lysates were separated by electrophoresis on 12.5% (A) or 7.5% (B) acrylamide gels. Tyrosine-phosphorylated proteins were detected by immunoblotting total cell lysates with antiphosphotyrosine antibodies. (C) RBL cells that were mock infected or infected with vaccinia virus recombinants expressing the wild-type 16:7:Syk chimera or kinase-inactive 16:7:Syk k-chimera were stimulated, lysed, and analyzed as described above. Proteins whose phosphorylation on tyrosine is induced by cross-linking of Syk and FcεRI are indicated by black circles. The open circle in panel A marks the 33-kDa protein, likely to be the β chain of FcεRI, whose phosphorylation is induced by FcεRI but not Syk aggregation. The approximately 15-kDa γ chain of FcεRI is not visible in these gels. The asterisk in panel C indicates the position of the 16:7:Syk chimera. Sizes of molecular weight standards are indicated in kilodaltons.

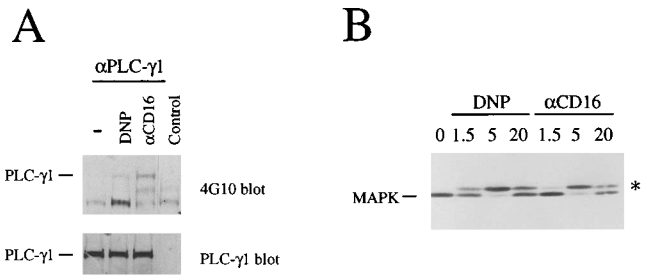

FIG. 6. Clustering of Syk induces tyrosine phosphorylation of PLC-γ1 and p42^{mapk}. (A) RBL cells infected with a vaccinia virus recombinant expressing the wild-type 16:7:Syk chimera were presensitized with anti-DNP IgE and incubated with medium alone (−) or medium containing DNP-BSA (0.1 μg/ml) (DNP) or a combination of anti-CD16 (0.5 μg/ml) and anti-IgG (2.5 μg/ml) (αCD16) for 5 min. Cells were then lysed in RIPA buffer, and immunoprecipitations were performed with anti-PLC-γ1 (αPLC-γ1) or anti-IgG (control) antibodies as indicated. Immunoprecipitates were then immunoblotted with antiphosphotyrosine (top) or anti-PLC-γ1 (bottom) antibodies. (B) RBL cells stably transfected with a vector expressing the 16:7:Syk chimera were sensitized and stimulated as described above for the indicated amount of time (minutes). Cells were then lysed in NP-40 lysis buffer, and total cell lysates were immunoblotted with anti-p42^{mapk} antibodies. The positions of unphosphorylated p42^{mapk} (MAPK) and the more slowly migrating tyrosine and threonine phosphorylated form (*) are indicated. The more slowly migrating form exactly comigrates with a band detected in an antiphosphotyrosine immunoblot of the same gel (data not shown).

ception of β, γ, and endogenous Syk, cross-linking of the Syk chimera induced a profile of tyrosine-phosphorylated proteins that was essentially identical to, and limited to, that induced by clustering of FcεRI. Therefore, we conclude that the observed effects of Syk aggregation were due to activation of pathways normally induced by FcεRI aggregation. Furthermore, these findings suggest that Syk acts at a very early step in the tyrosine phosphorylation pathway induced by FcεRI aggregation and that there is no major Syk-independent cascade of tyrosine phosphorylation.

These findings implicate Syk as an upstream activator of several known signaling pathways, including that leading to the tyrosine phosphorylation of PLC-γ1. It has been shown that phosphorylation of PLC-γ1 on critical tyrosine residues increases its activity (29), resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol, which in turn leads to the mobilization of intracellular calcium and activation of protein kinase C, respectively. In RBL cells, tyrosine phosphorylation of PLC-γ1 correlates with its activation (2, 41), suggesting that Syk-induced phosphorylation of PLC-γ1 also induces its activity. It remains to be determined whether PLC-γ1 is a direct substrate for Syk or whether Syk activates a tyrosine kinase that phosphorylates PLC-γ1. In addition, we have shown that clustering of Syk induces the rapid and transient tyrosine phosphorylation of MAP kinase, suggesting that Syk leads to activation of both the tyrosine kinase and the tyrosine phosphatase that act on MAP kinase. Since multiple pathways can lead to MAP kinase phosphorylation (9), it will be of interest to determine which of these pathways is stimulated upon clustering of Syk.

Activation of these specific signaling pathways is likely to play a role in the release of certain mediators. For example, synthesis of leukotrienes requires activation of phospholipase A_2 to release arachidonic acid from membrane phospholipids. In other systems, phospholipase A_2 activation and translocation to the membrane has been shown to require phosphorylation by MAP kinase and elevation of intracellular calcium levels (16, 17, 34). Activated MAP kinase may also play a role in inducing expression of c-*fos* and cytokine genes by phosphorylating and activating the transcription factor ELK-1 (35). Fos, as a component of the transcription factor AP-1, may then be involved in the activation of cytokine expression.

Our finding that the clustering of the Syk chimera is sufficient to induce mast cell activation suggests that a critical function of FcεRI aggregation is to promote the clustering of Syk. Current evidence supports the general model that antigen-mediated clustering of FcεRI leads to activation of Lyn and that activated Lyn, which is basally associated with the β chain, then phosphorylates the ITAMs within the β and γ chains (18, 23, 26). Syk then binds to the phosphorylated γ ITAM through its two SH2 domains and becomes clustered as a result (7, 28, 48).

Although we have shown that the ability of Syk to activate downstream signaling pathways requires its kinase activity, the mechanism by which Syk becomes activated upon clustering is presently unclear. One possibility is that clustering of Syk allows it to transphosphorylate and thereby activate itself as occurs upon ligand-mediated activation of receptor tyrosine kinases (52). Another possibility is that aggregation of FcεRI results in the coclustering of Syk with another kinase that then phosphorylates and activates Syk. The possible involvement of Lyn is suggested by the finding that coexpression of Lyn with Syk in Cos cells increases the kinase activity of Syk (31). However, clustering of the chimeric Syk protein activates signaling events without an apparent requirement for Lyn. We cannot rule out the possibility, though, that clustering of Syk does result in some coclustering of Lyn which is present on the plasma membrane or that a requirement for Lyn in this system was circumvented by the artificial expression of Syk at the membrane as part of a chimeric protein. In this regard, it is of interest that whereas endogenous Syk is not phosphorylated on tyrosine in unactivated cells, the chimeric Syk protein is phosphorylated on tyrosine in the absence of antibody cross-linking (Fig. 4C). Basal phosphorylation of Syk at critical sites could also be responsible for abrogating a requirement for Lyn.

What is the mechanism by which aggregation of Syk leads to activation of downstream signaling pathways? An aggregation-induced increase in Syk's kinase activity may induce Syk to phosphorylate downstream signaling molecules directly. Alternatively, phosphorylation of Syk on critical tyrosine residues may provide binding sites for recruitment and activation of other SH2 domain-containing proteins. Future studies aimed toward resolving this issue will focus on mapping the sites at which Syk becomes phosphorylated upon aggregation and identifying substrates and proteins that associate with Syk.

Analysis of signaling by the membrane-localized chimeric Syk also gives insight to the role that localization of Syk plays in activation. The absence of elevated tyrosine phosphorylation or mediator release prior to aggregation indicates that localization of Syk to the membrane is not sufficient to activate downstream signaling pathways. Furthermore, since a membrane-tethered Syk is able to induce mediator release upon aggregation, critical substrates must be located at or become recruited to the membrane. In addition, the data raise the question of whether cytoplasmic Syk, which represents the majority of Syk in both unstimulated and activated RBL cells (48), has any function in mast cell mediator release.

The critical role for Syk in activation is not restricted to signaling from FcεRI on mast cells. Syk is also expressed in B and T cells and binds to the phosphorylated ITAM-containing subunits of the corresponding antigen receptors (12, 54). In T cells, clustering of Syk has been shown to be sufficient to induce tyrosine phosphorylation, calcium mobilization, and cytolytic effector function (30). In an avian B-cell line, disruption of Syk expression blocks tyrosine phosphorylation and calcium

mobilization induced by cross-linking of the B-cell receptor (50). The presence of Syk and ITAM-containing receptors such as FcγRI and FcγRIII in other cells of the hematopoetic system (1, 21) suggests that clustering of Syk plays an important role in signaling a variety of cellular responses to receptor aggregation.

An important implication of our work is that interfering with the binding of Syk to the phosphorylated γ ITAM should block clustering of Syk and thereby block mast cell activation. This suggestion has been supported by experiments demonstrating that introduction of an excess of Syk's tandem SH2 domains into permeabilized RBL cells, to interfere with the binding of endogenous Syk to the γ ITAM, blocks tyrosine phosphorylation and mediator release elicited by cross-linking of FcεRI but has no effect on activation induced by clustering of chimeric Syk (47). Taken together with the studies in this report, these results suggest that clustering of Syk is both necessary and sufficient to induce the allergic response to antigen.

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