




Differences in the dynamics of the tandem-SH2 modules of the Syk and ZAP-70 tyrosine kinases

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Abstract

The catalytic activity of Syk-family tyrosine kinases is regulated by a tandem Src homology 2 module (tSH2 module). In the autoinhibited state, this module adopts a conformation that stabilizes an inactive conformation of the kinase domain. The binding of the tSH2 module to phosphorylated immunoreceptor tyrosine-based activation motifs necessitates a conformational change, thereby relieving kinase inhibition and promoting activation. We determined the crystal structure of the isolated tSH2 module of Syk and find, in contrast to ZAP-70, that its conformation more closely resembles that of the peptide-bound state, rather than the autoinhibited state. Hydrogen–deuterium exchange by mass spectrometry, as well as molecular dynamics simulations, reveal that the dynamics of the tSH2 modules of Syk and ZAP-70 differ, with most of these differences occurring in the C-terminal SH2 domain. Our data suggest that the conformational landscapes of the tSH2 modules in Syk and ZAP-70 have been tuned differently, such that the autoinhibited conformation of the Syk tSH2 module is less stable. This feature of Syk likely contributes to its ability to more readily escape autoinhibition when compared to ZAP-70, consistent with tighter control of downstream signaling pathways in T cells.

KEYWORDS

conformational landscape, evolution, hydrogen–deuterium exchange mass spectrometry, protein dynamics, tyrosine kinase signaling

1 | INTRODUCTION

The adaptive immune system facilitates highly specific and robust responses to a wide variety of antigens. The

primary drivers of this complex system are lymphocytes, known as B and T cells.¹ Together, they work to mount responses to antigens, as well as to store memories of those antigens. B cells are primarily involved in humoral

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immunity through the production and secretion of antibodies, while T cells play various roles in cell-mediated immunity.^{1,2} This intricate system arose approximately 450 million years ago in a common ancestor of jawed vertebrates.^{3,4} Two genome-wide duplication events resulted in the acquisition of many paralogous genes with essential roles in B and T cell-mediated immunity.³ These genes encode important proteins found in signal-transduction pathways that are activated following the engagement of cell-surface antigen receptors, such as the B cell receptor (BCR) or T cell receptor (TCR). The regulation of these signaling proteins is critical for B and T cell-mediated immunity.

One such set of paralogous genes encodes the two Syk-family kinases, spleen tyrosine kinase (Syk) and zeta-chain-associated protein kinase 70 (ZAP-70).⁵ Syk and

ZAP-70 are critical triggers of early signaling events in B cells and T cells respectively. In B cells, following BCR engagement, the Src-family kinase Lyn phosphorylates the cytoplasmic tails of the BCR on tyrosine residues located in immunoreceptor tyrosine-based activation motifs (ITAMs) (Figure 1a, left).^{6,7} Syk is recruited to these phosphorylated ITAMs through its tandem Src homology 2 module (tSH2 module) and is activated by ITAM binding. In T cells, the Src family kinase Lck phosphorylates the ITAM-containing cytoplasmic tails associated with the TCR leading to recruitment and activation of ZAP-70 (Figure 1a, right).⁸ Syk differs from ZAP-70 in that it can directly phosphorylate ITAMs and also phosphorylates itself on the kinase activation loop, further increasing activity.^{9,10} While not required, the activity of Lyn has been shown to increase BCR sensitivity to

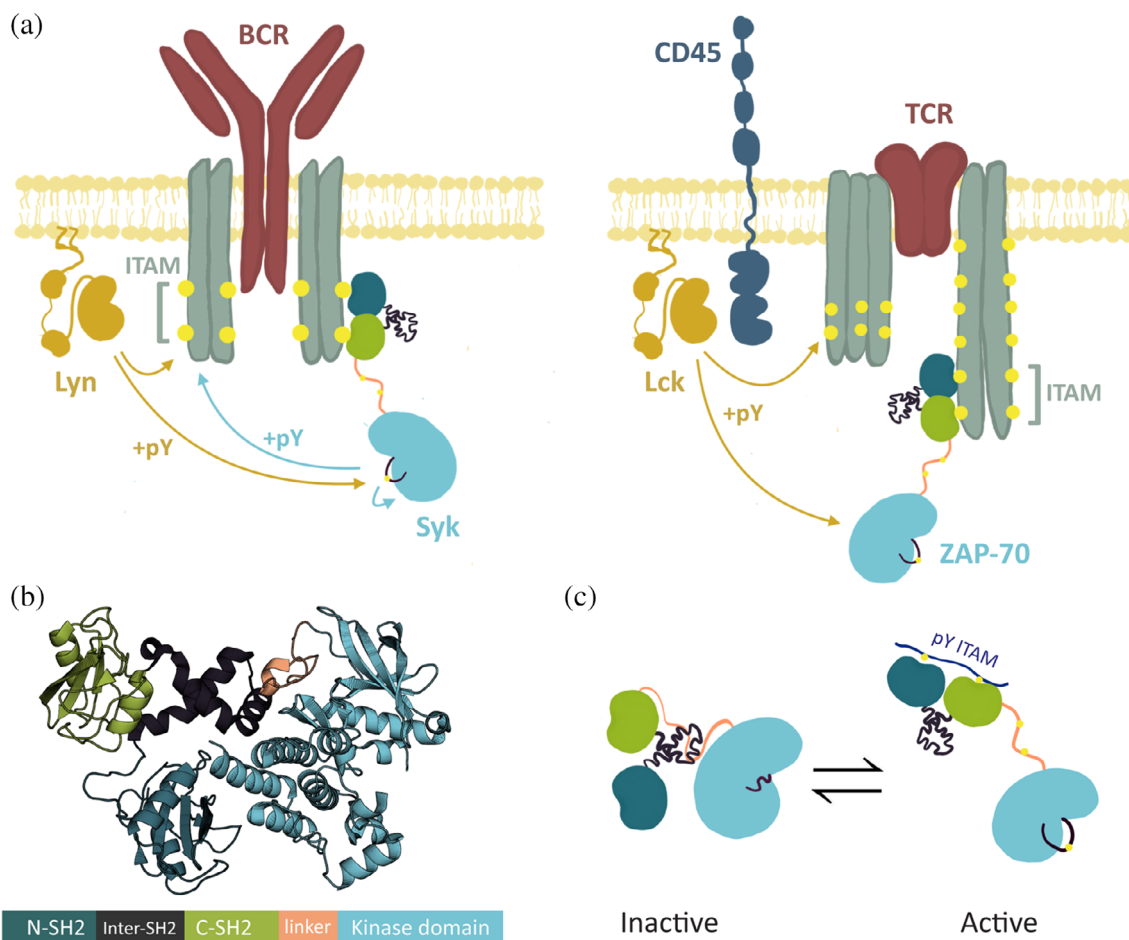


FIGURE 1 Structure and function of the Syk family kinases. (a) Early B cell signaling events, left. Early T cell signaling events, right. In B and T cells, a Src family kinase (yellow) phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) (phosphotyrosine denoted by yellow spheres) near the receptor. Binding of the tandem-Src-homology 2 module (tSH2 module) to phosphorylated ITAMs recruits the kinase to the receptor. Zeta-chain-associated protein kinase 70 (ZAP-70) signaling requires Lck, the Src family kinase, but Syk can initiate some signaling, even in the absence of Lyn, the B cell Src family kinase. (b) Domain architecture and autoinhibited structure of Syk family kinases (PDB: 4FL2, autoinhibited human Syk). (c) Activation of Syk family kinases by conformational change in the tSH2 module and phosphorylation. The tSH2 module binds to a doubly phosphorylated ITAM (blue) and phosphorylation of tSH2-kinase linker stabilizes the open, active conformation

monomeric antigens and to mediate more rapid signaling than what would occur from Syk activity alone.¹¹ In contrast, ZAP-70 requires a Src-family kinase for activation, reflecting stricter regulation of the activation of T cells. Additionally, it has been shown that the catalytic activity of Syk is higher than that of ZAP-70.¹²

Syk and ZAP-70 share a common domain architecture (Figure 1b), which includes two SH2 domains connected by a helical linker, comprising the tSH2 module. A flexible linker (SH2-kinase linker) connects the tSH2 module to the kinase domain. Like most protein kinases, Syk and ZAP-70 can switch from an autoinhibited conformation (“OFF state”) to a catalytically competent conformation (“ON state”).^{13–15} As an example, the OFF state for Syk is depicted in Figure 1b (PDB: 4FL2).¹³ In this conformation, the docking of the SH2-kinase linker on the face of the kinase domain distal to the active site impedes the inward rotation of the α C-helix.¹⁵ The rotation of the α C-helix is key to the conformational transition between the ON and OFF states and is coupled to the conformation of the activation loop at the active site cleft.¹⁶ In particular, the side chain of Tyr 352 in the SH2-kinase linker of human Syk (Tyr 319 in human ZAP-70) is wedged in the interface between the α C-helix and the N-lobe of the kinase domain, stabilizing the OFF state.^{14,15} This state is further stabilized by the tSH2 module, which adopts a conformation in which the relative orientation of the two SH2 domains is inconsistent with ITAM binding.^{17,18} ITAM binding triggers a conformational change toward the ON state, which is further enforced by phosphorylation of the SH2-kinase linker at Tyr 348 and Tyr 352 (Tyr 315 and Tyr 319 in ZAP-70) (Figure 1c).^{13,14} The crystal structure of the ITAM-bound tSH2 module has been reported for both Syk (PDB: 1A81) and ZAP-70 (PDB: 2OQ1).^{17,18} Overall, these structures are very similar. For ZAP-70, the crystal structure of the ITAM-free tSH2 module has also been reported (PDB: 1M61). In this structure, the C-terminal SH2 (C-SH2) domain is rotated substantially from its position in the ITAM-bound structure.¹⁹ This conformation is similar to that observed in autoinhibited ZAP-70.¹³

We now present the crystal structure of the isolated, ITAM-free tSH2 module of Syk, which has not been previously reported. Unlike ZAP-70, the Syk tSH2 module crystallizes in a conformation similar to that in the ITAM-bound structure and distinct from its conformation in the autoinhibited structure. To further understand how the tSH2 module differs between Syk and ZAP-70, we studied the dynamics of these proteins using hydrogen–deuterium exchange mass spectrometry (HDX-MS) and also ran all-atom molecular dynamics simulations. The resulting data are in agreement with previous studies that showed that the C-SH2 of ZAP-70 undergoes

a large conformational change upon binding of an ITAM and that it is dynamic in the absence of a bound peptide.^{20,21} Conversely, we find that the C-SH2 of Syk is relatively rigid, even in the unbound state, and does not undergo a similar conformational rearrangement upon binding a peptide. The bias of the Syk tSH2 module toward a conformation resembling that found in the ON state of the kinase, even in the absence of ITAM peptides, suggests that the autoinhibited state of Syk is less stable than that of ZAP-70.

2 | RESULTS

2.1 | Crystal structure of the ITAM-free Syk tSH2 module

We determined the structure of the ITAM-free Syk tSH2 module at 3.2 Å resolution by X-ray crystallography (deposited as PDB: 7SA7). The structure was solved by molecular replacement using Chain F from the ITAM-bound structure of Syk (PDB: 1A81).¹⁸ The crystal conditions, space group, and unit cell dimensions, described in Table S1, differ from those reported for the ITAM-bound structure.¹⁸ Additionally, while some of the crystal packing interfaces are similar to those of the ITAM-bound complex, not all of the interfaces are the same, suggesting that similarity in conformation is not due to crystal packing. As in the structure of the ITAM-bound Syk tSH2 module (PDB: 1A81), we observe a small degree of conformational heterogeneity in the six molecules in the asymmetric unit. When all six tSH2 modules are aligned on their N-SH2 domains, as in Figure 2a, the positions of the C-SH2 domains and the inter-SH2 linker are slightly variable. The most extreme differences between molecules in the asymmetric unit, represented by the darkest and lightest red structures in Figure 2a, results in a displacement of the C-SH2 of 2.7 Å.

We find that, unlike the ZAP-70 tSH2 module, the ITAM-free Syk tSH2 module adopts a conformation remarkably similar to that observed in the ITAM complex and distinct from the conformation observed in the autoinhibited kinase structure (Figure 2b,c). When the molecules from the ITAM-bound structure (PDB: 1A81) and the molecules from the ITAM-free structure are aligned on their N-SH2 domain, the displacement of the C-SH2 domain ranges between \sim 0.8 and 6.6 Å (value varies based on the molecules used in the alignment). Overall, the tSH2 modules in our ITAM-free structure represent a set of conformations in which the SH2 domains have moved apart, with respect to the ITAM-bound structure (Figure 2b). When an alignment on the C-SH2 domain is

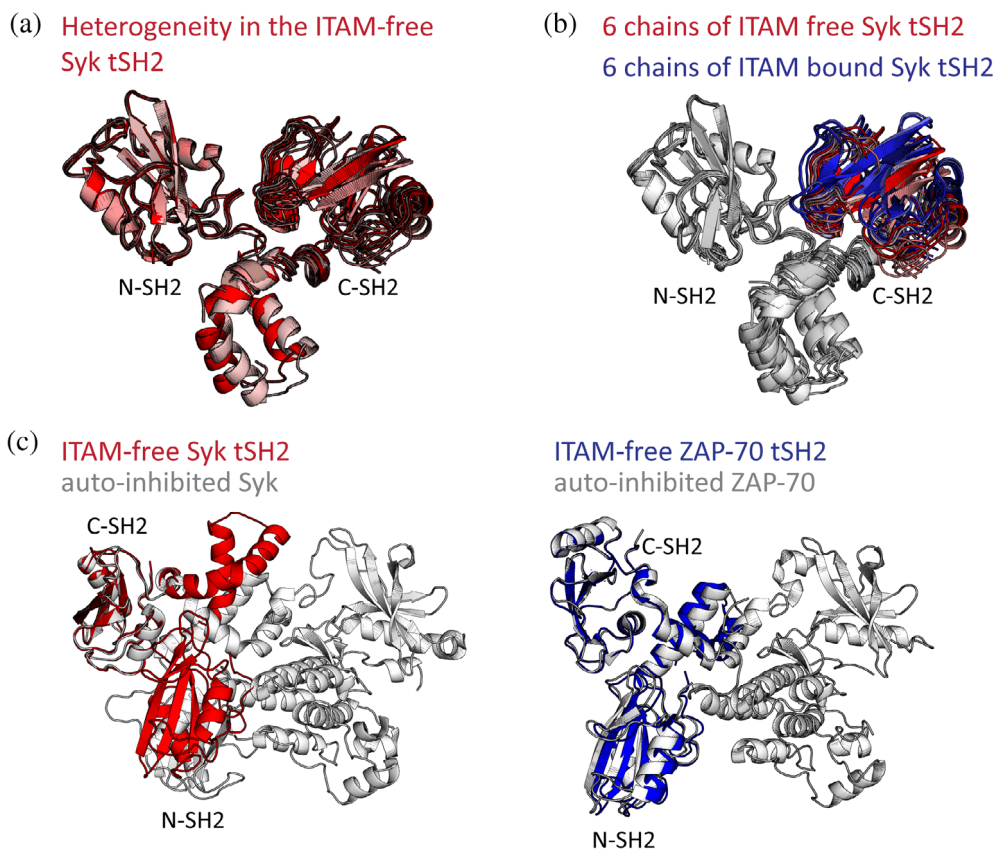


FIGURE 2 The crystal structure of the immunoreceptor tyrosine-based activation motif (ITAM)-free tandem-*Src*-homology 2 module (tSH2 module) is similar to that of the ITAM-bound Syk tSH2 module. (a) The six molecules of ITAM-free Syk tSH2 aligned on the N-SH2 domain, showing the observed conformational heterogeneity. (b) Alignment on N-SH2 of all molecules in the ITAM-bound structure (PDB: 1A81, blue) and all in the ITAM-free structure (red). Only the C-terminal SH2 (C-SH2) is colored. (c) Left, alignment on the C-SH2 domain of the isolated, ITAM-free Syk tSH2 module (red) to that in the full-length autoinhibited Syk structure (PDB: 4FL2, white), resulting in a ~ 36 Å displacement of the N-SH2 domain of the ITAM-free tSH2 module with respect to its position in the autoinhibited state. Right, the ITAM-free zeta-chain-associated protein kinase 70 (ZAP-70) tSH2 module (PDB: 1M61, blue) aligns well to the full-length autoinhibited structure of ZAP-70 (PDB: 4K2R, white), right

performed using the tSH2 module from the autoinhibited, full-length structure of Syk (PDB: 4FL2), the displacement for the C-SH2 in the ITAM-free structure is ~ 30.5 Å, reflecting a large change in the conformation. The structure of the ITAM-free Syk tSH2 module described here suggests that it favors the conformation corresponding to the ON state, unlike the tSH2 module of ZAP-70, which prefers the conformation of the OFF state.

2.2 | Analysis of the dynamics of the tSH2 modules by HDX-MS

To further interrogate the conformational ensembles of the Syk and ZAP-70 tSH2 modules we turned to HDX-MS. This technique monitors the time course of exchange of backbone amide hydrogens with heavier deuterons in the solvent. The ability of an amide to undergo exchange is directly related to its local

structure and stability.²² Briefly, in our experiment, a protein is diluted into deuterated buffer and allowed to undergo HDX. After various amounts of time, the exchange is quenched by lowering the pH and temperature. The quenched sample is then cleaved into peptides using acid proteases, and the extent of exchange for each peptide is monitored by MS by following the increase in molecular weight of each peptide (see Section 4). We carried out HDX-MS on both the Syk and ZAP-70 tSH2 modules in the ITAM-bound and ITAM-free states. A comparison of deuterium uptake for the same peptide in different states reports on changes in the secondary structure and/or stability. For each protein state, we analyzed approximately 300 individual peptides (average length of ~ 11 amino acids), corresponding to 100% sequence coverage for Syk with an average redundancy (number of peptides reporting on a residue) of 11.6 and 99% sequence coverage for ZAP-70 with an average redundancy of 12.5.

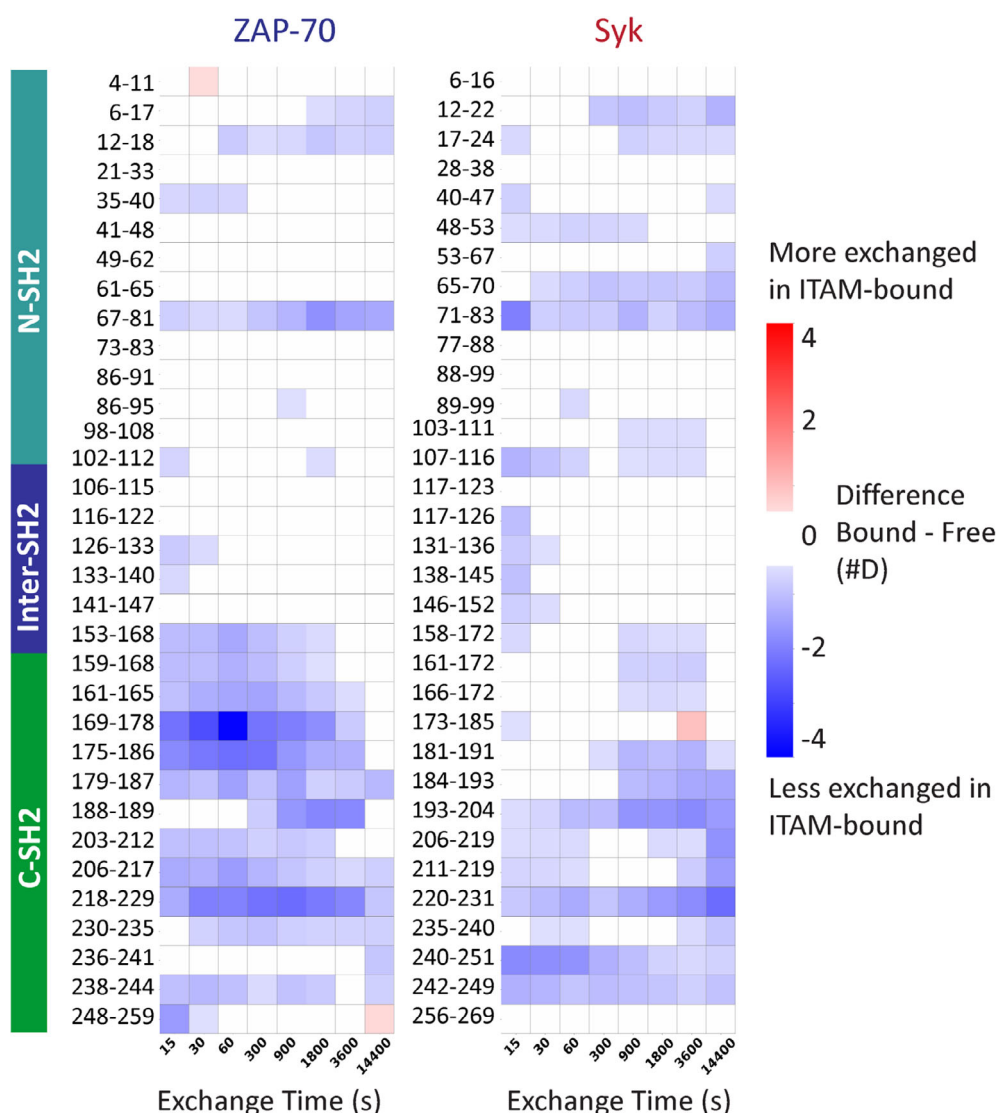
The effect of ITAM binding on the deuterium uptake is depicted for a select set of peptides as a difference heatmap in Figure 3; these peptides represent the typical uptake pattern observed for all peptides in each region. These data indicate that, as expected, the amide groups in both Syk and ZAP-70 are more protected from exchange when bound to an ITAM (blue on the heatmap in Figure 3), suggesting a ligand-induced global stabilization of the entire domain, as was previously observed for the Syk tSH2 module.²³ The extent and pattern of increased protection in Syk and ZAP-70 due to ITAM-binding differ most noticeably in regions in and around the C-SH2 domain, corresponding to residues 161–192 in Syk and 156–187 in ZAP-70 (Figure 4a). In Syk, the ITAM-free and ITAM-bound conformations show comparable exchange patterns in this region, with a possible hint of protection in the bound state. In ZAP-70, however, peptides from this region exchange rapidly in the absence of ITAM, and

binding of ITAM slows the exchange, similar to what is observed in both Syk states (Figure 4b). Together these data suggest that even in the absence of ITAM, the Syk tSH2 module adopts a conformation akin to what would be observed in the ON state of the kinase and distinct from the conformation observed in the OFF state. This tuning of the conformational landscape of Syk likely contributes to the instability of its autoinhibited state.

2.3 | Molecular dynamics simulation of the tSH2 modules of Syk and ZAP-70

We ran all-atom molecular dynamics simulations starting from the ITAM-bound structures to gain insight into the differences in dynamics between the tSH2 modules of Syk and ZAP-70. After removing the ITAM, we generated five trajectories for each protein, three extending for

FIGURE 3 The Syk and zeta-chain-associated protein kinase 70 (ZAP-70) tandem-*Src*-homology 2 modules (tSH2 modules) show different deuterium uptake in and around the C-terminal SH2 (C-SH2). Heat maps from representative peptides depicting differences in deuteration between the immunoreceptor tyrosine-based activation motif (ITAM)-bound and ITAM-free states of both tSH2 modules as measured by hydrogen–deuterium exchange mass spectrometry (HDX-MS) ($n = 3$). Red squares indicate increased deuterium exchange in the ITAM-bound state and blue squares indicate decreased deuterium uptake in the ITAM-bound state. A difference of at least ± 0.5 Da was considered significant, and white squares indicate no change in deuterium uptake



500 ns and two for 1 μ s. For both Syk and ZAP-70, over the course of simulation, the N- and C-SH2 domains relax so that they are positioned farther apart than observed in the starting structure, as measured by the distance between the centers of mass of each SH2 domain

(Figure 5). This movement occurs to a much greater extent in ZAP-70 than in Syk. Despite the significant separation of the SH2 domains in ZAP-70, the final structure achieved during simulation does not have the same rotation of the C-SH2 domain as observed in the ITAM-free

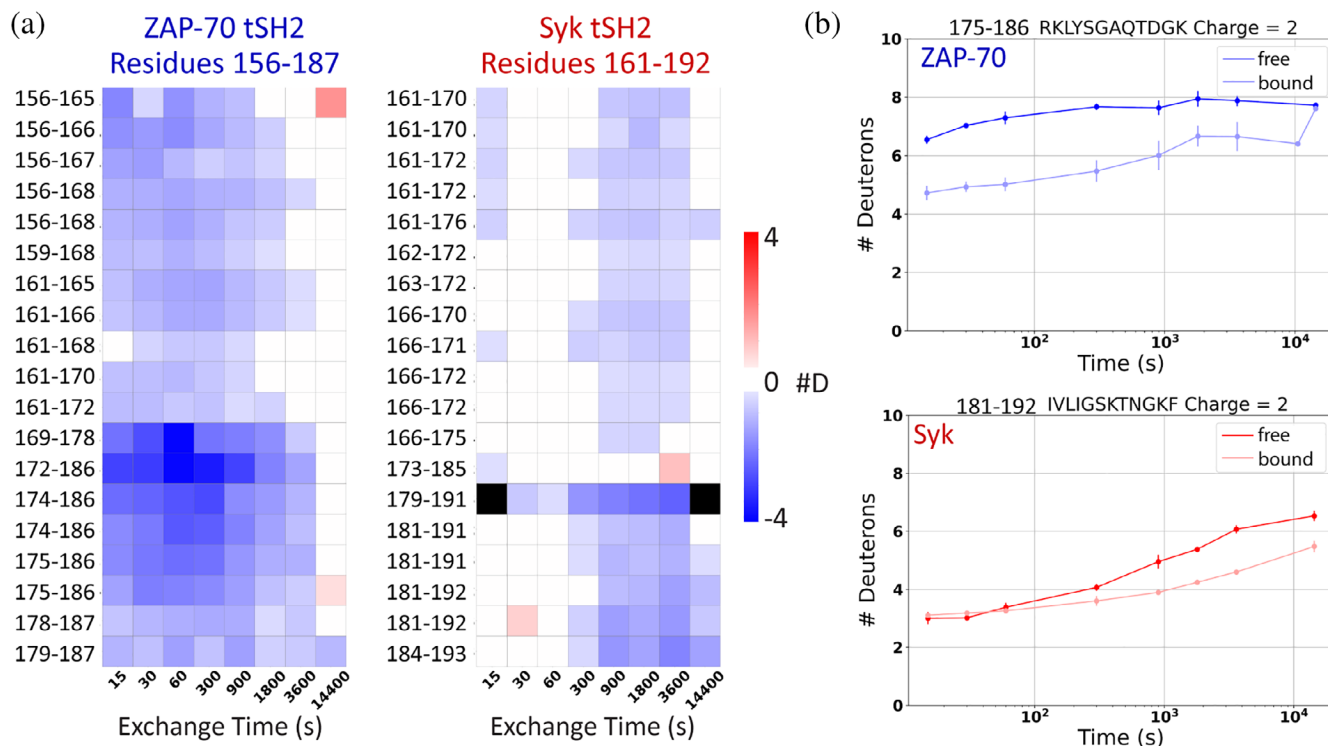


FIGURE 4 The more flexible C-terminal Src homology 2 (C-SH2) domain of zeta-chain-associated protein kinase 70 (ZAP-70) is rigidified by immunoreceptor tyrosine-based activation motif (ITAM) binding. (a) Deuteration difference heatmaps of all peptides, including different charge states, from the region spanning residues 161–192 in Syk (156–187 in ZAP-70). Peptides in ZAP-70 show a greater difference in deuteration between the ITAM-free and bound state. (b) Uptake plots from similar peptide in ZAP-70 and Syk. The peptides in Syk exchange similarly in the ITAM-free (dark blue for ZAP-70 and dark red for Syk) and bound states (light blue for ZAP-70 and light red for Syk). The corresponding peptides in ZAP-70 exchange similarly to Syk in the ITAM-bound state but are more flexible in the absence of peptide

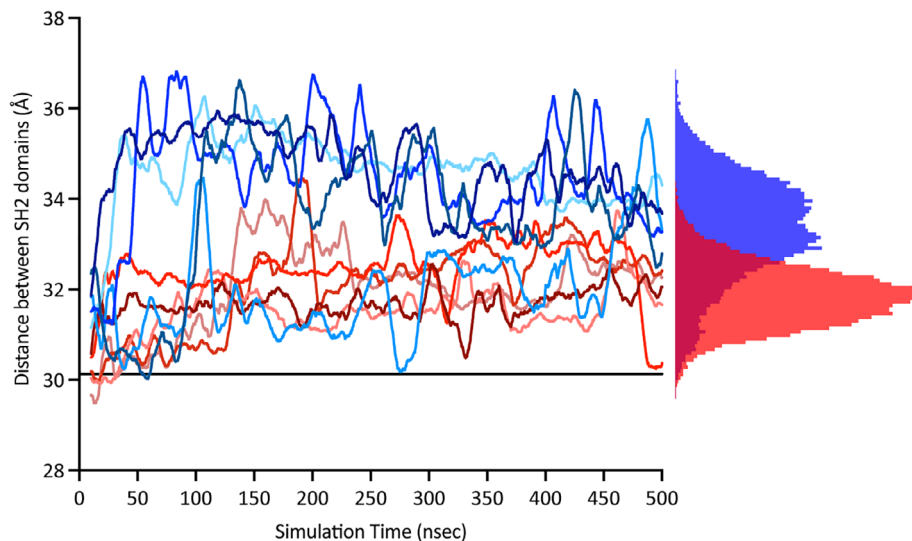


FIGURE 5 The Src-homology 2 (SH2) domains of the immunoreceptor tyrosine-based activation motif (ITAM)-free zeta-chain-associated protein kinase 70 (ZAP-70) move apart following the removal of the bound peptide. The distance between the center of mass of the N- and C-terminal SH2 (C-SH2) domains in Syk tandem-SH2 (tSH2) (red) and ZAP-70 tSH2 (blue). Simulations are smoothed over 10 ns. The histogram depicts the distribution of inter-SH2 distances sampled by either Syk (red) or ZAP-70 (blue)

ZAP-70 crystal structure, presumably because of the limited timescale of our simulations. In Syk, the two SH2 domains drift apart from their initial positions but still remain close together; ZAP-70 accesses a broader range of inter-SH2 distances than Syk (Figure 5). These data suggest that the tSH2 module of ZAP-70 adopts a conformation incompatible with peptide binding in the absence of ITAM.

2.4 | Sequence differences between the ZAP-70 and Syk tSH2 modules are conserved

We compiled a list of extant Syk-family kinase sequences through homology searches against databases of

metazoan proteins.²⁴ The organisms analyzed had zero, one, or two distinct Syk-family kinases (Figure 6a). Based on our analysis, the appearance of two Syk-family kinases in the genomes of modern organisms occurs at the transition from jawless fish to bony fish. This transition corresponds to one of the two genome-wide duplication events that correlate with the emergence of the adaptive immune system.³ In total, we compiled 183 sequences from 96 organisms, which were used to make a multiple sequence alignment (MSA).²⁵

In those metazoans with two Syk-family kinases, each protein could reasonably be assigned as a Syk or ZAP-70 ortholog based on percent identity to the corresponding human sequence. Notably, although this sequence analysis used the full-length protein sequences, the tSH2 module sequence alone can be used to predict whether the

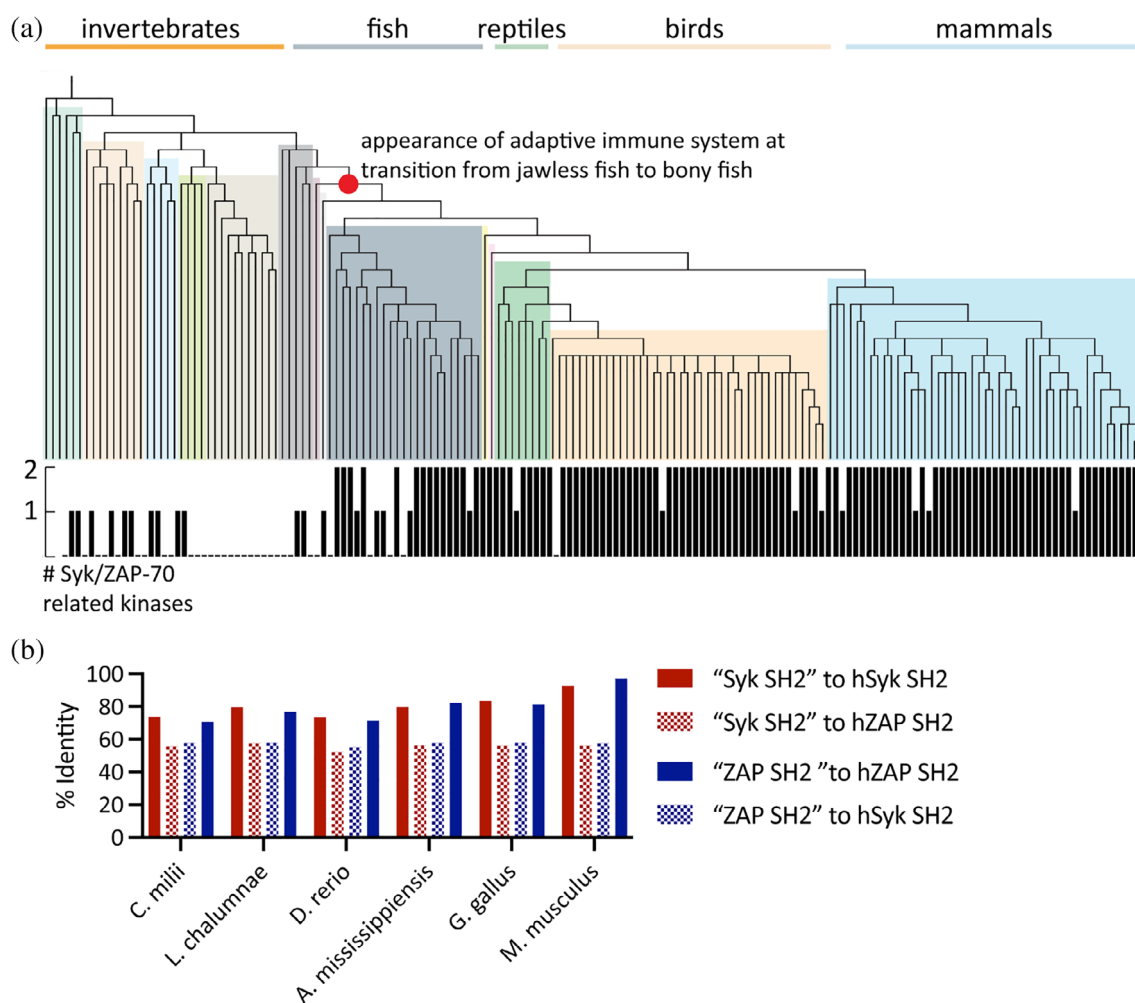


FIGURE 6 The differences between tandem-*Src*-homology 2 modules (tSH2 modules) are conserved in extant organisms. (a) Species tree generated based on the taxonomic relationships between organisms searched for Syk-family kinases.³⁰ The occurrence of Syk-family kinases in each organisms is denoted on the bar graph below the tree. The occurrence of two Syk-family kinases in the genome corresponds to the emergence of jawed vertebrates (denoted by red circle). (b) Alignment of only the tSH2 modules to either the human Syk or human zeta-chain-associated protein kinase 70 (ZAP-70) SH2 modules, from a representative organism from that order (common names: shark, lungfish, zebrafish, alligator, chicken, mouse)

gene corresponds to a Syk or a ZAP-70 (Figure 6b). From cartilaginous fish (*Callorhynchus milii*) to mice (*Mus musculus*), the tSH2 modules of Syk and ZAP-70 can be readily differentiated, with one aligning better to human Syk and the other to human ZAP-70. The tSH2 modules within a species remain approximately 55% identical to each other in all these organisms. These data suggest that observed differences between the regulatory tSH2 modules are the result of evolutionary selection and ancient specialization.

Using the MSA, the rate of sequence change at each position was calculated for both the Syk and ZAP-70 lineages. This rate is characterized by its Armon score; a lower score signifies a lower evolutionary rate, or a higher degree of conservation, at that position.²⁶ In Figure 7, the Armon score is plotted for positions at which the human Syk and ZAP-70 residues have different properties (e.g., lysine and arginine are not considered to be different). Overall, the ZAP-70 tSH2 module is more conserved than that of Syk, with many residues in ZAP-70 having a low Armon score. However, there are positions at which the variance in ZAP-70 is high and the corresponding position in Syk is conserved, such as residues 246, 247, and 249. There are also many sequence differences that are conserved in both lineages. Notably many of these are located in the C-SH2 domain, which we have identified as a region where the two proteins show significant differences in dynamics.

A set of residues that are conserved in the Syk lineage but not in the ZAP-70 lineage was noted while considering the MD simulations. The root-mean-square-fluctuation (Å) (RMSF) of each C α atom about its average

position in the tSH2 modules is shown in Figure S1. The RMSF values calculated for residues in ZAP-70 are higher than those in Syk in several regions of the C-SH2. This region corresponds to the ends of three β strands as well as the loops connecting those strands (Figure 8a). Throughout the simulations, two of these β strands (depicted in the lower portion of Figure 8b) frequently separate, and this separation is more sustained in the ZAP-70 simulations (Figures 8b and S2). In the corresponding region of Syk we noted the presence of several charged amino acids, that are not present in ZAP-70 (Figure 8a). These charged amino acids may allow for the formation of cross β strand salt bridges, which stabilize a more closed conformation of these strands and contribute to the attenuated dynamics of the C-SH2 domain of Syk compared to those of the same domain in ZAP-70. Notably, these charged amino acids are highly conserved in Syk, but not in ZAP-70 (Figure 8c), hinting at the specialization of not only the more globally conserved ZAP-70 but also Syk.

3 | DISCUSSION

We have determined the structure of the ITAM-free tSH2 module of Syk and found that its conformation differs from that of the ITAM-free tSH2 module of ZAP-70. The isolated ITAM-free Syk tSH2 module adopts a conformation like the ITAM-bound form, while the ZAP-70 tSH2 module adopts one similar to that seen in the structure of the full-length, autoinhibited kinase. Previously reported NMR residual dipolar coupling data for the ITAM-free

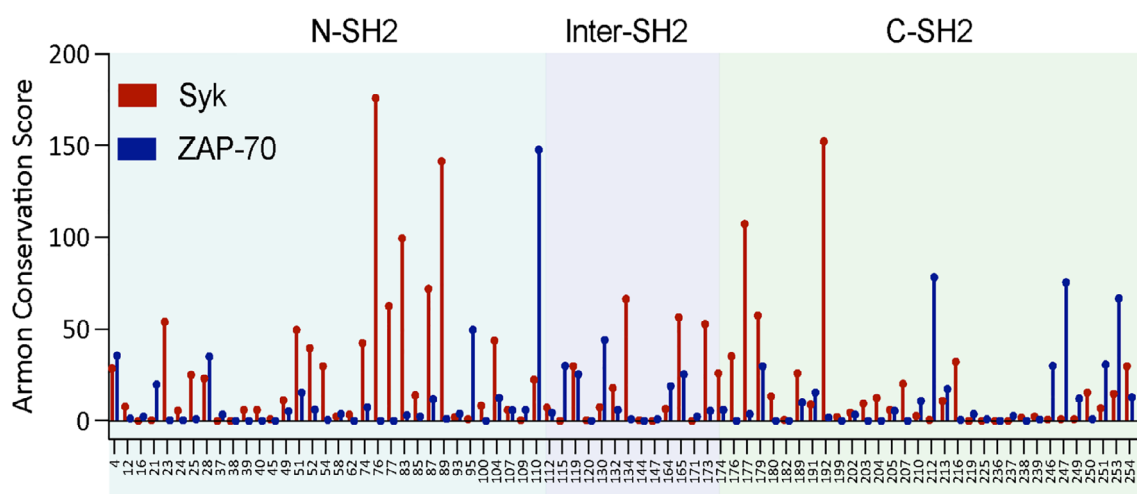


FIGURE 7 Sequence differences are conserved in both the Syk and zeta-chain-associated protein kinase 70 (ZAP-70) lineage. The Armon conservation score at each position (Syk numbering) where the human Syk (red) and ZAP-70 (blue) sequence differ significantly. Overall, ZAP-70 is more conserved than Syk, but there are positions which are conserved in only the Syk lineage or equally conserved in both lineages

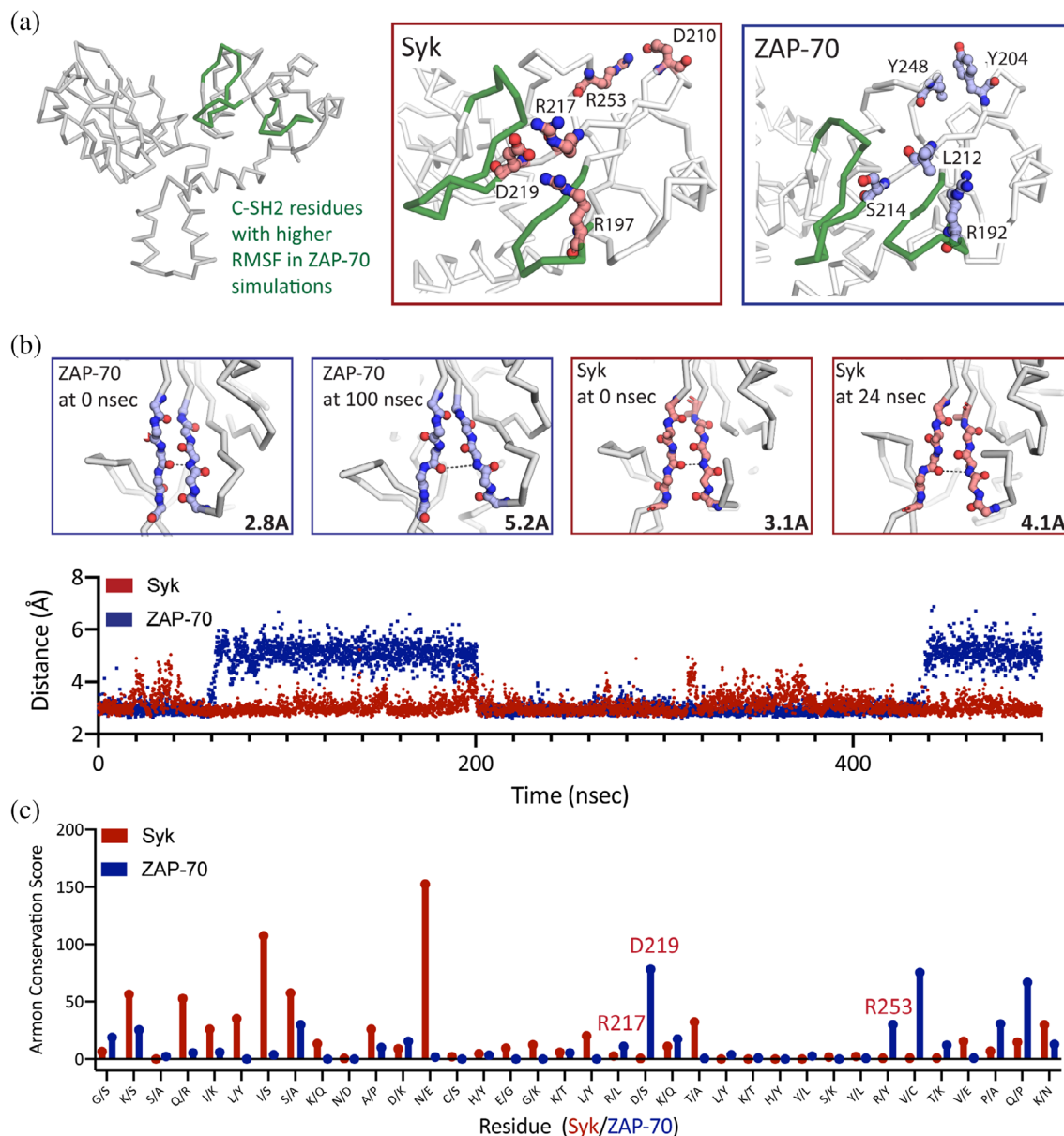


FIGURE 8 Charged residues conserved in Syk but not zeta-chain-associated protein kinase 70 (ZAP-70) may contribute to increased stability of the C-terminal Src-homology 2 (C-SH2). (a) The residues in ZAP-70 with higher root-mean-square-fluctuation (RMSF) values than the corresponding regions of Syk are colored in green. Boxes highlight the C-SH2 domains of Syk (left) and ZAP-70 (right). In the Syk C-SH2, charged residues (red sticks) may be involved in stabilizing the fluctuations in this domain. D219 can interact with R217 and R197, forming cross β -strand interactions. These residues are not conserved in ZAP-70 (blue sticks). In Syk, R253 and D210 are positioned to interact. (b) Snapshots of the β strands discussed in (a) at different points in the simulation. In both Syk and ZAP-70, these β -strands peel apart. The distance between two of the β -strand residues is noted in the corner of each box and is depicted by the dashed line. The lower panel shows this distance throughout one of the simulations (plots of other simulations can be found in Supplemental Figure S2). The β -strands in ZAP-70 move apart to a greater extent and this separation is sustained for longer than in Syk. (c) All positions in the C-SH2 domain in which human ZAP-70 and human Syk differ are noted along the x-axis. Residues discussed in (a and b) are conserved in the Syk lineage but are not completely conserved in the ZAP-70 lineage, pointing to a potential constraint on this region during the evolution of Syk

Syk tSH2 module have shown that the SH2 domain-orientation observed in solution is similar to the orientation observed in the ITAM-bound state.²⁷ This observation, as well as our HDX-MS data, indicate that our crystal structure reflects the solution structure of the

Syk tSH2 module. Additionally, HDX-MS has previously been used to probe full-length ZAP-70 and the isolated tSH2 module in various states representing steps of the activation pathway.²¹ Our HDX-MS data for the tSH2 module corresponds well to theirs; in particular, our

conclusion that ZAP-70 is tuned toward an OFF state is consistent with the earlier work.

It has been established that although the Syk and ZAP-70 tSH2 modules bind to most ITAMs with comparable affinities, the ZAP-70 tSH2 module binds cooperatively while the Syk tSH2 module does not.^{20,28} Recently, Gangopadhyay et al. proposed a model in which the C-SH2 domain of ZAP-70 binds the phosphorylated ITAM first with a low affinity, thus allowing the binding pocket of the N-SH2 to be fully formed.²⁰ Binding of the second phosphorylated tyrosine in this pocket remodels the dynamics of the C-SH2 domain binding pocket, resulting in a higher affinity for the phosphorylated ITAM. Our data are consistent with the C-SH2 of ZAP-70 being more flexible in the ITAM-free state, with binding of a peptide rigidifying this domain. We find that the same is not true for Syk, as its C-SH2 domain is comparatively rigid even in the ITAM-free state. This is consistent with earlier work showing that the dynamics of the individual SH2 domains do not significantly differ between the ITAM-bound and ITAM-free states and it is the flexibility of the inter-SH2 linker that influences binding affinity.^{29,30} We identified several, conserved polar residues in the C-SH2 of Syk that may contribute to its stability in the absence of a bound phosphotyrosine. However, additional studies are needed to elucidate how sequence contributes to the differences in conformation reported here.

Previous studies of the catalytic activity of the Tec family kinases, another kinase family involved in adaptive immune system signaling, as well as the Syk family kinases, have suggested that T cell signaling proteins are subject to stricter regulation than their B cell counterparts.^{12,31} We propose that the regulatory domains of the Syk family kinases may also contribute to this proposed difference in regulation. Our data support a model in which the tSH2 module of Syk is less stable in its autoinhibited conformation, while the opposite is true for the tSH2 module of ZAP-70, which prefers the conformation observed in the autoinhibited state. The additional conformational change in the tSH2 module of ZAP-70 likely provides another energetic barrier preventing spurious activation of ZAP-70 and thereby contributes to strict regulation over the activation of T cells. Other modes of regulation governing B cell activation, such as the involvement of helper T cells, may allow for Syk to be relatively less regulated, and could be important to its roles in B cells and other signal transduction pathways.

4 | MATERIALS AND METHODS

4.1 | Protein constructs and purifications

Both the Syk and ZAP-70 tSH2 modules were expressed and purified as described in Visperas et al.³²

4.2 | Sequence curation and alignment

The ZAP-70 and Syk sequences were compiled through a series of BLAST searches, partially described in Shah et al.³³ In total, there are 184 sequences, 87 corresponding to ZAP-70, 89 to Syk, and 8 originating from hagfish and lamprey. Assignment as a Syk or ZAP-70 was determined based on highest homology to human Syk or ZAP-70. The sequences were aligned using the T-coffee sequence alignment software.²⁵

4.3 | ITAM-free Syk crystallization, data collection, and structure determination

The Syk tSH2 was purified as described and then buffer exchanged via dialysis to a crystallography buffer containing 10 mM HEPES pH 7.0, 50 mM NaCl, 5% glycerol, and 1 mM TCEP. The dialyzed protein was immediately concentrated to 35 mg/ml. Then, 1 μ l of the concentrated protein was mixed with 1 μ l of well solution (0.2 M sodium nitrate and 10% wt/vol PEG 3350) and equilibrated in a hanging drop set-up \sim 20°C. Crystals grew over the course of 2 weeks after which they were harvested. The crystals were soaked in a cryoprotectant containing 0.2 M sodium nitrate, 20% PEG 3350, and 20% glycerol and flash cooled in liquid nitrogen. The data were collected at the Advanced Light Source (Lawrence Berkeley National Laboratory) on beamline ALS 8.2.2. Detailed data collection and structure determination procedures can be found in the Supporting Information.

4.4 | Hydrogen–deuterium exchange mass spectrometry

Deuterated buffer was prepared by three rounds of lyophilization of buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM TCEP, and 5% glycerol) followed by resuspension in D₂O. ITAM-free experiments were carried out by diluting 30 μ M tSH2 module 10-fold into the deuterated buffer. At the following timepoints (15 s, 30 s, 1 min, 5 min, 15 min, 30 min, 1 hr, and 4 hr) 80 μ l of the exchange protein was removed and quenched in a 5x quench buffer containing 4.8 M Gdn-Cl, 12% glycerol, and 2% formic acid. Quenched samples were immediately flash frozen in liquid nitrogen and stored at -80° C. For the ITAM-bound experiments, lyophilized peptide with the sequence corresponding to ITAM 1 of the T cell antigen receptor ζ -chain (CGNQL(pY)NELNLGRREE(pY)DVLD, where pY is phosphotyrosine (prepared by David King, UC Berkeley/Howard Hughes Medical Institute) was resuspended in the same pH 7 buffer to a final concentration of 2.9 mM. The concentrated peptide was

mixed with 30 μM protein to a final concentration of 260 μM , well above the reported K_d of 80–100 nM.²⁸ This sample was allowed to equilibrate for 4 hr at room temperature. The equilibrated sample was then diluted 10x into deuterated buffer and timepoints collected and stored in the same way as the ITAM-free samples. Detailed descriptions of the LC-MS methods can be found in the Supporting Information as well as the source data for all peptides analyzed.

4.5 | MD simulations

For the MD simulations, we began with the ITAM-bound tSH2 structures of Syk (PDB: 1A81 Chain A) and ZAP-70 (PDB: 2OQ1). To prepare these structures for simulation, we edited the PDB files to remove the ITAMs as well as revert heavy atoms to their wild-type identity. We used the LEaP program in AMBER to solvate the proteins and add the counter ions (Na^+ or Cl^-) required to bring the net charge of the system to zero. For ZAP-70, the system size was 89,564 atoms and 82,514 atoms for Syk. For all simulations, we used the AMBER ff14SB for protein atoms and the TIP3P for water and ion atoms.

All simulations were performed using the AMBER18 software and the Compute Unified Device Architecture version of Particle-Mesh Ewald Molecular Dynamics on graphical processing units. The system energy was minimized in three rounds using the steepest-descent method and then heated to 300 K. Heating was followed by 200 ps of constant number/pressure/temperature equilibration. Then, 500 or 1,000 ns unconstrained simulations were started after energy minimization and equilibration, with coordinates saved every 2 ps. Replicate simulations were based on the same minimization model, but new equilibrations were performed in order to generate unique initial atom velocities. Additional methods in the Supporting Information.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Helen T. Hobbs: Conceptualization (equal); data curation (equal); investigation (lead); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Neel H. Shah:** Investigation (supporting); visualization (supporting); writing – review and editing (supporting). **Jean M. Badroos:** Investigation (supporting); writing – review and editing (supporting). **Christine L. Gee:** Data curation (supporting); validation (equal); visualization (supporting); writing – review and editing (supporting). **Susan Marqusee:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal). **John Kuriyan:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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