An evolutionary divergent thermodynamic brake in ZAP-70 fine-tunes the kinetic proofreading in T cell

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Abstract

T cell signaling starts with assembling several tyrosine kinases and adaptor proteins to the T cell receptor (TCR), following the antigen binding. The lifetime of the TCR: antigen complex and the time delay between the recruitment and activation of each kinase determines the T cell response. The mechanism by which the time delays are implemented in TCR signaling is not fully understood. Combining experiments and kinetic modeling, we here report a thermodynamic-brake in the regulatory module of ZAP-70, which determines the ligand selectivity, and may delay the ZAP-70 activation in TCR. Phylogenetic analysis revealed that the evolution of the thermodynamic-brake coincides with the divergence of the adaptive immune system to the cell-mediated and humoral responses. Paralogous kinase Syk expressed in B cells, does not possess such a functional thermodynamic brake, which may explain higher basal activation and lack of ligand selectivity by Syk.

Introduction

The activation and quiescence in the cell-mediated immune response by T cell is regulated by a kinetic proofreading mechanism¹⁻³. According to this mechanism, a time delay separates the binding of an antigen to the T cell receptor (TCR) from the subsequent downstream signaling^{4,6}. TCR lacks intrinsic catalytic activity, and the downstream signaling starts with the recruitment of multiple kinases and adaptor proteins to the complex (Figure S1a)⁷⁻⁹. Each recruitment step introduces a delay between the ligand binding and activation of the enzyme. The nonspecific

interaction between self-antigen and TCR is short-lived and does not signal because the antigen: TCR complex dismantles before the activation of the downstream kinases. The interactions arising from foreign antigens are long-lived and get enough time to signal by activating the kinases. Paralogous kinases mediate the early events in B cell receptor (BCR) signaling of the humoral immune response employ conceptually similar mechanisms¹⁰. Nevertheless, the mechanism of differential activation of early T cell signaling compared to B cell remains unclear^{11,12}.

The Syk family of non-receptor tyrosine kinases, ZAP-70 and Syk, are indispensable in the early stage of TCR and BCR signaling, respectively^{10,13}. Both the kinases are activated by recruiting to the membrane following antigen binding (Figure S1a). The dwell time of the kinases at the membrane determines their response¹⁴. ZAP-70 and Syk, both shares a modular structure composed of an N-terminal regulatory module and a C-terminal kinase domain (Figure 1a)^{10,13}. The regulatory module is made up of tandem Src homology 2 (tSH2) domains connected by a helical linker called interdomain A (Figure 1a, c). In the inactive state (apo-state), the two SH2 domains adopt an ‘L’ like open conformation making them incompatible to ligand binding (Figure 1c)^{15,16}. The Syk kinases are activated by binding to the doubly-phosphorylated immunoreceptor tyrosine-based activation (ITAM-Y2P) motifs at the TCR or BCR, respectively, through the tSH2 domain (Figure 1b, S1a)^{17,18}. The tSH2 domain adopts a closed conformation upon binding to ITAM-Y2P, releasing the autoinhibitory interactions leading to the activation of the kinase domain (Figure 1c, S1a)^{19,20}. The active conformation of ZAP-70 is stabilised by phosphorylating two key residues Y315 and Y319, by Lck, a Src family kinase, recruited to the antigen: TCR complex²¹⁻²³.

Several lines of evidence suggest that the ZAP-70 and Syk behave differently in the cell. Notably, ZAP-70 does not display basal activation, whereas Syk mediated basal signaling is essential for cell survival²⁴⁻²⁹. A ligand-independent closed conformation of the Syk tSH2 domain is proposed to facilitate high basal activation^{30,31}. Despite the high sequence homology with Syk, it is not understood why the tSH2 domain of ZAP-70 could not adopt a stable closed conformation in the *apo*-state. Moreover, ZAP-70 displays a delayed Ca²⁺ response upon activation compared to Syk¹². The tSH2 domain of ZAP-70 binds in a biphasic manner with a high degree of selectivity to a conserved ITAM-Y2P sequence, compared to hyperbolic binding in Syk³²⁻³⁵. The functional significance of biphasic ligand binding for T cell signaling remains unclear.

We present a kinetic model from a comparative study of the tSH2 domain of ZAP-70 and Syk, that explains the differential ligand-binding. We observed that the tSH2 domain of ZAP-70 binds to ITAM-Y2P in two-step kinetics, fast and slow, compared to one-step binding in Syk. The slow binding to the ZAP-70 tSH2 domain arises from a thermodynamic penalty (brake) that determines the ligand-selectivity and biases the conformational equilibrium of the *apo*-tSH2 domain towards the open conformation. Conversely, such thermodynamic break is non-functional in Syk tSH2-domain. The emergence of the thermodynamic brake coincides with the evolution of the BCR-TCR-MHC like immune system at the divergence of jawless and jawed fish approximately 500 million years ago³⁶.

Results and Discussion

The tSH2 domain of ZAP-70 is sensitive to the subtle changes in the ITAM peptide sequence

The ZAP-70 tSH2 domain binding to the doubly-phosphorylated ITAM-ζ1 peptide (ITAM-Y2P-

90 $\zeta 1$) produce a biphasic curve with three distinct dissociation constants, K_{d1} , K_{d2} , and K_{d1}^* (Figure
 91 1b-d) ³⁵. First, the N-terminal phosphotyrosine residue from ITAM-Y2P binds uncooperatively to
 92 the C-SH2 phosphate-binding pocket (PBP) with a low nanomolar affinity (K_{d1}) to form an
 93 encounter complex (Figure 1c-e). The formation of the tSH2: ITAM-Y2P encounter complex
 94 allows the assembly of the N-SH2 PBP. Subsequently, C-terminal phosphotyrosine residue from
 95 ITAM-Y2P binds weakly to the newly formed PBP with micromolar affinity (K_{d2}). In the steady-
 96 state, the two binding events are interlinked by a plateau (Figure 1d). The second binding event
 97 remodels the C-SH2 PBP to an intermediate binding pocket (K_{d1}^*) producing a *hill-coefficient* of
 98 3.4 ± 0.36 .

99 It was reported previously, the tSH2-domain of ZAP-70 displays hierarchical preference
 100 in binding to different ITAM sequences^{17,33,37-39}. We begin by asking which part of the biphasic
 101 binding isotherm, in the steady-state, is sensitive to the ITAM peptide sequence (Figure 1b). We
 102 comparatively studied the binding of ITAM-Y2P- $\zeta 3$ to the tSH2 domain by intrinsic tryptophan
 103 fluorescence spectroscopy and isothermal titration calorimetry (ITC) (Figures 1b, d-e, S1b-f). We
 104 probed K_{d1} , and K_{d1}^* by fluorescence spectroscopy, and K_{d2} and K_{d1}^* by ITC. To probe the K_{d2} ,
 105 we used ^{tSH2}R190A mutant that impairs phosphotyrosine binding to C-SH2 PBP (Figures 1e, S1f).

106 We overserved that ITAM-Y2P- $\zeta 3$ binds weakly to the ZAP-70 tSH2 domain, compared
 107 to ITAM-Y2P- $\zeta 1$, consistent with the previous reports^{17,40} (Figure 1e). Our data revealed that the
 108 C-SH2 domain does not distinguish between the two ITAM-Y2P while forming the encounter
 109 complex. Both the peptides, ITAM-Y2P- $\zeta 1$ and ITAM-Y2P- $\zeta 3$, bind to the C-SH2 domain with
 110 low nanomolar affinity (K_{d1}) of 3.3 ± 0.5 nM and 6.8 ± 1.5 nM, respectively. However, we noted
 111 a significant increase in the plateau width (Figure 1d). The ITAM-Y2P- $\zeta 3$ binding perturbed the
 112 K_{d2} , and K_{d1}^* contributing to the overall increase in the dissociation constant (Figure 1e). We ask

why the plateau-width in the steady-state binding (Figure 1d) is sensitive to the subtle changes in ligand type?

A multistep ligand-receptor model explains the binding kinetics of ITAM-Y2P to ZAP-70

We developed a multistep mathematical-kinetic model to explain the biphasic bindings of ITAM-Y2P and tSH2 domains. This model comprised of different tSH2 domain conformations, open and closed, connected by a complex network (Figure 2a). The tSH2-*apo* state (open) ultimately reaches the tSH2-*holo* state (closed) through different pathways associated with distinct rates. The receptor adopts an *apo*-state, partially bound state (either C-SH2 or N-SH2 PBP are occupied), or *holo*-state, depending on the ligand concentration or type. A single binding event of one phosphotyrosine residue to either of the two PBP was modeled as first-order kinetics, meaning, the corresponding forward binding rate is proportional to the ligand concentration. We assumed, the transition between the open and closed conformations is ligand-independent (Figure 2a), and the formation of the encounter complex is the fastest ($K_{d1} = \frac{k_b}{k_f} = 3 - 7 \text{ nM}$; Figure 1e). To explain the biphasic binding (Figure 1d), we further assumed that the transitions to the *holo*-state from the partially bound states exhibit negative cooperativity. These steps represent kinetic penalties⁴¹, and occur with much slower rates ($w_1 k_f$ and $w_2 k_f$, with $0 < w_i < 1$, $i = 1$ or 2) with dissociation constants $K_{d2} (= \frac{k'_b}{w_1 k_f})$ and $K_{d1}^* (= \frac{k'_b}{w_2 k_f})$, respectively. We anticipate, the variation in K_{d1} , K_{d2} , or K_{d1}^* determine the steady-state response.

We derived Ordinary Differential Equations (ODEs) for concentrations of chemical species using the law of mass-action (see Eq. S1). The ODEs were solved numerically to calculate the

ligand bound fraction (defined in Eq. S2), and to predict the steady-state response and the kinetic behavior. In the steady-state, the K_{d1} determines the receptor sensitivity (initial rising slopes of the bound fractions) in low ligand concentration regime (nM level) and also modulates the plateau width (Figure 2b). The kinetic behavior mostly showed a single exponential decay except in a narrow intermediate range of K_{d1} (around 8nM – 20nM), where a two-step decay was observed (Figure 2c and 2d).

Next, we assessed the effect of K_{d2} (Figure 1c) variation by changing the penalty factor, w_1 (Figure 2a). The K_{d2} variation modulated the ligand selectivity by altering the plateau width in the steady-state (Figure 2e). With a higher penalty (equivalently lower w_1 or higher K_{d2}), the plateau width became broader and displayed two-step kinetics with a sharp initial decay and a slow subsequent decrease of the unbound fraction. (Figure 2f-g).

The variation of K_{d1}^* marginally alters the plateau width in the steady-state (Figure S2). Therefore, the slowest binding step to the N-SH2 PBP (K_{d2}) mainly controls the plateau width. This conclusion correlates with the observed K_{d2} , which is orders of magnitude lower than the K_{d1} and K_{d1}^* (Figure 1e), suggesting the corresponding step may impart a significant penalty in the dynamic binding of the ligand. However, measurement of free energy change is necessary to determine if a thermodynamic cost manifests as a kinetic penalty, as elucidated in the model.

Finally, for a particular set of parameter choices, the model prediction reasonably agreed with the experimental data of ITAM-Y2P- ζ 1 and ITAM-Y2P- ζ 3 bindings to the tSH2 domain (Figure 2h). In the model, reported values of K_{d1} , K_{d2} , and K_{d1}^* (Figure 1e) were used for the quantitative matching, but other parameters were unknown and chosen arbitrarily to fit the data (Table S5).

The tSH2 domain of ZAP-70 binds ITAM-Y2P in two kinetic steps

We next probed the binding kinetics of ITAM-Y2P- ζ 1 or ITAM-Y2P- ζ 3 to the tSH2 domain by stop-flow fluorescence spectroscopy. We started with mixing excess ITAM-Y2P- ζ 1 to the tSH2 domains at 10°C and measured the change in tryptophan fluorescence intensity for 200 seconds (Figure 3a). We observed that the fluorescence intensity decay in two steps, fast (<200 ms) and slow (>20 s) (Figure 3a). Hence, we recorded all the kinetic experiments at two-time scales. All kinetic data were first normalized against the highest intensity and then subtracted by the blank (protein only sample) (Figure 3a-b) and fitted to a one-site association kinetics (Table 1).

We observed that the ITAM-Y2P- ζ 1 binds to the tSH2 domain with two observed rates of $k_{obs}^{fast} = 23.87 \pm 1.01 \text{ s}^{-1}$, and $k_{obs}^{slow} = 0.262 \pm 0.098 \text{ s}^{-1}$ (Figure 3a-b, Table 1). Our mathematical model suggests that the fast-binding kinetic may arise (Figure 2d, 2g) during the formation of the encounter complex (K_{d1}). To test that, we turn to two samples, one ^{tSH2}R39A mutant that has an impaired N-SH2 PBP and second, a single phosphotyrosine ITAM- ζ 1 peptide (ITAM-YP- ζ 1) that will show only one binding event (Figure 3c). As expected, the steady-state fluorescence titration of ^{tSH2}R39A to ITAM-Y2P- ζ 1 and tSH2 domain to ITAM-YP- ζ 1 showed that the first binding step is preserved with (K_{d1}) of $8 \pm 1.05 \text{ nM}$ and $3.7 \pm 0.1 \text{ nM}$, respectively, and no subsequent binding was observed (Figures 3c, S3a-b). In the kinetics experiment, both the samples showed a k_{obs}^{fast} of $24.82 \pm 2.29 \text{ s}^{-1}$ and $28.60 \pm 5.84 \text{ s}^{-1}$, respectively, with no detectable slow binding (Figure 3d-e).

To check if our proposed model (Figure 2a) could explain the above data (Figure 3c-e), we introduced a modification in the model (Figure 3f). The N-SH2 binding is almost absent in both cases, and the partially bound state does not transform to the final *holo*-state. Hence, we ignore all

pathways except the first encounter complex and the subsequent conformational change (Figure 3f). This assumption was sufficient for a quantitative matching between the model prediction and the experimental data in the steady-state (Figure 3g). The corresponding kinetic behaviour also showed a single exponential decay as observed in our experiment (Figure 3h).

Our model suggests that the tSH2 domain forms the encounter complex (K_{d1}) with a fast-kinetic step, whereas the phosphate binding to the N-SH2 PBP (K_{d2}) is the rate-limiting step and may determine the plateau width (Figure 2e-f). To validate, we measured the binding kinetics of the tSH2 domains and ITAM-Y2P- ζ 3 (Figure 1d). Indeed, the ITAM-Y2P- ζ 3 binds slower ($k_{obs}^{low} = 0.012 \pm 0.005 \text{ s}^{-1}$) to the N-SH2 PBP compared to ITAM-Y2P- ζ 1, with no significant change in the fast-kinetic step (Figure 2h, 3i-j, Table 1).

Since the K_{d2} seems to be the rate-limiting step, a reduced version of the full model (Figure 2a) is sufficient to capture essential features of the two-step kinetics. This reduced model has three basic steps: (i) a fast encounter to C-SH2 pocket (ii) followed by slower conformational changes of intermediates (open $\leftrightarrow$ closed), and (iii) subsequent transition of intermediates to the final *holo*-state by imposing a kinetic penalty (Figure 3k). The minimal model was solved mathematically, and it produced qualitatively the same results as the full model (see Equations S4-S5, Figure S3 c-f). However, incorporating the finer details in the full model was necessary for quantitative matching with the data. We now ask, what determines the nature of the slow-kinetic step?

The nature of the slow-kinetic step is determined by the structural coupling between the two SH2 domains

The ‘open to closed’ structural transitions of ZAP-70 tSH2 domain upon ligand binding requires cooperative interaction between the ITAM peptide, and the allosteric network residues in the tSH2 domain (Figure 1c)^{17,35,42}. Analyzing the crystal structure of ZAP-70 tSH2 domain in complex with ITAM-Y2P- ζ 1¹⁹, we identified a salt-bridge between the ^{ITAM- ζ 1}E13 and ^{tSH2}K245 residue that may be critical for the structural coupling between the N- and C- SH2 domain during ligand binding (Figure 4a). The corresponding residue in ITAM- ζ 3 is an aspartic acid, which may increase the distance between the ion-pair in the salt-bridge. This may reduce the transition rate to the final *holo*-state (k_{obs}^{slow}), and increase the plateau width in the steady-state (Figure 1b, d, 3i). To test the role of the salt-bridge in determining the plateau width, we modulate the strength of the salt-bridge by changing the pH or by ^{ITAM- ζ 1}E13A mutation (Figure 4a-e, S4d, 1b). We observed that lowering the pH to 6.5 changes the surface potential of the tSH2 domain (Figure S4d) and marginally increases the plateau width, suggesting that the ^{ITAM- ζ 1}E13 and ^{tSH2}K245 salt-bridge may be essential in the structural coupling (Figure 4b). To further investigate the role of the salt bridge, we measure the steady-state and kinetics of the tSH2 domain binding to the ^{ITAM- ζ 1}E13A. We observed that the ^{ITAM- ζ 1}E13A increases the plateau width in the steady-state binding with a significant increase in the K_{d2} , and K_{d1}^* compared to the ITAM-Y2P- ζ 1 (Figure 1e, 4c-f). The binding kinetics shows that the mutation in the ITAM-Y2P- ζ 1 does not perturb the rate of encounter complex formation (k_{obs}^{fast}), but slows down the transitioning to the closed-conformation ($k_{obs}^{slow} = 0.022 \pm 0.005 \text{ s}^{-1}$), similar to ITAM-Y2P- ζ 3 binding (Figure 3i-j, 4d-e, and Table 1). Our data suggest that the coupling between the two SH2 domains may determine the rate of the slow kinetic step (k_{obs}^{slow}).

A network of allosteric residues comprised of aromatic-aromatic stacking interaction allosterically couple the two SH2 domains of ZAP-70 during the transition to the final *holo*-conformation (Figure 4g).³⁵ Mutating the residues in the allosteric network uncouples the formation of the encounter complex to the phosphotyrosine binding to the N-SH2 PBP (K_{d2}). To determine if the mutation in the allosteric network will reduce the rate of conformational transition to the closed state (k_{obs}^{slow}), we studied the effect of ^tSH2R43P and ^tSH2F117A mutants on the ITAM-Y2P- ζ 1 binding kinetics (Figure 4g-i, table 1). We observed that the allosteric mutant did not perturb the rate of encounter complex formation (k_{obs}^{fast}) compared to the wild-type tSH2 domain. As anticipated, the allosteric mutants, ^tSH2R43P, and ^tSH2F117A, either inhibit or reduce the k_{obs}^{slow} , respectively (Figure 4h-i, Table 1). Analysis of the change in Gibb's free-energy due to ligand binding ($\Delta G_{binding}$) suggests that the final transition to the close-conformation may impart a thermodynamic penalty (Figure 4j-l). Therefore, weakening the allosteric coupling led to a higher penalty at the transitions to the closed-state (Figure 3k). We conclude that the residues in the allosteric network collectively constitute a thermodynamic brake which may impart a delay between ZAP-70 binding and activation at the membrane (Figure 4m). It is widely believed that TCR shares a common ancestry and design principle with the B cell receptor (BCR). Is the thermodynamic brake present in Syk?

Thermodynamic penalty coincides with the evolutionary-divergence of humoral and cell-mediated immune response

Syk is less selective than ZAP-70 and activated by a wide range of ITAM sequences in cells of innate and adaptive immune systems^{10,17,20,34,43-45}. Sequence analysis shows that most PBP and allosteric network residues in the regulatory module are conserved between Syk and ZAP-70

(Figure 5a, b). The exception is ^{ZAP-70}R43; the corresponding residue in Syk is glutamine (Figure 5a, b). Phylogenetic mapping using the ZAP-70 as reference revealed that the ZAP-70 is conserved in all vertebrates and may appear first in the jawed fish (cartilage fish) (Figure 5c)^{30,46}. Syk and Syk-related proteins are present in vertebrates and some invertebrates, including sponges and hydra (Figure 5c, S6a). This indicates, ZAP-70 may appear along with the evolution of the BCR-TCR-MHC like adaptive immune system at the divergence of jawless and jawed fish^{36,47}. Intriguingly, the allosteric network residues comprising the thermodynamic brake in ZAP-70 are conserved, except residue L191 (Figure 5b-d). In amphibians and jawed fish, the Leu is often replaced by similar residues like Ile, Val, and Met. In Syk, two key residues in the allosteric network, N46, and W238 (corresponds to ZAP-70 R43 and W235, respectively), are not conserved (Figure 5b-d, S6b), suggesting that the thermodynamic brake may be non-functional.

To evaluate the role of the thermodynamic-brake in the Syk tSH2 domain, we characterized the steady-state binding of the tSH2 domain to ITAM-Y2P- ζ 1, and ITAM-Y2P- ζ 3, respectively. As reported previously, we observed that the Syk tSH2 domain could not distinguish between the ITAM sequences and bind with a similar K_d (~ 65 nM) (Figure 6a-b)³⁴. Unlike ZAP-70, the tSH2 domain of Syk binds ITAM-Y2P in a single fast kinetic step ($k_{obs}^{fast} = 32.81 \pm 0.005$ s⁻¹), without any subsequent slow binding (Figure 6b-d). To evaluate if the absence of this slow binding correlates with the integrity of the allosteric network³⁵, we inspected the open and closed structures of the Syk tSH2 domain (Figure 4g and 6e). Analysis of the crystal structures showed that the ZAP-70 tSH2 domain could adopt only two conformations, *open (apo)* and closed (*holo*) (Figure 1c)^{19,31}. In comparison, the Syk tSH2 domain adopts three conformations: two structures in the *apo*-state, open and closed, and one ITAM-Y2P bound closed conformation^{16,20,30}. In the apo-

state of both Syk and ZAP-70, the SH2 domains are separated, preventing the aromatic amino acid residues to form the stacking interaction, which is central in coupling the N- and C- SH2 domains (Figure 4g, 6e). Surprisingly, the aromatic-aromatic stacking interaction does not form in the Syk tSH2 *holo*-state (Figure 6e). This indicates that the final transition to the *holo*-state does not require the allosteric network to assemble. To test that, we determine the steady-state binding of ITAM-Y2P- ζ 1 to Syk tSH2 mutant, ^{tSH2}F120A, and ^{tSH2}W168C, respectively (Figure 5a, 6f-g). None of the mutants altered the ITAM-Y2P binding, implying that the allosteric network is non-functional in Syk.

To explain if the absence of a penalty in Syk could lead to the hyperbolic binding in steady-state (Figure 6a) and one-step binding kinetics (Figure 6b-c), we remove the kinetic penalty in all pathways ($w_i = 1$) in the model described before (compare Figure 2a and 6h). We further assumed that the closed conformation in the *apo*-state (the central species in the network, Figure 6i) is short-lived. The experimental data of ITAM-Y2P binding to Syk matched with the modified model (Figure 6i), producing a single exponential decay (Compare Figure 6j and 2e). Therefore, the same kinetic model used for ZAP-70, in principle, also explains ligand binding in Syk if we consider independent binding in two SH2 domains^{20,32}. This suggests that Syk tSH2 may have little or no penalty, explaining the lack of specificity for ITAM sequence^{34,48}. Indeed, titration with ITAM-YP showed that Syk tSH2 domain binds with relatively stronger affinity ($K_d = 27.7 \pm 2.3$ nM) compared to ITAM-Y2P ($K_d = 65 \pm 12$ nM) (Figure 6a, S7a-b), suggesting the presence of negative cooperativity, as reported previously.⁴⁹ We conclude, the thermodynamic brake is unique to ZAP-70, which may have coevolved in the adaptive immune cells during the divergence of the BCR-TCR-MHC like the immune system in the jawed fish.

Conclusions

In summary, we have presented a unified kinetic model that explains the ZAP-70 and Syk tSH2 domain recruitment to the ITAM motifs at the membrane. Our model explains the recently observed closed conformation of an isolated *apo*-tSH2 domain structure of Syk³⁰. Due to the absence of a penalty step, the Syk tSH2 domains could spontaneously adopt a closed conformation like in the *holo*-state, allowing the tSH2 domain to bind a wide range of ITAM-Y2P sequences with less selectivity. Absence of thermodynamic penalty facilitates binding of ITAM-Y2P even at lower concentrations observed under the basal level in B cells.

Our model suggests that incorporating a penalty step (thermodynamic brake) during the final transitioning to the *holo*-state (Figure 4m) is enough to explain the biphasic ITAM-Y2P binding and selectivity in the ZAP-70 tSH2 domain. The residues constituting the thermodynamic brake are conserved in all ZAP-70 kinases in the vertebrates, which coincides with the evolution of the humoral and cell-mediated immune system in jawed fish.

We propose that the thermodynamic brake is an essential component in the kinetic proofreading mechanism of ZAP-70 regulation. Several rate-limiting steps kinetically regulate the initiation of the ZAP-70-depended TCR signalling, including recruitment of coreceptor molecules coupled to Lck, dwell-time of ZAP-70, and phosphorylation of Y132 in LAT by ZAP-70^{46,50,51}. In the T cell quiescence, the thermodynamic brake shifts the conformational equilibrium of the *apo*-tSH2 domain of ZAP-70 towards the open state. The additional energy barrier further stabilizes the compact inactive conformation of the kinase domain, which may shorten ZAP-70 dwell time at the TCR, causing reduced basal activation. The penalty step introduces a delay between the

encounter complex formation and subsequent transition to the *holo*-tSH2 structure required for the activation of ZAP-70, which most likely explains delayed Ca^{2+} release in T cell¹². The thermodynamic brake may provide an added layer of regulation in the kinetic proofreading, fundamentally differentiating TCR from BCR response.

Materials and Methods

Details of materials and method comprising of the Purification of tSH2 domains, steady-state and pre-steady state Fluorescence experiments, Isothermal Titration Calorimetry, phylogenetic analysis, and Mathematical modeling can be found in Supplementary file.

Supporting Information

The Supporting Information contain Material and Methods including Mathematical model, FigureS1- S7 and Table S1-S7.

Author Contributions:

The manuscript was written through the contribution of all authors. All authors have approved the final version of the manuscript. RD, and KG designed the experiments. KG, ACC, SR, OD, SSG and SC performed the Biochemical experiments and data analysis. ACC did the phylogenetic analysis of ZAP-70, OD, KG and RD performed the network analysis of tSH2 domain structure. DD, AR and ACC developed the mathematical model. RD, DD, KG and AR wrote the manuscript.

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349 **Data Availability Statement**

350 All the relevant data are contained within this article and in the supporting information.

351

352 **Ethics Declaration:**

353 *Competing interests*

354 The authors declare that they have no competing interests.

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Table 1: Observed rate for the ZAP-70 tSH2 domain and ITAM-Y2P binding

Construct	$f_{obs}^{fast} (s^{-1})$	$f_{obs}^{slow} (s^{-1})$
tSH2: ITAM-Y2P-ζ1	23.87 ± 1.01	0.262 ± 0.0977
tSH2: ITAM-Y2P-ζ3	21.44 ± 1.83	0.012 ± 0.0048
tSH2 R39A: ITAM-Y2P-ζ1	24.82 ± 2.29	---
tSH2: ITAM-YP-ζ1	28.60 ± 5.84	---
tSH2: ITAM-Y2P-ζ1 E13A	24.4 ± 2.04	0.022 ± 0.0045
tSH2 F117A: ITAM-Y2P-ζ1	21.83 ± 1.49	0.0517 ± 0.0062
tSH2 R43P: ITAM-Y2P-ζ1	27.96 ± 3.45	2.25 ± 0.032

Figure 1 Binding of tSH2 domain of ZAP-70 to the ITAM-Y2P peptides: (a) Domain architecture of ZAP-70 full-length and the regulatory module. (b) Sequence alignment between ITAM-Y2P- ζ_1 and ITAM-Y2P- ζ_3 peptides. (c) Space-filled representation of tSH2-*apo* (PDB ID: 1M61) and tSH2-*holo* (PDB ID: 2OQ1) structure, the intermediate step is represented as cartoon.

501 The N- and C-terminal SH2 domain, phosphate-binding pocket, and the respective binding
 502 constants are labeled. (d) Plot of change in intrinsic tryptophan fluorescence as a function of
 503 indicated ITAM-Y2P: tSH2 domain ratio. The Hill coefficients were calculated using Hill Plot.
 504 (e) Table summarizing the respective binding constants for the indicated tSH2 domain and ITAM-
 505 Y2P measured by ITC and fluorescence spectroscopy. Also see Figure S1.

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Figure 2. A kinetic-mathematical model explaining the biphasic ligand binding (a) Schematic diagram of the model showing different reaction pathways. Arrow-widths represent distinct weightages of kinetic rates (bold arrows: higher rates, thin arrows: lower rates, and dotted arrows: negligible rates). (b-c) Effects of K_{d1} variation on the bound and unbound fraction under steady-state and pre-steady state, respectively. (d) Two-step decay is shown with two distinct exponential fits (dashed line) in a semi-log plot. (e-f) Effects of K_{d2} variation on the bound and unbound fraction under steady-state and pre-steady state, respectively. (g) Similar to panel d, two exponential fits (dashed line) represent distinctive two-step kinetics. (h) Comparison of theoretical

519 prediction with the experimental data for binding of indicated ITAM-Y2P to the ZAP-70 tSH2
520 domains. Fitted parameters are in Table S5. Also see Figure S2.

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Figure 3 Binding kinetic of tSH2 domain of ZAP-70 and ITAM-Y2P. (a) and (b) the plot of time-dependent binding kinetics of tSH2 domain and 30 μ M ITAM-Y2P- ζ 1 in the slow and fast time scales, respectively. Inset represents the single exponential fitting of the blank subtracted data. The error bar represents the standard deviation from three independent experiments. (c) The steady-state binding of the indicated tSH2 construct to ITAM-Y2P- ζ 1 and ITAM-YP- ζ 1. (d) and (e) The plots of slow and fast binding kinetics of indicated tSH2 construct to ITAM-Y2P- ζ 1 and

533 ITAM-YP- ζ 1, respectively. (f) A modified version of the full model (Figure 2a). Forbidden
 534 intermediate conformations are highlighted by shadow boxes and dotted arrows. (g-h) The bound
 535 and unbound fractions of tSH2:ITAM-YP and ^{tSH2}R39A:ITAM-Y2P under the steady state and
 536 pre-steady state conditions, respectively. In panel g, experimental steady-state data are also shown
 537 for comparison. (i-j) Binding kinetics of ITAM-Y2P- ζ 1 and ITAM-Y2P- ζ 3 to the ZAP-70 tSH2
 538 domain. (k) A reduced kinetic model of ITAM binding. Also see Figure S3.

Figure 4. Structural evaluation of the tSH2 domain in determining the penalty step. (a) Space filled model of tSH2-holo structure of ZAP-70 (PDB ID: 2OQ1) highlighting the salt-bridge

between the tSH2 domain and ITAM-Y2P- ζ 1. (b) and (c) The steady-state binding of the tSH2 domain to ITAM-Y2P- ζ 1 at indicated pH and ITAM-Y2P- ζ 1 mutant, respectively. (d) and (e) The plots of slow and fast binding kinetics of tSH2 domain to indicated ITAM-Y2P- ζ 1 peptides, respectively. (f) Table summarizing the dissociation constants for the indicated tSH2 domain and ITAM-Y2P samples. (g) Structure representing the allosteric network coupling the two SH2 domains in ZAP-70 (PDB ID: 2OQ1). (h) and (i) Slow and fast binding kinetics of indicated tSH2 domain mutants, respectively. The error bar represents the standard deviation from three independent experiments. (j) represents the change in Gibb's free energy upon ligand binding ($\Delta G_{binding}$) in tSH2:ITAM-Y2P- ζ 1 (blue), tSH2:ITAM-Y2P- ζ 3 (green), tSH2:ITAM-Y2P- ζ 1E13A (grey), and tSH2F117A:ITAM-Y2P- ζ 1 (yellow). (k) and (l) are the $\Delta G_{binding}^{total}$ and $\Delta \Delta G_{binding}^{total}$, for the indicated tSH2 domain: ITAM-Y2P interactions. (m) Schematic model showing the binding kinetics and the thermodynamic penalty in the tSH2 and ITAM-Y2P interaction. Also see Figure S4.

Figure 5: Phylogenetic analysis of allosteric network in the Syk family kinases (a) Sequence Alignment of tSH2 domain of Syk and ZAP-70. The residues at the PBP are marked with red-star, and the allosteric network residues are marked with blue-arrow. (b) Schematic representation of undirected allosteric network in the tSH2 domain of ZAP-70. (c) Phylogenetic tree depicting the evolution of Syk family kinases, and Syk-related kinases. The emergence of ZAP-70 is marked as a red dot. (d) Sequence logo representing the conservation of allosteric network residue in ZAP-

70 (top) and in Syk (including the Syk-related kinases) (bottom). Also see Table S1, Table S2, Figure S5 and S6.

Figure 6. Steady-state and kinetic binding of Syk tSH2 domain to ITAM-Y2P: (a) Steady-state binding of ITAM-Y2P- ζ_1 and ITAM-Y2P- ζ_3 to the Syk tSH2 domain. (b) & (c) Slow and fast binding kinetics of indicated tSH2 domains to ITAM-Y2P peptides. (d) Table summarizing

570 the dissociation constant (K_d) and observed rate constant (k_{obs}) for the Syk tSH2 domain. (e)
571 Structure highlighting the allosteric network in the Syk tSH2 domain in *apo*- (PDB ID: 4FL2) and
572 *holo*- state (PDB ID: 1A81), respectively. (f) Steady-state binding of indicated tSH2 construct to
573 ITAM-Y2P- ζ 1. (g) Tabulation of dissociation constant (K_d) for the tSH2 allosteric mutant. The
574 error bar represents the standard deviation from three independent experiments. (h) A modified
575 version of the full model (Figure 2a) explaining Syk ITAM-Y2P interaction. Forbidden
576 intermediate conformations are highlighted by shadow boxes and dotted arrows. (i) Comparison
577 of the bound-fraction with the experimental data (filled circles and triangles) in the steady-state.
578 (j) Pre-steady state kinetic profiles showing single-step decay. Also see Figure S7.

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