

ZAP-70: An Essential Kinase in T-cell Signaling

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ZAP-70 is a cytoplasmic protein tyrosine kinase that plays a critical role in the events involved in initiating T-cell responses by the antigen receptor. Here we review the structure of ZAP-70, its regulation, its role in development and in disease. We also describe a model experimental system in which ZAP-70 function can be interrupted by a small chemical inhibitor.

Approximately 18 years ago, a T-cell receptor (TCR)- ζ chain-associated 70 kDa tyrosine phosphoprotein (therefore termed ZAP-70) was identified in TCR-stimulated Jurkat cells (Chan et al. 1991). The importance of ZAP-70, a cytoplasmic tyrosine kinase mainly expressed in T cells, was rapidly revealed by the following observations: ZAP-70-deficient patients have no functional T cells in their peripheral blood and suffer from severe combined immunodeficiency (SCID) (Arpaia et al. 1994; Chan et al. 1994; Elder et al. 1994); T-cell development in ZAP-70 deficient mice is arrested at the transition from the CD4 + CD8 + double positive (DP) stage, where positive selection occurs (Negishi et al. 1995b; Kadlecik et al. 1998); and a Jurkat-derived cell line (p116) deficient in ZAP-70 expression fails to activate downstream signaling events after TCR stimulation (Williams et al. 1998). Because ZAP-70 plays a critical role in T-cell development

and activation, much effort has been focused on studying the regulation, structure, and function of ZAP-70.

How does ZAP-70 mediate TCR signaling? Our lab proposed a model by which the TCR initiates signaling via sequential interactions with cytoplasmic tyrosine kinases (Weiss 1993). When the TCR interacts with peptide antigen bound to a MHC complex molecule on antigen presenting cells, coreceptor-associated Lck is brought into proximity of the CD3 complex and phosphorylates tyrosines in the immunoreceptor tyrosine-based activation motifs (ITAMs). When doubly phosphorylated, ITAMs recruit ZAP-70 via a relatively high affinity interaction by binding to the tandem SH2 domains of ZAP-70. Recent studies suggest that this binding event leads to the release of ZAP-70 from its autoinhibited conformation, which exposes the regulatory phosphorylation sites for Lck-mediated



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H. Wang et al.

phosphorylation. In addition, tyrosines in the activation loop of the ZAP-70 kinase domain are then phosphorylated by Lck or by ZAP-70 itself in *trans* to further promote its catalytic activity. A number of signaling proteins, including the linker for the activation of T cells (LAT) and the SH2-domain-containing leukocyte protein of 76 kDa (SLP-76), are subsequently phosphorylated by active ZAP-70. The phosphorylated LAT and SLP-76 proteins function as scaffolds to recruit many other signaling molecules (Fig. 1). The consequences of these early signaling events eventually lead to T-cell activation, proliferation, and differentiation.

Here we discuss the structure and regulation of ZAP-70, as well as the role of ZAP-70 in T-cell development and disease. We also highlight a recently developed chemical-genetic approach of inhibiting the ZAP-70 kinase activity and its potential usage in studying the role of ZAP-70 in T-cell responses, both *in vitro* and *in vivo*, which will facilitate and justify further effort in developing an inhibitor of wild-type ZAP-70 protein.

STRUCTURE OF ZAP-70

ZAP-70 is composed of two SH2 domains and a carboxy-terminal kinase domain (Fig. 2A). Unlike Src-family kinases with an amino-terminal SH3-domain and a SH2-domain, the amino terminus of the spleen tyrosine kinase (Syk) family of kinases, of which ZAP-70 is a member, consists of two tandem SH2 domains that are separated by a linker region, termed interdomain A. In ZAP-70, these SH2 domains bind to doubly phosphorylated ITAMs of TCR ζ chain, and are responsible for bridging ZAP-70 to the activated TCR. Another linker region, interdomain B, connects the SH2 domains to the kinase domain (Chan et al. 1992).

The crystal structure of a truncated version of ZAP-70 containing the tandem SH2 domains complexed with a doubly tyrosine phosphorylated ITAM peptide reveals interesting features about its structure and clues to its regulation. The most unusual feature of the ZAP-70 SH2 ITAM complex is that the phosphotyrosine binding (PTB) pocket of the amino-terminal

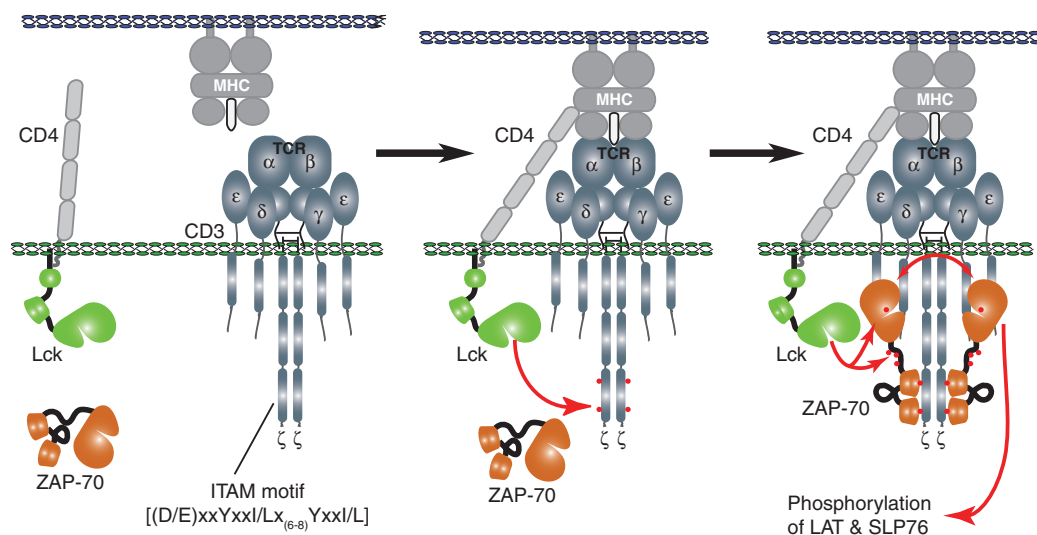


Figure 1. A sequential model for T-cell activation. Following TCR engagement, CD4-associated Lck is brought into proximity of the CD3 complex and phosphorylates the ITAMs (phosphorylation depicted as red dots). Doubly phosphorylated ITAMs then interact with the tandem SH2 domains of ZAP-70. After ITAM binding, ZAP-70 can be phosphorylated by Lck, which results in activation of ZAP-70 catalytic activity and its autophosphorylation. Active ZAP-70 subsequently phosphorylates LAT and SLP-76, which function as scaffolds to recruit many other signaling molecules and lead to T-cell activation, proliferation, and differentiation (not shown).

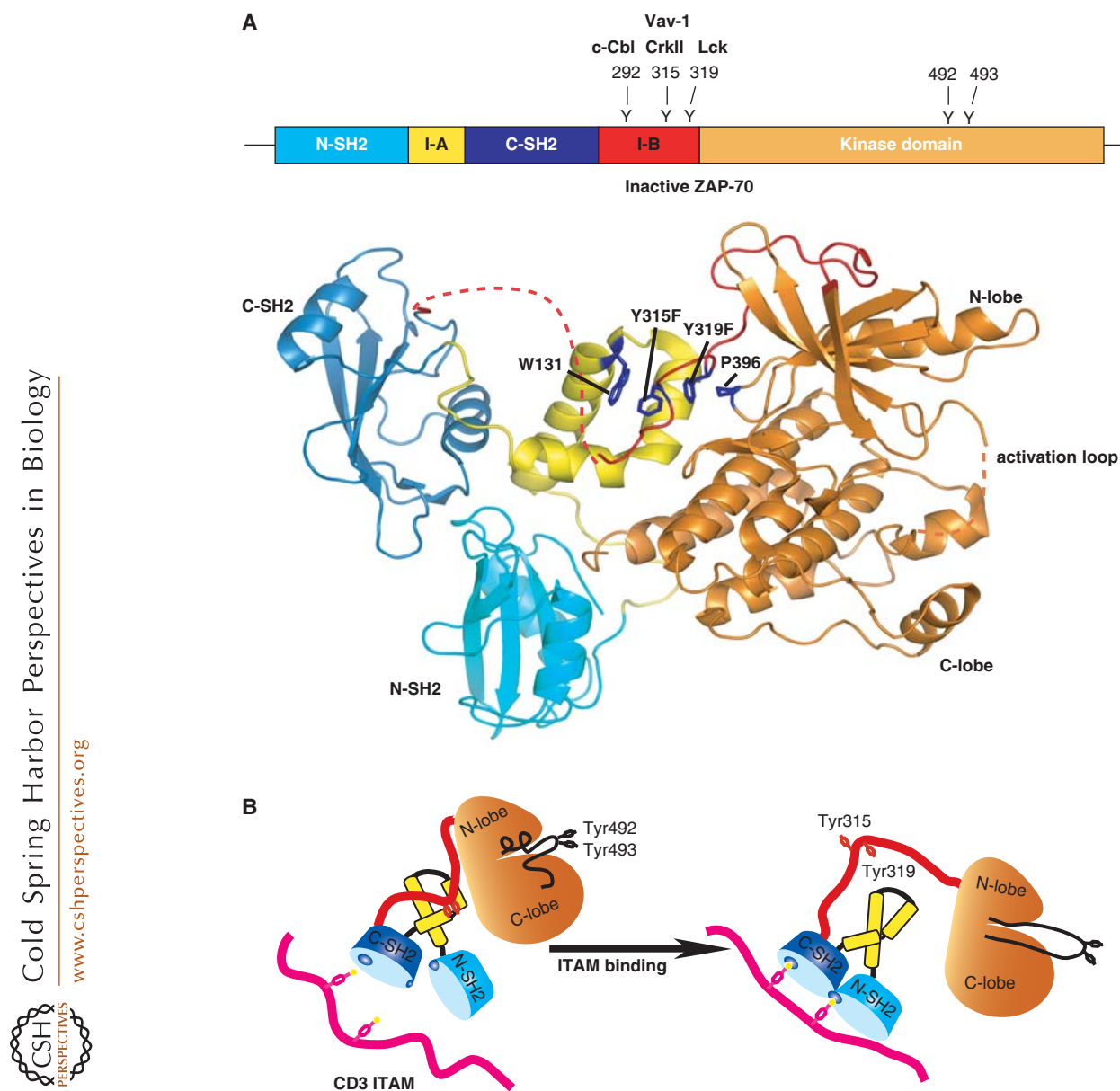


Figure 2. Structural organization of autoinhibited ZAP-70, and model of autoinhibition. (A) Schematic of ZAP-70 domains and phosphorylated tyrosine residues with binding molecules (*top*) and a crystal structure of autoinhibited ZAP-70 (PDB code 2OZO, *bottom*). The amino-terminal SH2 domain, interdomain A, carboxy-terminal SH2 domain, interdomain B, and the kinase domain are shown in cyan, yellow, blue, red, and orange, respectively. The side chains of residues involved in the aromatic–aromatic interactions between interdomain A, interdomain B, and the kinase domain are labeled and colored in dark blue. Dotted lines represent disordered regions. (B) Model for the activation of ZAP-70 following ITAM binding. The SH2 domains, interdomain A, interdomain B, and kinase domains are colored as in (A). The doubly phosphorylated ITAM is depicted in pink. When ZAP-70 shows an autoinhibited conformation it is incompatible with binding to phosphorylated ITAMs. Following ITAM binding, conformational changes in ZAP-70 promote disassembly of the interface mediating the autoinhibited conformation, and exposure of tyrosines in interdomain B, leading to their phosphorylation that further destabilizes the interface.

H. Wang et al.

SH2 domain is formed by residues contributed from both the amino- and carboxy-terminal SH2 domains that are brought into juxtaposition by ITAM binding. As in most SH2 domains, the carboxy-terminal SH2 PTB pocket is completely composed of residues within the carboxy-terminal SH2 domain. In addition, interdomain A appears to form a coiled-coil domain that facilitates extensive contact between the two SH2 domains and stabilizes their interaction (Hatada et al. 1995). This structure suggests that the SH2 domains do not function independently, and that interactions between the domains are important for specific recognition of the phospho-ITAMs of the activated TCR. Mutational studies addressing minimal spatial requirements between tyrosines in ITAM sequences for signaling also support this model (Ottinger et al. 1998).

Comparison of the crystal structures and NMR analysis of the bound and unbound SH2 domains reveals two very different conformations. Binding to phospho-ITAM peptide induces large movements between the SH2 domains, and the formation of the complete amino-terminal PTB pocket. Compared to a more rigid structure in the bound form, interdomain A shows a flexible structure in the unbound form. This more rigid structure is likely to contribute to the specificity of binding to the appropriately spaced and phosphorylated ITAM (Folmer et al. 2002). Additionally, studies using antibodies against regions within interdomain A show binding to ZAP-70 only when it is not associated with the ζ chain, further enforcing the idea of a conformational change upon docking to the ITAM (Grazioli et al. 1998).

Following TCR engagement, two tyrosine residues located in the activation loop of the kinase domain (Y492 and Y493) are phosphorylated. It is thought that this phosphorylation displaces the activation loop from the catalytic site of the kinase, leading to complete activation of the kinase (Watts et al. 1994). The crystal structure of a protein consisting of the kinase domain (aa327-606) in complex with the ATP-competitive inhibitor Staurosporin, shows an active-like kinase conformation, despite the

fact that Y492 and Y493 are unphosphorylated. Its bilobed structure is well conserved compared to the catalytic domains of many protein kinases. Common to these kinases, ZAP-70 is composed of an amino-terminal lobe containing a five-stranded antiparallel β -sheet and a single helix, and a carboxy-terminal lobe that is predominantly helical with short strands of β -sheets. An extended linker connects these lobes, and the ATP-binding site is located at the junction between the two lobes (Jin et al. 2004). The ability of the catalytic domain to adopt an active conformation without activation loop phosphorylation suggests that additional mechanisms are used in the full-length protein to regulate its activation.

ZAP-70 contains several tyrosine residues that are phosphorylated following TCR stimulation and are known to be important in its regulation, activity, and association with other signaling molecules. Y292, Y315, and Y319 are located within interdomain B and when phosphorylated are thought to serve as docking sites for downstream signaling molecules (Fig. 2A). Regulation of ZAP-70 kinase activity is also dependent on tyrosine residues in interdomain B. Mutation of both Y315 and Y319 to phenylalanine renders the kinase inactive. In contrast, mutation of these same residues to alanine results in increased basal kinase activity (Brdicka et al. 2005). Additionally, some function is maintained in a mutant lacking most of interdomain B, suggesting that it may be involved in an autoinhibitory mechanism (Zhao and Weiss 1996). It was hypothesized that mutation of Y315 and Y319 to phenylalanine stabilizes an autoinhibited conformation and tyrosine phosphorylation may impede the autoinhibited conformation (Brdicka et al. 2005).

Indeed, the crystal structure of autoinhibited ZAP-70 has also been solved at 2.6 Angstrom resolution using a recombinant protein with Y315 and Y319 in interdomain B mutated to phenylalanine (Fig. 2A). In the crystal structure the catalytic domain in autoinhibited ZAP-70 adopts a conformation that is characterized by many of the features of the inactive conformation of Src. Helix α C in the N-lobe of the kinase domain is rotated away from the

active site, the salt bridge between Glu386 and Lys369 that is required for catalytic activity is disrupted, and a short 3_{10} -helix at the base of the activation loop packs against the displaced helix α C. Although this structure contains many common features of autoinhibited Src family kinases, the mechanism for its autoinhibitory regulation is novel. A hydrogen bonding network involving interdomains A and B and the back of the kinase domain serves to stabilize the hinge region of the catalytic domain, thereby stabilizing the inactive conformation. This notion is supported by comparison of energy displacement data from the active and inactive kinase domains. Two interactions serve to stabilize the autoinhibited conformation. First, two α -helices from interdomain A (viewed as a single helix in the tandem SH2-ITAM bound structure) are docked onto the carboxy-terminal lobe of the kinase domain. A proline residue at the junction of these two is wedged between two tyrosines on the back of the C-lobe of the catalytic domain. Interdomain B is positioned between the kinase domain and interdomain A. Additionally, critical residues stabilizing this conformation include a set of perpendicular aromatic-aromatic interactions between W131 and P296 with F315 and F319 that create a rather stable hydrophobic environment (Deindl et al. 2007). The hydroxyls of Y315 and Y319 in the wild-type kinase would presumably decrease the stability of the hydrophobic interactions in this region and allow for more conformational flexibility in wild-type ZAP-70. Of considerable interest, the alignment of the tandem SH2 domains in the autoinhibited structure is incompatible with binding to phosphorylated ITAMs, because the PTB pocket of the amino-terminal SH2 domain is not appropriately apposed to the carboxy-terminal SH2 domain to complete the PTB site. Studies of mutant kinases, predicted to interfere with the interface interactions, that were overexpressed in 293T epithelial cells and in the Jurkat cell line deficient in ZAP-70 validate this structure. In vitro kinase activity, TCR-induced tyrosine phosphorylation, intracellular Ca^{2+} response, as well as ITAM peptide binding were all increased compared to WT ZAP-70 in

mutants when the interface is disrupted (Deindl et al. 2009).

A multistep model for activation of ZAP-70 has been proposed that involves spatial and temporal coordination of several key events (Fig. 2B). In resting cells ZAP-70 is in a dynamic equilibrium, and transitions in and out of the autoinhibited conformation. Following TCR stimulation and Lck-mediated phosphorylation of tyrosine residues of the ITAM, the tandem SH2 domains of ZAP-70 can then bind the doubly phosphorylated ITAM. The binding of the two SH2 domains requires their realignment from the autoinhibited conformation. Thus, a conformation of ZAP-70 that disrupts the interactions stabilizing interdomain B interaction with interdomain A as well as with the catalytic domain is favored, thereby destabilizing the interactions key to the maintenance of the autoinhibited state of ZAP-70. This allows for increased accessibility and Lck-mediated phosphorylation of Y315 and Y319 in interdomain B, phosphorylation of Y493 of the activation loop of the kinase, and subsequent full activation of the kinase.

REGULATION OF ZAP-70

Recruitment of ZAP-70 to the TCR

ZAP-70 activation can be regulated both by binding to phosphorylated ITAMs of the TCR and by phosphorylation of multiple tyrosine residues on ZAP-70 (Fig. 2A). Imaging studies have shown that in resting T cells, ZAP-70 is distributed throughout the cytoplasm, but is rapidly recruited to the plasma membrane following TCR stimulation and ITAM phosphorylation (Sloan-Lancaster et al. 1997; Sloan-Lancaster et al. 1998). ZAP-70 mobility in the cytoplasm is rapid, and decreases as ZAP-70 associates with the plasma membrane (Sloan-Lancaster et al. 1998). Whereas the recruitment to the plasma membrane is enhanced by Lck activity and phosphorylation of the ITAMs, involvement of other proteins such as the activated forms of ezrin and RhoH, a member of the Rho GTPase family, has also been reported

H. Wang et al.

(Sloan-Lancaster et al. 1997; Gu et al. 2006; Ilani et al. 2007).

Following TCR ligation, TCR microclusters are formed in which signaling is initiated and sustained. The microclusters continue to form in the periphery of the immune synapse (IS) and later translocate to the center of the synapse. ZAP-70 accumulates at these microclusters. Although the initial clustering of TCR is independent of tyrosine phosphorylation events, the activity of Lck and ITAM phosphorylation are required for ZAP-70 accumulation (Sloan-Lancaster et al. 1998; Campi et al. 2005; Yokosuka et al. 2005; Varma et al. 2006).

Positive Regulation of ZAP-70 Function

Although critical, binding to TCR- ζ and perturbation of the autoinhibitory conformation alone is insufficient for complete ZAP-70 activation (van Oers et al. 1994; Chan et al. 1995; Zhao et al. 1999; Jin et al. 2004). Phosphorylation of tyrosine residues, by the action of Src family kinases such as Lck, and/or by ZAP-70 itself, is also important. Requirements for full catalytic activity include phosphorylation of tyrosine residue Y493 in the activation loop of the kinase domain of ZAP-70. Y493 is a preferred phosphorylation site for Lck but can also be a substrate for ZAP-70 autophosphorylation (Watts et al. 1994; Chan et al. 1995; Kong et al. 1996). If phosphorylation is prevented by mutation of this residue to phenylalanine, Lck is unable to activate ZAP-70 (Chan et al. 1995; Wange et al. 1995). Reconstitution studies of the Syk deficient DT40 B-cell line showed that Lck mediates phosphorylation of Y493 and that catalytic activity of ZAP-70 is required for downstream signaling (Chan et al. 1995; Kong et al. 1996). When overexpressed in the Jurkat T-cell line, ZAP-70 mutated both on Y492 and Y493 to phenylalanines (Y492E, Y493F) impaired TCR-mediated signaling. However, the mutant was able to bind Lck, suggesting that Lck association with ZAP-70 is not dependent on activation loop phosphorylation (Mege et al. 1996).

Following TCR engagement, the tyrosine residues Y315 and Y319 in interdomain B are

phosphorylated. In addition to stabilization of the active conformation, these are docking sites for other molecules controlling the function of ZAP-70 and downstream signaling. Phosphorylated Y319 is a positive regulator of ZAP-70 function and is well documented to bind Lck. Overexpression of Y319F ZAP-70 mutant in Jurkat cells resulted in a dominant-negative effect, with reduced TCR-triggered activation of NFAT and IL-2 induction. Further, it conferred impaired up-regulation of ZAP-70 catalytic activity and reduced phosphorylation of its substrates LAT and SLP-76 (Di Bartolo et al. 1999). Additionally, Y319F ZAP-70 expressed in ZAP-70 deficient Jurkat cells failed to bind Lck and to reconstitute TCR triggered signaling (Williams et al. 1999). Further, mutations of the Lck SH2 domain abrogated binding of Lck to ZAP-70 and T-cell activation (Pelosi et al. 1999; Williams et al. 1999). The binding of Lck to phosphorylated Y319 may thus help prevent reversion to the autoinhibitory conformation, promote Lck mediated phosphorylation of Y493, and facilitate activation of downstream signaling. The importance of Y319 phosphorylation in positive regulation of ZAP-70 was confirmed in a mouse model expressing Y319F ZAP-70, which resulted in severe defects in calcium mobilization and thymocyte selection (Gong et al. 2001).

Vav-1 has been shown to interact with ZAP-70 via its SH2 domain, and mutation of Y315 to phenylalanine abrogates binding of Vav to ZAP-70 in DT40 cells and causes a decrease in antigen receptor mediated tyrosine phosphorylation of Vav, SLP-76, Shc and ZAP-70 itself (Katzav et al. 1994; Wu et al. 1997). Further, although not having large effects on intrinsic kinase activity, the Y315F mutation was reported to alter ZAP-70 binding affinity for phosphorylated ITAMs, reducing its capacity to trigger Erk activation in murine T cells (Di Bartolo et al. 1999). This suggested that the Vav/ZAP-70 interaction via phospho-Y315 promotes ZAP-70 function. However, because Vav phosphorylation was not affected by the ZAP-70 mutation in vivo, the importance of Vav SH2 binding to ZAP-70 at Y315 for its phosphorylation is not clear (Magnan et al. 2001).



Actin polymerization may be influenced by ZAP-70 interaction with members of the CT10 regulator of kinase (Crk) family. Crk adaptor protein can bind to phospho-Y315 on ZAP-70. A model has been proposed in which phosphorylated ZAP-70 associates with Crk-like (CrkL)-WIP-WASP complexes allowing redistribution of the complex to lipid rafts and the IS following TCR triggering. PKC θ , whose activity is indirectly dependent on Lck and ZAP-70, can phosphorylate WIP thereby releasing WASP from WIP-mediated inhibition. WASP can then be activated by membrane bound Cdc42, leading to actin polymerization, whereas WIP binds newly formed actin and thus helps to stabilize the IS (Sasahara et al. 2002). Studies of mouse models have further confirmed a detectable, though modest, positive regulatory role of Y315 in T-cell activation and thymocyte selection (Gong et al. 2001; Magnan et al. 2001). Whether this is mediated by specific interactions of ZAP-70 with Vav, CrkL or other molecules, in addition to stabilization of an active conformation, has yet to be clarified.

Negative Regulation of ZAP-70 Function

Phosphorylation of specific tyrosine residues has also been implicated in negative regulation of ZAP-70 function. Y492 is phosphorylated following TCR triggering. Mutation of this tyrosine to phenylalanine elevates ZAP-70 kinase activity, showing a negative regulatory role for this site. Furthermore, Y492F mutant ZAP-70 was able to restore antigen receptor signaling in Syk deficient DT40 B cell line, indicating that phosphorylation of this tyrosine residue is not required for ZAP-70 function (Watts et al. 1994; Chan et al. 1995; Wange et al. 1995). Y492, like Y493, is positioned in the activation loop of the kinase domain, and could thus potentially influence accessibility of the catalytic site. However, the exact mechanism for Y492 influence on kinase activity has yet to be revealed (Chan et al. 1995; Wange et al. 1995; Jin et al. 2004).

Although able to recruit positive effectors of TCR signaling, Y315 was also shown to be

involved in ZAP-70 association with the Crk family member, CrkII, which has been implicated in negative regulation of TCR signaling. Lck-dependent CrkII interaction with ZAP-70 was detected after pervanadate treatment of Jurkat cells, and mutation of Y315 to phenylalanine eliminated this association (Gelkop and Isakov 1999; Gelkop et al. 2005). One possibility is that negative regulation by CrkII is exerted by complex formation between Crk, p85 PI3K, and the E3 ubiquitin ligase Cbl (Gelkop et al. 2001).

Several studies have also described a negative regulatory role for Y292 in interdomain B. Mutation of Y292 to phenylalanine enhanced NFAT induction in DT40 cells in response to BCR stimulation, without directly affecting kinase activity or TCR- ζ binding. Phosphorylation of Y292 may therefore promote association of ZAP-70 with a negative regulatory protein (Zhao and Weiss 1996). One strong candidate is c-Cbl, known to diminish TCR-mediated activation (Murphy et al. 1998; Naramura et al. 1998). ZAP-70 has been reported to associate with Cbl following TCR stimulation and phosphorylation of Y292 was required for this interaction, as well as for the negative effect of Cbl on signaling (Lupher et al. 1996; Lupher et al. 1997; Rao et al. 2000; Magnan et al. 2001). Cbl may promote ubiquitination and degradation of TCR- ζ using ZAP-70 as an adaptor protein, possibly also affecting ZAP-70 function (Wang et al. 2001; Naramura et al. 2002; Davanture et al. 2005). However, because of discrepancies in phenotypes of c-Cbl knockout mice, mice expressing the ZAP70 Y292F mutation, and mice expressing a mutation in the tyrosine kinase binding domain of c-Cbl, the mechanism for the regulatory effect of Y292 remains undefined (Murphy et al. 1998; Naramura et al. 1998; Magnan et al. 2001; Thien et al. 2003).

SYK FAMILY KINASES AND THEIR ROLE IN T-CELL DEVELOPMENT

ZAP-70 and Syk, the only two members of the Syk kinase family, provide both overlapping and distinct functions in immunoreceptor signaling. Both ZAP-70 and Syk consist of two tandem N-terminal SH2 domains and a

H. Wang et al.

carboxy-terminal kinase domain. Although the tandem SH2 domains of the two kinases share 57% sequence identity, crystal structures of the tandem SH2 domains complexed with a phosphorylated ITAM peptide reveal important differences between ZAP-70 and Syk. As discussed earlier, in the ITAM-bound state, both SH2 domains of ZAP-70 are tightly apposed, making extensive contacts with each other and the phosphorylated ITAM. The amino-terminal SH2 domain of ZAP-70, unlike its carboxy-terminal SH2 domain, has an incomplete PTB pocket. The phosphotyrosine of the ITAM

peptide in the amino-terminal PTB pocket is closely associated with the side chain of Y238 and K242 from the C-terminal SH2 domain (Fig. 3A), revealing that the amino-terminal SH2 domain requires residues of the carboxy-terminal SH2 domain to complete this PTB pocket. These features of the ZAP-70 structure confer high selectivity for interaction with the activated TCR:CD3 complex (Hatada et al. 1995). Surprisingly, the PTB site of Syk is self-contained within the amino-terminal SH2 domain. The interaction between phosphotyrosine in the amino-terminal PTB site and

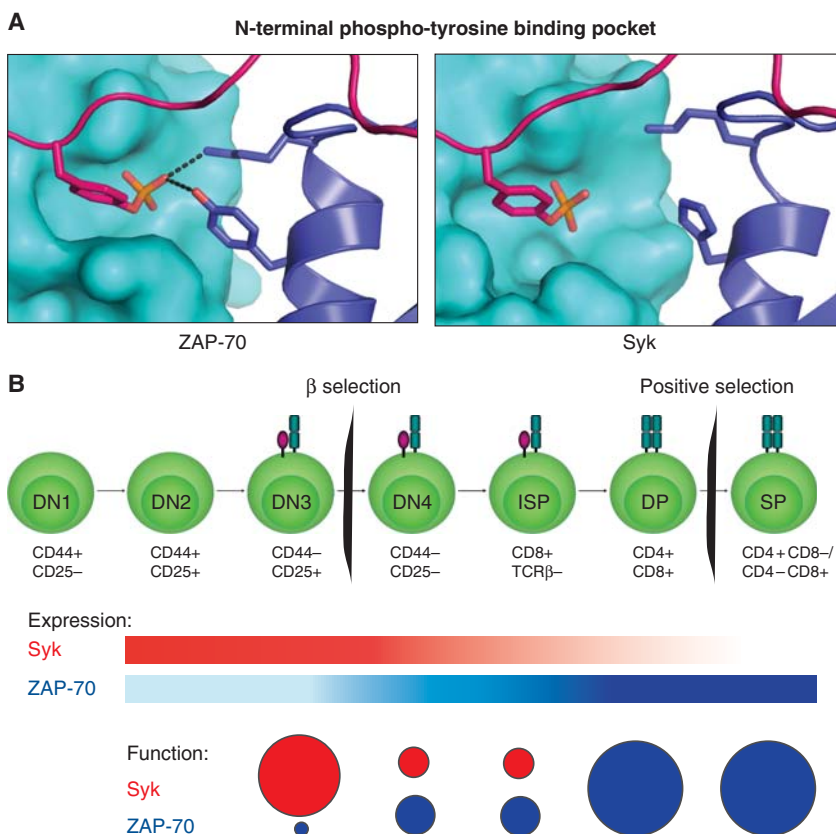


Figure 3. Comparison of ZAP-70 with Syk. (A) Cartoon structures of the amino-terminal phosphotyrosine binding pocket in ZAP-70 (PDB code 2OQ1, left) and in Syk (PDB code 1A81, right). The amino-terminal SH2 domain, carboxy-terminal SH2 domain, and phosphorylated ITAM peptide are shown in cyan, blue, and pink, respectively. There are six different conformations in the crystal asymmetric unit of Syk. The Syk structure shown here is representative of three of six conformations. In the other three conformations, only one residue (Lysine) in the carboxy-terminal SH2 domain seems to interact with the phosphotyrosine of the ITAM. However, the electron density for the side-chain of this Lysine is absent in all three conformations (not shown), suggesting that this interaction may not contribute significantly to the binding. (B) Expression and function of Syk family kinases throughout T-cell development.



residues of carboxy-terminal SH2 domain is significantly reduced compared to ZAP-70 (Fig. 3A), suggesting that the amino- and carboxy-terminal SH2 domains of Syk can function independently (Futterer et al. 1998; Kumaran et al. 2003). In addition, the asymmetric unit of Syk crystals contains six different conformations, reflecting a remarkable flexibility in the relative orientation of the two tandem SH2 domains. These features may provide the molecular basis for Syk interaction with a greater variety of ITAMs and other binding partners that vary considerably in the length of the spacer region between the two phosphotyrosines (Futterer et al. 1998; Kumaran et al. 2003).

Although both Syk and ZAP-70 are expressed during T-cell development, they have distinct expression patterns and play different roles. The early thymic progenitors transit through four stages as CD4/CD8 double negative (DN) cells (DN1–4) before up-regulating CD8 and CD4 to become double positive (DP) thymocytes, and finally become single positive (SP) thymocytes. Thymocyte maturation requires two critical checkpoints: pre-TCR mediated β -selection at the DN3 stage and TCR-mediated positive/negative selection at the DP stage. Syk and ZAP-70 proteins are inversely expressed across T-cell development. Whereas Syk is highly expressed in early development (DN1–DN3), ZAP-70 is highly expressed in later development (after DN4). However, there is overlapping expression of both kinases during some transitory stages, including the DN3, DN4, and “immature single-positive” (ISP) stages (Palacios and Weiss 2007). The positive/negative selection at the DP stage is completely arrested in ZAP-70 knockout mice but is not affected by Syk deficiency (Negishi et al. 1995b). Neither ZAP-70- nor Syk-deficient thymocytes have any obvious defect at the β -selection stage; however, the double knockout of both kinases results in an absolute block at this stage, indicating that Syk and ZAP-70 kinases can be functionally redundant during pre-TCR signaling at the DN3 stage (Cheng et al. 1997).

To determine whether Syk or ZAP-70 is preferentially used to mediate pre-TCR signaling,

competitive repopulation assays using ZAP-70-deficient or Syk-deficient versus WT hematopoietic stem cells were performed in irradiated host mice. The Syk-deficient, but not ZAP-70-deficient, thymocytes are specifically impaired in initial pre-TCR signaling and cell-cycle entry at the DN3 stage. Despite overlapping expression of both kinases, only ZAP-70 is responsible for sustained pre-TCR/TCR signaling and cellular proliferation during the DN4, ISP and DP stages (Palacios and Weiss 2007). These data suggest a model where Syk-dependent pre-TCR signaling is replaced with ZAP-70-dependent pre-TCR signaling in early T-cell development (Fig. 3B). To further test this model, the generation of a knockin mouse in which Syk is targeted into the ZAP-70 locus could provide further insights. ZAP-70-like expression of Syk within DN4, ISP and DP stages would allow testing of whether ZAP-70 has a unique function in generating DP thymocytes before positive selection.

ZAP-70 AND DISEASE

Chronic Lymphocytic Leukemia (CLL)

B-chronic leukemia (B-CLL) is a B-cell malignancy caused by accumulation of monoclonal CD5+ B lymphocytes in the blood, bone marrow, lymph nodes, and spleen (Chiorazzi et al. 2005). Early studies showed that the mutational status of the immunoglobulin heavy chains of B-CLL was associated with a distinctly different prognosis, with ~50 percent of cases in each group. Subsequent gene expression profiling showed that ZAP-70 expression was the most discriminating feature between the two subtypes (Rosenwald et al. 2001). In fact, ZAP-70 protein expression in B-CLL patients appears to have more predictive value than IgVh mutations, and its expression level appears to be constant during the course of disease (Rassenti et al. 2004).

Despite the prognostic value of ZAP-70 expression in B-CLL, the presence of what might be considered a T-lineage specific gene in B-cell lineage derived leukemic cells is unexpected. Whether ZAP-70 expression plays a functional

H. Wang et al.

role in B-CLL progression is unclear. ZAP-70 expression in B-CLL is associated with enhanced BCR signaling (Chen et al. 2002). ZAP-70 can couple to the BCR and up-regulate Syk phosphorylation and activation as well as downstream signaling events. Transduction of ZAP-70-deficient B-CLL cells with adenoviral vectors expressing ZAP-70 increased BCR-induced tyrosine phosphorylation of several BCR signaling molecules as well as mobilization of intracellular calcium (Chen et al. 2005). Together, these results suggest that the function of ZAP-70 in CLL cells may be to enhance BCR signaling.

The finding that ZAP-70 can enhance BCR signaling in CLL B cells seems counterintuitive, given that there are similar levels of Syk expression in both ZAP-70+ or ZAP-70- CLL B cells and Syk has approximately 100-fold greater *in vitro* kinase activity than does ZAP-70 (Latour et al. 1996). A recent study showed that ZAP-70 promotes BCR signaling independently of its kinase activity, as both wild-type ZAP-70 and a catalytically inactive ZAP-70 mutant induced similar increases in calcium and protein tyrosine phosphorylation (Chen et al. 2008). Consistent with these observations, the interaction between ZAP-70 and downstream effector molecules appeared unchanged in the CLL B cells (Gobessi et al. 2007). Together, these results suggest that ZAP-70 may function as an adaptor protein to facilitate BCR signaling or compete for a negative regulator of Syk.

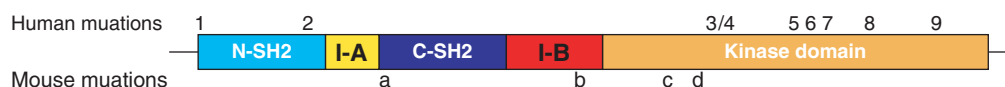
How might kinase-inactive ZAP-70 enhance Syk activation? Previous studies of DP thymocytes showed that ZAP-70, rather than Syk, promotes more efficient TCR- ζ chain phosphorylation by recruiting Lck into the TCR- ζ complex (Ashe et al. 1999). The role of ZAP-70 in augmenting TCR ITAM phosphorylation occurs when expression of Lck and the TCR are limiting in DP thymocytes, and is independent of its kinase activity. Similarly, B-CLL cells express lower levels of IgM and CD79b. Therefore, it is conceivable that, when limiting numbers of CD79b ITAMs are available in B-CLL cells, ZAP-70 positively affects Syk activity by

facilitating BCR recruitment to Lyn. In support of this notion, ZAP-70 expression in B-CLL is associated with efficient translocation of BCR to lipid rafts in response to BCR engagement. As a result, phosphorylation of the BCR by lipid-raft-associated Lyn kinase is significantly stronger in ZAP-70+ CLL cells (Allsup et al. 2005). Signaling enhancement of Syk by ZAP-70 in B-CLL may represent an amplification mechanism that compensates for the low expression of IgM and CD79 in B-CLL.

In summary, ZAP-70 can enhance the intracellular signaling capacity of the Ig expressed in CLL. Whether such increased intracellular signaling influences the survival or proliferation of CLL cells, leading to disease progression remains unknown. However, ZAP-70 is an attractive candidate for treatment, owing to its participation in BCR signaling in CLL.

SCID

Loss of function or expression of ZAP-70 in humans leads to SCID. This form of SCID is characterized by normal numbers of nonfunctional CD4+ T cells and an absence of CD8+ T cells in the peripheral blood of these patients (Elder et al. 1994; Hivroz and Fischer 1994). As shown in Figure 4, the ZAP-70 mutations occurring in human SCID, which abolish ZAP-70 expression, are mostly located in the kinase domain (Arpaia et al. 1994; Chan et al. 1994; Elder et al. 1994; Gelfand et al. 1995; Matsuda et al. 1999; Noraz et al. 2000; Toyabe et al. 2001). Mutations causing transcriptional loss or destabilized protein have also been reported (Fig. 4) (Gelfand et al. 1995; Matsuda et al. 1999; Noraz et al. 2000). A patient with a missense mutation within the highly conserved DLAARN motif of kinase domain has been reported, resulting in a moderate decrease in ZAP-70 stability and complete absence of kinase activity (Elder et al. 2001). Interestingly, an identical spontaneous mutation in DLAARN motif has also been reported in mice (Wiest et al. 1997), but still leads to a discordant phenotype of the peripheral T-cell compartment of humans and mice.

**Human SCID mutations**

Mutant allele	DNA mutation	Consequence to protein
1	transcript defect	No protein
2	C to A transition at nucl. 448	Missense (P to Q at residue 80, temperature-sensitive allele)
3	C to T transition at nucl. 1602	Missense (R to C at residue 465)
4	G to A transition at nucl. 1603	Missense (R to H at residue 465)
5	13 bp deletion (nucl. 1719-1731)	Deletion (premature termination at residue 538)
6	C to T transition at nucl. 1729	Missense (A to V at residue 507)
7	C to A transition at nucl. 1763	Missense (S to R at residue 518)
8	G to A transition at nucl. 1833	Splicing error (9 bp in-frame addition; L-E-Q 3 addition at residue 541)
9	A to T transition at nucl. 1923	Missense (M to L at residue 572, temperature-sensitive allele)

Hypomorphic ZAP-70 mutants

Mutant mouse	Mutant Name	Mutation	Autoimmune phenotype
a	SKG	W to C at residue 163	autoimmune arthritis, hyperglobulinemia, RF autoantibodies
b	YYAA	Y to A at residue 315 and 319	no arthritis, RF autoantibodies
c	ZAP-70 ^{msd/msd}	I to F at residue 367	no anti-DNA autoantibody, no hyper-IgE
d	ZAP-70 ^{msl/msl}	W to R at residue 504	no anti-DNA autoantibody, no hyper-IgE
c&d	ZAP-70 ^{msd/msl}	both I367F and W504R	anti-DNA autoantibody, hyper-IgE

Figure 4. Mutations of ZAP-70 in Human SCID and hypomorphic mutant mice.

Regardless of the mutation, the lack of ZAP-70 or complete loss of its function affects T-cell maturation differently in humans and in mice. In ZAP-70-deficient mice, T-cell development is blocked at the DP stage of thymocyte differentiation, resulting in no mature T cells in the periphery (Negishi et al. 1995a; Kadlecik et al. 1998). The selective deficiency of peripheral CD8⁺ cells in humans is caused by a defect in thymic T-cell development. In a thymic section from a ZAP-70 deficient SCID patient, DP T cells are present in the thymic cortex, but only CD8⁺SP cells are absent from the medulla (Arpaia et al. 1994). This observation suggests that positive selection of CD8⁺ cells is specifically blocked. It is possible that ZAP-70 plays different roles in selection of human CD4⁺ and CD8⁺ T cells and is indispensable for maturation into CD8⁺ T cells.

Although positive selection of CD4 SP cells occurs in these patients, the peripheral CD4⁺ T cells do not respond normally to mitogens or to stimulation by anti-CD3 antibodies, as shown by a failure to proliferate and produce IL-2. Interestingly, unlike peripheral CD4⁺ T cells,

CD4⁺ thymocytes from the patients are able to transduce signals via their TCRs as measured by calcium flux and tyrosine phosphorylation (Gelfand et al. 1995). The differential signaling capacity between CD4⁺ thymocytes and peripheral T cells may be explained by the observations that Syk is present at higher levels and later in development in human thymocytes compared to mouse thymocytes (Chu et al. 1998; Chu et al. 1999). These findings suggest that higher levels of Syk in human thymocytes may be able to partially compensate for the loss of ZAP-70 in SCID patients' CD4⁺ thymocytes but not in peripheral T cells. Further down-regulation of Syk expression in the human peripheral T cells and/or the absolute requirement for ZAP-70 may explain the signaling defect seen in peripheral CD4⁺ T cells lacking ZAP-70. The mechanism for the preferential selection of CD4⁺ cells remains unclear. It is possible that greater association of Lck with CD4 rather than CD8 may allow more efficient activation of ZAP-70, sufficient to overcome a threshold leading to CD4 but not CD8 lineage selection (Wiest et al. 1993). Taken

H. Wang et al.

together, studies on human ZAP-70 immunodeficiency reveal the critical role of ZAP-70 signaling both in mature T cells and in thymic development.

Hypomorphic ZAP-70 Mutant Mice

Although ZAP-70 deficiency typically leads to the complete absence of T cells in the periphery of mice, several ZAP-70 mutant mice with partial defects in TCR signaling have been reported. The SKG mouse has a spontaneous missense mutation (W163C) in the C-terminal SH2 domain of ZAP-70 and develops autoimmune arthritis in response to innate immune activation (Sakaguchi et al. 2003). This mutation greatly impairs the binding of ZAP-70 to the TCR- ζ chain, thereby altering TCR signaling and affecting thymic development, particularly positive and negative selection, and the differentiation of T cells. As a consequence of impaired negative selection, it is thought that the thymocytes showing the strongest affinity for self-peptide/MHC ligands are positively selected instead of being deleted. This presumably leads to a shift of T-cell repertoire that would otherwise have been eliminated during negative selection in WT mice. Adoptive transfer studies have suggested that expansion of these autoreactive CD4⁺ T cells that escaped negative selection is a key cause of autoimmune arthritis in SKG mice. SKG arthritis resembles human rheumatoid arthritis (RA) in many ways, such as presentation of autoimmune arthritis, hyper- γ -globulinemia, vasculitis, nodule formation, and rheumatoid factor production (Sakaguchi et al. 2006). Therefore, SKG mice may be a suitable model for understanding the cellular mechanism for autoimmune arthritis and might also provide useful insights into the development of autoimmunity in general.

Another hypomorphic ZAP-70 mutant mouse has recently been reported by our lab and has revealed mechanistic insights into how signaling defects may result in autoimmunity or autoimmune disease (Hsu et al. 2009). This ZAP-70 YYAA strain has knockin alanine mutations of Y315 and Y319 in the interdomain

B of ZAP-70. YYAA mice show defects similar to SKG mice. For instance, YYAA mice show diminished TCR signaling, impaired T-cell development, and defective positive and negative selection. However, in contrast to the SKG mice, which develop both arthritis and rheumatoid factor autoantibodies on the Balb/c background, YYAA mice only develop rheumatoid factor autoantibody. Quite surprisingly, in addition to impaired negative selection seen in the YYAA mice, the suppressive activity of T regulatory cells in these mice is also profoundly defective. This raises the question of why very limited autoimmunity is observed in the YYAA mice. Based on the extent of deletion of V β TCR genes to endogenous superantigens, our findings suggest that TCR-mediated negative selection is more impaired in the SKG than in the YYAA mice. Thus, it appears that different TCR repertoires result from different sensitivity to negative selection in these mice. The difference in TCR repertoires may be responsible for their different susceptibility to arthritis.

Recently, a hypomorphic allelic series of ZAP-70 have been generated through *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations, and these mutations are located within the kinase domain of ZAP-70 (Siggs et al. 2007). A graded change in TCR signaling potential created by combining two ZAP-70 hypomorphic mutations (*mrd* and *mrt*) has revealed that the balance between thymic selection and function of effector T cells is differentially regulated. In these ZAP-70 allelic mutants, this results in the coincident occurrence of both autoimmunity (spontaneous production of anti-DNA autoantibodies) and immune dysregulation (hyper-IgE syndrome). Interestingly, although both SKG mice and ZAP^{*mrt/mrd*} mice show defective negative selection, they show different autoimmune syndromes. SKG mice develop arthritis but not anti-DNA autoantibody, whereas ZAP^{*mrt/mrd*} mice develop anti-DNA antibody but not arthritis. There are two possibilities that might explain the difference in autoimmune syndromes in SKG, YYAA and ZAP^{*mrt/mrd*} mice. First, the genetic background could play a role in the resultant phenotypes.

Alternatively, quantitative and qualitative differences in TCR signaling resulting from ZAP-70 mutations may skew the TCR repertoire in ways that manifest as different autoimmune syndromes in these mice. Collectively, ZAP-70 hypomorphic alleles extend our understanding of how a quantitative difference in T-cell signaling may influence the outcome of immune responses as well as identify a threshold between susceptibility toward autoimmunity versus autoimmune disease.

ZAP-70 AS A THERAPEUTIC TARGET

Because of its critical role in TCR signaling, ZAP-70 is an attractive target for therapy. A specific inhibitor for ZAP-70 could potentially block T-cell activation at a level of signal transduction very proximal to the TCR. Additionally, ZAP-70 expression is relatively restricted to T cells, NK cells, and basophils (Au-Yeung et al. 2009). Thus, effects of specific ZAP-70 inhibition would be limited to these cell types. However, NK cells and basophils also express Syk, which may be able to compensate for the lack of ZAP-70. This appears to be the case for ZAP-70 deficient NK cells, which are still able to exert cytolytic activity (Negishi et al. 1995b). This suggests that inhibition of ZAP-70 activity may specifically target T-cell responses, while leaving innate immunity intact. Such an inhibitor could be particularly useful in settings of allograft rejection or T-cell-mediated autoimmunity.

To date, the development of a highly specific, cell permeable small molecule inhibitor of ZAP-70 has been difficult. Although some potential ZAP-70 inhibitors have been described (Hirabayashi et al. 2009), their specificity and potency in T cells or in vivo have not been shown.

In the absence of an available inhibitor, our lab has engineered a ZAP-70 mutant that retains catalytic activity, yet can be inhibited by an analog of the kinase inhibitor PP1. This chemical-genetic approach takes advantage of the presence of a conserved, bulky “gatekeeper” residue within the ATP-binding region of tyrosine kinases. Mutation of the bulky gatekeeper residue to glycine or alanine allows the

mutant kinase to accommodate a larger analog of PP1, one that would be too large to inhibit the WT kinase (Bishop et al. 1998). This technique has allowed our lab to study the requirements for ZAP-70 kinase activity by T cells for productive TCR signaling leading to T-cell activation.

An analog-sensitive ZAP-70 (ZAP-70^{AS}) mutant was generated by mutating the gatekeeper residue M414, to alanine, along with a second mutation, C405V, to stabilize the ATP-binding domain (Levin et al. 2008) (Fig. 5). Expression of ZAP-70^{AS} in ZAP-70 deficient P116 Jurkat T cells showed that, indeed, expression of ZAP-70^{AS} could reconstitute TCR signaling. Additionally, stimulation of ZAP-70^{AS}-expressing Jurkat cells in the presence of the inhibitor 3-MB-PP1 resulted in a rapid, dose-dependent inhibition of downstream signals, including phosphorylation of LAT and SLP-76, as well as impaired calcium mobilization and ERK phosphorylation. Furthermore, the ZAP-70^{AS} system showed the dynamic role of ZAP-70 catalytic activity in the regulation of the calcium flux, as inhibitor addition at the plateau of the calcium response resulted in a rapid decrease in intracellular free Ca²⁺ down to basal levels within seconds.

These studies further highlight the critical importance of ZAP-70 kinase activity in the transduction of signals from the TCR. In the future, it will be of great interest to test the ZAP-70^{AS} system in mature, primary T cells. Such experiments will enable investigation of whether ZAP-70 activity is required for naïve, effector, and memory T-cell responses. Additionally, using the ZAP-70^{AS} system may eventually allow us to model the effects of a ZAP-70 inhibitor in pathological T-cell responses in vivo. Until then, however, the analog-sensitive approach could be applied to the study of other signaling kinases to analyze the temporal requirements for kinase activity in TCR signal transduction and T-cell activation.

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H. Wang et al.

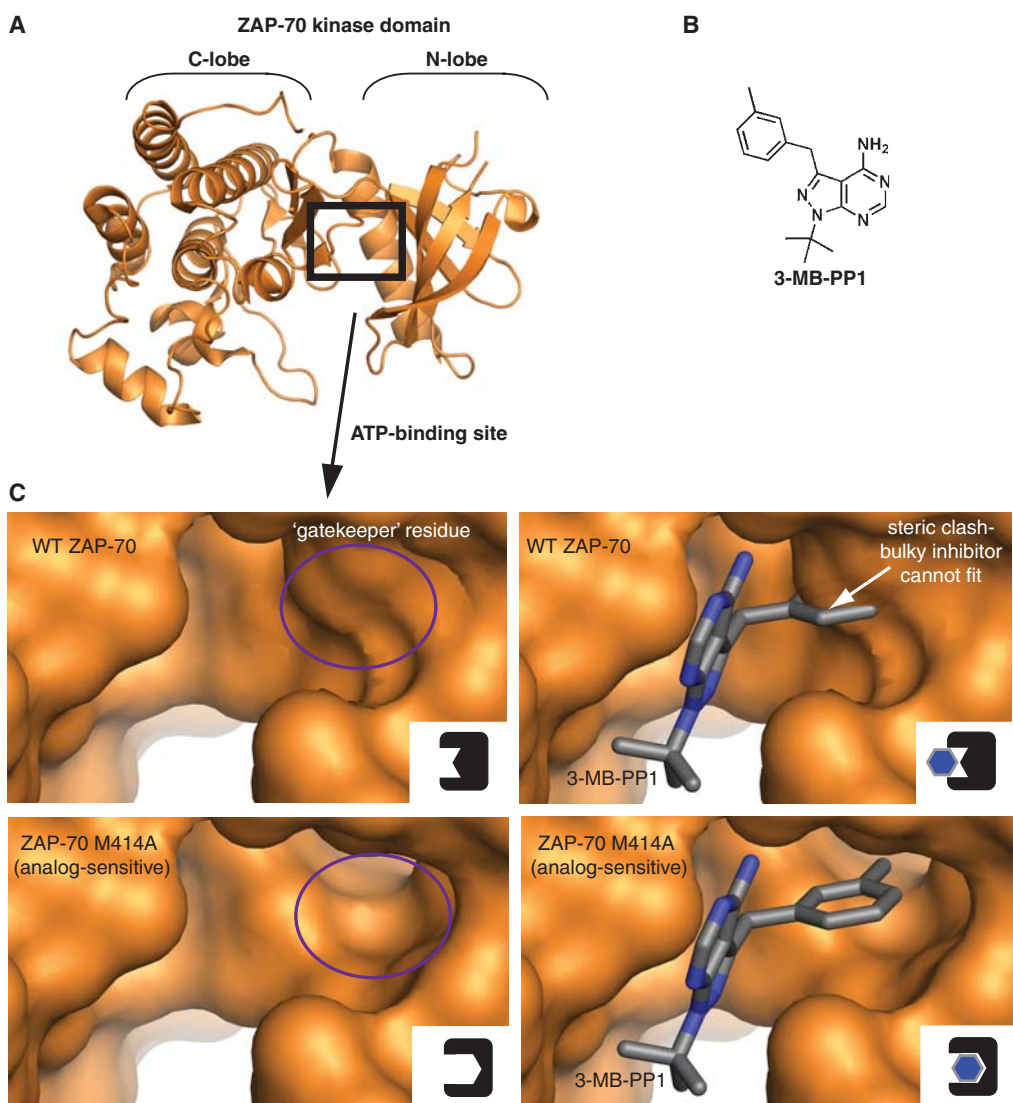


Figure 5. Chemical-genetic strategy to generate an inhibitor-sensitive ZAP-70. (A) Structure of the ZAP-70 kinase domain (PDB code 1U59). C- and N-lobes are indicated. The ATP-binding site is boxed. (B) Chemical structure of the inhibitor 3-MB-PP1. (C) Schematic of the analog-sensitive strategy. *Left*, Diagrams show the WT (*top*) and analog-sensitive mutant (*bottom*) ZAP-70 ATP-binding regions, modeled using PyMol. Circles identify the region of the gatekeeper residue. *Right*, Diagrams show the model of 3-MB-PP1 binding within the WT (*top*) and analog-sensitive mutant (*bottom*) ZAP-70. The presence of the “gatekeeper” residue in the ATP-binding site of WT ZAP-70 prevents the binding of the bulky 3-MB-PP1 inhibitor. In the analog-sensitive ZAP-70 mutant (*bottom*), the enlarged ATP-binding pocket allows binding of 3-MB-PP1. The 3-MB-PP1 model is derived from the crystal structure of the kinase domain of cryptosporidium parvum calcium dependent protein kinase in complex with 3-MB-PP1 (PDB code 2WEI). Top, white arrow points to the steric clash between 3-MB-PP1 and the gatekeeper residue, indicating that 3-MB-PP1 is not compatible with binding to WT ZAP-70.

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H. Wang et al.

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