Many immune signaling pathways require activation of the Syk tyrosine kinase to link ligation of surface receptors to changes in gene expression. Despite the central role of Syk in these pathways, the Syk activation process remains poorly understood. In this work we quantitatively characterized the molecular mechanism of Syk activation in vitro using a real time fluorescence kinase assay, mutagenesis, and other biochemical techniques. We found that dephosphorylated full-length Syk demonstrates a low initial rate of substrate phosphorylation that increases during the kinase reaction due to autophosphorylation. The initial rate of Syk activity was strongly increased by either pre-autophosphorylation or binding of phosphorylated immune tyrosine activation motif peptides, and each of these factors independently fully activated Syk. Deletion mutagenesis was used to identify regions of Syk important for regulation, and residues 340–356 of the SH2 kinase linker region were identified to be important for suppression of activity before activation.

Comparison of the activation processes of Syk and Zap-70 revealed that Syk is more readily activated by autophosphorylation than Zap-70, although both kinases are rapidly activated by Src family kinases. We also studied Syk activity in B cell lysates and found endogenous Syk is also activated by phosphorylation and immune tyrosine activation motif binding. Together these experiments show that Syk functions as an “OR-gate” type of molecular switch. This mechanism of switch-like activation helps explain how Syk is both rapidly activated after receptor binding but also sustains activity over time to facilitate longer term changes in gene expression.

Syk is a tyrosine kinase that functions immediately downstream of antigen receptors in immune cells including B lymphocytes, mast cells, and macrophages (1–3). The central role of Syk in cell types associated with disorders such as rheumatoid arthritis and allergic rhinitis suggests that strategies to block Syk activation may have therapeutic benefit (4–6). After receptor ligation and phosphorylation, Syk becomes localized to immune receptors and proceeds to phosphorylate downstream targets leading to Ca2+ mobilization, initiation of the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades, and activation of transcription factors such as NF-κB (7). Although increased Syk activity is known to be indispensable for each of these cellular events, a molecular-level understanding of the steps leading to Syk activation has not been clearly defined.

The Syk domain structure consists of an N-terminal pair of Src homology 2 (SH2) domains separated by an inter-SH2 linker, an SH2-domain-kinase linker, and a C-terminal kinase domain. Recruitment of Syk to immune receptors involves binding of the tandem SH2 domains of Syk to motifs in the receptor known as immune tyrosine activation motifs (ITAMs), which are two YXXL sequences typically separated by 7–12 intervening residues (8, 9). Syk is known to have multiple sites of phosphorylation which both regulate activity and serve as docking motifs for other proteins (1). These sites include Tyr-348 and Tyr-352 within the SH2-linker region (10), Tyr-525 and Tyr-526 within the activation loop of the kinase domain (11), Tyr-630 in the C terminus of Syk (12), and other sites. Many of the key structural features and sites of phosphorylation found in Syk are conserved in its homolog Zap-70 (13).

A greater understanding of the activation process for Syk and Zap-70 was recently gleaned from the crystal structure of a full-length version of Zap-70 (14). In this structure a unique network of interactions involving the kinase-SH2 linker, the inter-SH2 linker, and the kinase hinge region was observed that appeared to stabilize an inactive Zap-70 conformation. Disruption of this network was proposed to cause kinase activation, and indeed mutations in this region increased Zap-70 activity. The similarity between Syk and Zap-70 and the electron microscopy structure of Syk (15) suggest that Syk may adopt a similar inactive conformation, and hence, show a similar activation mechanism, as Zap-70. However, for Syk, functional data directly testing this model is lacking.

Previous studies have linked both binding to phosphorylated ITAM sequences and phosphorylation at several sites to increased Syk activity (16). However, many questions still remain regarding the molecular mechanism of Syk activation. For instance, how much do phosphorylation and ITAM binding each contribute to Syk activation? What is the magnitude of activation? Does maximal activation require both phosphorylation and ITAM binding, or is each process itself sufficient to fully activate? What are the similarities and differences between activation of Syk and Zap-70?

The abbreviations used are: SH2, Src homology 2; ITAM, immune tyrosine activation motif; PTP1B, protein-tyrosine phosphatase 1B; FcεRIγ, γ chain of the FcεRI receptor; DTT, dithiothreitol; BCR, B cell receptor.
In this study we set out to better understand the molecular basis of Syk activation. Full-length Syk was studied in vitro using both a real time kinase assay and Western blotting with phosphospecific antibodies. It was observed that either Syk phosphorylation or binding of phosphorylated ITAM peptides was sufficient to fully activate Syk. Mutagenesis indicated that residues 340–356 of the SH2 kinase linker are important for phosphorylation-dependent regulation. Furthermore, endogenous Syk from B cell lysates was found to demonstrate similar activation as recombinant Syk in vitro. These experiments suggest a model whereby multiple different stimuli can each fully activate Syk, and hence, Syk can be viewed as an OR-gate type of kinase switch (17).

**EXPERIMENTAL PROCEDURES**

*Reagents*—The following enzymes were obtained from Invitrogen: Syk (catalog # PV4089), Zap-70 (catalog # PR3017A), Lck (catalog # P3043), Lyn (catalog # PR3722A), PTP1B (catalog # PR4454A). Syk from Invitrogen was used for the studies described here. Syk was also obtained from Carna Biosciences (Kobe, Japan) (catalog # 08-761) and SignalChem (Richmond, BC, Canada) (catalog # s52) and confirmed to have the same regulation by ITAM binding and phosphorylation as Syk from Invitrogen (data not shown). All Syk proteins contained an N-terminal glutathione S-transferase tag for purification purposes. ITAM phosphopeptides were custom synthesized by Quality Controlled Biochemicals (Hopkinson, MA) and obtained at 95% purity level. The following antibodies were employed: Syk monoclonal from BD Biosciences, Syk phosphospecific 525/526 polyclonal from Cell Signaling Technologies (Danvers, MA), Syk phosphospecific 348/352 and Zap-70 315/319 phosphospecific polyclonal from Cell Signaling Technologies. Sox-fluorophore-containing peptide substrates for Syk (Y7, KNZ3071) and Zap-70 (Y8, KNZ3081) were obtained from Invitrogen.

**Dephosphorylation and Autophosphorylation Conditions**—To dephosphorylate the basal state of Syk (which was observed to be phosphorylated following purification), 4 μM Syk was incubated together with 0.08 μg/μl phosphatase PTP1B for 60 min at 27 °C in a buffer of 20 mM Hepes, pH 7.15, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mg/ml bovine serum albumin, 10 mM MgCl2. After dephosphorylation with PTP1B, all subsequent evaluations of Syk activity were performed in a buffer containing 10 μM phosphatase inhibitor sodium vanadate. We demonstrated that adding 10 μM sodium vanadate to the buffer blocked the basal phosphatase activity of PTP1B (supplemental Fig. 1) and, hence, prevented PTP1B from affecting evaluations of Syk activity. Furthermore, we demonstrated that 10 μM sodium vanadate had no affect on basal Syk activity in the absence of PTP1B (supplemental Fig. 1). To re-autophosphorylate Syk before analysis of activity, 0.8 μM Syk was incubated with 100 μM ATP for 15 min at 27 °C in a buffer of 20 mM Hepes, pH 7.15, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mg/ml bovine serum albumin, 10 mM MgCl2, and 10 μM sodium vanadate.

**Enzyme Activity Assay**—Syk enzyme activity was monitored as described previously (18). Briefly, activity was assessed by monitoring the increase in fluorescence after phosphorylation of a commercially available peptide substrate (pep7) that contains the phosphorylation-sensitive amino acid Sox (19, 20). Assays were performed in 20 mM Hepes, pH 7.15, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mg/ml bovine serum albumin, 10 mM MgCl2, 10 μM sodium vanadate, and typically 25 μM ATP in 96-well black plates (Corning Inc., Corning, NY) using a final assay volume of 50 μl. Data were collected on either a SpectraMax GeminiXS fluorescence plate reader (Molecular Devices, Sunnyvale, CA) or PheraStar microplate reader (BMG LABTECH, Durham, NC) using excitation and emission wavelengths of 360 and 455 nm, respectively. The concentration of Syk in the assay was typically 5 or 10 nM (except for experiments with Syk356 and Syk360, which employed 1 or 2 nM Syk due to the high activity of these particular Syk variants).

**Michaelis-Menten Analysis**—The initial velocity (v<sub>i</sub>) of Syk product formation measured in μM/min was assessed by evaluating the rate of product formation from the initial phase of the fluorescence progress curve. This time course was typically linear until ~50% of substrate was converted to product for autophosphorylated Syk, Syk with ITAM peptides, and truncated Syk variants. For dephosphorylated Syk, v<sub>i</sub> was estimated from the first few time points (~2 min) of the reaction time course. The product formation rate of a given experiment (k<sub>cat</sub>) was calculated by dividing v<sub>i</sub> by the concentration of enzyme used in the experiment. To determine the kinetic constants of maximum turnover rate (k<sub>cat</sub>) and Michaelis constant (K<sub>m</sub>) of different Syk constructs with respect to ATP, the ATP concentration was varied at quarter log scale from 1 to 300 μM at a constant pep7 concentration of 5 μM. Likewise, to determine the kinetic constants (k<sub>cat</sub>, K<sub>m</sub>) with respect to pep7, the pep7 concentration was varied at quarter log scale from 1 to 30 μM at a constant ATP concentration of 25 μM. These data were evaluated using the Michaelis-Menten equation. Data analysis was performed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

**Western Blotting**—Syk samples containing 0.1 μg of protein were analyzed by electrophoresis using the NuPAGE gel system and transferred to nitrocellulose via Iblot transfer (Invitrogen). Membranes were probed with antibodies against total Syk at a 1:10,000 dilution, phosphorylated Tyr residues 348 and 352 at 1:500 dilution, or phosphorylated Tyr residues 352 and 526 at 1:500 dilution. Blots were probed with horseradish peroxidase-conjugated secondary antibodies at a 1:10,000 dilution and developed with ECL<sup>+</sup> solution (GE Healthcare). Other Western blot procedures were performed as described (21).

**Surface Plasmon Resonance**—Surface plasmon resonance experiments were carried out using a Biacore S1 biosensor instrument (GE Healthcare). Interactions between the protein immobilized on a biosensor surface and peptides flowed over the surface were monitored in real time as a change in surface plasmon resonance as measured in resonance units (22). An anti-glutathione S-transferase (GST) antibody was captured to the surface of a CMS sensor chip using a commercially available anti-GST kit and the manufacturer’s instructions (GE Healthcare). Full-length glutathione S-transferase-Syk was capture by flowing freshly thawed protein aliquots over the antibody surface until saturation of the antibody surface was achieved. Peptides were diluted in running buffer (50 mM Hepes, pH 8.0, 150 mM NaCl, 10 mM MgCl2, 1 mM Tris[2-carboxyethyl]phosphine, and 0.01% Tween 20) to the top concentrations in the dilution
series. From this, a 2-fold dilution series was made, and the peptides were injected over the surface at a flow rate of 100 μl/min. Raw sensogram data were reduced and double referenced using the Scrubber II software package (BioLogic Software, Campbell, Australia). Equilibrium fits to a 1:1 binding model were insufficient to describe the data, but data were well described by a 2-site equilibrium model, reporting two $K_D$ values. Data for the $\gamma$ chain of the FceRI receptor (FceRI$\gamma$) ITAM exhibited measurable kinetics and were fit using the CLAMP99 software package (23). $K_D$ values derived from the kinetics are very similar to those obtained from the equilibrium fits.

Expression and Purification of Truncated Syk Protein—Truncated versions of human Syk were cloned into a PVL1392 vector and expressed by baculovirus in Sf9 insect cells. Cells were harvested 3 days after infection and resuspended in 250 ml of disruption buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5% glycerol, 200 mM arginine, 0.1% Ipegal) and disrupted on ice using a microfluidizer with 2 passes at 15,000 p.s.i. Supernatant was then collected after centrifugation at 13,000 × g, and the lysate was filtered using a 0.45-μm SuporCap Capsule. Material was then incubated for 3 h with a nickel-Sepharose FF resin that had been pre-equilibrated in disruption buffer with 20 mM imidazole. Resin was collected at 1000 × g and then washed 4 times using disruption buffer before bound proteins were eluted twice with 20 ml of elution buffer (25 mM Hepes pH 7.5, 5% glycerol, 150 mM NaCl, 10 mM methionine, 200 mM imidazole). The sample was then concentrated to 5 mg/ml and passed through a His-trap column to remove uncleaved protein. Flow-through was collected and concentrated to 2 ml using a Superdex XK16/60 column equilibrated with buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM DTT, 10 mM methionine), and samples were run at 1 ml/min. 1-min fractions were then collected before being analyzed on 8–16% Tris-glycine gels. The clean fraction was pooled and concentrated to 1 mg/ml and stored at −80 °C.

Zap-70 Activation—The enzyme activity of Zap-70 was assessed using 10 μM Sox-containing substrate pep8 at a final concentration of 50 nM Zap-70. Other buffer conditions included 20 mM Hepes, pH 7.15, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mg/ml bovine serum albumin, 10 mM MgCl$_2$, 10 μM sodium vanadate, and typically 500 μM ATP using a final assay volume of 50 μl. To preactivate Zap-70 before enzymatic analysis, Zap-70 was incubated with 1 mM ATP for varying times at 30 °C in a buffer of 20 mM Hepes, pH 7.15, 0.1 mM EGTA, 0.1 mM DTT, 10 mM MgCl$_2$, and 10 μM sodium vanadate. Note that the requirement for a high ATP concentration in the preactivation conditions prevents determination of Zap-70 activity at very low ATP concentration in the Michaelis-Menten analysis of Zap-70. Zap-70 samples were analyzed by Western blot as described above for Syk using the dual Syk 348/352 and Zap315/319 phosphospecific antibody. Here, total Zap-70 concentration was determined by Coomassie staining. To assess how the activity of Zap-70 and Syk is modulated by Src family kinases, 10 nM concentrations of either Lck or Lyn was added to the standard enzyme activity mixture for either Zap-70 or Syk, respectively, and the time course of product formation was monitored.

Monitoring Syk Activity in Ramos B Cell Lysates—Ramos B cells were cultured in media containing RPMI 1640 with l-glutamate, 10% fetal bovine serum, and 1 mM sodium pyruvate and incubated in 5% CO$_2$ at 37 °C. To prepare lysates, cells were harvested at a density of 1 million cells/ml by centrifugation at 100 × g for 5 min. Cells were then resuspended at 100 million cells/ml in RPMI 1640 and 10 mM Hepes, pH 7.4. Cells were then incubated for 10 min at 27 °C and centrifuged at 600 × g for 10 min, and the pellet was snap-frozen at stored at −80 °C. On the day of the experiment, cells were reconstituted and lysed with cold buffer containing 0.5% Triton-X, 50 mM Tris, pH 7.5, 150 mM NaCl, and protease inhibitor tablet. Samples were centrifuged at 3600 × g for 10 min at 4 °C. The supernatant and pellet were both retained. For activity analysis in whole lysates, a dilution of the final prepared Ramos lysate of 1:10 was typically employed into the standard enzyme assay buffer containing sodium vanadate. To isolate endogenous Syk by immunoprecipitation for analysis of activity, lysates were incubated with the total Syk monoclonal antibody at 1:100 for 1 h at 4 °C then with protein A-conjugated agarose beads (Thermo Scientific) for 1 h at 4 °C, and the beads were isolated by centrifugation at 1000 × g.

RESULTS

Phosphorylation-dependent Syk Activity—In this study we first set out to explore the relationship between Syk kinase activity and the phosphorylation state of the kinase. To monitor the enzymatic activity of Syk, a recently developed fluorescence kinase assay was employed. This assay monitors phosphorylation of a peptide substrate, pep7, in real time using the change in fluorescence of a non-natural amino acid (the “Sox” amino acid) incorporated directly into the peptide substrate (19, 20). Utilizing Syk that was not treated after purification (“basal Syk”), it was found that product formation was initially linear with time and then plateaued after substrate was completely converted to product (Fig. 1A). In contrast to basal Syk, Syk that was treated with protein-tyrosine phosphatase PTP1B before activity analysis (dephosph Syk) demonstrated a clear lag phase in product formation (Fig. 1A). This nonlinear time course is consistent with Syk being in a less active conformation at the outset of the experiment, which then converts to a more active conformation during the reaction due to autophosphorylation. If PTP1B-treated Syk was incubated with Mg$^{2+}$/ATP before activity analysis (autophosph Syk), the lag in the reaction time course was eliminated (Fig. 1A). The product formation rate (calculated from the initial velocity) of the basal Syk, dephos Syk, and autophos Syk conditions were determined, respectively, to be 33 ± 1, 2.8 ± 0.6, and 52 ± 1 min$^{-1}$ (Table 1), indicating that phosphorylation causes a greater than 10-fold increase in Syk activity.

The preceding results suggest that autophosphorylation increases Syk activity. To confirm that Syk undergoes a change in phosphorylation after treatment with PTP1B, we used Western blotting with phosphospecific antibodies. Antibodies against Tyr-352 and Tyr-525/526 were employed. Both the basal and autophosphorylated forms of Syk were observed to be phosphorylated at Tyr-352 and Tyr-525/526, whereas neither site showed significant phosphorylation in the PTP1B-treated
Activation Mechanism of Syk

**FIGURE 1.** Autophosphorylation regulates Syk activity. A, the time course of phosphorylation of Syk substrate peptide pep7 (μM Product) as a function of time (min) for basal Syk (open squares), dephosphorylated Syk (open circles), and pre-autophosphorylated Syk (crosses). Syk enzyme concentration in this experiment was 10 nM, and the initial velocity (v₀) for each condition was 0.33 ± 0.1 μM/min for basal Syk, 0.028 ± 0.006 μM/min for dephosphorylated Syk, and 0.52 ± 0.1 μM/min for autophosphorylated Syk. B, Western blot of basal Syk (lane 1), dephosphorylated Syk (lane 2), and pre-autophosphorylated Syk (lane 3). Samples were probed with antibodies against Syk anti-phosphotyrosine (pY) 348/352 (top), anti-phosphotyrosine 525/526 (middle), and total Syk (bottom). C, Western blot of the time course of phosphorylation of Syk that was previously incubated with PTP1B. Shown is the amount of reaction against anti-phosphotyrosine 348/352 (top), anti-phosphotyrosine 525/526 (middle), and total Syk (bottom) after 10 min (lane 4) and 30 min (lane 5) of reaction.

**TABLE 1**

Activity of Syk in different states

| Enzyme form | ITAM peptide | k_{cat} (min⁻¹) |
|-------------|--------------|-----------------|
| Basal Syk   | None         | 33 ± 1          |
| Dephos Syk  | None         | 2.8 ± 0.6       |
| Autophos Syk| None         | 52 ± 1          |
| Dephos Syk  | FcεRIγ       | 50 ± 1          |
| Dephos Syk  | FcγRIIα      | 51 ± 1          |
| Autophos Syk| FcγRIIα      | 54 ± 1          |

**FIGURE 2.** Syk is activated by binding of ITAM peptides. A, the sequences of the ITAM peptides used in this study. pY represents phosphoryrosine. B, the time course of pep7 phosphorylation by previously dephosphorylated Syk (open circles) and by dephosphorylated Syk with 1 μM of BCR ITAM (closed squares). Shown is the concentration of product (μM) as a function of time (min). Syk enzyme concentration in this experiment was 5 nM, and the initial velocity (v₀) for each condition was 0.014 ± 0.002 μM/min for dephosphorylated Syk and 0.25 ± 0.1 μM/min for dephosphorylated Syk plus BCR ITAM. C, dose-response relationship of ITAM peptide activation of Syk. Shown is the initial velocity of pep7 phosphorylation (μM/min) as a function of ITAM concentration (M). Data are shown for BCR (open squares), FcεRIγ (closed diamonds), and FcγRIIα (open circles). Solid lines represent the nonlinear least squares best fit to a sigmoidal dose response function providing the EC_{50} (μM) for each peptide.

Sample (Fig. 1B). Western blotting also demonstrated that, during the course of an enzyme assay, PTP1B-treated Syk became autophosphorylated at both Tyr-352 and Tyr-525/526 (Fig. 1C), confirming that the increase in Syk activity over time observed is attributable to Syk autophosphorylation. Together, these findings indicate that 1) dephosphorylated Syk has low activity toward the pep7 substrate, 2) Syk can autophosphorylate and autophosphorylation increases Syk activity, and 3) a lag phase in product formation reflects Syk being in the inactive conformation at the outset of the experiment.

**ITAM Activation of Syk**—In the cell Syk binds dual-phosphorylated regions within receptors known as ITAM motifs. To explore if binding of phosphorylated ITAM sequences activates Syk in vitro, we studied three dually phosphorylated ITAM peptides based on Syk docking sites on the B cell receptor (BCR), FcεRIγ, and FcγRIIα receptor (Fig. 2A). Incubation of dephosphorylated Syk with either 1 μM BCR, FcεRIγ, or FcγRIIα peptide eliminated the lag phase in PTP1B-treated Syk activity and resulted in a linear rate of product formation (see Fig. 2B and supplemental Fig. 2, A and B), indicating that ITAM binding activates Syk. The product formation rate under each condition was determined to be 50 ± 1, 56 ± 1, and 51 ± 1 min⁻¹ for the BCR, FcεRIγ, and FcγRIIα peptides, respectively (Table 1). To ascertain the potency of Syk for the ITAM peptides, the concentration of each peptide was varied, whereas the initial component of the reaction velocity was monitored. Fitting the data to a sigmoidal dose-response function ascertained that BCR, FcεRIγ, and FcγRIIα peptides had EC_{50} values of 72 ± 6, 5 ± 1, and 14 ± 1 nM, respectively (See Table 2). This finding indicates that Syk is potently activated by binding of dually phosphoryl-
activated ITAM sequences and that the sequence context of the ITAM peptide can modulate Syk potency. It should be noted that above 10 μM ITAM concentration we observed that each peptide began to inhibit Syk activity (see Fig. 2C and below).

To directly characterize the binding interaction between ITAM peptides and Syk, surface plasmon resonance was employed (see supplemental Fig. 3). In these experiments all 3 ITAM peptides were observed to bind to PTP1B-treated Syk with 2 binding modes; a potent binding mode and a weak binding mode. For the more potent binding mode, a potency pattern similar to the enzyme activation assay was obtained; the $K_d$ values of FceRIγ and FcγRIIa were similar ($K_d \approx 40$ nM), whereas the BCR peptide was 10-fold weaker (Table 2). Potent binding presumably occurs between the ITAM peptide and the tandem SH2 domains of Syk. The weaker binding mode observed in the surface plasmon resonance experiments demonstrated $K_d$ values in the range of 0.64–32 μM (Table 2). The observation of a weak binding mode between Syk and ITAM peptides in surface plasmon resonance experiments (Table 2) and the finding of inhibition of Syk activity at high ITAM peptide concentrations (Fig. 3C) suggest that ITAM peptides can weakly interact with Syk in a previously uncharacterized way that inhibits enzyme activity.

**Syk Activation Is an OR-gate Switch**—The experiments above indicate that autophosphorylation and ITAM binding each activate Syk by the same magnitude (Table 1). However, they do not reveal whether phosphorylation and ITAM binding activate through the same mechanism or different mechanisms. If phosphorylation and ITAM binding operate through different mechanisms, it would be expected that simultaneous application of both stimuli could further increase Syk activity. In contrast, if both stimuli worked through the same mechanism, application of both autophosphorylation and ITAM binding would be expected to result in little further increase in activity. The latter type of mechanism is referred to as an OR-gate switch as either one stimulus OR the other is sufficient to cause full activation (17, 24, 25). An OR-gate switch is distinguished from an “AND-gate” switch where multiple stimuli must be present simultaneously to get activation of the molecular switch (25, 26). To ascertain the relationship between autophosphorylation and ITAM binding, the activity of autophosphorylated Syk in the presence of ITAM peptide was evaluated. The activity in the presence of both stimuli was observed to be identical to either stimulus individually (Fig. 3A, Table 1). This finding indicates that Syk activation is an OR-gate switch and implies that ITAM binding and autophosphorylation activate through the same molecular mechanism (see “Discussion”). Identical results were obtained with each ITAM studied and using multiple autophosphorylation conditions (data not shown).

The ITAM-stimulated increase in Syk activity could potentially be due to 2 different molecular mechanisms; that is, direct stimulation of Syk activity by ITAM or rapid ITAM-mediated autophosphorylation. To address if ITAM binding directly stimulates Syk activity, we monitored the time course of autophosphorylation at Tyr-348/Tyr-352 in the presence and absence of ITAM. Using Western blotting, we observed in the presence of ITAM that the level of autophosphorylation increased throughout the 30-min time course (Fig. 3B). Hence, the autophosphorylation process is likely too slow to account for the immediate increase in Syk peptide phosphorylation observed in the presence of ITAM. Therefore, it is probable than ITAM binding directly stimulates Syk. However, it was also observed that the level of autophosphorylation at Tyr-348/Tyr-352 was lower in the absence of ITAM (Fig. 3B), suggesting that ITAM binding does increase the rate of phosphorylation at Tyr-348/Tyr-352. The time course of phosphorylation at Tyr-525/Tyr-526 was also monitored; it was found that autophosphorylation at Tyr-525/Tyr-526 occurs more slowly than at Tyr-348/Tyr-352 and is also increased by ITAM binding.
Truncation Analysis of the SH2 Kinase Linker—The structure of Zap-70 suggests that the SH2 kinase linker of Syk (residues 258–360) might be critical for regulation of Syk activation. To functionally define the regions of the SH2 kinase linker that impact Syk activation, we constructed a panel of Syk truncation mutants within the linker (Fig. 4A) and measured the kinetic parameters ($k_{cat}$, $K_m$) of the dephosphorylated and phosphorylated states of the proteins (supplemental Fig. 4, Table 3). It should be noted that, unlike dephosphorylated full-length Syk, none of the Syk truncation mutants demonstrated a lag phase in product formation, facilitating straightforward kinetic analysis.

Overall, the tested constructs can be categorized into three groups based on their kinetic properties (Fig. 4B). The first group (Syk-(360–635) and Syk-(356–635)) demonstrated the highest level of activity and showed no regulation at all by phosphorylation, confirming previous findings (18). The second group (Syk-(340–635), Syk-(320–635), Syk-(300–635), and Syk-(280–635)) showed 3–5-fold less activity than the first group and also ~2-fold enhancement in activity after phosphorylation. The third group consisted solely of full-length Syk, which showed low initial activity in the dephosphorylated state and profound regulation by phosphorylation. The difference in kinetic behavior between Syk-(356–635) and Syk-(340–635) highlights residues 340–355 (Fig. 4C), a region containing Tyr-348, Tyr-352, and other residues that may interact with the kinase hinge region, as important for Syk regulation. The dissimilarity between full-length Syk and Syk-(280–635) also suggests important regulatory interactions in the N terminus of the protein.

Comparison of Syk with Zap-70—The discovery of the importance of residues Syk 340–355 suggested that a direct kinetic comparison of Syk and Zap-70 activation might be insightful. We examined nonphosphorylated Zap-70 and found that, as for Syk, substrate phosphorylation displayed a distinct lag phase (Fig. 5A). Interestingly, the lag phase was much longer for Zap-70 than for Syk, indicating that Zap-70 is self-activated more slowly than Syk. Similar to Syk, the lag phase in Zap-70 substrate phosphorylation could be completely eliminated by pre-autophosphorylation. However, full activation of Zap-70 required pre-incubation with 1 mM ATP for a full hour at 30 °C (Fig. 5A), which contrasts with Syk, which is fully activated after a 15-min preincubation with 0.1 mM ATP at room temperature. It was observed that the time-dependent increase in Zap-70 kinase activity after autophosphorylation mirrored the time-dependent increase in the phosphorylation level at Tyr-315/319 (homologous to Syk Tyr-348/352) as assessed by Western blotting (Fig. 5, A and B). Hence, like Syk, the activity of Zap-70 is strongly regulated by phosphorylation level, although Syk is more easily activated by autophosphorylation than Zap-70. We determined the kinetic parameters of preactivated Zap-70 using Michaelis-Menten analysis (supplemental Fig. 5) and observed that $k_{cat}$ for Zap-70 was ~3-fold less than activated Syk (54 ± 4 min$^{-1}$ for Zap-70 versus 171 ± 5 min$^{-1}$ for Syk), whereas the $K_m$ for ATP was ~2-fold greater (57 ± 6 μM for Zap-70 versus 37 ± 2 μM for Syk).

To address whether Zap-70 could be activated more rapidly than occurs by autophosphorylation, we studied Zap-70 activity in the presence of the kinase Lck, which is known to be an

**FIGURE 4.** Truncation mutagenesis reveals that residues 340–356 of Syk regulate activity. A, truncated Syk mutants employed in the kinetic analysis. B, summary of the kinetic parameters of truncated Syk. Shown are the values of $k_{cat}$ (min$^{-1}$), $K_m$(ATP) (μM), and $K_m$(pep7) (μM) for both dephosphorylated (white bars) and phosphorylated (black bars) Syk. Data are boxed into groups based on the pattern of kinetic behavior. Full-length Syk (long dashed line) has significantly higher $K_m$(ATP) and more regulation by phosphorylation than other constructs; Syk280, Syk300, Syk320, and Syk340 show low ATP $K_m$ values and modest regulation of activity by phosphorylation; Syk356 and Syk360 show the highest activity and no regulation by phosphorylation. C, residues 340–356 of Syk. Shown in italics are Tyr-348 and Tyr-352, which are known to be important for Syk regulation.
upstream activator of Zap-70 in T cells (27, 28). In the presence of Lck, Zap-70 demonstrates robust activity from the initiation of the experiment (Fig. 5C), indicating that Lck rapidly activates Zap-70 in vitro. This indicates that the slow rate of Zap-70 self-activation is attributable to a poor ability to autophosphorylate. We also observed a rapid activation process for Syk using its upstream kinase in B cells, Lyn (Fig. 5D) (29). Neither Lck nor Lyn demonstrated any activity toward the peptide substrate in the absence of Zap-70 or Syk, indicating that the increased activity observed in the experiment was not due to direct peptide phosphorylation by these kinases. These results indicate that both Syk and Zap-70 can be activated in vitro by Src family kinases in addition to by autophosphorylation and ITAM binding.

Activation of Syk from Ramos B Cells—To expand our analysis of Syk activation to a more physiologically relevant context, we studied endogenous Syk activity in Ramos B cell lysates. It has recently been reported that Syk activity can be monitored in Ramos B cell lysates using pep7 as a substrate (30). We showed that phosphorylation of pep7 by the lysate was due solely to Syk by using immunoprecipitation to by autophosphorylation and ITAM binding.

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**DISCUSSION**

Syk is a key mediator of signal transduction in B cells, mast cells, macrophages, and several non-immune cell types (2, 3, 31). It is believed that Syk inhibition could be beneficial for...
Mg²⁺ is stimulated by the addition of ITAMs but little by a 15-min pretreatment with Mg²⁺. Shown is the time course of product formation for the basal lysate of Ramos B cells, Syk immunoprecipitated from the lysate (open circles), and Syk immunoprecipitated from the lysate (IPed Syk, open squares). B, Syk activity in Ramos B cell lysates is stimulated by the addition of Mg²⁺/ATP but not by ITAMs. Shown is the time course of product formation for the basal lysate (open circles), the lysate pretreated with Mg²⁺/ATP (closed triangles), with 1 μM BCR ITAM peptide (open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds). C, immunoprecipitated Syk (IPed Syk) activity is stimulated by addition of ITAMs but little by a 15-min pretreatment with Mg²⁺/ATP. Shown is the time course of product formation for the basal Syk (open circles), Syk pretreated with Mg²⁺/ATP for 15 min (closed triangles), with 1 μM BCR ITAM peptide (ITAM, open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds). D, immunoprecipitated Syk activity is stimulated by the addition of ITAMs and also by a 2-h pretreatment with Mg²⁺/ATP. Shown is the time course of product formation for the basal Syk (open circles), Syk pretreated with Mg²⁺/ATP for 2 h (closed triangles), with 1 μM BCR ITAM peptide (open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds).

**TABLE 4**

| Conditions                  | Figure | V₉₀/min |
|-----------------------------|--------|---------|
| B cell lysate               | Fig. 6A| 4.4 ± 0.6 |
| Depleted B cell lysate      | Fig. 6A| 1.5 ± 0.5 |
| IPed Syk                    | Fig. 6A| 5.5 ± 0.5 |
| B cell lysate               | Fig. 6B| 8.4 ± 0.8 |
| Lysate + 1 μM ATP           | Fig. 6B| 48 ± 0.6  |
| Lysate + 1 μM BCR ITAM     | Fig. 6B| 9.4 ± 0.4  |
| Lysate + ATP + BCR ITAM    | Fig. 6B| 25 ± 0.5  |
| IPed Syk                    | Fig. 6C| 2.0 ± 0.4  |
| IPed + 1 μM ATP, 15 min     | Fig. 6C| 9.5 ± 0.5  |
| IPed Syk + 1 μM BCR ITAM   | Fig. 6C| 37 ± 1.6  |
| IPed Syk + ATP, 15 min + 1 μM BCR ITAM | Fig. 6C| 46 ± 1.0  |
| IPed Syk                    | Fig. 6D| 0.7 ± 0.3  |
| IPed + 1 μM ATP, 120 min    | Fig. 6D| 19 ± 0.8  |
| IPed Syk + 1 μM BCR ITAM   | Fig. 6D| 26 ± 1.1  |
| IPed Syk + ATP, 120 min + 1 μM BCR ITAM | Fig. 6D| 27 ± 1.3  |

FIGURE 6. Activation of endogenous Syk by phosphorylation and ITAM binding. A, activity toward pep7 from Ramos B cell lysates is exclusively Syk. Shown is the time course of pep7 product formation (μM) as a function of time (min) for a whole cell lysate prepared from Ramos B cells (open circles), the same lysate depleted of Syk by immunoprecipitation (closed triangles), and Syk immunoprecipitated from the lysate (IPed Syk, open squares). B, Syk activity in Ramos B cell lysates is stimulated by the addition of Mg²⁺/ATP but not by ITAMs. Shown is the time course of product formation for the basal lysate (open circles), the lysate pretreated with Mg²⁺/ATP (closed triangles), with 1 μM BCR ITAM peptide (open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds). C, immunoprecipitated Syk (IPed Syk) activity is stimulated by addition of ITAMs but little by a 15-min pretreatment with Mg²⁺/ATP. Shown is the time course of product formation for the basal Syk (open circles), Syk pretreated with Mg²⁺/ATP for 15 min (closed triangles), with 1 μM BCR ITAM peptide (ITAM, open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds). D, immunoprecipitated Syk activity is stimulated by the addition of ITAMs and also by a 2-h pretreatment with Mg²⁺/ATP. Shown is the time course of product formation for the basal Syk (open circles), Syk pretreated with Mg²⁺/ATP for 2 h (closed triangles), with 1 μM BCR ITAM peptide (open squares), and with both Mg²⁺/ATP and 1 μM BCR ITAM peptide (closed diamonds).

**TABLE 4**

Activity of endogenous Syk from B cells

| Conditions                  | Figure | V₉₀/min |
|-----------------------------|--------|---------|
| B cell lysate               | Fig. 6A| 4.4 ± 0.6 |
| Depleted B cell lysate      | Fig. 6A| 1.5 ± 0.5 |
| IPed Syk                    | Fig. 6A| 5.5 ± 0.5 |
| B cell lysate               | Fig. 6B| 8.4 ± 0.8 |
| Lysate + 1 μM ATP           | Fig. 6B| 48 ± 0.6  |
| Lysate + 1 μM BCR ITAM     | Fig. 6B| 9.4 ± 0.4  |
| Lysate + ATP + BCR ITAM    | Fig. 6B| 25 ± 0.5  |
| IPed Syk                    | Fig. 6C| 2.0 ± 0.4  |
| IPed + 1 μM ATP, 15 min     | Fig. 6C| 9.5 ± 0.5  |
| IPed Syk + 1 μM BCR ITAM   | Fig. 6C| 37 ± 1.6  |
| IPed Syk + ATP, 15 min + 1 μM BCR ITAM | Fig. 6C| 46 ± 1.0  |
| IPed Syk                    | Fig. 6D| 0.7 ± 0.3  |
| IPed + 1 μM ATP, 120 min    | Fig. 6D| 19 ± 0.8  |
| IPed Syk + 1 μM BCR ITAM   | Fig. 6D| 26 ± 1.1  |
| IPed Syk + ATP, 120 min + 1 μM BCR ITAM | Fig. 6D| 27 ± 1.3  |

To further characterize the molecular mechanism of the OR-gate switch, we compared the time required for ITAM binding to activation (14). Here we present biochemical and enzymatic data that support the model whereby molecular interactions between the SH2 kinase linker, the inter-SH2 domain linker, and the kinase hinge region are important for autoinhibition of Syk and Zap-70 and that disruption of these interactions leads to activation (14). We observed that Syk kinase activity is equivalently increased by phosphorylation, ITAM binding, or both stimuli together. Truncation mutagenesis defined regions of Syk important for activation including residues 340–356 of the SH2 kinase linker. Furthermore, Zap-70 and Syk both demonstrated strong activation through autophosphorylation and by Src family kinases.

In cells, Syk is a key component of a signaling network of proteins. In general, signaling networks are organized with important molecules like Syk located at nodes within the network (32). Nodes can be characterized as logic gates such as AND-gates (which signal when one stimulus and the other is present) or OR-gates (which convey information when one stimulus or the other is present) (17, 24, 25). An OR-gate is a switch that signals equivalently in the presence of either stimuli or both simultaneously (17). OR-gate behavior often results from the different stimuli acting through the same molecular mechanism. In this study we observed that Syk functions as an OR-gate switch with respect to phosphorylation and ITAM binding, suggesting that phosphorylation and ITAM binding act through a common molecular pathway. It is likely that both phosphorylation and ITAM binding disrupt the network of interactions involving the SH2 kinase linker required to maintain the Syk inactive conformation (See Fig. 7).

Treatment of disorders including rheumatoid arthritis, allergic rhinitis, and asthma (4–6). Fully understanding both the biological role of Syk and its attractiveness as a drug target necessitates characterizing the different conformations of Syk. The recent crystal structure of Zap-70 has provided a conceptual framework to better understand the molecular basis of Syk activation (14). Here we present biochemical and enzymatic data that support the model whereby molecular interactions between the SH2 kinase linker, the inter-SH2 domain linker, the kinase hinge region are important for autoinhibition of Syk and Zap-70 and that disruption of these interactions leads to activation (14). We observed that Syk kinase activity is equivalently increased by phosphorylation, ITAM binding, or both stimuli together. Truncation mutagenesis defined regions of Syk important for activation including residues 340–356 of the SH2 kinase linker. Furthermore, Zap-70 and Syk both demonstrated strong activation through autophosphorylation and by Src family kinases.
Activation Mechanism of Syk

increase Syk peptide phosphorylation activity with how ITAM modulated the time course of Syk autophosphorylation. Although ITAM led to an immediate full activation of Syk peptide phosphorylation activity (Fig. 2B), ITAM-stimulated autophosphorylation occurred over many min (Fig. 3B). Hence, it appears that ITAM binding is sufficient to activate Syk independent of autophosphorylation. Further evidence that Syk activity is directly stimulated by ITAM binding (rather than by ITAM-stimulated autophosphorylation) would be obtained if ITAM binding was found to strongly activate a non-phosphorylatable Tyr-348/Tyr-352 mutant enzyme.

The OR-gate behavior of Syk may help to explain recent findings that Syk activity is maintained over time after transient activation of the B cell receptor. Many signaling events after BCR ligation are rapidly reversed, but it has recently been found that Syk activity is required for more than 1 h to induce activation of NFAT (nuclear factor of activated T cells) transcription (33). Furthermore, sustained Syk activity has also been linked to store-operated Ca\(^{2+}\) entry, which is important for longer term signaling events (12). Having both ITAM binding and phosphorylation cause Syk activation provides redundancy, which likely helps maintain Syk activity over time in the cell. The sustained activation of Syk probably also facilitates the docking of SH2 domain-containing proteins to phosphorylated sites on Syk. Docking sites within the SH2 kinase linker region include Tyr-348, Tyr-352, and Tyr-317, which form binding sites for Cbl, phosphatidylinositol 3'-kinase, phospholipase C\(\gamma\)2, and other proteins (34–36). Syk activation also exposes Tyr-630 in the C terminus of Syk, which has been reported to interact with BLNK/SLP-65 (12).

Previous investigations have found that autophosphorylation increases Syk activity and leads to a conformational change in Syk (37). The contribution of individual phosphorylation sites to modulation of full-length Syk activity has yet to be determined, but several pieces of data suggest that activation loop tyrosines play a lesser role than tyrosine residues located in the SH2 kinase linker (38). For instance, mutation of Tyr-525 and Tyr-526 to phenylalanine in the context of the Syk kinase domain was demonstrated to have no effect on kinase activity (18). Although other investigations have mainly focused on Syk activation within the cell, in this study we present the first kinetic characterization of full-length Syk in vitro and how kinetic parameters are regulated by phosphorylation state. The kinetic parameters of Syk truncation mutants (Fig. 4, Table 3) have provided information regarding how phosphorylation regulates the interaction between domains in Syk. For instance, full-length dephosphorylated Syk was distinguished from all other constructs not only by a lag phase in product formation but also by a higher \(K_m\) for ATP compared with truncated proteins (Fig. 4B, Table 3). The higher \(K_m\) suggests that the presence of the tandem SH2 domains hinders ATP binding at the active site. In addition, the maximal turnover rate (\(k_{\text{cat}}\)) was 5-fold higher for Syk356 and Syk360 compared with other constructs, indicating that residues 340–356 interact with the kinase domain to hinder catalysis (Fig. 4B). Furthermore, unlike all other constructs, Syk356 and Syk360 were not regulated by phosphorylation (Fig. 4B). A crystal structure of the Syk kinase domain supports the lack of regulation of Syk356 and Syk360 by phosphorylation because the structure shows the kinase domain in an active conformation despite the absence of phosphorylation at Tyr-525 or Tyr-526 (39).

In this study, we determined that Syk binds phosphorylated ITAM sequences from the B cell receptor, the gamma chain of the FceRI receptor, and Fc\(\gamma\)RIa receptor all in the nanomolar range (Table 2). Because the BCR and Fc\(\gamma\)RIa ITAMs have 7 residues separating the pYYXL motifs, but the spacing for the Fc\(\gamma\)RIa peptide is 12 residues (Fig. 2), these results indicate that Syk can bind ITAM motifs of different length with high affinity. The crystal structure of the tandem SH2 domains of Syk indicates how different length ITAM sequence can be accommodated (40). Specifically, the structure demonstrates that the tandem domains of Syk have sufficient conformational flexibility to bind different length ITAMs. Conformational flexibility was also demonstrated in thermodynamic binding studies with the Syk tandem SH2 domains (41) and also by introducing a disulfide bond between the tandem SH2 domains to constrain flexibility (42). These previous studies together with our findings indicate that Syk can bind a variety of differently spaced ITAM motifs within the cell.

To understand the similarities in activation between Syk and Zap-70, the activation of Zap-70 was also investigated. Like Syk, Zap-70 also demonstrated a lag phase in product formation that was eliminated by preincubation with Mg\(^{2+}\)/ATP (Fig. 5A). The kinetics of Zap-70 activation and substrate phosphorylation have been studied previously, and similar results were obtained (43). Despite the slow rate of autophosphorylation, Zap-70 (like Syk) was rapidly activated by Src family kinases. This finding indicates that the slow activation of Zap-70 is likely due to Zap-70 being a poor substrate for itself rather than more thermodynamically stable in its inactive conformation than Syk.

It is feasible that factors found in a cellular context could modulate Syk activation compared with what is observed in vitro. Hence, we employed an assay to monitor activation of endogenous Syk obtained from B cell lysates (30). We discovered that many of the features of the activation process observed with recombinant Syk also applied to Syk obtained from lysates. For instance, a lag phase in product formation was observed (Fig. 6A). Immunoprecipitated Syk also demonstrated comparable activation by both pretreatment with Mg\(^{2+}\)/ATP and with ITAM peptides (Fig. 6D). However, some differences between activation of recombinant and endogenous Syk were found that might provide insight into the regulation of Syk in a cellular context. Specifically, it was found that the addition of ITAM peptides failed to stimulate the activity of Syk within the lysate, which was surprising (Fig. 6B). Furthermore, pretreatment with Mg\(^{2+}\)/ATP for 2 h was required to fully activate immunoprecipitated Syk, whereas only 15 min was required to activate Syk expressed recombinantly (Fig. 6, C and D). It is possible that endogenous Syk is more stabilized in its inactive conformation than recombinant Syk. Overall, these experiments make it clear that the ability using pep7 to monitor Syk activity in a cellular context provides a powerful approach to studying the kinetics of Syk activation. Greater insight into Syk function could be garnered by additional studies using this technique, and information provided by these studies would
provide an even greater understanding of the role of Syk in immune cell signaling pathways.

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