Distinct Roles for the Linker Region Tyrosines of Syk in FceRI Signaling in Primary Mast Cells*

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Stimulation of FcRI, the high affinity IgE receptor of mast cells results in the rapid binding of the Syk tyrosine kinase to cytoplasmic domains of FcRI and to its subsequent activation. Syk plays an essential role in signal transduction from FcRI as shown by Syk-deficient mast cells, which are defective in receptor-induced degranulation, cytokine synthesis, and intracellular pathways. However the mechanism by which Syk activates these pathways remains unclear. Activation of Syk is associated with its phosphorylation on several tyrosine residues, including the linker tyrosines Tyr342, Tyr346, and Tyr346. These residues have been proposed to play important roles in the transduction of signals by binding to other signaling proteins. To test these hypotheses in primary murine mast cells, we used retroviral infection of Syk-deficient mast cells to generate cells expressing Syk proteins bearing mutations in the linker tyrosines. We show that Tyr342 and Tyr346 contribute positively to the function of Syk and have both overlapping as well as distinct functions. Mutations in either Tyr342 or Tyr346 alone had no effect on FcRI-induced degranulation or calcium flux, whereas mutation of both residues caused a significant reduction in both pathways. In contrast, phosphorylation of PLCγ1, PLCγ2, and Vav1 was strongly decreased by a mutation in Tyr346 alone, whereas phosphorylation of ERK and Akt was more dependent on Tyr346. Finally we show that Tyr317 functions as a negative regulatory site and that its mutation can partially compensate for the loss of both Tyr342 and Tyr346.

The activation of mast cells by complexes of antigen and immunoglobulin E (IgE) is a key component of the allergic response. Binding of IgE-antigen to FcRI, the high affinity IgE receptor of mast cells activates biochemical signaling pathways resulting in the release of three classes of allergic mediators (1). First, the cells degranulate, releasing allergic mediators such as serotonin and histamine from preformed granules. Second, they synthesize leukotrienes and prostaglandins, and finally, they induce expression and secretion of cytokines.

Signaling from FcRI is initiated by IgE-antigen-induced aggregation, resulting in the activation of the associated Src family kinases Lyn and Fyn, which phosphorylate tandem tyrosines of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of the β- and γ-subunits of FcRI (2). These ITAM phosphotyrosines in turn form binding sites for the tandem SH2 domains of the Syk tyrosine kinase. Binding of Syk to the phosphorylated ITAMs causes a conformational change in the enzyme resulting in its activation, autophosphorylation, and Lyn-dependent phosphorylation (3, 4). Syk is essential for signal transduction from FcRI, as we demonstrated using Syk-deficient primary murine mast cells. In the absence of Syk, mast cells failed to degranulate, synthesize leukotrienes, and secrete cytokines following FcRI stimulation (5). Similar results were obtained by others using a Syk-deficient variant of the rat basophilic leukemic cell line RBL-2H3 (6).

Analysis of FcRI signaling pathways in Syk-deficient mast cells showed that, as expected, Syk was not required for the activation of Lyn, or for the phosphorylation of FcRI-β and -γ (5). In contrast, the FcRI-induced rise in intracellular Ca2+, and activation of the ERK and JNK MAP kinase pathways was completely abrogated in the absence of Syk. Furthermore, phosphorylation of phospholipase Cγ1 (PLCγ1) and of the Vav1 exchange factor was also Syk-dependent.

A number of tyrosine residues in Syk have been shown to undergo phosphorylation following stimulation of FcRI or other immune receptors (7). There has been much interest in determining the significance of these modifications, in particular of tyrosines in the kinase domain and in the linker domain, which lies between the SH2 domains and the kinase domain. Two tyrosine residues in the activation loop of the kinase domain of Syk (Tyr317 and Tyr520) have been shown to be essential for FcRI signaling in RBL-2H3 cells (8). Mutation of both tyrosines does not affect the kinase activity of Syk, but blocks FcRI-induced degranulation, ERK activation, and PLCγ1 phosphorylation. Three conserved tyrosine residues in the linker domain, Tyr317, Tyr342, and Tyr346, lie within consensuses sequences for recognition by SH2 domains, suggesting that they may play important roles in transducing signals. Phosphorylation of Tyr317 creates a binding site for c-Cbl, an E3 ubiquitin ligase, whose binding has been proposed to cause the ubiquitination and subsequent degradation of Syk (9). Thus Tyr317 was proposed to function as a negative regulatory site on Syk. In agreement with this, expression of the SykY317F mutant...
protein in Syk-deficient RBL-2H3 cells resulted in a large increase in FcεRI-induced degranulation, and phosphorylation of PLCγ1 and PLCγ2 (10).

In contrast, Tyr342 and Tyr346 have been proposed to function as positive regulatory sites. In COS cells, Vav1 has been reported to bind to phosphorylated Tyr342 (pY342) through its SH2 domain, and this binding may be enhanced by pY346 (11). Furthermore, also in COS cells, mutation of Tyr342 and Tyr346 abolished both the binding of PLCγ1 to Syk and the Syk-induced phosphorylation of PLCγ1 (12). Thus it has been proposed that pY342 and pY346 may function as binding sites to recruit substrates for Syk, such as Vav1, PLCγ1, and potentially other signaling proteins. This idea has been tested in RBL-2H3 cells, where expression of Syk with a Y342F mutation resulted in decreased FcεRI-induced degranulation, calcium flux, and phosphorylation of PLCγ2, LAT, and SLP-76 but, surprisingly, normal phosphorylation of PLCγ1 and Vav1 (13). In contrast, a Y346F mutation had little effect.

In this study we examine the role of Tyr342 and Tyr346 in transducing FcεRI signals in primary murine mast cells by reconstituting Syk-deficient cells with Syk genes mutated at Tyr342, Tyr346, or both. We show that while mutation of both residues is required to show significant reductions in FcεRI-induced degranulation and calcium flux, single mutations in either Tyr342 or Tyr346 cause reductions in FcεRI-induced phosphorylation of PLCγ1, PLCγ2, LAT, SLP-76, Vav1, ERK, and Akt. Interestingly, the effects of the two mutations are distinct with different pathways showing different dependence on the two tyrosines.

**EXPERIMENTAL PROCEDURES**

**Mast Cell Culture**—Mice bearing a gene disruption of the Syk gene on a 129S8 background have been described before (129S8-Slyk−/−; Syk−/−) (14). Syk−/− mice were intercrossed, and fetal liver cells from resulting 16.5-day wild-type or Syk−/− embryos were cultured at 2 x 10^5 cells/ml in mast cell medium (CM; RPMI 1640, 10% fetal calf serum, glutamine (2 mM), non-essential amino acids (Invitrogen, Life Technologies), sodium pyruvate (1 mM), 2-mercaptoethanol (50 mM), penicillin/streptomycin, and 10% conditioned medium from WEHI-3B cells as a source of IL-3). After 7 days of culture, the adherent cells were scraped off, and together with the suspension cells were reseeded at 2 x 10^5 cells/ml in fresh MCM. This was repeated every week. After 4 weeks of culture, only suspension cells were propagated. These were confirmed as being >95% mast cells by staining for FcεRI (5).

**Retroviral Vectors—**pcDNA3 plasmids containing wild-type murine Syk cDNA and SykY342F, SykY346F, and SykY317F/Y342F/Y346F have been described before (15). The SykY342F/Y346F mutant was generated by site-directed mutagenesis using the following primers: 5'-GGA-CACAGAGGTGTGGAGACCTTTGAGG-3', and 5'-GAAAAAGGGC-TTCTGGAACACCTGTTGC-3', and the site-directed mutagenesis kit (Stratagene, cat. no. 200618). Wild-type and mutant Syk cDNAs were excised from pcDNA3 as a 2.5-kb BamHI/EcoRI fragment and cloned between the BamHI and NotI sites of the pcMXpuro retroviral vector (T. Kawakami, La Jolla Institute for Allergy and Immunology) (16). All mutants were verified by sequencing.

**Retroviral Transduction of Cells—**pcMXpuro retroviral vectors were transfected into the BOSC23 packaging cell line (17) using Lipofectamine (Invitrogen, Life Technologies). For infection of NIH-3T3 cells, medium containing the retrovirus was harvested from the BOSC23 cells 48 h after transfection, filtered (0.45 μm), and 2 ml of this retroviral supernatant containing polybrene (10 μg/ml, Sigma-Aldrich) were placed onto 10-cm plates of 70% confluent NIH-3T3 cells from which medium had been removed. The cells were cultured for 4 h at 37 °C, then fresh medium was added (10 ml/plate). After a further 48 h of culture, the NIH-3T3 cells were selected with puromycin (2 μg/ml). For infection of mast cells, the transfected BOSC23 cells (5 x 10^5 cells) were mitotically inactivated (3000 rad of γ-irradiation from a ^137Cs source), and then co-cultured for 24 h in MCM, stem cell factor (SCF, 100 ng/ml, R&D Systems), and polybrene (10 μg/ml) with 4-week-old mast cell cultures (10^4 cells) that had been grown for the previous 2 weeks in MCM with SCF. Following exposure to retrovirus, the mast cells were harvested away from the adherent BOSC23 cells and put into fresh MCM plus SCF for 48 h, after which they were selected by culturing in MCM, SCF, and puromycin (2 μg/ml) for at least a week. Cells used for experiments were taken out of puromycin selection at least 2 weeks prior to the experiment.

**Degranulation**—To measure degranulation by release of serotonin, mast cells were preloaded overnight with anti-DNP-IgE (5 μg/ml, SPE-7, Sigma) and with 5-hydroxy[3H]tryptamine (serotonin) (66 nM, 15.1 Ci/mmol, Amersham Biosciences). Washed cells were challenged with DNP-HSA (Sigma) diluted in RPMI 1640 (Sigma) for 30 min. Percentage degranulation was defined as the fraction of [3H]serotonin released into the supernatant. Typical maximal degranulation response of mast cells expressing wild-type Syk (usually observed at 10 ng/ml DNP-HSA) was 50–70%. Within each experiment each stimulation was carried out in duplicate or triplicate, and the mean degranulation calculated. To combine data from experiments carried out on different days, degranulation was normalized to the maximum response observed for cells expressing wild-type Syk, which was set at 100%.

**Intracellular Calcium Flux**—Mast cells were preloaded with anti-DNP-IgE (5 μg/ml) for 1 h in RPMI 1640, then washed into RPMI 1640 containing Indo-1-AM (1 mM) and incubated at 37 °C for 45 min in the dark. Analysis was undertaken on a FACSVantage flow cytometer (BD Biosciences). Indo-1 emission was detected using 405/40 nm (violet) and 495/20 nm (blue) bandpass filters. To determine relative calcium concentration, cells were warmed to 37 °C, analyzed for 1 min to establish baseline calcium levels and stimulated by the addition of the agonist (10 ng/ml). Acquisition was continued for a further 5 min. Relative intracellular calcium concentration was calculated from a ratio of the violet/blue emissions.

**Biochemical Analysis of Signaling Pathways**—Cells were preloaded overnight with anti-DNP-IgE. To make total cell lysates, cells were challenged at 10^6 cells/ml with 10 ng/ml DNP-HSA in RPMI, and lysates prepared by directly boiling cells in reducing group-containing Laemmli sample buffer. For immunoprecipitations, cells were challenged in RPMI at 10^5 cells/ml with DNP-HSA (10 ng/ml) and lysed in lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 10 mM NaF, 10 mM disodium pyrophosphate, pH 7.5, 2% n-octyl β-D-glucopyranoside, 2 mM sodium orthovanadate, 1:50 (v/v) protease inhibitor mixture (P8340; Sigma-Aldrich)). Lysates were cleared by centrifugation at 14,000 rpm for 30 min. Immunoprecipitations and immunoblots were carried out as standard procedures. Anti-phosphotyrosine mAb RC20 conjugated directly to horseradish peroxidase (HRP, BD Transduction Labs), anti-phospho-ERK(1/2) (E-4) mAb (sc-7383, Santa Cruz Biotechnology, Inc.), anti-PLCγ2 rabbit polyclonal Ab (sc-407; Santa Cruz Biotechnology, Inc.), anti-LAT rabbit polyclonal Ab M41 (M. Turner, Babraham Institute), anti-SLP-76 sheep polyclonal Ab (G. Koretzky, University of Pennsylvania), anti-Vav1 rabbit polyclonal Ab (6B-25), Upstate Biotechnology), anti-ERK mAbs, with protein A-HRP (Amersham Biosciences) for rabbit monoclonal antibodies, and with goat anti-sheep IgG-HRP (Serotec) for sheep polyclonal antibody.

For densitometric analysis, the blots were scanned, bands of interest were quantitated and in-lane background was subtracted. To determine specific phosphorylation, the signal from phosphorylated bands was divided by the signal from the appropriate loading control, and all values were normalized to the maximum response (set to 100%).

**In Vivo Syk Kinase Assay**—2 x 10^7 NIH-3T3 cells retrovirally transduced with the different forms of Syk were lysed in lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 10 mM NaF, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine hydrochloride (Sigma-Aldrich), 0.1 mM TLCK (Sigma-Aldrich), 0.1 mM TPCK (Sigma-Aldrich), protease inhibitor mixture (Sigma-Aldrich)). Lysates were cleared by centrifugation at 14,000 rpm for 30 min. Lysates were preincubated with protein A-agarose beads (sc-2001, Santa Cruz Biotechnology, Inc.) for 30 min at 4 °C. Syk was immunoprecipitated from the lysates using an anti-Syk rabbit polyclonal (sc-1077, Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C, and then protein A-agarose beads for 2 h at 4 °C. Beads were washed twice with kinase buffer (30 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.1% Nonidet P-40), and then resuspended in 40 μl of kinase buffer containing 5μM ATP, 5μM of γ-32P-ATP, 5 μg of cd3 (cytoplastmic domain of erythrocyte band 3 protein expressed in Escherichia coli and purified by ion exchange chromatography and ammonium sulfate precipitation), and incubated at 25 °C for 20 min.
Syk mutants were all expressed at similar levels in the mycin. Immunoblot analysis showed that wild-type Syk and the Y346F double mutant showed a reduced efficiency of degranulation in Y342F-expressing cells was normal. Nonetheless, it is clear that while Tyr342 and Tyr346 play a role in transducing FcεRI signals leading to degranulation, they are not essential, since at the optimal dose of DNP-HSA, degranulation in Y342F/Y346F-expressing cells was normal.

A third tyrosine residue in the linker region of Syk which becomes phosphorylated following receptor activation is Tyr317. When phosphorylated, this residue has been postulated to bind Cbl, an E3 ubiquitin ligase, which acts as a negative regulator of immune receptor signaling by causing the ubiquitination and subsequent degradation of Syk (9, 10). Expression of SykY317F in RBL-2H3 basophilic cells leads to increased FcεRI-induced degranulation and phosphorylation of PLCγ1 and PLCγ2 (10). The effect of this mutation is presumably to cause less ubiquitination and degradation of Syk. We wondered whether the Y917F mutation could compensate for the signal defects in mast cells expressing Syk mutants in Tyr342 and Tyr346. To evaluate this, we generated mast cell cultures expressing SykY342F/Y346F (3F), and compared their responses to cells expressing the double mutant Y342F/Y346F. We found that the 3F mutant restored normal FcεRI-induced degranulation (Fig. 2), consistent with Tyr317 playing a negative regulatory role.

**RESULTS**

**Expression of Syk with Mutations in Linker Region Tyrosines in Syk+/− Mast Cells—**To generate primary mast cells expressing Syk with mutations in linker region tyrosines, we infected fetal liver-derived Syk+/− mast cells with the pMXpuro retroviral vector. Both the empty pMXpuro vector (E), or pMXpuro expressing wild-type Syk (WT), SykY342F (Y342F), SykY346F (Y346F), SykY324F/Y346F (Y342F/Y346F), or SykY317F/Y342F/Y346F (3F), Blot was immunoblotted (IB) with antiserum specific for Syk, then stripped and reprobed with antiserum against Lyn as a loading control. Note that there are two isoforms of Lyn. B, Syk was immunoprecipitated (IP) from NIH-3T3 cells infected with retroviral vectors described in A, and used in an *in vitro* kinase (IVK) reaction with [*γ-32P]*ATP to phosphorylate cdb3. An autoradiograph of the immunoblot shows incorporation of [*32P]* into cdb3 as a measure of Syk kinase activity (IVK). The blot was probed with antiserum specific for Syk as a loading control.

Control experiments showed that the kinase reaction was still linear at this time point (not shown). An equal quantity of 2× sample buffer was added to the samples, which were denatured at 100 °C for 5 min before analysis by SDS-PAGE, autoradiography, and immunoblotting.

Mutation of Tyr342 and Tyr346 Results in Defective FcεRI-Induced Calcium Flux—A rapid rise in intracellular calcium is a hallmark of FcεRI signaling and is required for degranulation (18). Once again we had shown earlier that Syk is absolutely required for the FcεRI-induced calcium flux in primary mast cells (5). Examination of FcεRI-induced calcium flux in mutant mast cells showed that while mutations in either Tyr342 or Tyr346 alone had no effect, the Y342F/Y346F double mutant showed no delay (Fig. 3). However, despite the delay, cells expressing SykY342F/Y346F still achieved a normal maximum of intracellular calcium, showing that these linker tyrosines are not absolutely required for this response. In cells expressing the 3F Syk mutant, FcεRI-induced calcium flux was the same as that in cells expressing wild-type Syk, showing that, as with degranulation, the Y317F mutation reversed the effects of the Y342F/Y346F double mutant.

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Mutation of Tyr342 and Tyr346 Results in Ineffective FcεRI-Induced Phosphorylation of PLCγ1, PLCγ2, LAT, SLP-76, and Vav—In mast cells, the FcεRI-induced intracellular calcium flux is induced by the second messenger inositol-3,4,5-trisphosphate, which in turn is generated following the hydrolysis of phosphatidylinositol-4,5-bisphosphate by PLCγ1 and PLCγ2. We had previously shown that Syk was absolutely required for the FcεRI-induced phosphorylation of PLCγ1, a modification that correlates with its activation (5). In this study we found that the phosphorylation of the related PLCγ2 isoform was also strictly Syk-dependent (Fig. 4, A and B). Examination of cells expressing the mutant Syk proteins showed that the Y342F mutation alone was sufficient to substantially reduce FcεRI-induced phosphorylation of both PLCγ1 and PLCγ2 (Fig. 4, A and B). In contrast, the effect of the Y346F mutation was less
pronounced. In cells expressing the Y342F/Y346F double mutant, the FcεRI-induced phosphorylation of both PLCγ1 and PLCγ2 was largely abrogated, similar to the Y342F single mutation. Expression of the 3F Syk mutant restored some FcεRI-induced phosphorylation of PLCγ1 and PLCγ2, once again consistent with a negative signaling role for Tyr317.

Studies of primary mast cells deficient in the LAT or SLP-76 adapter proteins have shown that both proteins are required for FcεRI-induced calcium flux and phosphorylation of PLCγ1 and PLCγ2 (19, 20). Furthermore, Jurkat T cells deficient in the Syk family kinase ZAP-70 show no T cell receptor (TCR)-induced phosphorylation of either LAT or SLP-76 (21, 22). Taken together, these studies suggest that in mast cells, Syk may transduce FcεRI signals to the phosphorylation of PLCγ1 and PLCγ2 in part by phosphorylating LAT and SLP-76. To examine this possibility, and to determine if the effect of the Tyr342 and Tyr346 mutations on PLCγ phosphorylation may be caused by reduced phosphorylation of the adapter proteins, we examined this pathway in mast cells deficient in Syk, or expressing the mutant proteins. We found that FcεRI-induced phosphorylation of both LAT and SLP-76 absolutely required Syk (Fig. 5A and data not shown). Furthermore, mutation of either Tyr342 or Tyr346 resulted in a reduction of both LAT and SLP-76 phosphorylation (Fig. 5A and data not shown). In these cases, the Y342F/Y346F double mutant was not affected any further than the single mutants. Expression of the 3F Syk mutant partially restored FcεRI-induced phosphorylation of SLP-76, but not that of LAT (Fig. 5A and data not shown).

Mast cells deficient in the Rho-family GTPase exchange factor Vav1 show reduced FcεRI-induced calcium flux and phosphorylation of PLCγ1 and PLCγ2 (23). Furthermore, we have shown that Syk is required for the FcεRI-induced phosphorylation of Vav1 (5). It has been proposed that through its SH2 domain, Vav1 may bind to phosphorylated Tyr315 of Syk or the related Tyr312 of ZAP-70, and that this binding may be required for Vav1 phosphorylation (11, 24, 25). In view of this, we asked whether mutation of the linker tyrosines perturbs Vav1 phosphorylation. Indeed, we found that FcεRI-induced Vav1 phosphorylation was substantially reduced in cells expressing the Y342F mutation, and to a lesser extent in cells expressing Y346F (Fig. 5B). The double mutant also showed a large reduction in Vav1 phosphorylation.

Tyr346 Is More Important Than Tyr342 for FcεRI-induced Phosphorylation of ERK and Akt—Another critical pathway in the allergic and inflammatory response induced by FcεRI is the activation of the ERK MAP kinases. Once again, in earlier work we had shown that Syk is required for FcεRI-induced ERK activation (5). Examination of cells expressing the mutant forms of Syk showed that the Y342F mutation did not perturb the initial phosphorylation of ERK, though the signal was not maintained as long (Fig. 6A). By 20 min, ERK phosphorylation had dropped to baseline in the Y342F mutant whereas phosphorylation was still detectable in cells expressing wild-type Syk. In contrast, the Y346F mutation had a much stronger effect, with little or no ERK phosphorylation visible in the mutant cells. Similarly, the double mutant shows essentially no FcεRI-induced ERK phosphorylation. It is interesting to note that while the phosphorylation of PLCγ1, PLCγ2, and Vav1 is affected more by the Y342F mutation than by Y346F, the reverse is true for ERK phosphorylation, which is more dependent on Tyr346. Expression of the 3F Syk mutant once
again showed rescue of the FcRI-induced ERK phosphorylation, consistent with a negative role for Tyr317.

A second pathway, which shows the same greater dependence on Tyr346 rather than Tyr342, is the activation of the serine/threonine kinase Akt. Akt activation requires the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of the action of phosphoinositide 3-kinase (PI3K), and hence is often used as a surrogate of PI3K activation (26, 27). Activation of Akt was monitored by phosphorylation of serine 473, and showed that FcRI-induced Akt phosphorylation was completely dependent on Syk (Fig. 6B). Furthermore, mutation of Tyr346 led to a substantial reduction in Akt phosphorylation. In contrast, as with ERK activation, the Y342F mutation had only a partial effect, resulting in a more transient phosphorylation of Akt. Once again, the double mutant showed almost no detectable Akt phosphorylation. Thus like ERK, Akt phosphorylation downstream of FcRI is more dependent on Tyr346 than on Tyr342 of Syk. In contrast to many of the other pathways we had looked at, expression of the 3F Syk mutant did not rescue FcRI-induced Akt phosphorylation.

DISCUSSION

We have studied the function of Tyr317, Tyr342, and Tyr346 of Syk by expressing mutant forms of Syk in Syk-deficient murine mast cells using retroviral gene transduction. While the vector-derived wild-type and mutant Syk proteins were expressed at equivalent levels, this was about 10-fold higher than the level of endogenous Syk in wild-type mast cells. The effect of this was to cause slightly higher levels of FcRI-induced degranulation and calcium flux (not shown). It is possible that this overexpression of Syk might have obscured some of the phenotypes of the tyrosine mutations. Nonetheless, since the levels of the transduced wild-type and mutant Syk proteins were equivalent, we think it is likely that the phenotypes observed are a true reflection of the function of these tyrosines in primary mast cells.

Analysis of kinase activity of the mutant proteins showed no changes. In contrast, earlier work in RBL-2H3 cells suggested that SykY342F had reduced basal and FcRI-stimulated kinase activity (13). The difference between these studies is unclear, but we note that in the RBL-2H3 work, the kinase activity was measured by autophosphorylation. It is possible that the observed reduced signal was because of the lack of phosphate incorporation into Tyr342 itself. In contrast, in our studies, we used an exogenous substrate (cdb3) and saw no differences in enzymatic activity.

Our studies demonstrate that both Thr342 and Tyr346 contribute positively to Syk function in FcRI signaling within primary mast cells. For some pathways, the two residues appear to have overlapping redundant functions. So while mutation of either Tyr342 or Tyr346 alone had no effect on FcRI-induced degranulation or calcium flux, mutation of both residues together partially compromised both processes. Notably, however, even the Y342F/Y346F double mutant was still capable of FcRI-induced degranulation and calcium flux, demonstrating that while these tyrosines may contribute to these signaling pathways, they are not essential.
This conclusion is further reinforced by the effect of the Y317F mutation. It has been previously reported in RBL-2H3 cells, that Tyr317 is a negative regulatory site for Syk function downstream of FcγRI, which may act by recruiting c-Cbl to Syk, causing ubiquitination and degradation of the kinase (9, 10).

We found that in primary mast cells, the Y317F mutation was capable of reversing the effects of the Y342F/Y346F double mutant on degranulation and calcium flux. If the effect of Y317F is to maintain higher levels of active Syk protein, this result emphasizes that even with mutations in Tyr342 and
Tyr346, Syk is capable of transducing FcεRI signals to degranulation and calcium flux. In contrast to their redundant function in degranulation and calcium flux, our data show for the first time that Tyr342 and Tyr346 also have unique and distinct functions. We found that FcεRI-induced phosphorylation of PLCγ1, PLCγ2, and Vav1 was strongly reduced by the Y342F mutation, but only weakly affected by Y346F. In contrast, FcεRI-induced phosphorylation of ERK and Akt was decreased strongly by Y346F, but only partially perturbed by Y342F. Finally, FcεRI-induced phosphorylation of LAT and SLP-76 was affected equally by both mutations.

How might tyrosines 342 and 346 be functioning? Our data support the hypothesis that PLCγ1, PLCγ2, and Vav1 may need to bind directly to pY342-Syk in order to become efficient substrates for Syk. Earlier work in COS cells had shown that PLCγ1 binding to Syk and its subsequent phosphorylation is abolished by simultaneous mutation of both Tyr342 and Tyr346 (12). More recently, a model in which TCR stimulation leads to phosphorylation of ZAP-70 on Tyr319, and low level phosphorylation of LAT. Subsequently, PLCγ1 is proposed to bind to pY319-ZAP-70 through its C-terminal SH2 domain and to a pTyr on LAT through its N-terminal SH2 domain, thereby facilitating further LAT phosphorylation by ZAP-70. PLCγ1 (or PLCγ2) may play such an adapter function in mast cells, bridging pY342/pY346-Syk with LAT, thereby allowing more efficient phosphorylation of LAT. Potentially a similar explanation may hold for the role of Tyr342 and/or Tyr346 in controlling SLP-76 phosphorylation, since SLP-76 is brought into a complex with LAT and PLCγ1 by binding to SH3 domains on PLCγ1 and Gads, an adapter which in turn binds to pTyr residues on LAT (29).

One surprising conclusion from our studies is that phosphorylation of PLCγ1 and PLCγ2 may not be required for FcεRI-induced calcium flux since the Y342F and Y342F/Y346F mutations strongly decrease phosphorylation of PLCγ1 and PLCγ2, yet have only a small effect on calcium fluxes. Alternatively, the small residual phosphorylation of PLCγ1 and PLCγ2 may

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**FIG. 6.** Defective FcεRI-induced phosphorylation of ERK and Akt in mast cells expressing Syk with mutations in either Tyr342 or Tyr346. Syk−/− mast cells infected with empty or Syk-expressing retroviruses as in Fig. 1A were stimulated and analyzed as indicated in Fig. 4. A, phosphorylation of ERK1 and 2 was analyzed by immunoblotting total cell lysates with antisera specific for phosphotyrosine 204 of ERK1 and 2 (p-ERK1 and p-ERK2), a modification which is required for their activation (33). B, phosphorylation of Akt was analyzed by immunoblotting total cell lysates with antisera specific for phosphoserine 473 of Akt (p-Akt).
be sufficient to activate the enzymes and result in a calcium flux. It may even be the case that at least some of the FceRI-induced calcium flux is independent of PLCγ1 and PLCγ2. PLCγ2-deficient mast cells show only a mild defect in FceRI-induced calcium flux (30), but there is currently no published study of mast cells deficient in PLCγ1 or both PLCγ isoforms. One potential PLCγ-independent pathway for FceRI-induced calcium flux is via sphingosine kinase (31).

Our studies have also revealed distinct functions for Tyr317. Mutation of Tyr317 in the context of mutations in Tyr342 and Tyr346 partially restores FceRI-induced phosphorylation of PLCγ1, PLCγ2, SLP-76, and ERK. These results are consistent with the model that when phosphorylated, Tyr317 binds Cbl, an E3 ubiquitin ligase resulting in the ubiquitination and subsequent degradation of Syk (9, 10). Thus the effect of the Y317F mutation is presumably to cause less ubiquitination and degradation of Syk, and hence increase Syk-dependent signaling leading to the phosphorylation of PLCγ1, PLCγ2, SLP-76, and ERK. In contrast to these results, the 3F mutant did not restore FceRI-induced phosphorylation of Akt. One possibility is that Tyr342 and Tyr346 may be absolutely essential for the ability of Syk to transduce signals leading to the phosphorylation of Akt, and thus stabilization of Syk by mutation of Tyr317 cannot rescue the defect. Alternatively, Tyr317 may have a positive role in Syk function in transducing signals to Akt. Recent work from one of us suggests that the latter possibility may be true. We have shown that the p85α adapter subunit of PI3K binds through its C-terminal SH2 domain to pY317, whereas its N-terminal SH2 domain may bind to pY342 or pY346 (32). These results predict that mutation of Tyr317 would reduce the recruitment to Syk of p85α and its associated catalytic PI3K subunit, thereby decreasing the amount of phosphatidylinositol 3,4,5-trisphosphate (PIP3) synthesized and hence inhibiting Akt activation. Our observation that the Y317F mutation fails to rescue FceRI-induced phosphorylation of Akt is in agreement with this prediction.

In conclusion, we have shown that Tyr317 plays a negative role in transducing FceRI signals, however this effect is specific for only some pathways and not others. In addition we have found that while Tyr342 and Tyr346 are not essential for the function of Syk downstream of FceRI, they nonetheless make an important positive contribution to the ability of Syk to transduce signals. We have shown that the two residues have both overlapping and distinct functions, making contributions to a number of key FceRI signaling pathways including degranulation, calcium flux, and activation of Akt and the ERK MAP kinases.

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