Tyrosines in the Carboxyl Terminus Regulate Syk Kinase Activity and Function

The Syk tyrosine kinase family plays an essential role in immunoreceptor tyrosine-based activation motif (ITAM) signaling. The binding of Syk to tyrosine-phosphorylated ITAM subunits of immunoreceptors, such as FcεRI on mast cells, results in a conformational change, with an increase of enzymatic activity of Syk. This conformational change exposes the COOH-terminal tail of Syk, which has three conserved Tyr residues (Tyr-623, Tyr-624, and Tyr-625 of rat Syk). To understand the role of these residues in signaling, wild-type and mutant Syk with these three Tyr mutated to Phe was expressed in Syk-deficient mast cells. There was decreased FcεRI-induced degranulation, nuclear factor for T cell activation and NFκB activation with the mutated Syk together with reduced phosphorylation of MAP kinases p38 and p42/44 ERK. In non-stimulated cells, the mutated Syk was more tyrosine phosphorylated predominantly as a result of autophosphorylation. In vitro, there was reduced binding of mutated Syk to phosphorylated ITAM due to this increased phosphorylation. This mutated Syk from non-stimulated cells had significantly reduced kinase activity toward an exogenous substrate, whereas its autophosphorylation capacity was not affected. However, the kinase activity, the protein and the autophosphorylation capacity of this mutated Syk were dramatically decreased when the protein was dephosphorylated before the in vitro kinase reaction. Furthermore, mutation of these tyrosines in the COOH-terminal region of Syk transforms it to an enzyme, similar to its homolog ZAP-70, which depends on other tyrosine kinases for optimal activation. In testing Syk mutated singly at each one of the tyrosines, Tyr-624 but especially Tyr-625 had the major role in these reactions. Therefore, these results indicate that these tyrosines in the tail region play a critical role in regulating the kinase activity and function of Syk.

Syk and ZAP-70 are members of a tyrosine kinase family one or both of which are expressed in most hematopoietic cells (1–5). Structurally Syk/ZAP-70 consists of two Src-homology 2 domains (SH2) and a kinase domain followed by a short COOH-terminal extension (tail). The inter-SH2 domain is located between the NH2-terminal SH2 (SH2-N) and COOH-terminal SH2 (SH2-C), whereas the linker region is between the SH2-C and the kinase domains (6, 7). An alternatively spliced form of Syk, termed SykB, lacks a 23-amino acid sequence in the linker domain (8). Although both isoforms are expressed in immune cells such as the RBL-2H3 rat mast cell line, Syk is more efficient than SykB in immunoreceptor signaling (8, 9).

In mast cells, aggregation of the high affinity receptor for IgE (FcεRI) initiates a series of biochemical changes that results in release of pre-formed mediators from granules (histamine, β-hexosaminidase, heparin, and others), together with the synthesis and release of lipid mediators and cytokines (14). FcεRI is a tetrameric receptor formed by an α-chain responsible in binding IgE to the cell surface, a β-chain, and a homodimer disulfide-linked γ-chain (15). Because FcεRI has no intrinsic enzymatic activity, the activation of non-receptor protein tyrosine kinases are critical for cell activation. Aggregation of FcεRI results in phosphorylation of the tyrosines in the ITAM of the β- and γ-chains by Lyn that are associated with the receptor. These phosphorylations create a docking site for binding by the two SH2 domains of Syk (16–18). This binding of Syk to the phosphorylated ITAM results in a conformational change of Syk, with an increase in its enzymatic activity (19, 20). Activation of Syk leads to downstream propagation of signaling with tyrosine phosphorylation of linker for activation of T cells (LAT), Vav, phospholipase Cγ, and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76). Phosphorylated LAT functions as a platform providing binding sites directly for growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor protein (Gads), and phospholipase Cγ; also indirectly for SLP-76, and several other adaptors. Activated phospholipase Cγ catalyzes generation of inositol triphosphate, which
release Ca\(^{2+}\) from intracellular stores and activates store-operated calcium channels for the influx of Ca\(^{2+}\). All these events culminate with mast cell degranulation, and activation of nuclear factor for T cell activation (NFAT) and nuclear factor \(\kappa B\) (NF\(\kappa B\)), which turn-on cytokine synthesis (21, 22).

Several tyrosines of Syk/ZAP-70 are phosphorylated after immunoreceptor activation. Phosphorylation of these residues regulates the activity of Syk/ZAP-70 and provides binding sites for other molecules. Phosphorylation mapping has identified 10 tyrosines in Syk that are autophosphorylated after an in vitro kinase reaction (23). Among these sites is the Tyr-317 of rat Syk, analogous to the Tyr-292 in human ZAP-70, phosphorylation of which creates a binding site for Cbl, a negative regulator of protein-tyrosine kinases (24–27). In addition, substitution of this tyrosine with phenylalanine results in a gain of function in signaling by Syk or ZAP-70 (28, 29). Similarly, the two adjacent tyrosines in the activation loop of the kinase domain of Syk (Tyr-519 and Tyr-520) are critical for downstream propagation of signals after immunoreceptor activation (30). However, the substitution of Tyr-519 and Tyr-520 with phenylalanine resulted in a major loss in the kinase activity of ZAP-70 but not of Syk (4, 30, 31). The COOH-terminal region of Syk has three conserved tyrosines (Tyr-623, Tyr-624, and Tyr-625 in rat Syk) that is the last two of which are also conserved in ZAP-70. Some of these tyrosines are phosphorylated in both Syk and ZAP-70 by autophosphorylation or following receptor stimulation (23, 32, 33). For example, in mast cells the last two, Tyr-624 and Tyr-625, are phosphorylated after FceRI aggregation (34). When B cell signaling is reconstituted in 26 insect cells, Tyr-630 of human Syk (analogous to Tyr-624 of rat Syk) is phosphorylated following BCR activation and this creates a binding site for SLP-65 (33). Furthermore, structural studies suggest that the two COOH-terminal tyrosines of ZAP-70 stabilize the autoinhibitory form of the kinase (35). These results suggest that tyrosines of the COOH-terminal region are phosphorylated after receptor stimulation and could play a role in signal transduction.

The purpose of this study was to characterize in vitro and in vivo the roles of tyrosines 623, 624, and 625 in the tail region in regulating Syk activity and function. Therefore, these tyrosines were mutated to phenylalanine and a plasmid with these mutations was transiently expressed in Syk negative mast cells. Compared with the wild-type protein, expression of the mutant with these three tyrosines replaced with phenylalanine (Y623F, Y624F, and Y625F) resulted in decreased FceRI-induced degranulation, together with reduced NFAT and NF\(\kappa B\) activation. In non-stimulated cells, this mutated Syk was more tyrosine phosphorylated predominantly as a result of autophosphorylation; this increased phosphorylation included both the activation loop and the negative Tyr-317 sites. In vitro this mutated Syk had dramatically reduced kinase activity and capacity for autophosphorylation unless it had been tyrosine phosphorylated by other tyrosine kinases in vivo. These results indicate that these tyrosines in the tail region play a critical role in regulating the kinase activity and function of Syk.

**EXPERIMENTAL PROCEDURES**

*Materials and Antibodies*—Triton X-100, Tween 20, protein A-agarose beads, streptavidin-agarose beads, aprotinin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), \(p\)-nitrophenyl-N-acetyl-\(\beta\)-d-glucosaminide were from Sigma. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride, bovine serum albumin fraction V (BSA), DTT, and leupeptin were from MP Biomedicals (Solon, OH). Reacti-Bind NeutrAvidin-coated clear strip plates were from Pierce. TMB substrate was from Cell Signaling Technology Inc. (Danvers, MA).

Horse-radish peroxidase (HRP)-conjugated anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p/44/42 ERK, anti-phospho-p/44/42 ERK (Thr-202/Tyr-204), anti-p38, anti-phospho-p38 (Thr-180/Tyr-182), anti-phosphotyrosine-Syk 327 (to rat Syk Tyr-317), and anti-phosphotyrosine Syk 525/526 (to rat Syk Tyr-519/520) were from Cell Signaling Technology Inc. (Danvers, MA). Anti-Syk (N-19) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-Syk (26BC1A6) was purified by GammaBind Plus-Sepharose from GE Healthcare. Rabbit anti-mouse IgG, HRP-conjugated donkey anti-mouse, and HRP-conjugated donkey anti-rabbit were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

**Plasmids and Constructs**—The pEAK12 plasmid containing the rat Syk sequence (NCBI Reference Sequence NM_012758.1) has been described previously (36). Syk with triple mutations Y623F/Y624F/Y625F (triple mutation, 3F) cloned into pSVL vector was transferred to pEAK12 vector containing KpnI and NotI. Tyrosines 130, 623, 624, 625, or both 624/625 (termed 2F) of Syk were mutated to phenylalanine using the QuikChange\textsuperscript{\textregistered} Lightning Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA). Dead kinase (DK) Syk (K396R) described previously (31) was transferred to pEAK12 vector containing KpnI and NotI. Syk-DK combined with the 3F mutation was made using XbaI and FspI. Syk-Y130F mutation was combined with the 3F mutation using KpnI and NotI. The full-length of Syk was sequenced to confirm these mutations.

**Cell Culture, cDNA Transfection, and Cell Activation**—Syk-negative C4A2 and the Syk-negative NFAT reporter mast cells have been described previously (37). These cells were cultured as monolayers in minimum essential medium from Invitrogen, supplemented with 15% heat-inactivated FBS, penicillin, streptomycin, amphotericin, and glucose. A Syk-negative NF\(\kappa B\) reporter cell line (C4A2-IC2) was prepared by cotransfecting a transcriptional reporter vector carrying four copies of the consensus NF\(\kappa B\) binding site located upstream of the minimal cytomegalovirus promoter that regulates NF\(\kappa B\)-dependent GFP expression pTRF1-NF\(\kappa B\)-dscGFP (System Biosciences, Mountain View, CA) and pSV-Neo plasmids at a ratio of 10:1 into Syk-negative C4A2 cells using the Nucleofector II program T20 and Amaca Cell Line Nucleofector Kit T (Biosystems, Middletown, CT). Transfected cells were selected by genetin (500 \(\mu g/ml\)), and then cloned to select cells with maximum GFP fluorescence response after stimulation with calcium ionophore.

For transient transfection, Syk-negative C4A2 cells were transfected using the Nucleofector II program T20 and Amaca Cell Line Nucleofector Kit T. After 32 h in culture, anti-trini-

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K. Sada, J. Zhang, and R. P. Siraganian, unpublished observations.
tyrosine-specific IgE (0.32 μg/ml) was added to the cells for 16 h. Cell monolayers were then washed two times with minimum essential medium containing 0.1% BSA and 10 mM Tris-HCl, pH 7.4, and activated with several concentrations of antigen (DNp31-HSA; 31 molecules of dinitrophenyl coupled per molecule of human serum albumin).

Measurement of β-Hexosaminidase Release—Cells were grown in 48-well plates (103/well), sensitized, and activated for 45 min as described above. Degranulation was determined by measuring the release of the granule marker, β-hexosaminidase. After activation, duplicate aliquots from supernatants of the cells (50 μl) were mixed with equal amount of substrate (8 mM p-nitrophenyl-N-acetyl-β-d-glucosaminide), in 0.1 M citrate-phosphate, pH 3.5, buffer (0.1 M citric acid, 0.2 M sodium phosphate dibasic). After incubation for 60 min at 37 °C, 50 μl of 0.4 M glycine, pH 10.5, buffer (0.4 M glycine, 0.4 M NaOH, 0.4 M NaCl) was added to stop the reaction and absorbance was measured at 405 nm. Values were expressed as percentage of intracellular β-hexosaminidase released into the medium.

Flow Cytometric Measurements of NFAT and NFκB Activation—Fluorescence of the GFP reporter was used as a marker of FcεRI-induced NFAT or NFκB activation after transient transfection with Syk. IgE-sensitized cells were washed and stimulated with antigen for 6 (for NFκB activity) or 16 h (for NFAT activity). These stimulation periods were found to result in maximal GFP expression. The percent of GFP positive cells was determined by a FACScan analysis and processed by CellQuest software (BD Bioscience).

Immunoprecipitation and Immunoblotting—Cell monolayers were rinsed with ice-cold PBS containing 2 mM Na3VO4 and protease inhibitors (2 mM PMSF, 200 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 76 milliunits/ml of aprotinin, 50 μg/ml of leupeptin, 5 μM pepstatin A) and solubilized in Triton lysis buffer (1% Triton, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, protease inhibitors and Na3VO4). In the indicated experiments, the lysis buffer was with or without Na3VO4 and the lysates were then kept for 4 h at 4 °C to dephosphorylate the proteins. The postnuclear supernatants were immunoprecipitated with anti-Syk mAb pre-bound with rabbit anti-mouse IgG to protein A-agarose beads. After rotation at 4 °C for 2 h, the beads were washed four times with ice-cold lysis buffer, and the bound proteins were eluted by boiling for 5 min with SDS-PAGE sample buffer (4% SDS, 160 mM Tris–HCl, pH 6.8, 20% glycerol, 2 mM Na3VO4, 100 mM DTT, and 0.005% bromphenol blue). To prepare total cells lysates, the cell monolayers were washed twice with ice-cold PBS containing 2 mM Na3VO4 and solubilized with boiling SDS-PAGE sample buffer. Total cell lysates or immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions (Criterion Precast Gel from Bio-Rad) and electrotransferred to polyvinyldene difluoride membranes (Millipore, Bedford, MA). The blots were probed with individual primary antibodies, and then incubated with HRP-conjugated donkey anti-mouse or rabbit antibody as required. In all blots, proteins were visualized by enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer Inc., Waltham, MA). The membranes were stripped for reprobing when indicated.

RESULTS

Conserved Tyrosines of Syk COOH-terminal Region Are Required for Optimal Mast Cell Secretion—The Syk and ZAP-70 tyrosine kinases are highly homologous proteins (3), which are implicated in antigen and Fcε receptor signaling (5). Analysis of the amino acid sequence of the COOH-terminal region of Syk and ZAP-70 show three adjacent tyrosines (Tyr-623, Tyr-624, and Tyr-625) were replaced with phenylalanine either singly or together and cloned into a mammalian expression vector (Fig. 1A). The two residues, Tyr-624 and Tyr-625, are autophosphorylated in vitro and in vivo they are phosphorylated in mast cells after FcεRI activation (23, 34). Structural studies of the ZAP-70 suggest that the COOH-terminal tyrosines interact with the kinase and the inter-SH2 domains resulting in autoinhibition of the enzymatic activity (Fig. 1B). To analyze the role of these tyrosines in FcεRI-mediated signaling, each of these three tyrosines (Tyr-623, Tyr-624, and Tyr-625) were replaced with phenylalanine either singly or together and cloned into a mammalian expression vector (Fig. 1B). Syk-negative mast cells were then transiently transfected with these plasmids containing...
shown in Fig. 1 were expressed at similar levels (a representative analysis is presented). In all experiments immunoblotting was used to ensure that the wild-type and mutant Syk proteins were expressed at similar levels (a representative analysis is shown in Fig. 1C).

As has been reported previously, the absence of Syk resulted in complete loss of mast cell degranulation (Fig. 1D), whereas FcεRI-induced secretion was reconstituted by transfection of wild-type Syk (11, 21, 30, 38). There was significant decreased degranulation in cells transfected with mutant Syk containing the triple tyrosine mutation (Y623F/Y624F/Y625F, termed 3F) compared with those that expressed the wild-type protein (Fig. 1D, left). The decrease was at all antigen concentrations tested (p < 0.0001). Syk mutated singly on each of these tyrosines (Y623F, Y624F, or Y625F) was also tested in this degranulation assay. There was reduced β-hexosaminidase release in cells transfected with Syk-Y625F or Syk-Y624F compared with wild-type (Fig. 1D, right). Among these two, there was a greater decrease in degranulation with Syk-Y625F than Syk-Y624F, whereas Syk-Y623F release was essentially identical to that of the wild-type. Transfection with Syk with a double mutation (Y624F/Y625F, named 2F) also had markedly reduced secretion (supplemental Fig. S1A). These results suggest that Tyr-624 and Tyr-625 of Syk play an important role in signaling for degranulation in mast cells.

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Syk-2F had dramatically reduced NFκB activation at all antigen concentrations (supplemental Fig. S1C). All together, these results indicate that the Tyr-624 and Tyr-625 of Syk are required for optimal mast cell degranulation and activation of NFAT and NFκB.

Tyr-625 Is Required for Optimal Activation of p42/44 ERK and p38 MAP Kinases—The MAP kinases are important signaling mediators between the cytosol and nucleus that regulate a variety of transcriptional factors (44). The activation of the MAP kinases p42/44 ERK and p38 are downstream of Syk (45–47). Accordingly, the phosphorylation of these MAP kinases was compared in cells expressing the wild-type or different mutants of Syk (Fig. 2C). Compared with wild-type, the Syk-3F expressing cells showed more transient FcεRI-induced p42/44 ERK phosphorylation. A stronger effect was observed on p38 activation; there was a dramatic decrease with Syk-3F in the extent of p38 phosphorylation. On analysis of the single mutants for both ERK and p38 phosphorylation, the stronger effects were with Syk-Y625F, with some changes with Syk-Y624F but essentially no difference from the wild-type for Syk-Y623F (Fig. 2C). These results suggest that Tyr-624 but especially Tyr-625 are important for the activation of the p38 and have some effects on stimulation of p42/44 ERK.

Enhanced Tyrosine Phosphorylation of Cellular Proteins by Expression of Syk-3F and Syk-Y625F—The increase in tyrosine phosphorylation of cellular proteins, in which Syk plays an important role, is a prominent response to FcεRI aggregation (11, 48). Therefore we investigated tyrosine phosphorylation of cellular proteins after transfection with wild-type or Syk mutants (Fig. 3A). There was dramatically increased tyrosine phosphorylation of proteins in non-stimulated cells expressing Syk-3F compared with wild-type; with prominent bands between 82 and 64 kDa. After FcεRI activation this strong phosphorylation slightly increased and persisted throughout the next 45 min. In contrast, receptor stimulation in cells expressing wild-type Syk resulted in tyrosine phosphorylation of proteins that subsided by 45 min. These effects on cellular tyrosine phosphorylations were also studied with Syk where these tyrosines were singly mutated; there were changes in both basal and receptor-induced phosphorylations in the Syk-Y624F and Syk-Y625F expressing cells that were similar but less in inten-

FIGURE 2. Tyrosines of Syk tail region are required for optimal mast cell activation. Syk-negative C4A2 cells with the NFAT-GFP (A) or NFκB-GFP (B) reporters were transiently transfected with empty plasmid (EV) or with plasmid containing wild-type (WT) or Syk mutants (3F, Y623F, Y624F, or Y625F) and cultured for 32 h. Antigen-specific IgE was added to the cultures and after another 16 h cells were washed and stimulated with the indicated concentrations of antigen and after further culture the percent of GFP-positive cells was determined by FACS. The results are the mean of three independent experiments; the error bar indicates the S.D. Statistical significance is shown on the graph for comparison of the release curves of the Syk mutants compared with wild-type Syk. C, MAP kinase activation; Syk-negative C4A2 transiently transfected and IgE-sensitized as described in the legend of Fig. 1. At the indicated times total cell lysates were collected and analyzed by immunoblotting with anti-phosphotyrosine antibodies (mAb-4G10, pY). Arrow marks the location of Syk. The bottom panel is the anti-Syk antibody re-probing of the membrane after stripping. Similar results were observed in four other experiments.

FIGURE 3. Total cellular phosphorylations. Syk-negative cells transiently transfected with the indicated Syk plasmids were stimulated as described in the legend of Fig. 1. At the indicated times total cell lysates were collected and analyzed by immunoblotting with anti-phosphotyrosine antibodies (mAb-4G10, pY). Arrow marks the location of Syk. The bottom panel is the anti-Syk antibody re-probing of the membrane after stripping. Similar results were observed in four other experiments.
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activation and are important in Syk-mediated signaling in mast cells (30, 31). Similarly, analysis using anti-phospho-p519/520 Syk antibodies showed that these residues were also phosphorylated in the Syk mutants in non-stimulated cells (Fig. 4B). Together, these results indicate that mutation at Tyr-624 and Tyr-625 leads to phosphorylation of Syk on tyrosines that have positive and negative regulatory functions.

**Basal Tyrosine Phosphorylation of Syk-3F Was Due to Both Other Tyrosine Kinases and Autophosphorylation**—Syk is capable of autophosphorylation when either expressed in vitro or immunoprecipitated from cells. We therefore, investigated the mechanism of the increased Syk-3F tyrosine phosphorylation in non-stimulated cells. Mutation of K396R in the catalytic domain of Syk abolishes its kinase activity (31). The Syk-3F was combined with K396R generating the mutant 3F dead kinase (Syk-3F/DK) similar to what has been reported previously (50). After expression in cells and immunoprecipitation, the Syk-3F/DK was more phosphorylated than its control, dead kinase protein (Syk-DK) suggesting that the 3F mutation was allowing the enzymatically inactive Syk to be phosphorylated by another tyrosine kinase (Fig. 4C). There was considerably reduced tyrosine phosphorylation of Syk-3F/DK compared with Syk-3F suggesting that most of the increase in this phosphorylation is due to autophosphorylation. Therefore, most of the increase in tyrosine phosphorylation of mutated Syk in the cell is due to Syk itself. Furthermore, these findings suggest that mutation of tyrosines in the COOH-terminal region of Syk result in a change in structure that allows easier access to the molecule by other tyrosine kinases, which then can phosphorylate Syk, activate it, and then results in autophosphorylation. Therefore, these results indicate that the tyrosines in the tail region prevent phosphorylation of Syk by other tyrosine kinases.

**FcεRI-induced Tyrosine Phosphorylation of Syk Mutants**—Receptor aggregation leads to increased tyrosine phosphorylation of Syk (19, 51). Because the mutations at tyrosines in the COOH-terminal region resulted in increased tyrosine phosphorylation of Syk in non-stimulated cells, it was important to determine whether receptor activation still induced tyrosine phosphorylation of the molecule itself. After receptor activation, Syk immunoprecipitated from lysates of cells transfected with the different mutants was analyzed by immunoblotting (Fig. 5A). Even though there was baseline tyrosine phosphorylation of Syk-3F, it increased after receptor aggregation. This increase was considerably greater in all the mutants compared with the wild-type protein. However, the pattern of changes was different for the phosphorylation of specific sites on Syk.

The phosphorylation of the negative regulatory Tyr-317 increased after receptor stimulation in the wild-type protein as well as in Syk-Y623F, whereas it was already well phosphorylated in the Syk-3F and other mutants with a minimal increase after stimulation (Fig. 5B). In contrast, receptor stimulation induced phosphorylation of the activation loop Tyr-519/Tyr-520 in wild-type and mutant Syk, with a stronger response in the 3F and Y625F proteins (Fig. 5C). Therefore, although Syk mutated at tyrosines in the COOH-terminal region have increased basal phosphorylation they still are efficiently further phosphorylated after receptor aggregation.

**FIGURE 4.** Mutations of tyrosines of Syk COOH-terminal region result in increased basal Syk tyrosine phosphorylation in non-stimulated cells. Syk-negative cells were transiently transfected with the indicated Syk constructs and cultured for 48 h. A, Syk was immunoprecipitated from the cells and analyzed by immunoblotting with anti-phosphotyrosine (pY). B, immunoblotting with anti-pY317 Syk or anti-pY519/pY520 Syk (activation loop). C, dead kinase Syk (DK) and Syk-3F combined with dead kinase (3F/DK) to abolish its kinase activity were transiently expressed in cells. Immunoprecipitated Syk was analyzed by immunoblotting with anti-phosphotyrosine (pY).

The bottom panel in all cases is the anti-Syk antibody re-probing of the membranes after stripping. Similar results were observed in at least three independent experiments.
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Binding of Mutated Syk to Phosphorylated ITAM—The binding of Syk through its two SH2 domains to phosphorylated ITAM of the γ-subunit of FceRI is important for receptor-mediated signaling (5, 17, 52). Such interactions result in a conformational change that exposes the COOH-terminal tail of Syk and increases its kinase activity (19, 20). Therefore, we tested if mutations of the tail tyrosines could affect binding of Syk to phosphorylated ITAM. Lysates from cells expressing equal amounts of wild-type or mutant Syk were incubated with peptides corresponding to non-phosphorylated (γYY) or phosphorylated (γPP) ITAM of FceRIγ. There was reduced binding to γPP of Syk-3F compared with wild-type (Fig. 6A, upper panel) also with some decrease in binding of Syk-Y625F. However, binding of Syk-Y623F and Syk-Y624F to γPP was similar to the wild-type. This reduced binding of Syk-3F and Syk-Y625F could be the direct result of the mutations or due to the secondary effect of the increased tyrosine phosphorylation of these molecules in non-stimulated cells. Therefore, binding experiments were repeated with lysates that were prepared without vanadate and incubated to allow for dephosphorylation of Syk (Fig. 6A, bottom panel). These conditions increased the binding of Syk-3F and Syk-Y625F to γPP. This result was further supported by observations with the mutant that combined 3F with a dead kinase (Syk-3F/DK) that had lower basal phosphorylation in non-stimulated cells. There was more binding of Syk-3F/DK than Syk-3F (Fig. 6B, upper panel), which was no different from wild-type Syk once the molecule was allowed to dephosphorylate. This indicates that tyrosine mutations in the COOH-terminal region do not directly influence ITAM binding. The decreased binding of Syk to phosphorylated ITAM is therefore due to increased phosphorylation of Syk caused by mutation of COOH-terminal region tyrosines.

Recent studies have demonstrated that phosphorylation of Tyr-130 of Syk prevents its binding to phosphorylated ITAM (53). To explore if Tyr-130 was involved in decreased Syk-3F binding to γPP, we mutated tyrosine 130 to phenylalanine in both wild-type and Syk-3F (Fig. 6C). The binding of Syk-Y130F/3F to γPP was similar to Syk-3F, whereas both were reduced when compared with the control wild-type or Syk-Y130F. Thus, this result suggests that not only Tyr-130 but other phosphorylation sites regulate binding of Syk to ITAM. Therefore, the increased basal phosphorylation at several sites of Syk-3F results in a decrease in its capacity to bind to phosphorylated ITAM.

COOH-terminal Tyrosines Regulate Syk Kinase Activity and Its Autophosphorylation—The enzymatic activity of Syk is regulated by phosphorylation of several tyrosines including those in the activation loop (28, 49, 54). The Syk-3F mutants were tested in an in vitro kinase reaction for their capacity to auto-phosphorylate and phosphorylate a substrate (Fig. 7). Compared with wild-type, the Syk-3F, Syk-Y624F, and Syk-Y625F had significantly decreased kinase activity toward an exogenous...
substrate, whereas Syk-Y623F was not significantly different from the control wild-type Syk (Fig. 7A). However, the in vitro autophosphorylation in these mutants was similar to that of the wild-type (Fig. 7B). There was also no difference in autophosphorylation of the activation loop Tyr-519/Tyr-520 among the different Syk proteins (supplemental Fig. S2A). These experiments were repeated with lysates collected and incubated in the absence of vanadate to allow for the dephosphorylation of the proteins, with the vanadate replaced during the kinase reaction. Under these conditions there was a dramatic loss of the capacity of the Syk-3F protein to phosphorylate a substrate or to autophosphorylate (Fig. 7, C and D). The kinase activity and autophosphorylation were also decreased with Syk-Y624F and Syk-Y625F, with a significantly stronger effect observed with Syk-Y625F. In the Syk-3F, there was essentially a complete loss in autophosphorylation of the activation loop Tyr-519/Tyr-520 with decreases also in Syk-Y624F and Syk-Y625F (supplemental Fig. S2B). As in the previous experiments, Syk-Y623F was similar to wild-type. Therefore, COOH-terminal tyrosines 624 and 625 are critical for regulating the kinase activity and therefore the function of Syk.

**DISCUSSION**

These results suggest a model for the role of the COOH-terminal tyrosines in regulation of Syk function (Fig. 8). Structural studies of ZAP-70 suggest that this family of kinases has an autoinhibitory state where there are interactions of Tyr in the tail with both the kinase and the inter-SH2 domains. The globular representation of the wild-type Syk (Fig. 8, left) shows the molecule in its autoinhibitory state with the COOH-terminal tail interacting with the inter-SH2 domains thereby contributing to keeping the molecule in a closed conformation. Due to the COOH-terminal Tyr mutations, the mutant Syk-3F (Fig. 8, right) has decreased autoinhibitory conformation depicted here by a more open structure. This decrease of autoinhibition in Syk-3F allows the molecule to be phosphorylated by another tyrosine kinase in cells. This then activates Syk which results in further autophosphorylation and phosphorylation of other substrates in cells. Without such phosphorylation by another tyrosine kinase, the Syk-3F has very low enzymatic activity and capacity for autophosphorylation. After FceRI activation the Syk-3F is less efficient in signaling because of decreased pITAM binding and lower enzymatic activity. This also suggests a role of the Tyr in the tail of Syk in ITAM-induced signaling. The binding of Syk to the pITAM results in a conformational change that exposes the COOH-terminal region. This leads to the phosphorylation of the two tyrosines (Tyr-624 and Tyr-625), which then keeps the molecule in an open conformation allowing for further phosphorylation of other tyrosine residues on Syk both by other tyrosine kinases but mostly by autophosphorylation. Therefore, the phosphorylation of Tyr-624 and Tyr-625 is important for the activation and regulation of the activity of Syk.

The Syk-3F protein when expressed in cells was more tyrosine phosphorylated than the wild-type molecule. Similarly the expression in cells of ZAP-70 with mutation of the two analogous tyrosines results in increased basal phosphorylation of the
molecule (32, 35). In the present experiments the increased tyrosine phosphorylation was also observed when dead kinase Syk was combined with the 3F mutation. These results indicate that mutation of the COOH-terminal tyrosines of Syk disrupts the native state of the molecule and makes other tyrosines on the protein accessible to phosphorylation. Expression of dead kinase Syk also mutated at tail tyrosines indicated that most of this increased in vivo phosphorylation was due to autophosphorylation. However, in vitro the Syk-3F, once dephosphorylated, was incapable of autophosphorylation suggesting that Syk-3F required an initial input from another tyrosine kinase for activation. The dependence of the Syk-3F on other tyrosine kinases for activation makes it similar to ZAP-70. In immunoreceptor signaling, Syk alone is sufficient to propagate downstream signaling, whereas ZAP-70 requires the additional activity of another tyrosine kinase, Lck (4, 55). Similarly, the in vitro enzymatic activity of ZAP-70 requires Lck (55). Syk has a ~100-fold greater capacity than ZAP-70 to autophosphorylate and phosphorylate a substrate; chimeric proteins that placed the COOH-terminal region of ZAP-70 on Syk had much reduced kinase activity demonstrating again the importance of the tail in regulating enzymatic activity (4).

In non-stimulated cells, Syk-3F was more tyrosine phosphorylated on positive and negative regulatory sites that are important in regulating Syk and ZAP-70 function (1, 2, 6, 7, 56–58). Among these tyrosines were Tyr-519/Tyr-520 of the activation loop and the Cbl interacting site Tyr-317. Phosphorylation of the activation loop is central to the regulation of the activity of many kinases. Expression of ZAP-70 in Syk-negative B cells does not reconstitute BCR signaling when the two activation loop tyrosines are mutated to phenylalanine (59). Similarly, tyrosines in the activation loop of Syk (Tyr-519 and Tyr-520) are critical for FcεRI-mediated signaling in mast cells (30). In contrast to the activation loop, substitution of Tyr-317 with phenylalanine results in a gain of function of Syk (28), suggesting that phosphorylation of this site serves as a docking site for Cbl that down-regulates intracellular signals. In the present experiments, the expression of Syk-3F also resulted in increased basal tyrosine phosphorylation of cellular proteins in non-stimulated cells. However, this did not affect the basal release of β-hexosaminidase or NFAT activation, but there was higher basal NFκB activation (data not shown). This could be due to the fact that NFκB is more susceptible to cellular stress, whereas there are more complex regulatory pathways for degranulation and NFAT activation.

Structural studies of ZAP-70 suggest that several tyrosine residues throughout the molecule can regulate its enzymatic activity (35); phosphorylation of these can stabilize the active form, whereas in the non-phosphorylated state the same residues may contribute to autoinhibition of the enzymatic activity. In the inactive ZAP-70 the hydrogen-bonding network is stabilized by a hydrophobic cluster formed by Pro-396, Tyr-397, and Tyr-474 in the kinase domain and Tyr-319 in the linker region; whereas in the active form, the hydrogen-bonding network and hydrophilic interactions are disrupted (35). This structural study of ZAP-70 was with a protein that had the tail region missing and the crystal structure of only the SH2 domains of Syk has been determined. However, it is predicted that the two

FIGURE 8. Model of COOH-terminal tyrosines regulation of Syk function. The globular model of the wild-type is on the left and that of 3F mutant Syk on the right. The COOH-terminal Tyr mutations result in a decrease of the autoinhibition that allows the molecule to be phosphorylated by another tyrosine kinase (TKs) in cells. This then results in further Syk autoprophosphorylation. In cells the Syk-3F is tyrosine phosphorylated, which decreased its binding to phospho-ITAM (pITAM) and therefore together with its reduced enzymatic activity contributes to its low receptor-induced signaling capacity. Without such phosphorylation by another tyrosine kinase, in vitro, Syk-3F has very low enzymatic activity.
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tyrosines in the tail of ZAP-70 interact with the α-helix of the C-lobe of the kinase domain and the inter-SH2 domain regions; these interactions help to stabilize the autoinhibitory form (56). These observations suggest that tyrosines in the tail of the Syk family kinases contribute in maintaining the autoinhibitory state.

The SH2 domain-mediated binding of Syk and ZAP-70 to phosphorylated ITAM in subunits of immune receptors is critical for activation of these cells (3, 17–19, 60). In mast cells Syk binds predominantly to the γ-subunit of FcεRI; the expression in cells of truncated Syk containing just the two SH2 domains has a dominant-negative effect that inhibits binding of native Syk and decreases receptor signaling (52). In the present experiments, the Syk-3F isolated from cells when tested in vitro had decreased binding to phosphorylated γ-ITAM peptide. This was due to in vivo tyrosine phosphorylation of the Syk-3F and was reversed by in vitro dephosphorylation before the binding assay. There are several tyrosine phosphorylation sites that regulate Syk-ITAM interactions, including Tyr-130 and Tyr-342 (53, 54). Phosphorylation of Tyr-130, located in the inter-SH2 domain, inhibits Syk binding to phosphorylated ITAM, whereas the Y130F mutation enhances this association (53, 61).

In summary, the results provide a model for the activation of Syk in immunoreceptor signaling. The tyrosines in the tail region, especially Tyr-624 and Tyr-625 are important to maintain the autoinhibitory form of Syk. Binding of Syk to phosphorylated ITAM induces conformational changes that exposes the COOH-terminal region of Syk and switches the protein from an inactive to active form. This allows phosphorylation of Tyr-624 and Tyr-625 by another kinase or by Syk itself to further destabilize the molecule and maintain the active state. All together, these events are essential for optimal signaling by Syk by ITAM containing immune receptors and indicate the important role for these tyrosines in the tail region in regulating Syk function.

Acknowledgments—We thank Alina Barbu, Ana Cristina Grodzki, Michel Guiraudelli, and Jacqueline Groves for helpful discussions and review of the manuscript; we also thank Elsa Berenstein and Lynda Weedon for technical help.

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