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Activation of the High-Affinity Immunoglobulin E Receptor FcεRI in RBL-2H3 Cells Is Inhibited by Syk SH2 Domains

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Antigen-mediated aggregation of the high-affinity receptor for immunoglobulin E, FcεRI, results in the activation of multiple signaling pathways, leading to the release of mediators of the allergic response. One of the earliest responses to receptor stimulation is the tyrosine phosphorylation of the β and γ subunits of FcεRI and the association of the tyrosine kinase Syk with the phosphorylated receptor. This association is mediated by the SH2 domains of Syk and is believed to be critical for activating signaling pathways resulting in mediator release. To examine the importance of the interaction of Syk with FcεRI in signaling events following receptor activation, we introduced a protein containing the SH2 domains of Syk into streptolysin O-permeabilized RBL-2H3 cells. The Syk SH2 domains completely inhibited degranulation and leukotriene production following receptor aggregation, and they blocked the increase in protein tyrosine phosphorylation observed after receptor activation. Inhibition was specific for FcεRI-mediated signaling, since degranulation of cells activated by alternative stimuli was not blocked by the Syk SH2 domains. A protein containing a point mutation in the carboxy-terminal SH2 domain which abolishes phosphotyrosine binding was not inhibitory. In addition, inhibition of degranulation was reversed by pretreatment of the SH2 domains with a tyrosine phosphorylated peptide corresponding to the tyrosine-based activation motif found in the γ subunit of FcεRI, the nonphosphorylated peptide had no effect. The association of Syk with the tyrosine-phosphorylated γ subunit of the activated receptor was blocked by the Syk SH2 domains, and degranulation in cells activated by clustering of Syk directly without FcεRI aggregation was not affected by the Syk SH2 domains. These results demonstrate that the association of Syk with the activated FcεRI is critical for both early and late events following receptor activation and confirm the key role Syk plays in signaling through the high-affinity IgE receptor.

Activation of the high-affinity receptor for immunoglobulin E (IgE), FcεRI, which is expressed on the surface of mast cells and basophils, plays a central role in the initiation of the allergic response. Following aggregation of the receptor by antigen, the mast cell releases a variety of potent biologically active molecules, including cytokines, lipid-derived mediators, amines, proteases, and proteoglycans (6, 52).

The receptor complex is composed of the α subunit (which contains the IgE-binding site), the β chain, and two disulfide-linked γ chains (10, 28, 42). A protein sequence motif designated the ITAM (immunoreceptor tyrosine-based activation motif) is found in the cytoplasmic domain of the β and γ subunits. This motif, which contains the consensus sequence D/E-X_2-Y-X_2-L/I-X_{6-7}-Y-X_2-L/I, is also found in subunits of other multisubunit antigen receptors, including the B-cell IgM receptor and T-cell receptor (BCR and TCR, respectively) (43), and has been shown to be important for receptor-mediated activation through these receptors (46, 60). Recent studies in which chimeric receptors were used to look at the function of the cytoplasmic domains of the TCR ζ chain, CD3 ε chain, or FcεRI γ chain in the absence of other subunits have demonstrated that the activation of these subunits alone is sufficient for induction of many of the events seen after stimulation of the wild-type receptors (18, 24, 32, 33). In addition, mutation of the conserved tyrosine or leucine residues or alteration in the spacing between the YXXL repeats abolishes cell signaling in response to receptor activation (32, 45), demonstrating the importance of the ITAMs in these events.

The activation of protein tyrosine kinase (PTK) activity is one of the earliest events detectable after antigen cross-linking of the IgE receptor. PTK activation results in the tyrosine phosphorylation of multiple cellular proteins, including the β and γ subunits of the receptor itself (7, 15, 17, 25, 34, 40). PTK inhibitors prevent signal transduction events following activation of the receptor, demonstrating the importance of PTK activation in signaling events leading to mediator release (25, 30, 47). Since the receptor subunits themselves do not possess intrinsic kinase activity, the increased PTK activity detectable after receptor aggregation is probably due to the activation of cytoplasmic tyrosine kinases which are associated with the receptor.

At least two families of PTKs are implicated as being responsible for the increase in tyrosine phosphorylation seen after FcεRI activation. Lyn, a member of the Src-like family of kinases, is reported to be associated with the receptor and to be activated following aggregation of the FcεRI (17, 18, 24). In addition, the Syk tyrosine kinase becomes phosphorylated on tyrosine and its activity is increased following receptor activation (24, 37). This kinase also associates with the activated IgE receptor, as demonstrated in coimmunoprecipitation experiments (8, 48).

Syk is a 70-kDa cytoplasmic tyrosine kinase which contains two SH2 domains in the amino-terminal half of the protein and a tyrosine kinase domain in the carboxy-terminal half of the protein (31, 55). Recent in vitro experiments have demonstrated that the Syk SH2 domains can directly associate with the tyrosine-phosphorylated form of the γ subunit of the IgE receptor (27, 48). These data suggest that Syk associates with

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the receptor through an interaction between the tandem SH2 domains of Syk and the phosphorylated tyrosines in the ITAM of the γ subunit. A similar association/activation of Syk-like tyrosine kinases following receptor stimulation has been found in other systems. For example, in T cells, cross-linking of the TCR results in the tyrosine phosphorylation and activation of ZAP-70 (12, 13, 53, 58). In addition, ZAP-70 associates with the ζ subunit of the TCR via a direct interaction between the SH2 domains of ZAP-70 and the ITAMs in ζ in a manner similar to the association of Syk with the γ subunit of FcεRI (23, 29, 56, 59). Similarly, in B cells, activation of the BCR results in the tyrosine phosphorylation, activation, and association of Syk with the Igα and Igβ subunits (21, 22, 31, 62). A model for activation of this family of receptors suggests that receptor cross-linking results in activation of Src-family kinases and tyrosine phosphorylation of the ITAM, followed by an interaction of Syk or ZAP-70 with the receptor tails. This binding may either activate Syk/ZAP-70 or translocate the kinase to the membrane so that its targets become accessible to phosphorylation.

The functional importance of Syk in signal transduction following activation of FcεRI is still unclear. Syk-deficient B cells are defective in tyrosine phosphorylation of phospholipase C-γ2 (PLC-γ2), resulting in a loss of both inositol 1,4,5-triphosphate generation and calcium mobilization after stimulation of the BCR (54). In addition, several groups have recently demonstrated that a human immunodeficiency syndrome is correlated with mutations in the T cell which abolish ZAP-70 protein expression, demonstrating that ZAP-70 is required for a functional T-cell response (4, 14, 19). By analogy with this system, it is likely that Syk is also necessary for signaling through the FcεRI receptor. Recent results have shown that clustering of Syk in the absence of FcεRI stimulation is sufficient to induce mast cell activation (44), demonstrating the central role that Syk activation plays in mediating signaling events following aggregation of the IgE receptor.

In the present study, we examined the importance of the interaction between Syk and the FcεRI receptor in signal transduction following receptor activation. Here we demonstrate that introduction of the Syk tandem SH2 domains into streptolysin O (SLO)-permeabilized RBL cells blocked the activation of these cells after receptor cross-linking. This inhibition was specific for the Syk SH2 domains, was reversed by pretreatment of the SH2 domain with γITAM peptides phosphorylated on tyrosine (but not by the analogous nonphosphorylated peptides), and was not seen in cells activated by alternative pathways. In addition, the Syk SH2 domains inhibited the association of endogenous Syk with the tyrosine-phosphorylated γ chain of the receptor following activation. These results demonstrate that the Syk SH2 domains play a critical role in assembling the complexes necessary to get full activation of the cell following aggregation of the IgE receptor and that blocking these critical SH2 interactions is sufficient to inhibit activation and mediator release in the mast cell.

MATERIALS AND METHODS

Expression of SH2 domains. The DNA sequence encoding residues 6 to 265 of human Syk was cloned into the pET expression vector and transformed into *Escherichia coli* BL21(DE3) (48). The tandem Syk protein with a single-site mutation, R195A, was constructed as a glutathione *S*-transferase (GST) fusion protein as previously described (48). The PLC-γ1 tandem SH2 domains (bovine, residues 547 to 752) (3) and the Src SH2 domain (human, residues 147 to 252) were also expressed as GST fusion proteins. In a typical preparation, protein was produced from the growth and induction of 2 liters of culture in brain heart infusion medium supplemented with 200 μg of ampicillin per ml. The culture was grown at 25°C to an optical density at 595 nm of 1 to 2, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and harvested 4 h later.

Purification of SH2 domains. All operations were performed at 4°C unless otherwise described.

(i) Wild-type Syk tandem SH2 domain. Cells were lysed with a French pressure cell and 2 volumes of lysis buffer [20 mM Tris (pH 8), 500 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. The supernatant was collected by high-speed centrifugation, diluted twofold with buffer A (20 mM Tris [pH 8], 5 mM DTT), and applied to a 20-ml polyethyleneimine anion-exchange column (Baker) equilibrated with the same buffer. The flowthrough was dialyzed overnight against buffer B (20 mM Tris [pH 7.4], 5 mM DTT, 50 mM NaCl) and then loaded onto a 50-ml phosphotyrosine-agarose column (Sigma). The tandem Syk protein bound to the column and was eluted with a salt gradient, 50 mM to 1.5 M NaCl in 200 ml. The tandem Syk protein, eluting between 50 and 100 ml, was collected, diluted 1:1 with cold 3 M ammonium sulfate, and then applied to a 20-ml phenyl-Sepharose column (Pharmacia) equilibrated with buffer C (1.5 M ammonium sulfate, 20 mM Tris [pH 8], 5 mM DTT). The column was washed with a reverse salt gradient (0 to 100% buffer A), and the tandem Syk eluted at 100% buffer A.

(ii) GST fusion proteins. The GST fusion proteins containing the Syk R195A, PLC-γ1, and Src SH2 domains were purified on glutathione-agarose (Sigma) as previously described (48). The proteins were then cleaved with thrombin (Enzyme Research Laboratories), using 1 to 3 U/mg of protein, for 2 h at 25°C or 16 h at 4°C. The cleavage reaction was monitored by sodium dodecyl sulfate (SDS)-gel electrophoresis. The GST protein was separated from the PLC-γ1 and Src SH2 domains by applying the mixture to a phosphotyrosine-agarose column as for the wild-type Syk tandem SH2 protein. Since the Syk R195A SH2 domain does not stick to phosphotyrosine resin, it was passed over a Q Sepharose column (Pharmacia) equilibrated with 100 mM Tris (pH 8)–100 mM NaCl–2 mM DTT. The tandem Syk R195A eluted in the flowthrough just prior to the GST protein. SDS-gel electrophoresis indicated that all proteins were >95% pure. N-terminal sequencing and mass-spectrometric analysis confirmed the expected sequence. The protein concentration was determined by measuring the A_{280}.

SLO permeabilization and activation of RBL-2H3 cells. RBL-2H3 cells were grown in minimal essential medium (GIBCO-BRL) supplemented with 15% fetal bovine serum (GIBCO-BRL), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine (GIBCO-BRL) at 37°C in 5% CO_2. For activation, cells were loaded overnight with 0.5 μg of rat IgE against 2,4-dinitrophenol (DNP) (Zymed) per ml. SLO permeabilization was performed as described previously (16) with some modifications. Briefly, cell monolayers were washed twice with permeabilization assay buffer [138.7 mM potassium glutamate, 5 mM glucose, 20 mM potassium salt of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 7 mM magnesium acetate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM ATP, 1 mg of bovine serum albumin (BSA) per ml, and 710 μM $CaCl_2$ to give a calculated free calcium concentration of 1 μM], and then SLO (Murex Diagnostics Ltd.) was added in permeabilization assay buffer at a final concentration of 0.2 U/ml. The cells were incubated with SLO for 10 min at 37°C. The SLO mixture was then removed from the cells, and the appropriate dilution of the Syk tandem SH2 domain was added to each well. After a brief incubation at room temperature (2 to 5 min), 1 μg of DNP-BSA (Sigma) per ml was added to the wells to stimulate the FcεRI receptor. In some experiments, DNP-BSA stimulation was replaced by stimulation with 1 mM guanosine 5′-(3-*O*-thio)triphosphate (GTP-γS) or a mixture of phorbol 12-myristate 13-acetate (PMA) (0.01 μM) and the Ca^{2+} ionophore A23187 (1 μM).

The RBL-2H3 cell line, which stably expresses a CD16/Syk chimera, has been described previously (44). These cells were permeabilized as described for the wild-type cells and were activated either by treatment with DNP-BSA (to cross-link endogenous FcεRI) or by treatment with a combination of 0.5 μg of anti-CD16 antibody (3G8; Medarex) per ml and 2.5 μg of goat anti-mouse IgG1 antibodies (Southern Biotechnology) per ml to cross-link the CD16/Syk chimera. Activated cells were analyzed as described below for wild-type RBL cells.

Degranulation assay: measurement of β-hexosaminidase release. After 40 min of stimulation, culture supernatants were harvested and aliquots (25 μl) were mixed with an equal volume of 8 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M citrate–phosphate buffer (pH 4.5). After incubation at 37°C for 30 min, color was developed by addition of 50 μl of 0.2 M glycine buffer (pH 10.0) (0.2 M glycine, 0.2 M NaCl, 0.2 M NaOH) and the optical density was read at 405 nM. For determination of the total cellular β-hexosaminidase content, supernatants from mock-treated cells were harvested and cell lysates prepared by either sonication or lysis in 0.2% Triton X-100 were assayed as described above. Percent release (mean ± standard error) was determined for each set of wells.

Leukotriene assay. Culture supernatants were harvested as described above and assayed in a leukotriene $C_4/D_4/E_4$ enzyme immunoassay (Amersham) as specified by the manufacturer.

Preparation of cell lysates and immunoprecipitation. Permeabilized cells were activated with DNP-BSA for 5 min at 37°C and immediately lysed on ice by addition of buffer containing 1% Nonidet P-40 (NP-40), 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 25 μg of leupeptin per ml, 10 U of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed for 30 min, and the lysates were clarified at 16,000 × *g* for 30 min. The supernatant was collected, and the protein concentration was determined (BCA protein assay; Pierce). Lysates were then either used directly for immu-

noblotting to detect phosphotyrosine-containing proteins (50 to 100 μg of total protein per lane) or used in immunoprecipitations.

For Syk immunoprecipitations, 0.5 to 1 mg of total protein from detergent lysates was incubated overnight with a rabbit polyclonal anti-Syk antibody. This antiserum was generated by injecting a rabbit with a Syk peptide containing residues 318 to 330 of rat Syk. The peptide was conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce). The rabbit was given seven injections at a concentration of 100 μg per boost on a 14-day boost-and-bleed schedule. For PLC-γ1 or FcεRIγ immunoprecipitations, protein from detergent lysates was incubated overnight with anti-PLC-γ1 antibody (Upstate Biotechnology Inc.) or rabbit polyclonal anti-γ antibody (a gift from J. Bolen, Bristol-Myers Squibb). Antibodies were collected by the addition of Pansorbin (CalBiochem). Pansorbin pellets were washed three times with lysis buffer and then resuspended in sample buffer.

Immunoblotting. Protein samples in SDS sample buffer containing 10% β-mercaptoethanol were boiled for 5 min and then separated by SDS-polyacrylamide gel electrophoresis (PAGE) or on a 4 to 20% Tris-glycine gradient gel (Novex). Proteins were transferred to polyvinylidene difluoride membranes (0.2-μm pore size; Bio-Rad) for 1.5 h at 0.5 mA. The membranes were blocked in 3% BSA–150 mM NaCl, 10 mM Tris (pH 8.0)–0.1% Tween 20 and probed with primary antibody (1:1,000) and horseradish peroxidase-coupled secondary antibody (1:20,000; Bio-Rad) in the same buffer. Washes were performed in 0.2% NP-40–50 mM Tris (pH 7.4)–150 mM NaCl. Immunoreactivity was detected by enhanced chemiluminescence (Amersham Corp).

Synthesis of peptides. N-acetylated peptides corresponding to FcεRI γ-chain cytoplasmic domain were synthesized and characterized as described previously (48). Tyrosine residues were phosphorylated by the global phosphorylation procedure.

RESULTS

Inhibition of degranulation and leukotriene production by Syk tandem SH2 domains. To investigate the role of the Syk tyrosine kinase in FcεRI-mediated signaling, we performed a series of studies with SLO-permeabilized RBL-2H3 cells treated with the Syk SH2 domains prior to receptor activation. RBL-2H3 cells, a rat basophilic leukemia cell line, have been shown to mimic many of the responses seen with primary mast cells and provide a useful in vitro model for the study of mucosal mast cells (38). Previous studies have demonstrated that RBL cells permeabilized with SLO are still responsive to receptor-mediated activation (2, 39, 61) and that the pores created have diameters exceeding 30 nm, which allows free passage of proteins across the membranes (9, 39). Interestingly, there is no detectable leakage of endogenous Syk from the permeabilized cells (results not shown). The Syk construct used for these experiments includes both SH2 domains of Syk, as well as the spacer region contained between the SH2 domains (48). There is no difference in binding of the tandem SH2 domains to tyrosine-phosphorylated targets in constructs containing Syk residues 1 to 265 or residues 6 to 265 (11); the latter construct was used exclusively in this study.

Activation of the FcεRI results in the release of preformed biogenic amines, proteases, and highly sulfated proteoglycans from the secretory granules present in RBL cells. To determine if the tandem SH2 domains of Syk had any effect on the ability of the cell to degranulate, we measured β-hexosaminidase activity in the culture supernatants of permeabilized cells following receptor activation. β-Hexosaminidase is an acid hydrolase which is stored in the secretory granules and is released along with other mediators after receptor stimulation; it is a convenient marker for monitoring degranulation in RBL cells. In SLO-permeabilized cells, DNP stimulation of RBL cells results in approximately 10 to 20% release of the total amount of β-hexosaminidase contained in the cells. This is approximately one-half to one-third the extent of degranulation routinely detected in nonpermeabilized cells and may reflect the more fragile state of the cells following permeabilization.

The effect of the Syk tandem SH2 domains on degranulation of RBL cells is presented in Fig. 1A. SLO-permeabilized cells were treated with either the Syk tandem SH2 domain or a wild-type GST protein as a control prior to receptor activation. After activation by addition of DNP-BSA, cells were incubated for 40 min and the supernatants were assayed for β-hexosaminidase activity. We found that the Syk tandem SH2 domain inhibited degranulation from RBL cells at 50% inhibitory concentration (IC_{50}) of approximately 1 μM. The GST protein, although of a similar size to the tandem SH2 domains, had no effect on degranulation. This suggested that the effect of tandem Syk was not an artifact resulting from the introduction of large bacterially produced proteins into RBL cells. Interestingly, although SLO permeabilization itself causes a slight activation of the cells (resulting in release of approximately 2 to 4% of total β-hexosaminidase contained in the cell), the tandem Syk is able to inhibit this low level of activation and reduces degranulation to that seen in nonpermeabilized cells (1 to 2% of total).

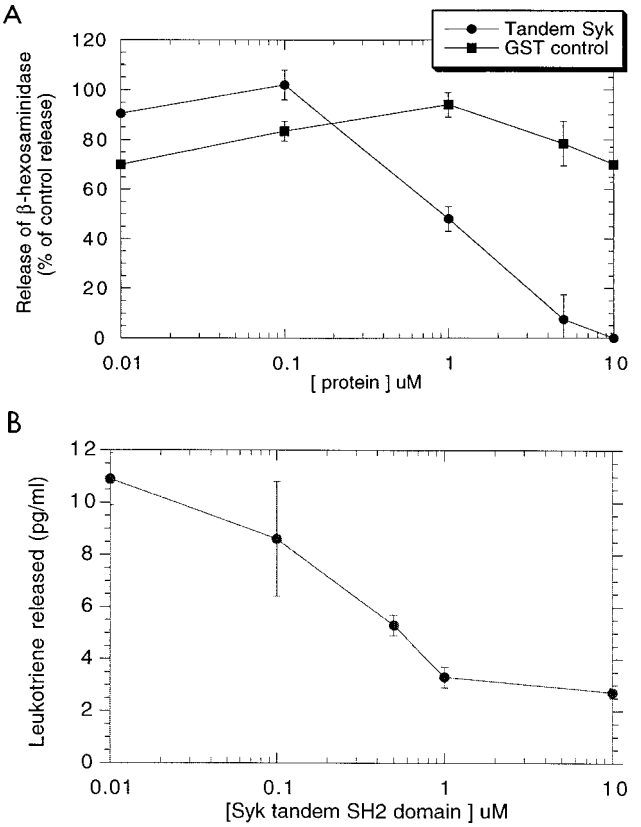

FIG. 1. The Syk tandem SH2 domains inhibit degranulation and leukotriene release. (A) Cells preloaded with anti-DNP IgE were permeabilized with SLO and treated with increasing concentrations of the Syk SH2 domains or GST protein for 5 min. Cells were then stimulated with DNP-BSA (final concentration, 1 μg/ml) and incubated for 40 min at 37°C. Supernatants from duplicate wells were assayed for β-hexosaminidase activity. The results are presented as a percentage of control release in the absence of test protein. (B) Cells were treated with the Syk SH2 domains as described above, and supernatants were assayed for the presence of released leukotrienes. Results from duplicate wells were averaged, and the results are expressed as picograms of leukotriene released per milliliter. The graphs presented are representative of the results of multiple experiments.

We also measured the inhibitory effect of the Syk tandem SH2 domain on leukotriene production (Fig. 1B). Leukotrienes are potent mediators of the allergic response and are newly synthesized in RBL cells following receptor activation. The cells were activated as described for the degranulation assay, and the supernatants were assayed for leukotriene B_4/

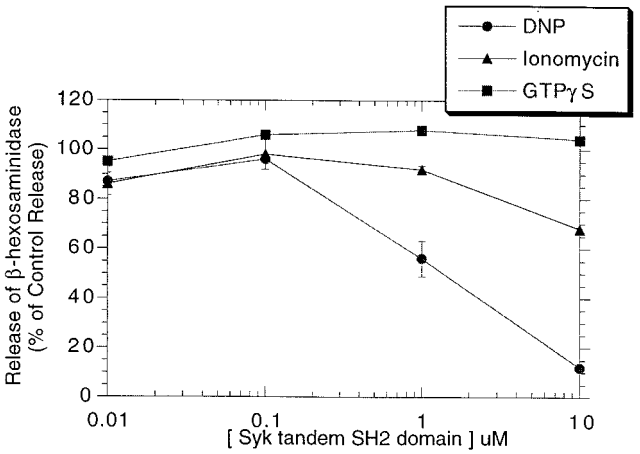

FIG. 2. The Syk tandem SH2 domains do not inhibit degranulation in cells activated by alternative stimuli. Cells preloaded with anti-DNP IgE were permeabilized with SLO and treated with increasing concentrations of the Syk SH2 domains. The cells were then stimulated with either 1 μg of DNP-BSA per ml, 1 mM GTPγS, or a combination of PMA (0.01 μM) and the Ca^{2+} ionophore A23187 (1 μM). Supernatants from duplicate wells were assayed for β-hexosaminidase as described in the text. The graphs presented are representative of the results of multiple experiments.

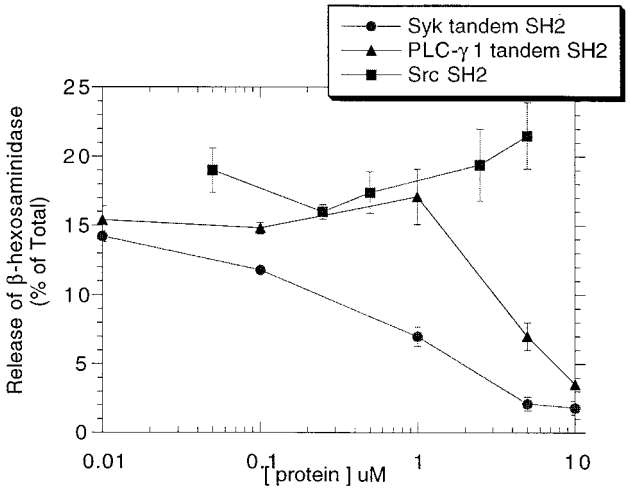

FIG. 3. Effect of other SH2 domains on degranulation in FcεRI-activated cells. Cells preloaded with anti-DNP IgE were permeabilized with SLO and treated with increasing concentrations of the Syk SH2 domains, the PLC-γ1 SH2 domains, or the Src SH2 domain. Supernatants from duplicate wells were assayed for β-hexosaminidase as described in the text. The graphs presented are representative of the results of multiple experiments.

C_4/E_4. Again, the Syk tandem SH2 domain inhibited leukotriene release from activated RBL cells at an IC_{50} similar to that seen for inhibition of degranulation (approximately 1 μM). Introduction of the GST protein as control had no effect on leukotriene production after receptor stimulation (results not shown). These results demonstrate that the Syk tandem SH2 domain can inhibit the release of two different mediators of the allergic response from RBL cells, presumably by blocking the interaction of endogenous Syk SH2 domains with their ligands.

The inhibition of Syk tandem SH2 domains is specific for FcεRI-mediated signaling. To further understand the mechanism by which the Syk tandem SH2 domain inhibits degranulation and leukotriene production, we measured the effect of the Syk tandem SH2 domain on degranulation induced by alternative stimuli. First, cells were stimulated with GTPγS, a nonhydrolyzable analog of GTP which activates stimulatory G proteins and induces degranulation in RBL-2H3 cells (1, 2, 16). As shown in Fig. 2, the Syk tandem SH2 domain was unable to inhibit degranulation induced by GTPγS at concentrations which completely blocked degranulation following FcεRI activation. In addition, cells were stimulated by a combination of PMA, which will activate protein kinase C, and the Ca^{2+} ionophore A23187, which elevates intracellular Ca^{2+} levels (1, 16). Again, the Syk tandem SH2 domain was unable to inhibit the degranulation induced by PMA-A23187. These results demonstrated that the inhibition by the Syk SH2 domains is specific for signaling through the IgE receptor and is not due to a nonspecific effect on alternative pathways leading to degranulation. In addition, these results suggest that the Syk tandem SH2 domains act at a step in the signaling cascade prior to Ca^{2+} mobilization.

Inhibition of degranulation by other SH2 domains. A number of proteins involved in signaling through the IgE receptor, including Lyn, PLC-γ1, Btk, and Vav, contain SH2 domains (5, 17, 20, 26, 36, 41). SH2 domains may be involved in assembling signaling complexes at several different points in the activation pathway leading to mediator release, so it is possible that the mechanism of tandem Syk SH2 inhibition of degranulation involves SH2 interactions at one or more of these different pathways. To examine the specificity of the inhibition observed with the Syk SH2 domains, two additional SH2 domains from proteins found in RBL cells were assayed. The first construct, tandem SH2 domains from PLC-γ1, was found to inhibit degranulation in RBL cells (Fig. 3). However, the PLC-γ1 SH2 domains inhibited degranulation with an IC_{50} approximately 10-fold higher than the IC_{50} for the Syk tandem SH2 domain. This finding provides evidence that there is specificity in the inhibition by the Syk SH2 domains and that it is not due to increased ligand binding by constructs containing two SH2 domains. In addition, the SH2 domain from the Src tyrosine kinase was tested. The kinase activity of Src is increased following FcεRI activation but is not found to be associated with the receptor after activation (17). The Src SH2 domain did not inhibit degranulation when tested at concentrations which were completely inhibitory for the Syk SH2 domains (Fig. 3). The apparent IC_{50} of the Src SH2 domain was 100-fold higher than the IC_{50} of tandem Syk (results not shown). These results suggest that the inhibition by the Syk tandem SH2 domains is due to interaction with Syk-specific ligands, not to a nonspecific SH2 interaction.

The Syk tandem SH2 domain does not inhibit activation following cross-linking of a CD16 Syk chimera. The results presented thus far indicate that the Syk tandem SH2 domains inhibit FcεRI-mediated signaling in RBL cells, possibly by blocking the interaction of endogenous Syk with a ligand(s) required for activation of Syk kinase activity. Alternatively, the Syk SH2 domains may inhibit the binding of endogenous Syk to potential substrates, thus acting downstream of Syk activation to prevent signal transduction. To examine these possibilities in greater detail, an RBL cell line which stably expresses a chimeric molecule containing the extracellular domain of CD16 and the transmembrane domain of CD7 linked to the complete coding region of the Syk tyrosine kinase was used (44). Cross-linking of the chimera with anti-CD16 antibodies results in activation of the kinase and release of mediators from RBL cells, demonstrating that Syk activation alone is sufficient for the downstream events necessary for activation of the cells (44).

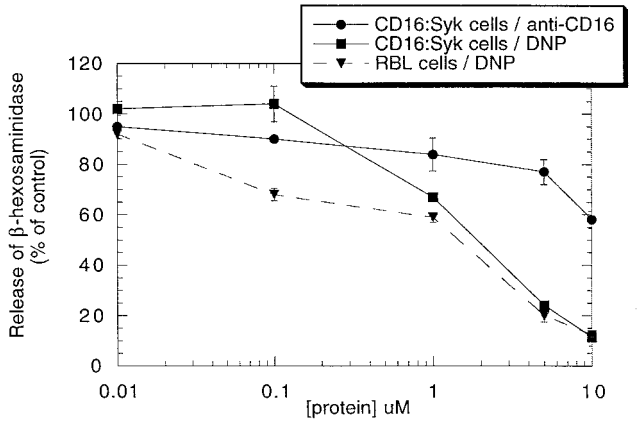

FIG. 4. The Syk SH2 domains do not inhibit activation following cross-linking of a CD16 Syk chimera. Wild-type RBL cells or RBL cells stably transfected with a vector expressing the 16:7:Syk chimera were preloaded with anti-DNP IgE and permeabilized with SLO as described in the text. Cells were stimulated with either 1 μg of DNP-BSA per ml or a combination of 0.5 μg of anti-CD16 per ml and 2.5 μg of anti-IgG per ml (αCD16). After a 40-min incubation at 37°C, supernatants were harvested and assayed for release of β-hexosaminidase. The graphs presented are representative of the results of multiple experiments.

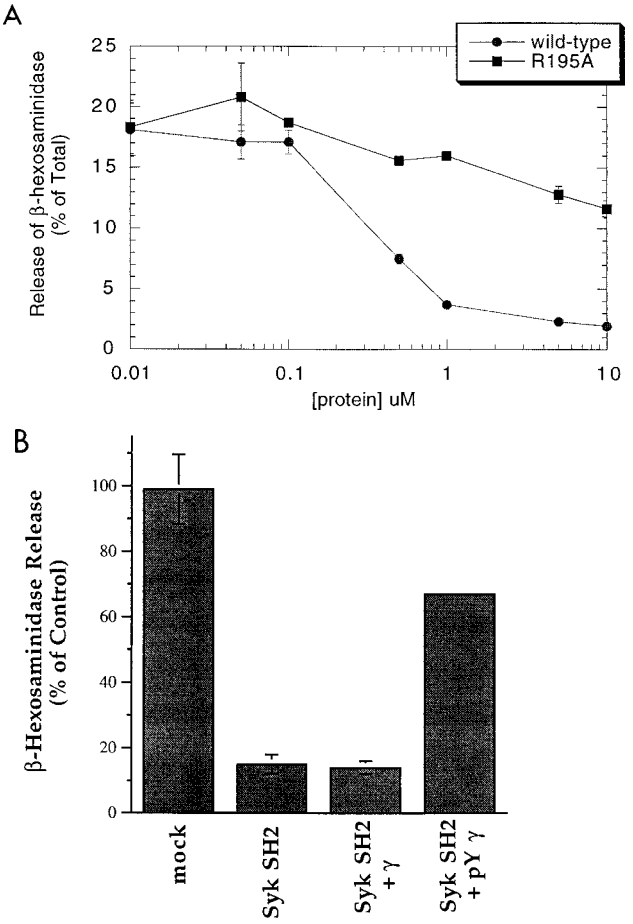

FIG. 5. Inhibition of degranulation requires SH2 interactions. (A) SLO-permeabilized cells were prepared as described in the text and treated with increasing concentrations of either the wild-type Syk tandem SH2 domains or a protein containing a mutation in the carboxy-terminal SH2 of Syk (R195A). Activation of the cells and the β-hexosaminidase assay were then performed as described in the legend to Fig. 1. (B) The Syk tandem SH2 domains (5 μM) were preincubated for 1 h at 4°C with either buffer alone, nonphosphorylated γITAM peptide (10 μM), or the analogous peptide tyrosine phosphorylated at the 4 and 15 positions in the ITAM (pY-γ). The Syk proteins were then introduced into the SLO-permeabilized cells as usual, and their effect on degranulation was monitored by assaying for release of β-hexosaminidase. The graphs presented are representative of the results of multiple experiments.

RBL cells expressing the CD16 chimera can be activated to induce degranulation either through the IgE receptor by addition of antigen or by activation of Syk directly by cross-linking with CD16 antibodies. The Syk tandem SH2 domain displayed the same inhibitory activity on either wild-type RBL cells or CD16/Syk RBL cells following activation of FcεRI by addition of DNP (Fig. 4). When the Syk tyrosine kinase was activated by cross-linking the CD16/Syk chimera with anti-CD16 antibodies, the Syk tandem SH2 domains exhibited little inhibitory activity, with only a slight reduction in degranulation at the highest concentration. Taken together with the data presented in Fig. 2, these results confirm our observations that the inhibition by the SH2 domains is not due to the inability of the cell to respond to stimuli but is limited to specific signaling events following FcεRI activation. In addition, the data indicate that a significant portion of the inhibitory activity of the Syk SH2 domains comes from blocking cell activation at a point in the pathway prior to Syk activation. Thus, likely targets for the Syk SH2 domains include the tyrosine-phosphorylated ITAMs found in the β and γ subunits of the receptor.

The inhibition of degranulation requires SH2 domain interactions. To determine if the inhibition by the Syk construct requires SH2 domain interactions, we tested a variant tandem Syk SH2 domain containing a point mutation at position 196 in the C-terminal SH2 domain. This mutation has been shown to significantly reduce the binding of tandem Syk to a tyrosine-phosphorylated γ peptide and to tyrosine-phosphorylated proteins in activated RBL cell lysates (48). As shown in Fig. 5A, the R195A mutation in tandem Syk significantly reduced the inhibitory activity of the tandem SH2 domains. The small amount of inhibition seen at the highest concentration may reflect residual phosphotyrosine binding by the N-terminal SH2 domain.

As an alternative approach, we used peptides containing the ITAM from the cytoplasmic domain of the γ subunit of FcεRI to block the inhibition by the Syk SH2 domains (Fig. 5B). A peptide which was tyrosine phosphorylated at the 4 and 15 positions in the ITAM significantly reduced the inhibition by tandem Syk. The nonphosphorylated ITAM peptide, which does not bind to the SH2 domains of Syk, had no effect on the inhibition by the Syk tandem SH2 domain. Together, these results demonstrate that the effects of the tandem SH2 domains on signaling after FcεRI activation require functional Syk SH2 interactions.

Tyrosine phosphorylation of cellular proteins after receptor activation is blocked by the Syk tandem SH2 domain. The results presented have suggested that inhibition of the interaction of the Syk SH2 domains with their critical targets is sufficient to block degranulation and leukotriene production in RBL-2H3 cells. Since one of the earliest events following activation of FcεRI is the tyrosine phosphorylation of multiple cellular proteins, we examined whether treatment of cells with the Syk SH2 domains inhibited this early event following receptor stimulation.

Lysates prepared from cells activated with DNP in the presence or absence of the Syk SH2 domains were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with the anti-phosphotyrosine antibody 4G10. As previously described by a number of laboratories (7,

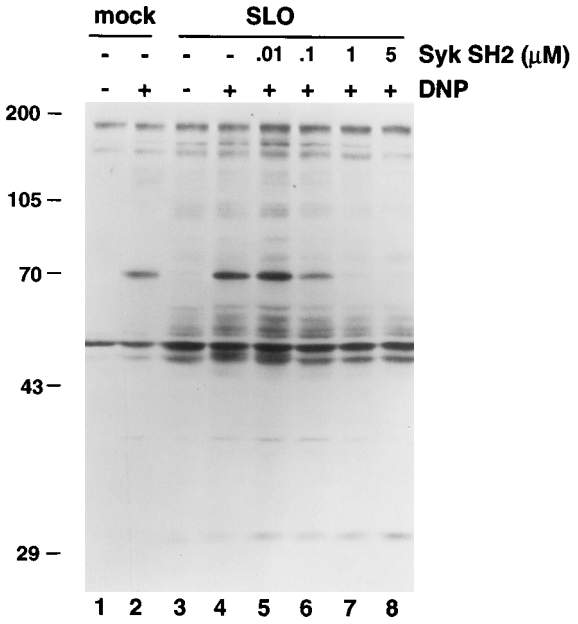

FIG. 6. The Syk tandem SH2 domains inhibit tyrosine phosphorylation in activated RBL cells. All cells were preloaded with anti-DNP IgE. Untreated RBL cells (lanes 1 and 2) or SLO-permeabilized RBL cells were incubated with buffer (lanes 3 and 4) or treated with increasing concentrations of the Syk tandem SH2 domain (lanes 5 through 8). The cells were then activated by addition of buffer alone (lanes 1 and 3) or 1 μg of DNP-BSA per ml (lanes 2 and 4 through 8) for 5 min at 37°C. Activation buffer was removed, and cell lysates were prepared in 1% NP-40 buffer. Proteins were separated by SDS-PAGE in 10% gels and transferred to polyvinylidene difluoride membrane; tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antibodies.

15, 17, 25, 34, 40), activation of RBL cells by cross-linking the FcεRI receptor induces the tyrosine phosphorylation of a number of cellular proteins (Fig. 6, lanes 1 and 2). Although SLO permeabilization alone slightly increased the tyrosine phosphorylation of several of these proteins (lane 3), DNP activation of the SLO-permeabilized RBL cells resulted in a significant increase in the phosphorylation of proteins with similar mobilities to those observed phosphorylated in intact cells (lane 4). This result confirmed that SLO permeabilization does not significantly alter the induction of tyrosine phosphorylation in response to FcεRI stimulation and that these cells are an appropriate model for looking at the effects of tandem Syk on tyrosine phosphorylation of cellular proteins following receptor activation.

Treatment of cells with increasing concentrations of the Syk tandem SH2 domain prior to receptor activation significantly reduced the phosphorylation of most of the proteins observed in immunoblots following DNP addition (lanes 4 to 8). The inhibition of phosphorylation occurred in a dose-dependent manner, with maximal inhibition at 1 μM Syk SH2 domain. As expected, proteins which are constituitively phosphorylated in RBL cells (lane 3) were not affected by the Syk SH2 domain. In addition, the phosphorylation of a 32- to 35-kDa protein detected in DNP-stimulated cells is not reduced by treatment with the Syk SH2 domain, indicating that the inhibition is not a global effect on tyrosine phosphorylation in activated cells.

After receptor stimulation, the Syk tyrosine kinase itself is rapidly phosphorylated on tyrosine, coincident with the activation of its kinase activity (24, 37). To determine if the Syk SH2 domains inhibited this phosphorylation, Syk was immunoprecipitated from cell lysates prepared as described above. Antiphosphotyrosine immunoblotting showed a low basal level of phosphorylation, which was rapidly induced after DNP addition (Fig. 7A, lanes 1 and 2). Treatment of the cells with the Syk SH2 domains inhibited the phosphorylation of Syk on tyrosine in a dose-dependent manner (lanes 3 to 5), with complete inhibition at 1 μM. This result suggests that the SH2 domains inhibit the signaling cascade at a step prior to Syk phosphorylation.

PLC-γ1 is also rapidly tyrosine phosphorylated following receptor aggregation (5, 20, 34, 41); activation of PLC-γ1 is critical for generation of downstream signal mediators inositol 1,4,5-triphosphate and diacylglycerol. Recent results have demonstrated that Syk is an upstream regulator of the PLC-γ1 signaling pathway (44). As shown in Fig. 7B, the Syk SH2 domain completely blocked the tyrosine phosphorylation of PLC-γ1 at the same concentrations which inhibited tyrosine phosphorylation of Syk as well as other cellular proteins.

The Syk SH2 domains inhibit the association of Syk with the activated FcεRI. Following activation of the FcεRI, Syk becomes associated with the activated receptor; this association is probably mediated by the Syk SH2 domains binding to the tyrosine-phosphorylated ITAMs of the γ subunit (8, 27, 48). We used coimmunoprecipitation experiments to determine if the Syk SH2 domains prevented this interaction.

As shown in Fig. 8A, the tyrosine phosphorylation of the γ and β subunits of FcεRI following receptor activation was not inhibited by the Syk SH2 domains. Interestingly, we observed higher levels of tyrosine phosphorylation of γ and β subunits in

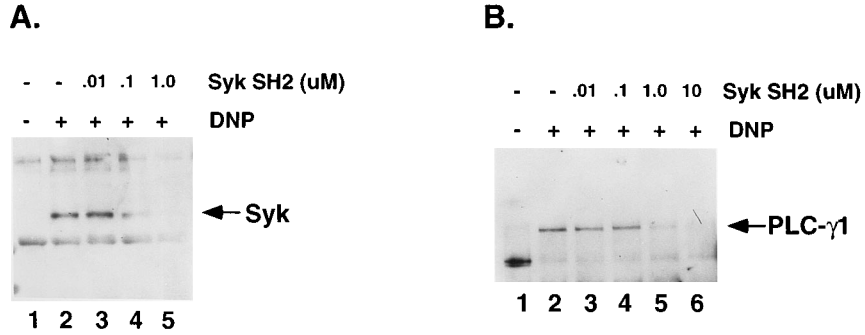

FIG. 7. The Syk SH2 domains inhibit tyrosine phosphorylation of the Syk kinase and PLC-γ1. NP-40 cell lysates were prepared from cells as described above and immunoprecipitated with antibodies directed against either Syk (A) or PLC-γ1 (B). Immunoprecipitates were analyzed by anti-phosphotyrosine immunoblotting as described in the text.

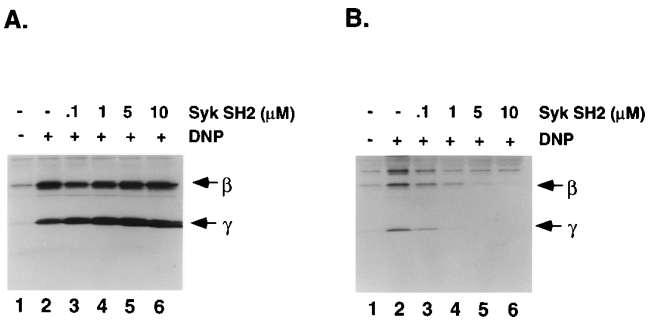

FIG. 8. The Syk SH2 domains prevent the association of Syk with the activated FcεRI. NP-40 lysates of permeabilized cells treated with the Syk SH2 domains were immunprecipitated with anti-γ antibodies (A) or anti-Syk antibodies (B). Immunoprecipitates were run on a 4 to 20% gradient gel, transferred to polyvinylidene difluoride membrane, and analyzed by anti-phosphotyrosine immunoblotting.

cells treated with the SH2 domains than in untreated cells (for example, compare lanes 2 and 6), suggesting that the SH2 domains introduced into the cell may be binding to the tyrosine-phosphorylated receptor subunits and protecting them from dephosphorylation. In contrast, however, the amount of tyrosine-phosphorylated γ and β found associated with Syk after receptor activation was dramatically reduced in cells treated with the Syk SH2 domains (Fig. 8B). These results support a model suggesting that the Syk SH2 domains are blocking cell activation by preventing the association of Syk with the activated receptor and demonstrate the importance of these interactions for mast cell activation.

DISCUSSION

In this report, we have shown that treatment of SLO-permeabilized RBL cells with the Syk tandem SH2 domains inhibits signaling through FcεRI. This inhibition is mediated by an SH2 interaction, since a Syk tandem SH2 domain which no longer binds phosphotyrosine was unable to block degranulation in activated RBL cells; in addition, the effect of the wild-type Syk SH2 domains was reversed by a phosphorylated peptide containing the ITAM from the FcεRIγ subunit of the receptor, while the analogous nonphosphorylated peptide had no effect. Within the cell, the inhibition by the Syk SH2 domains is probably due to the competitive inhibition of a critical interaction between the SH2 domains of endogenous Syk and a tyrosine-phosphorylated ligand. These results confirm the key role that Syk plays in mediating signaling through the IgE receptor on mast cells (44, 51) and demonstrate the importance of its SH2 domains in mediating these signaling events.

The specificity of the inhibition for interactions mediated by the endogenous Syk SH2 domains was validated in several ways. First, treatment of cells with the Syk SH2 domains had little effect on degranulation of RBL cells following activation by two alternative stimuli, GTPγS and PMA-A23187, which induce degranulation by either activation of a stimulatory G-protein pathway or direct mobilization of Ca^{2+} and activation of protein kinase C. In addition, the Syk SH2 domains exhibited little inhibitory activity on degranulation resulting from the direct activation of a CD16/Syk chimera in the absence of FcεRI cross-linking. In each of these cases, induction of degranulation is through stimulation of pathways which bypass the requirement for FcεRI aggregation and activation. The lack of inhibition by the Syk tandem SH2 domains under these circumstances demonstrates that the effect of the SH2 domains is specific for signaling through the IgE receptor and is not due to a global nonspecific effect on the ability of the cells to release granule contents.

Additional evidence supporting the specificity of the inhibition provided by the Syk SH2 was provided by experiments with SH2 domains from other signaling proteins expressed in RBL cells. We found that the SH2 domains from PLC-γ1 and Src inhibited degranulation with IC_{50}s approximately 10- and 100-fold higher, respectively, than the IC_{50} of the Syk SH2 domains. In vitro binding data demonstrate that the Syk, PLC-γ1, and Src SH2 domains bind with high affinity to different phosphopeptide sequences (50, 51) and that the biological activity of an SH2 domain can be correlated with its binding specificity (35). The results presented here suggest that there is selectivity in the ability of the different SH2 domains to inhibit signaling through the IgE receptor and that this selectivity may reflect a higher-affinity binding of the Syk SH2 domains to a target critical for activation.

It has been shown that in RBL-2H3 cells, tyrosine phosphorylation of PLC-γ1 is correlated with an increase in activity following FcεRI stimulation, resulting in the activation of pathways leading to mobilization of intracellular calcium and activation of protein kinase C. It is interesting that the PLC-γ1 SH2 domains did have some activity in blocking signaling following FcεRI activation. It is possible that this inhibition is due in part to competition with a PLC-γ1 SH2 ligand. Further experiments are necessary to identify the mechanism of this inhibition. Introduction of domains from other signaling proteins into permeabilized cells will provide a useful approach for examining signaling events in other systems as well.

Activation of mast cells induces the release of multiple mediators of the allergic response. These mediators fall into three classes: preformed molecules released by degranulation of secretory vesicles, newly synthesized mediators generated from lipid precursors, and newly transcribed cytokines. The Syk tandem SH2 domains inhibited two of these pathways, degranulation and leukotriene production. This finding is important because it demonstrates that blocking the interaction of the Syk SH2 domains with its target is sufficient to prevent late events associated with receptor activation. Although it is not possible to measure cytokine production in permeabilized cells, recent work has demonstrated that Syk activation in the absence of FcεRI cross-linking is sufficient to induce degranulation, leukotriene production, and synthesis of the cytokines interleukin-3 and tumor necrosis factor alpha (44). It is therefore likely that inhibition of mast cell activation by the Syk tandem SH2 domains would block cytokine synthesis as well as degranulation and leukotriene production.

One of the earliest events following FcεRI activation is the tyrosine phosphorylation of multiple cellular proteins, including Syk and the β and γ subunits of the receptor itself. Treatment of permeabilized cells with the Syk SH2 domains completely inhibited the tyrosine phosphorylation of most of these proteins. Since essentially all of the increase in tyrosine phosphorylation on cellular proteins can be attributed to activation of Syk (44), these results suggest that the block in cell activation by the Syk SH2 domains can be attributed to an inhibition of Syk activation. This conclusion is strengthened by the observation that tyrosine phosphorylation of PLC-γ1, a downstream target for Syk activation (44), is significantly reduced by the Syk SH2 domains. In addition, the rapid tyrosine phosphorylation of Syk itself, which is coincident with receptor binding to FcεRI and an increase in kinase activity (8, 37), was not observed in Syk SH2-treated cells. Taken together, the data demonstrate that the tandem Syk SH2 domains block RBL cell activation at a very early step in the pathway, most probably by inhibiting Syk activation.

Interestingly, the β and γ subunits of the FcεRI receptor which are tyrosine phosphorylated in response to receptor cross-linking did not exhibit a detectable decrease in phosphorylation after Syk SH2 treatment. Oliver et al. have recently described a similar insensitivity of receptor phosphorylation to treatment of RBL cells with the Syk-selective inhibitor piceatannol (37a). The results observed in the CD16 chimera cells are consistent with these data, suggesting that the SH2 domains inhibit Syk activation and that signaling events prior to that are not affected.

The current model for the early events following FcεRI aggregation suggests that aggregation of the receptor results in activation of Lyn kinase activity. The subsequent tyrosine phosphorylation of the receptor subunits by activated Lyn creates high-affinity binding sites for Syk (27, 48). Syk becomes phosphorylated on tyrosine, and its kinase activity is increased, resulting in the phosphorylation of downstream substrates. The results of this study suggest that the Syk SH2 domains inhibit mast cell activation by blocking a step in the signaling pathway prior to activation of Syk kinase activity and that the inhibition required an SH2 interaction.

What is the critical target for the Syk SH2 domains in mediating the inhibition of mast cell activation? A growing body of evidence would suggest that this target is the tyrosine-phosphorylated γ subunit of the FcεRI receptor. In vitro binding experiments have recently shown that the Syk SH2 domains selectively bind only the tyrosine-phosphorylated β and γ subunits in lysates of RBL cells and that the preferred ligand is the γITAM (27, 48). In addition, recent work has demonstrated that phospho-γITAM peptides can specifically stimulate both Syk autophosphorylation and phosphorylation of an exogenous substrate in vitro (49). The importance of the Syk SH2 interaction with the ITAMs can also be inferred from studies on the interaction of ZAP-70 with the ζ chain of the TCR. Recently, Wange et al. used a protein tyrosine phosphatase-resistant ζITAM peptide to block the interaction of ZAP-70 with the TCR, thus inhibiting ZAP-70 activation and tyrosine phosphorylation of a number of substrates (57). Taken together with the data presented here demonstrating that association of Syk with the activated receptor is blocked by the Syk SH2 domains, these studies support the model that binding of Syk to the tyrosine-phosphorylated γITAM is critical for Syk activation and subsequent steps leading to mediator release and that blocking the interaction of the Syk SH2 domain with γITAM is sufficient to prevent mast cell activation.

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