Biophysical and Mechanistic Insights into Novel Allosteric Inhibitor of Spleen Tyrosine Kinase

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Background: Spleen tyrosine kinase (Syk) is important for antigenic and inflammation immune responses. The abbreviations used are: BCR, B cell receptor; AMPPNP, adenosine-5’-(β,γ-imido)triphosphate; EC50, half-maximal effective concentration; FcεRI, the high affinity IgE receptor; ITAM, immunoreceptor tyrosine-based activation motif; SAXS, small-angle X-ray scattering; SH2, src homology domain 2; Syk, spleen tyrosine kinase; SykB, a natural splice variant of Syk; SykKD, construct of the kinase domain by itself (SykKD, residues 343–635). Furthermore a naturally occurring splice variant, SykB, lacking 23 residues in the flexible linker is not as efficacious an activator of immune cell signaling (10), possibly due to

Extracellular stimulation of the B cell receptor or mast cell FcεRI receptor activates a cascade of protein kinases, ultimately leading to antigenic or inflammation immune responses, respectively. Syk is a soluble kinase responsible for transmission of the receptor activation signal from the membrane to cytosolic targets. Control of Syk function is, therefore, critical to the human antigenic and inflammation immune response, and an inhibitor of Syk could provide therapy for autoimmune or inflammation diseases. We report here a novel allosteric Syk inhibitor, X1, that is noncompetitive against ATP (Kᵢ = 4 ± 1 μM) and substrate peptide (Kᵢ = 5 ± 1 μM), and competitive against activation of Syk by its upstream regulatory kinase LynB (Kᵢ = 4 ± 1 μM). The inhibition mechanism was interrogated using a combination of structural, biophysical, and kinetic methods, which suggest the compound inhibits Syk by reinforcing the natural regulatory interactions between the SH2 and kinase domains. This novel mode of inhibition provides a new opportunity to improve the selectivity profile of Syk inhibitors for the development of safer drug candidates.

The cornerstone of the human humoral immune system is induced immunity and inflammation, which arise from stimulation of either the B cell receptor (BCR) (immunity) or the mast cell high affinity IgE receptor (FcεRI) (inflammation). BCR or FcεRI receptor stimulation triggers a downstream cascade ultimately resulting in activation of immune gene response elements. Stimulated BCR or FcεRI recruit LynB, a Src family tyrosine kinase, that phosphorylates immune tyrosine activation motifs (ITAMs) on BCR or FcεRI-associated adaptor proteins. Spleen tyrosine kinase (Syk), a non-receptor-tyrosine kinase, binds to the phosphorylated ITAMs and is subsequently phosphorylated by LynB. ITAM binding (ITAM-Syk) or phosphorylation (P-Syk) results in Syk kinase activation (1, 2) and the subsequent phosphorylation of direct (PI3K, PLCγ, VAV, and SLP76) and indirect (AKT, B-cell linker protein [BLNK], PKCα and PYK2 among others) downstream targets (3–5). Syk activation commits B cells and mast cells to an immune response (6, 7) and is, therefore, an important control point for therapeutic intervention of diseases arising from inflammation, such as the common allergic response, or autoimmune responses, such as rheumatoid arthritis and asthma.

Syk has two adjacent N-terminal Src homology 2 (SH2) domains followed by a ~100 residue flexible linker connecting the second SH2 domain to the kinase domain (Fig. 1). ITAMs bind to the SH2 domains opposite the interface of the SH2 and kinase domains (8) and cause a 10-fold increase in the Syk kinase catalytic rate (1, 2). Phosphorylation of Syk at Tyr-348 and Tyr-352 by LynB (9) or autophosphorylation by Syk also results in a 10-fold increase in Syk kinase activity that is non-additive with ITAM activation (1, 2). Thus Syk is activated by an either/or mechanism where ITAM binding or phosphorylation results in maximal kinase activity.

Homology modeling of Syk with its closest relative, ζ-chain-associated protein of 70 kDa (Zap70), places Tyr-348 and Tyr-352 at the interface of the SH2 and kinase domains where phosphorylation would disrupt hydrophobic interactions between the SH2 and kinase domains (Fig. 1). Because ITAM binding or phosphorylation result in the same, non-additive, increase in kinase activity, the prevailing activation model is that ITAM binding or phosphorylation results in dissociation of the SH2 and kinase domains. Consistent with this model, Syk activated by ITAM binding or phosphorylation has similar catalytic rates as a construct of the kinase domain by itself (SykKD, residues 343–635). Furthermore a naturally occurring splice variant, SykB, lacking 23 residues in the flexible linker is not as efficacious an activator of immune cell signaling (10), possibly due to
tighter coupling between the SH2 and kinase domains from avidity affects (supplemental Fig. S1) (11–14); thus in the absence of ITAM binding or phosphorylation, the SH2 domains may down-regulate Syk kinase activity.

Kinases play essential roles in cell signal amplification and transduction. In the human genome the kinase domain motif can be found in 520 different proteins (15). Amazingly, although the kinase domain has been so multiply adapted, residues involved in ATP binding and catalysis are often conserved between even distant kinase relatives. Due to the conservation and prevalence of the kinase domain, it is a substantial challenge to develop ATP competitive inhibitors that strike a specific target while also simultaneously outcompeting physiological concentrations of ATP (16). This balance is exemplified by the natural product staurosporine, which has managed extreme potency (IC50 = 2.7 nM for PKCα) (17) at the cost of selectivity (efficacy against > 90% of kinases) (18). In contrast to classical ATP competitive inhibitors, noncompetitive kinase inhibitors bind outside of the highly conserved ATP binding pocket, promising far greater selectivity (19–21).

The potential for a Syk inhibitor to ameliorate both allergic and autoimmune human diseases has created considerable interest for the development of an inhibitor, but finding a selective Syk inhibitor has been limiting (22–27). We report here a novel allosteric inhibitor of Syk, compound X1 (see Fig. 2a).

Characterization of the mechanism of inhibition from fluorescence binding experiments and steady-state enzyme kinetics reveals X1 is noncompetitive against ATP and peptide substrates, is highly specific, inhibiting Syk and SykB but not SykKD or Zap70, inhibits activation by ITAM, and is a competitive inhibitor of phosphorylation by LynB. Finally, we use small angle x-ray scattering and analytical ultracentrifugation to investigate Syk activation and find structural evidence suggesting X1 inhibits Syk by reinforcing regulatory interactions between the SH2 and kinase domains.

EXPERIMENTAL PROCEDURES

Protein Preparation—The genes for the different Homo sapiens Syk constructs were cloned from an existing construct, pBacPAK9-WT (Invitrogen), then subcloned into the pFastBac HT-B baculovirus expression vector (Invitrogen). All Syk proteins included an N-terminal hexahistidine tag and a tobacco etch virus cleavage site; sequences were verified by automated sequencing. Recombinant genes were transformed into DH10Bac Escherichia coli-competent cells to form an expression bacmid, which were used for transfection into Spodoptera frugiperda (Sf-9) insect cells following manufacturer instructions (Invitrogen). In vivo homologous recombination between the bacmid DNA and the viral DNA transferred the recombinant gene to the viral genome to generate recombinant virus harboring Syk genes. Recombinant virus were purified by
plaque assay and amplified following the manufacturer instructions (Invitrogen).

Recombinant proteins were expressed in SF-9 host cell lines. Cells were grown in SF-9 serum-free insect cell medium (SF-900 III SFM, Invitrogen) at 27 °C to a density of 2.5 × 10⁶ cells ml⁻¹ before infection with virus. Cells were grown for 48 h at 27 °C with a shaker speed of 125 rpm and harvested at 75–90% cell viability. Cells were lysed using a Branson Ultrasonic Disintegrator (VWR Scientific Products, Chicago, IL) for 2 min at 60% duty cycle in buffer containing 20 mM Tris (pH 8), 150 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM Tris(2-carboxyethyl)-phosphine and EDTA-free Roche protease inhibitor mixture (Roche Applied Science), then centrifuged at 20,000 relative centrifugal force. The soluble fraction was applied to a HisTrap FF column (GE Healthcare) and eluted using a linear imidazole gradient (20 – 400 mM). Final purification of Syk constructs was done using two size-exclusion chromatography steps for Syk and SykB (HiLoad Superdex200 26/60 followed by HiLoad Superdex75 26/60, GE Healthcare) and one chromatography step for SykKD (Superdex75 26/60). Protein purity was verified by SDS-PAGE and electrospray ionization-TOF mass spectrometry: Syk, SykB, and SykKD were 7719 Da (7718.0 theoretical), SykB was 72,751.1 Da (72,750.6 theoretical), SykKD was 34,874.0 Da (34,874.1 theoretical). Protein concentrations were determined from sequence-based calculation of absorptivity at 280 nm (28).

H. sapiens Zap70 was purchased in purified form. C-terminal hexahistidine-tagged Zap70 (Invitrogen) was used in kinetics assays, and a catalytically incompetent Zap70 mutant (Y315F/D461N) (Jubilant Biosys, India) (29) was used in sedimentation velocity assays. The same assay inhibitor concentration was varied between 250 μM and 19.5 μM of compound X1. For ATP competition assays, ATP concentration was varied between 64 and 0.25 μM (2-fold dilutions) at a constant substrate peptide concentration of 50 μM. For substrate peptide competition assays, peptide concentration was varied between 48 and 12 μM (2-fold dilutions) at a constant ATP concentration of 250 μM. For LynB competition assays, LynB concentration was varied between 200 and 6.25 nm (2-fold dilutions) at a constant concentration of 250 μM ATP and 50 μM substrate peptide. The reaction velocity without LynB was subtracted from these data to account for contributions from Syk autoactivation.

Because incomplete inhibition of Syk was observed with the allosteric inhibitor, data were globally fit to a partial inhibition model using Equation 2,

\[
v = \frac{V_{\text{max}} - V_{\text{min}}}{1 + \frac{[I]}{K_i}} + V_{\text{min}}
\]

where \(v\) is reaction velocity, \(I\) is the inhibitor concentration, and \(H\) is the Hill coefficient.

The mechanism of inhibition experiments was performed using a variable concentration of ATP, peptide, or LynB against a constant concentration of 0, 6.5, 13, or 19.5 μM of compound X1. For ATP competition assays, ATP concentration was varied between 64 and 0.25 μM (2-fold dilutions) at a constant substrate peptide concentration of 50 μM. For substrate peptide competition assays, peptide concentration was varied between 48 and 12 μM (2-fold dilutions) at a constant ATP concentration of 250 μM. For LynB competition assays, LynB concentration was varied between 200 and 6.25 nm (2-fold dilutions) at a constant concentration of 250 μM ATP and 50 μM substrate peptide. The reaction velocity without LynB was subtracted from these data to account for contributions from Syk autoactivation.

For half-maximal inhibitory concentration (IC₅₀) assays, a constant concentration of 250 μM ATP and 50 μM substrate peptide was used against a variable concentration of X1 or an ATP competitive inhibitor, Kisei compound 9F (24). For X1, assay inhibitor concentration was varied between 100 and 0.19 μM (2-fold dilutions), and for 9F assays the inhibitor concentration was varied between 50 and 0.01 nm (2-fold dilutions). Changes in enzyme velocity were fit in GraphPad Prism v5 (San Diego, CA) using Equation 1,

\[
v = V_{\text{min}} + \frac{V_{\text{max}} - V_{\text{min}}}{1 + 10^{\frac{-\text{IC50} - \text{log}[I]}{\text{H}}}}
\]

where \(v\) is reaction velocity, \(I\) is the inhibitor concentration, and \(H\) is the Hill coefficient.
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\[ v = \frac{V_{\text{max}} - V_{\text{min}}}{1 + 10^{\log(K_{d} - x) / 10}} \]  

(Eq. 4)

where \( X \) is the ITAM concentration.

Fluorescence Binding Assay—Purified proteins were prepared at 5 \( \mu \)M. Compound X1 or 9F were prepared in DMSO, and increasing concentrations of inhibitor were mixed with protein by pipette for 15 s. Data were collected using a FluoroMax-3 (Horiba Jobin Yvon, Edison, NJ).

For fluorescence anisotropy measurements, data were collected in the L configuration with an excitation wavelength of 495 nm, 1-nm slit widths, a 0.5-s integration time, and emission monitoring at 520 nm.

Intrinsic tryptophan fluorescence experiments were performed using an excitation wavelength of 295 nm with 2-nm slit widths, a 0.5-s integration time, and emission monitoring between 330 and 370 nm. The fluorescence spectra were scanned in the ratio mode (signal/reference) to compensate for variations in lamp output as a function of wavelength. Changes in intrinsic tryptophan fluorescence in the absence of ligand, and \( E \) is the enzyme concentration, \( F_{\text{max}} \) is the maximal change in fluorescence upon saturation of ligand, and \( E \) is the enzyme and ligand complex, where ligand is either X1 or 9F (34).

Small-angle X-ray Scattering—SAXS data were collected at beamline X9 of the Brookhaven National Laboratory. Three 30-s data sets were collected using Syk concentrations between 0.5 and 3.5 g/liter in the absence or presence of 100 \( \mu \)M X1; no aggregation or radiation damage was observed for these samples. Scattering from identical solutions, but lacking Syk, were collected adjacent to sample data sets for use as buffer blanks.

Scattering data were circularly averaged over the detector and normalized against transmitted incident beam intensity using software developed at X9. Data at all concentrations were independently processed and compared before merging using PRIMUS (35). Radius of gyration and particle distance distribution function were determined using GNOM (36). Theoretical scattering of Zap70 (PDB accession 2OZO) was determined with CRYOSOL (37). A consensus model was determined from the average of 15 simulations using GASBOR (38), and rigid body fit of the Zap70 SH2 and kinase domains to the consensus model was performed using BUNCH (39).

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed using an OptimaXL-I centrifuge (Beckman Coulter, Brea, CA). Samples were loaded into two-channel (1.2 cm path length) carbon-Epon centerpieces in an An-50 Ti rotor and spun at 40,000 rpm, 15 °C with monitoring at 280 nm with 0.006 cm spacing in the continuous mode. Data were processed using SEDFIT v12.1 (40). Frictional coefficients from SEDFIT for ITAM-Syk experiment with ITAM at 3.33, 10, and 30 \( \mu \)M were affected by peak broadening due to exchange with excess ITAM in solution; consequently, frictional coefficients were estimated as the ratio of the sequence-based theoretical sedimentation (SEDNTERP v1.09) (41) to the experimentally determined weight average sedimentation.

Solubility Measurements—Compound solubility limits were measured using a DynaPro Plate Reader (Wyatt, Santa Barbara, CA). Solubility was defined as the concentration at which particle scattering intensity was 5-fold blank buffer, which was in excess of 100 \( \mu \)M for X1 and in excess of 50 \( \mu \)M for compound 9F.

RESULTS

A Novel Syk Inhibitor—Compound X1 was identified from a high throughput LANCE assay measuring inhibition of Syk at the apparent \( K_{m} \) of ATP (Fig. 2a). In these assays X1 inhibited Syk with an IC50 of 10 ± 5 \( \mu \)M. The solubility of X1 was subsequently tested and found to be above 100 \( \mu \)M, indicating Syk inhibition in the LANCE assay was not likely due to nonspecific association of X1 with components of the LANCE assay (data not shown). The compound inhibited the full-length enzyme but not a construct of the Syk kinase domain without the regulatory SH2 domains (SykKD, residues 343–635), which led to interest in understanding the mechanism of inhibition.

Solution-based binding studies monitoring the change in intrinsic Syk fluorescence upon binding to X1 or a previously characterized ATP competitive inhibitor, Kissel compound 9F (24) (Fig. 2b), were performed to correlate IC50 values with \( K_{d} \). Syk binds compound X1 with a \( K_{d} \) of 6 ± 2 \( \mu \)M and 9F with a \( K_{d} \) of 4 ± 2 \( \mu \)M, which is consistent with previous IC50 measurement for 9F (24) (Fig. 3).

Titration of X1 into a solution of Syk prebound with a saturating concentration of 9F or the reverse experiment where 9F was titrated into a solution of Syk prebound with a saturating concentration of X1 resulted in a change in intrinsic fluorescence, indicating separate binding sites for these two inhibitors, but due to combined inner filter effects the signal to noise was too low to robustly determine \( K_{d} \) values (data not shown). SykKD bound 9F with a \( K_{d} \) of 3 ± 1 nm, but no change in intrinsic fluorescence was observed for SykKD or phosphorylated Syk (P-Syk) upon X1 titration (data not shown).

Omnia peptide phosphorylation assays were used to determine the kinase parameters and inhibition strength of X1 for Syk and SykB as well as the mechanism of inhibition for Syk. Kinetic parameters for Syk in the absence of inhibitor are in good agreement with previous reports (2, 31) (Table 1). Compound X1 inhibits Syk and SykB (IC50 of 13 ± 5 and 12 ± 8 \( \mu \)M, respectively) but did not inhibit SykKD, Zap70, or P-Syk (Fig. 4, a and b; Table 2).

Experiments with varying concentrations of ATP or Omnia peptide show that X1 is not competitively displaced by either

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substrate, indicating either noncompetitive or uncompetitive inhibition. Inhibition data for peptide and ATP were well fit by a noncompetitive binding model, with a $K_i$ of $5 \pm 1$ and $4 \pm 1 \mu M$ for Syk against peptide and ATP, respectively. Data at the highest concentration of inhibitor (19.5 $\mu M$) and the lower concentrations of ATP (0.25 $\mu M$) deviate from the theoretical best fit line more than other data points. These results could be explained by reduced signal to noise inherent to high inhibitor and low substrate conditions or contributions from a secondary binding site populated at higher inhibitor concentrations (Fig. 5, a and b, Table 3).

Inhibition by X1 does not result in complete elimination of Syk activity. Instead the catalytic rate approximates the basal

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**TABLE 1**

Kinetic parameters for Syk and SykB

| Variable substrate | P-Syk | ITAM-Syk | SykB | P-SykB | ITAM-SykB |
|--------------------|-------|----------|------|--------|-----------|
| ATP$^a$            | $K_m$ (mM) | 30 ± 17 | 24 ± 12 | 23 ± 8 | 28 ± 4 | 17 ± 3 |
|                    | $k_{cat}$ (min$^{-1}$) | 91 ± 7 | 91 ± 6 | 8 ± 1 | 42 ± 1 | 44 ± 2 |
| Substrate peptide  | $K_m$ (mM) | 38 ± 18 | 42 ± 3 | 32 ± 13 | 42 ± 9 | 71 ± 6 |
|                    | $k_{cat}$ (min$^{-1}$) | 140 ± 34 | 146 ± 7 | 19 ± 8 | 68 ± 9 | 95 ± 7 |

$^a$ Variable ATP kinetic parameters obtained at the $K_m$ of substrate peptide.

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**FIGURE 3. Changes in intrinsic Syk fluorescence upon inhibitor binding.** Fluorescence emission of 5 $\mu M$ Syk upon binding to allosteric compound X1 (a) or the ATP competitive compound 9F (b) at 0 (red), 1 (orange), 5 (yellow), 15 (green), 25 (blue), 35 (indigo), or 50 $\mu M$ (violet). Change in fluorescence intensity versus inhibitor concentration (cyan) and best fit lines (black) for a binary association model are inset. Data are fit using Equation 5. RFU, relative fluorescence units; AU, absorbance units.

**FIGURE 4. Fluorescence time course for phosphorylated peptide production showing Syk activation and inhibition.** a, shown is the concentration of phosphorylated Omnia peptide (P-Peptide) versus time for Syk (blue), SykB (green), or Zap70 (pink) in an initially inactive state (solid line) preactivated by autophosphorylation (dashed line) or ITAM binding (dotted line). The inactive state has an initial lag phase that changes to a high activity state after autophosphorylation. b, shown is the concentration of P-Peptide versus time for Syk (blue), SykB (green), SykKD (red), or Zap70 (pink) in either the apo state (solid line) or in the presence of 100 $\mu M$ X1 (diamonds). c, shown is the concentration of P-Peptide versus time for Syk in an initially inactive (blue) or preactivated state due to autophosphorylation (purple) or ITAM binding (orange) in the absence (solid line) or presence of 100 $\mu M$ X1 (dashed line). d, shown are Syk concentration-dependent changes in autophosphorylation rates at 160 (red, light red), 80 (orange, light orange), 40 (yellow, light yellow), 20 (green, light green), 10 (blue, light blue), 5 (indigo, light indigo), and 2.5 nM (violet, light violet) with best fit lines (black) for a trans autophosphorylation mechanism using the method of Wu et al. (32) to yield a $K_{obs}$ of 3.4 ± 0.1 nM. A graph of $K_{obs}$ at each Syk concentration (cyan) and best fit lines (black) is inset.
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TABLE 2
Inhibition of different activation states of Syk and SykB by compound X1 or 9F
Values are the average ± S.D. of at least four experimental repeats.

| Inhibitor | Syk | P-Syk | ITAM-Syk | SykB | P-SykB | ITAM-SykB |
|-----------|-----|-------|----------|------|--------|-----------|
| Compound X1 | 13 ± 5 | NA | 8 ± 2 | 12 ± 8 | NA | 6 ± 1 |
| Compound 9F | 7E-3 ± 3E-3 | 3E-3 ± 2E-3 | 3E-3 ± 1E-3 | 4E-3 ± 1E-3 | 1E-3 ± 1E-3 | 10E-3 ± 1E-3 |

FIGURE 5. Inhibition of Syk by X1. Lineweaver-Burk plots of X1 are shown at a constant concentration of 0 (red), 6.5 (yellow), 13 (blue), or 19.5 μM (purple) with best fit lines (black) for a partial noncompetitive model versus a variable concentration of ATP (64 μM initial with 2-fold dilutions) at a constant substrate peptide concentration of 50 μM (a) or substrate peptide (48 μM initial with 2-fold dilutions) at a constant ATP concentration of 250 μM (b); c, shown is competitive inhibition of X1 by varied LynB (298 nM initial with 2-fold serial dilutions) at a constant ATP and substrate peptide concentration of 250 and 50 μM, respectively. Data were fit using Equations 2 and 3.

TABLE 3
Mechanism of inhibition of Syk and LynB by compound X1
Values are the average of the global fit plus or minus error of the fit. NA, not applicable; C, competitive; NC, noncompetitive.

| Protein | Inhibitor | Substrate | Pattern | $K_i$ (μM) | $\beta$ |
|---------|-----------|-----------|---------|------------|--------|
| Syk     | F9        | ATP       | Complete C | 3E-3 ± 1E-3 | NA     |
| Syk     | X1        | ATP       | Partial NC | 4 ± 1      | 0.87   |
| Syk     | X1        | Peptide   | Partial NC | 5 ± 1      | 0.81   |
| LynB    | X1        | ATP       | Complete C | 4 ± 1      | NA     |

Because Syk is activated by a trans mechanism, it is possible that X1 inhibition occurs by directly blockage of autophosphorylation sites. In vitro Syk is autophosphorylated at Tyr-348, Tyr-352, Tyr-525, and Tyr-526 (2), and in vivo Syk is phosphorylated by LynB at Tyr-348 and Tyr-352 (9); thus we set out to determine if X1 would inhibit not only Syk autophosphorylation but also phosphorylation by its physiological partner LynB. Experiments with variable concentrations of LynB demonstrate Syk activation is competitively inhibited by X1 with a $K_i$ of 3 ± 1 μM (Fig. 5c, Table 3). Compound X1 did not inhibit the kinase activity of LynB in peptide turnover assays (data not shown), demonstrating the nature of the competitive interaction against X1 is not global inhibition of LynB but is instead either overlapping binding sites or kinetic linkage between non-overlapping binding sites on the same form of Syk.

ITAM Binding to Syk Results in an Increase in the Solution Structure Radius of Syk—Radius of gyration calculated from SAXS gives a measure of the compactness of a structure in solution (42). The radius of gyration of apo- or X1-bound Syk were identical (30.0) and agree well with the theoretical radius of gyration of Zap70 (29.7) calculated from the full-length inactive structure (Fig. 6, a and b). Further analyses show good agreement between a rigid body fit of the Zap70 crystal structure to apo or X1-bound SAXS molecular envelopes, with a χ2 of 3.1 and 2.6, respectively (Fig. 6, c and d). Treatment of the SH2 and kinase domains as independent rigid bodies resulted in a modest improvement in χ2 but did not yield a unique solution relative to the intact Zap70 rigid body fit. These data are, there-
consistent with the inactive apo or inhibitor-bound Syk solution structures being monomeric and compact, with contact between the SH2 and kinase domains approximate to what is seen in the Zap70 crystal structure.

The $EC_{50}$ for ITAM activation of Syk is $4 \pm 2 \mu M$ (supplemental Fig. S4). To test the effect of ITAM binding on the solution structure of Syk, sedimentation velocity experiments were performed with Syk in complex with ITAM at concentrations near the $EC_{50}$. The sedimentation coefficient is consistent with an oblate monomer in solution; the frictional coefficient of apo-Syk is 1.65 and reached an approximate midpoint of 1.80 at 3.3 $\mu M$ ITAM, in good agreement with the ITAM $EC_{50}$ value, before a saturated frictional coefficient of 1.86 at 30 $\mu M$ ITAM. The correlation between the midpoint of the frictional coefficients determined from centrifugation and the $EC_{50}$ from kinetic assays is consistent with a Syk activation model where ITAM binding promotes dissociation of the SH2 and kinase domains. Parallel experiments with P-Syk also show an increase in the frictional coefficient, 1.77, suggesting activation by either ITAM or phosphorylation results in an expansion of Syk in solution (Fig. 7, a and b). The similar frictional coefficient of Syk activated by ITAM or phosphorylation may be due to similar overall solution structures.

In addition to an increase in the average frictional coefficient of Syk, ITAM binding results in a broadening of the Syk sedimentation distribution peak. Peak broadening could be due to free ITAM in exchange with the ITAM-Syk complex or ITAM-Syk sampling an ensemble of hydrodynamic radii due to dissociation of the SH2 and kinase domains. The addition of X1 does not dramatically change the frictional coefficient of Syk in apo, phosphorylated, or AMPPNP-bound states but does cause a large decrease in the frictional coefficient of ITAM-Syk and sharpening of the peak into a tighter distribution (Fig. 7c, Table 4). These results are consistent with X1 returning ITAM-Syk to an inactive state through compression of the hydrodynamic radii of ITAM-Syk to a form intermediate between apoSyk and

![Figure 6. Solution structure of apo- and X1-inhibited Syk determined using SAXS. SAXS data (squares) for apo (a) and X1-bound Syk (b) were fit with theoretical scattering (line) of the full-length Zap70 crystal structure. AU, absorbance units. Zap70 (cartoon) is well fit by the experimental molecular envelope (blue mesh) for apo- (c) and X1-bound (d) Syk. Zap70 domain coloring and orientation as in Fig. 1. Molecular envelopes were generated from the consensus of 15 molecular dynamics models. Data are consistent with the SH2 and kinase domains associating in the inactive apo or X1 inhibited Syk solution structures. Experimental radius of gyration for apo- and X1-bound Syk were both 30.0 with $\chi^2$ of 3.1 and 2.6 versus theoretical Zap70 scattering, respectively. Data were fit using PDB structure 2OZO (37).]

![Figure 7. Changes in hydrodynamics of Syk when activated by autophosphorylation or ITAM binding. a, sedimentation coefficient distribution profile for Syk (gray, solid) and P-Syk in the absence (gray, hollow dashes) or presence of 15 $\mu M$ X1 (black, dots). b, shown is a sedimentation coefficient distribution profile for Syk bound to 0 (gray, solid), 0.33 (gray, hollow dashes), 1.11 (black, dots), 3.33 (black, hollow dashes), 10 (gray, dots), or 30 $\mu M$ (black, dashes) ITAM. c, shown are sedimentation coefficient distribution profiles for apoSyk (gray, solid), Syk bound to 15 $\mu M$ X1 (black, dots), Syk bound to 60 $\mu M$ AMPPNP (black, dashes), Syk bound to 15 $\mu M$ ITAM (gray, dashes), or Syk bound to 15 $\mu M$ X1, 60 $\mu M$ AMPPNP, and 15 $\mu M$ ITAM (black, hollow dashes). The absorbance of Syk bound to 15 $\mu M$ X1 and Syk bound to 60 $\mu M$ AMPPNP were normalized to apoSyk. AU, absorbance units.]

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**TABLE 4**

Sedimentation parameters for Syk, SykB, and Zap70
Values have been adjusted in SEDFIT to give standard temperature and pressure.

| AMPPNP | X1  | ITAM | S_exp | S_calc | S_calc/exp | Frictional coefficient |
|--------|-----|------|-------|--------|------------|-----------------------|
|        | µM | µM  | µM    |        |            |                       |
| Zap70  |     | 3.63 | 5.96  | 1.64   | 1.70       |
| SykB   | 3.79 | 6.15 | 1.62   | 1.56   |
| Syk    | 3.85 | 6.29 | 1.63   | 1.60   |
| Syk    | 3.85 | 6.44 | 1.67   | 1.58   |
| Syk    | 3.86 | 6.40 | 1.66   | 1.66   |
| Syk    | 3.55 | 6.40 | 1.80   | ND*    |
| Syk    | 3.44 | 6.40 | 1.86   | ND*    |
| Syk    | 3.56 | 6.55 | 1.84   | 1.87   |
| Syk    | 3.70 | 6.55 | 1.77   | 1.77   |
| SykB   | 3.79 | 6.55 | 1.63   | 1.56   |
| Zap70  | 3.85 | 6.40 | 1.66   | 1.66   |

*Values could not be determined due to broad sedimentation distribution.

the ITAM-Syk complex. Because ITAMs do not displace X1 in a concentration-dependent manner, these results suggest X1 shifts the ITAM-Syk complex to a state off the normal activation path where ITAM is bound, but the kinase domain is not activated.

**DISCUSSION**

**Summary**—The signal from BCR or FcεRI receptor stimulation is relayed from the cell membrane to the cytosol by the kinase Syk. Syk rests in a quiescent state before receptor stimulation and is activated through binding to phosphorylated ITAM sequences on cell receptor-associated proteins or phosphorylation by LynB at residues sandwiched between the Syk SH2 and kinase domains. Activation of Syk through either ITAM binding or phosphorylation results in a non-additive order of magnitude increase in enzymatic activity (2). Activated Syk subsequently phosphorylates downstream targets to create an enzymatic cascade, potentially including other nonactivated Syk molecules, and is thus a committing step for immune mobilization; controlling Syk activation is therefore a potential therapy for human diseases resulting from inflammation or autoimmune responses.

Due to the critical role of Syk in both allergic and autoimmune human diseases, repeated efforts have been made to develop an inhibitor of Syk kinase activity (22–27). Despite the selectivity achieved by some ATP competitive inhibitors, the high conservation of key residues in the kinase domain make it challenging to find selective ATP competitive kinase inhibitors. However, by moving outside of the highly conserved ATP binding site specificity becomes easier to achieve as inhibitors bind regions unique to the kinase of interest.

There is increasing precedent for development of allosteric kinase inhibitors, which have proven to be much more selective than classical ATP competitive inhibitors (21, 43–49). Allosteric modulation of kinases is particularly promising because many soluble kinases have inter- or intra-domain associations that allosterically regulate kinase activity. Finding these sites is a challenge that must be considered when designing inhibitor screening assays. Traditional assays for kinase inhibitors have been performed against an isolated kinase domain, which bias inhibitors toward ATP competition. In contrast, our assays used full-length Syk, allowing for selection of both classical ATP competitive inhibitors as well as allosteric regulatory sites.

We report here a novel allosteric inhibitor of Syk that is noncompetitive against ATP and peptide substrates. Compound X1 inhibits activation of Syk through either auto-phosphorylation or phosphorylation by its physiological activator LynB to form phosphorylated Syk (P-Syk) but does not inhibit Syk after it has been phosphorylated. In contrast to P-Syk, X1 is able to inhibit Syk activated by ITAM binding (ITAM-Syk).

The sedimentation coefficient, radius of gyration, and solution state x-ray scatting of apo or X1 bound Syk are consistent with a compact monomer, where the SH2 and kinase domains remain associated in the inactive state (Fig. 6). Changes in the solution frictional coefficients between apoSyk and P-Syk or ITAM-Syk provides insight into the mechanism of Syk activation. Upon ITAM binding the frictional coefficient of Syk increases, consistent with an elongation of the solution structure of Syk, whereas binding of X1 to the ITAM-Syk complex causes a decreases in frictional coefficient. Because X1 is not competitive with ITAM binding, a decrease in frictional coefficient suggests contraction of the solution structure of Syk to a form intermediate between apoSyk and ITAM-Syk (Fig. 7c). Expansion of the solution structure of Syk upon activation is consistent with a model where dissociation of the SH2 domains from the kinase domain is responsible for Syk activation; therefore, contraction may represent a return to a form closer to nonactivated Syk. In keeping with this interpretation, P-Syk also has an increased fictional coefficient relative to apoSyk but does not contract upon the addition of X1 (Fig. 7a). ITAM binding or phosphorylation of Syk appears to cause an elongation of the Syk solution structure in addition to an equivalent increase in catalytic rates. However, whereas X1 can cause both inhibition and contraction of ITAM-Syk, it cannot do so for P-Syk.

X1 is a competitive inhibitor of LynB activation, suggesting X1 and LynB share the same binding site on Syk. Syk is phosphorylated at Tyr-348 and Tyr-352 by LynB (9); based on homology modeling, these residues are expected to pack between the SH2 domains and the hinge region of the kinase domain (Fig. 1), stabilizing interactions at this interface (29). In agreement with this model, constructs of the Syk kinase domain including linker residues N-terminal to Leu-341 have kinetic profiles similar to inactive Syk (2), whereas constructs beginning at M343 behave like activated Syk (Fig. 4b).
Further support of this model comes from the decreased catalytic rates of either Zap70 or SykB relative to Syk. Zap70 is a paralog of Syk expressed in T cells and in vitro is also activated by tyrosine autophosphorylation (1, 2). Syk and Zap70 have 53% sequence identity over 635 residues; the major sequence difference between these two paralogs is an extra 24 residues in the linker region between the SH2 and kinase domain in Syk. SykB is a natural splice variant of Syk with 23 residues missing from its linker region; comparison of the catalytic rates of Syk and SykB is, therefore, a measure of linker length on kinase function, where a shorter linker length should result in a tighter interdomain complex from increased avidity (11–14) (supplemental Fig. S1). Similarly, because SykB has only one residue more in its linker than Zap70, the effect of the linker on SykB activity bridges observations between Syk and Zap70 activity (1, 2, 31).

Nonactivated SykB and Zap70 have catalytic rates approximated to the reported values for nonactivated Syk (2, 31); however, both SykB and Zap70 are slower to autoactivate (Fig. 4a), and activated SykB is ~2-fold less active than activated Syk (Table 1). A previous comparison of Syk and Zap70 catalytic rates has been complicated by contributions from both sequence and linker length differences; however, comparison of SykB and Syk makes clear that linker length is a significant affects of kinase activity in this family. Immune cells expressing SykB have a reduced immune response efficacy relative to Syk expressing cells; the slower activation or reduced catalytic rates of SykB may play a role in the phenotypic difference of these cells (10). If this were the case, T cells may also have a less robust immune mobilization response based on the similarity of SykB and Zap70 catalytic rates.

Conclusions—Without regulation by the SH2 domains, Syk_{KD} is as active as full-length Syk after phosphorylation or ITAM binding. Tyr-348 and Tyr-352 are sandwiched between the SH2 and kinase domains; phosphorylation would introduce charge into a hydrophobic environment and may result in dissociation of the SH2 and kinase domains (Fig. 1). Similarly, changes in the interdomain linker between the SH2 domains upon ITAM binding are thought to result in dissociation of the SH2 and kinase domains (8, 50–53). Activation by phosphorylation or ITAM binding result in expansion of the Syk solution structure, whereas inhibition results in contraction of ITAM-Syk; thus activation and inhibition are correlated with expansion and contraction of Syk, which we propose results from dissociation and reassociation of the SH2 and kinase domains.

X1 is able to inhibit Syk activation by all three of its potential activation mechanisms: autophosphorylation, LynB phosphorylation, or ITAM binding. Noncompetitive inhibition against ATP and peptide substrates prevents autophosphorylation by locking Syk into its inactive basal state. Competitive inhibition by LynB activation could be due to a direct overlap of binding sites on Syk, although a more complicated kinetic model involving competitive linkage of non-overlapping sites cannot be dismissed. It is not known how far the LynB binding site extends around Tyr-348 and Tyr-352, but a reasonable interpretation of these data would place the X1 binding site near these residues, possibly directly interacting with the interdomain linker between the SH2 domains. This placement would allow X1 to redact ITAM activation and would explain why no inhibition is observed for Syk_{KD}.

Selectivity is the single largest problem facing the development of ATP competitive kinase inhibitors. Allosteric inhibitors have the potential to overcome selectivity issues and create high precision kinase inhibition therapy. If X1 inhibits Syk by reinforcing naturally occurring interdomain regulation in Syk, these results go a significant way toward demonstrating the feasibility of tailoring highly specific allosteric inhibition for the development of safer drugs.

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REFERENCES

1. Brdicka, T., Kadlecsek, T. A., Roose, J. P., Pastuszak, A. W., and Weiss, A. (2005) Intramolecular regulatory switch in ZAP-70. Analogy with receptor-tyrosine kinases. Mol. Cell. Biol. 25, 4924–4933
2. Tsang, E., Giannetti, A. M., Shaw, D., Dinh, M., Tse, J. K., Gandhi, S., Ho, H., Wang, S., Papp, E., and Bradshaw, J. M. (2008) Molecular mechanism of the Syk activation switch. J. Biol. Chem. 283, 32650–32659
3. Baba, Y., Hashimoto, S., Matsushita, M., Watanabe, D., Kishimoto, T., Kurotsuki, T., and Tsukada, S. (2001) BLNK mediates Syk-dependent Btk activation. Proc. Natl. Acad. Sci. U. S. A. 98, 2582–2586
4. Kawakami, Y., Kitaura, J., Yao, L., McHenry, R. W., Kawakami, Y., Newton, A. C., Kang, S., Kato, R. M., Leitges, M., Rawlings, D. J., and Kawakami, T. (2003) A Ras activation pathway dependent on Syk phosphorylation of protein kinase C. Proc. Natl. Acad. Sci. U. S. A. 100, 9470–9475
5. Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010) The SYK tyrosine kinase. A crucial player in diverse biological functions. Nat. Rev. Immunol. 10, 387–402
6. Reth, M., and Wienands, J. (1997) Initiation and processing of signals from the B cell antigen receptor. Annu. Rev. Immunol. 15, 453–479
7. Siraganian, R. P., Zhang, J., Suzuki, K., and Sado, K. (2002) Protein-tyrosine kinase Syk in mast cell signaling. Mol. Immunol. 38, 1229–1233
8. Fütterer, K., Wong, I., Grucza, R. A., Chan, A. C., and Waksman, G. (1998) Structural basis for Syk-tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. J. Mol. Biol. 281, 523–537
9. Keshtwar, L. M., Isaacson, C. C., Yankee, T. M., Sarac, R., Harrison, M. L., and Geahlen, R. L. (1998) Syk- and Lyn-dependent phosphorylation of Syk on multiple tyrosines following B cell activation includes a site that negatively regulates signaling. J. Immunol. 161, 5276–5283
10. Chu, D. H., Morita, C. T., and Weiss, A. (1998) The Syk family of protein-tyrosine kinases in T-cell activation and development. Immunol. Rev. 165, 167–180
11. Hall, J., Karplus, P. A., and Barbar, E. (2009) Multivalency in the assembly of intrinsically disordered Dynin intermediate chain. J. Biol. Chem. 284, 33115–33121
12. Kuriyan, J., and Eisenberg, D. (2007) The origin of protein interactions and allostery in colocalization. Nature 450, 983–990
13. Page, M. L., and Jencks, W. P. (1971) Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. Proc. Natl. Acad. Sci. U. S. A. 68, 1678–1683
14. Jencks, W. P. (1981) On the attribution and additivity of binding energies. Proc. Natl. Acad. Sci. U. S. A. 78, 4046–4050
15. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. Science 298, 1912–1934
16. Fischer, P. M. (2004) The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases. Curr. Med. Chem. 11, 1017–1031

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11, 1563–1583
17. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/ Ca2+-dependent protein kinase. Biochem. Biophys. Res. Commun. 135, 397–402
18. Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., Chan, K. W., Ciceri, P., Davis, M. I., Edeen, P. T., Farozi, R., Floyd, M., Hunt, J. P., Lockhart, D. I., Milanov, Z. V., Morrison, M. I., Pallares, G., Patel, H. K., Pritchard, S., Wodicka, L. M., and Zarrinkar, P. P. (2008) A quantitative analysis of kinase inhibitor selectivity. Nat. Biotechnol. 26, 127–132
19. Hirai, H., Sootome, H., Nakatsu, Y., Miyama, K., Taguchi, S., Tsujioka, K., Ueno, Y., Hatch, H., Majumder, P. K., Pan, B. S., and Kotani, H. (2010) MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol. Cancer Ther. 9, 1956–1967
20. Cherrin, C., Haskell, K., Howell, B., Jones, R., Leander, K., Robinson, R., Watkins, A., Bilodeau, M., Hoffman, J., Sanderson, P., Hartman, G., Mahan, E., Prueksaritanont, T., Jiang, G., She, Q. B., Rosen, N., Sepp-Lorenzino, L., Deleo-Jones, D., and Huber, H. E. (2010) An allosteric Akt inhibitor effectively blocks Akt signaling and tumor growth with only transient effects on glucose and insulin levels in vivo. Cancer Biol. Ther. 9, 493–503
21. Zhang, J., Adrián, F. I., Jahnke, W., Cowan-Jacob, S. W., Li, A. G., Iacob, R. E., Sim, T., Powers, J., Dierks, C., Sun, F., Guo, G. R., Ding, Q., Okram, B., Choi, Y., Wojciechowski, A., Deng, X., Liu, G., Fendrich, G., Strauss, A., Vajpai, N., Grzesiek, S., Tuntland, T., Liu, Y., Bursulaya, B., Azam, M., Manley, P., Engen, J. R., Daley, G. Q., Warmuth, M., and Gray, N. S. (2010) Targeting Bcr-Abl by combining allosteric with ATP binding site inhibitors. Nature 463, 501–506
22. Hisamichi, H., Naito, R., Toyohashi, A., Kawano, N., Ichikawa, A., Orita, M., Hamada, N., Takeuchi, M., Ohta, M., Takeuchi, M., Ozawa, M., Miyazawa, K., Misawa, K., Ohnota, H., and Isaji, M. (2008) An allosteric Syk kinase inhibitor. J. Med. Chem. 51, 7089–7095
23. Hirabayashi, A., Mukaiyama, H., Kobayashi, H., Shiohara, H., Nakayama, S., Ozawa, M., Miyazawa, K., Misawa, K., Ohnota, H., and Isaji, M. (2008) A novel Syk inhibitor. J. Med. Chem. 51, 7089–7095
24. Hirabayashi, A., Mukaiyama, H., Kobayashi, H., Shiohara, H., Nakayama, S., Ozawa, M., Tsuji, E., Miyazawa, K., Misawa, K., Ohnota, H., and Isaji, M. (2008) Structural basis for the inhibition of Syk kinase activity of Syk. J. Biol. Chem. 283, 15103–15114
25. Wang, H., and Wang, Z. X. (2003) The mechanism of p21-activated kinase 2 autoactivation. J. Biol. Chem. 278, 41768–41778
26. Sharma, A. S. S. G. (1999) Introduction to Fluorescence Spectroscopy, Wiley Interscience
27. Aulabaugh, A., Kapoor, B., Huang, X., Dollings, P., Hunt, W. T., Banker, A., Wood, A., and Elledest, G. (2007) Biochemical and biological characterization of inhibitor binding to caspase-3 reveals induced asymmetry. Biochemistry 46, 9462–9471
28. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. I., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. J. Appl. Crystallogr. 36, 1277–1282
29. Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. J. Appl. Crystallogr. 25, 495–503
30. Svergun, D., Barberato, C., and Koch, M. H. J. (1995) CRYSO--a program to evaluate x-ray solution scattering of biological Macromolecules from atomic coordinates. J. Appl. Crystallogr. 28, 767–777
31. Svergun, D. I., Petoukhov, M. V., and Koch, M. H. (2001) Determination of domain structure of proteins from X-ray solution scattering. Biophys. J. 80, 2946–2953
32. Vajpai, N., Grzesiek, S., Tuntland, T., Liu, Y., Bursulaya, B., Azam, M., Hwang, G. Q., Warmuth, M., and Gray, N. S. (2010) Targeting Bcr-Abl by combining allosteric with ATP binding site inhibitors. Nature 463, 501–506
33. Putnam, C. D., Hammel, M., Hura, G. L., and Tainer, J. A. (2007) X-ray solution scattering (SAXS) combined with crystallography and computation. Defining accurate macromolecular structures, conformations, and assemblies in solution. Q. Rev. Biophys. 40, 191–255
34. Russo, A. A., Jeffrey, P. D., Patten, A. K., Massagué, J., and Pavletich, N. P. (1996) Crystal structure of the p21Kip1 cyclin-dependent kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature 382, 325–331
35. Zhang, X., Pickin, K. A., Bose, R., Jura, N., Cole, P. A., and Kuriyan, J. (2007) Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. Nature 450, 741–744
36. Barnett, S. F., Defeo-Jones, D., Fu, S., Hancock, P. J., Haskell, K. M., Jones, R. E., Kahana, J. A., Kral, A. M., Leander, K., Lee, L. L., Malinowski, J., McAvoy, E. M., Nahas, D. D., Robinson, R. G., and Huber, H. E. (2005) Identification and characterization of pleckstrin homology domain-dependent and isoenzyme-specific Akt inhibitors. Biochem. J. 385, 399–408
37. Calleja, V., Laguerre, M., Parker, P. J., and Larijani, B. (2009) Role of a novel PH-kinase domain interface in PKB/Akt regulation. Structural mechanism for allosteric inhibition. PLoS Biol. 7, e17
38. Green, C. J., Göransson, O., Kular, G. S., Leslie, N. R., Gray, A., Alessi, D. R., Sakamoto, K., and Hundle, H. S. (2008) Use of Akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-dependent regulation of glucose and system A amino acid uptake. J. Biol. Chem. 283, 27653–27667
39. Hindle, V., Stroba, A., Zhang, H., Lopez-Garcia, L. A., Idrissova, L., Zeuzem, S., Hirschberg, D., Schaffner, F., Jorgensen, T. J., Engel, M., Alzari, P. M., and Biondi, R. M. (2009) Structure and allosteric effects of low molecular weight activators on the protein kinase PDK1. Nat. Chem. Biol. 5, 758–764
40. Wei, L., Gao, X., Warne, R., Hau, X., Bussiere, D., Gu, X. J., Uno, T., and Liu, Y. (2010) Design and synthesis of benzoazepin-2-one analogs as allosteric binders targeting the PIF pocket of PDK1. Bioorg. Med. Chem. Lett. 20, 3897–3902
41. Narula, S. S., Yuan, R. W., Adams, S. E., Green, O. M., Green, J., Phillips, T. B., Zydowsky, L. D., Botfield, M. C., Hatada, M., and Laird, E. R. (1995) Solution structure of the C-terminal SH2 domain of the human tyrosine kinase Syk complexed with a phosphoseryl pentapeptide. Structure 3, 1061–1073
51. Catalina, M. I., Fischer, M. J., Dekker, F. J., Liskamp, R. M., and Heck, A. J. (2005) Binding of a diphosphorylated-ITAM peptide to spleen tyrosine kinase (Syk) induces distal conformational changes. A hydrogen exchange mass spectrometry study. *J. Am. Soc. Mass Spectrom.* **16**, 1039–1051

52. Arias-Palomo, E., Recuero-Checa, M. A., Bustelo, X. R., and Llorca, O. (2007) Three-dimensional structure of Syk kinase determined by single-particle electron microscopy. *Biochim. Biophys. Acta* **1774**, 1493–1499

53. Arias-Palomo, E., Recuero-Checa, M. A., Bustelo, X. R., and Llorca, O. (2009) Conformational rearrangements upon Syk autophosphorylation. *Biochim. Biophys. Acta* **1794**, 1211–1217

54. Delano, W. L. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, San Carlos, CA