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# Autoinhibition and adapter function of Syk

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**Summary:** Development, survival, and activation of B lymphocytes are controlled by signals emanating from the B-cell antigen receptor (BCR). The BCR has an autonomous signaling function also known as tonic signaling that allows for long-term survival of B cells in the immune system. Upon binding of antigen to the BCR, the tonic signal is amplified and diversified, leading to alteration in gene expression and B-cell activation. The spleen tyrosine kinase (Syk) intimately cooperates with the signaling subunits of the BCR and plays a central role in the amplification and diversification of BCR signals. In this review, we discuss the molecular mechanisms by which Syk activity is inhibited and activated at the BCR. Importantly, Syk acts not only as a kinase that phosphorylates downstream substrates but also as an adapter that can bind to a diverse set of signaling proteins. Depending on its interactions and localization, Syk can signal opposing cell fate decisions such as proliferation or differentiation of B cells.

Keywords: *adapters, lymphocyte signaling, pre-B-cell receptor signaling, protein kinases, autoinhibition, immunoreceptor tyrosine-based activation motif*

## Introduction

B cells are part of the adaptive arm of the immune system and produce antibodies that establish humoral immunity against pathogens. B cells sense intruding pathogens through the B-cell antigen receptor (BCR), which is expressed in large numbers on the surface of mature B cells. The BCR is composed of the membrane-bound immunoglobulin (mIg) molecule and a heterodimer of the Igα (CD79a) and Igβ (CD79b) protein (1–3). Antigen binding to the mIg molecule induces an alteration of the BCR complex that is transmitted via the Igα/Igβ heterodimer to the cytoplasmic side, leading to the activation of intracellular signaling proteins. Essential for the signaling function of the heterodimer are the highly evolutionarily conserved cytoplasmic tails of Igα and Igβ. Both Igα and Igβ contain two tyrosines each in a particular sequence motif, $D/EX_2YXXL/IX_{7-10}YXXL/I$, known as immunoreceptor tyrosine-based activation motif (ITAM) (4). An ITAM sequence is part of many activating receptors of the Ig superfamily as well as several members of the integrin family

(5). The phosphorylation of the two ITAM tyrosines creates docking sites for the tandem Src homology 2 (SH2) domains of Syk family kinases. In this way, Syk can be recruited to the BCR and initiate several signaling pathways. For example, by phosphorylating the coreceptor CD19 and the adapter protein BCAP [B-cell adapter for phosphoinositide 3-kinase (PI3K)], Syk activates the PI3K pathway that controls proliferation and survival of B cells (6–8). Another well-known substrate of Syk is the adapter protein SH2 domain-containing leukocyte protein of 65 kDa (SLP-65), also known as B-cell linker (BLNK) or B-cell adapter containing SH2 domain (BASH) (9–11). Upon phosphorylation of several tyrosines in SLP-65 by Syk, the SH2 domains of Vav, Bruton's tyrosine kinase (Btk), and phospholipase C γ (PLCγ) can bind to the adapter (12). In this way, SLP-65 provides a platform for the organization of a signalosome leading to activation of downstream signaling and $Ca^{2+}$ release and the further differentiation of developing B cells (13).

The BCR and its signaling elements Syk and SLP-65 not only play an essential role in B-cell activation but also are required for B-cell development in the bone marrow (14–16). This aspect of BCR function has been extensively studied by knockout (KO) experiments of many BCR-signaling components. Our laboratory has supported this approach by the generation of the mb-1/Cre mouse line that allows the deletion or activation of floxed genes in early pro-B cells (17). However, these loss-of-function studies rarely give information about the molecular details of the regulation and activation of BCR-signaling proteins. To gain more insight into the mechanistic aspect of BCR signaling, we have developed, over the last decade, a synthetic biology approach that allows the reconstitution of minimal functional BCR-signaling systems in the genetically distant environment of the S2 *Drosophila* cell line. In the S2 system, we co-express the BCR and its signaling molecules in different combinations for only a few hours and monitor their co-localization inside the cell as well as their signaling activity and stability. The transient expression of these signaling proteins avoids any interference with the growth and survival program of the transfected S2 cells, which is a major problem with any B-cell transfectants. In combination with an extensive mutational analysis, the S2 cell system is a powerful approach to gain rapid insights into fundamental aspects of cellular signaling. For example, with this approach we have learned that the initiation and amplification of the BCR signal is regulated by a critical kinase–phosphatase equilibrium (18). Recent reviews have covered in detail the signaling pathways from the BCR as well as the structure and modifications of Syk (19–21). In this review, we focus on the allosteric regulation of Syk and the different activation states of this kinase ranging from autoinhibition in resting B cells, activation and amplification during signal initiation, sustained activity as part of different signalosomes, and finally the termination of Syk activity through degradative and non-degradative mechanisms.

## Expression and domain structure of Syk

Syk was initially identified as a 40 kDa protein with intrinsic kinase activity in lysates of bovine thymi and porcine spleens (22, 23). Later, it was found that the 40 kDa protein is a proteolytic fragment of Syk containing only the catalytic domain, whereas full-length Syk, is a 72 kDa multidomain protein (24). Syk is expressed in all cells of the hematopoietic cell linage and also in non-hematopoietic cells such as epithelial cells, fibroblasts, and neuronal and vascular endothelial cells (25). Defects in endothelial cells and vasculature are the reasons why Syk-deficient mice die prenatally by hemorrhaging, since Syk function in these cells is required for maintenance of vascular integrity (15, 26). Syk is activated downstream of ITAM-containing receptors in lymphocytes, mast cells, macrophages, dendritic cells, and natural killer cells (27–29). Reconstitution of irradiated mice with fetal livers from $Syk^{-/-}$ mice shows that B-cell development is blocked at the pro- to pre-B-cell transition resulting in reduced numbers of mature B cells (15, 26).

Syk is a multidomain protein that has two tandem SH2 domains and a kinase domain followed by a short C-terminal tail (*Fig. 1A*). The SH2 domains are connected to each other via a linker called interdomain A. The SH2 domains of Syk are separated from the kinase domain by a flexible 120 amino acid long linker termed interdomain B (30). The sequence of interdomain A and B contains several tyrosines that, upon Syk activation, become phosphorylated and play an important role in the regulation and activity of Syk (see below). These tyrosines include Y131 of interdomain A, Y323, Y348, and Y352 of interdomain B, and Y629-631 at the short C-terminal tail of Syk (numbering based on human Syk sequence) (31, 32). Several mammalian species, including mouse and humans, express an alternatively spliced form of Syk (SykB) that has a deletion of 23 amino acids at the beginning of interdomain B (33). A comparison of the function of Syk with SykB suggests that SykB binds less efficiently to phosphorylated ITAMs (34). The Syk family kinase ζ-associated protein of 70 kDa (ZAP-70) has a similar domain structure similar to Syk and also carries regulatory tyrosines in the interdomain A and B. Interestingly, ZAP-70 has a shorter interdomain B and is in this aspect more similar to

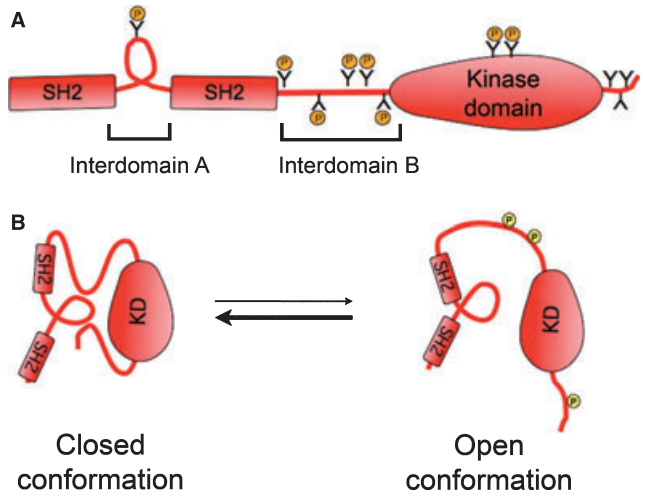


**Fig. 1. Domain organization and autoinhibition of Syk.** (A) Schematic representation of the modular domains and phosphorylated tyrosines in Syk. Protein domain structure of human Syk showing the two Src homology 2 (SH2) domains at the N-terminus separated from the catalytic domain by a flexible linker called interdomain B. The linker separating the two SH2 domains is termed interdomain A. Positions of tyrosines in Syk that are reported to be phosphorylated are indicated. (B) Model showing autoinhibited and active conformations of Syk. Intermolecular interactions mediated by the SH2 domains, interdomain A, interdomain B, and the kinase domain maintain the kinase in an inhibited conformation. Disruption of the inhibitory interactions results in an active conformation. In resting cells, Syk exists in a dynamic equilibrium between the two conformations.

SykB than to Syk (33). In contrast to Syk, the expression of ZAP-70 is more restricted to T cells and early pre-B cells, although some B-cell tumor cells such as B-CLL (B-cell chronic lymphoblastic leukemia) cells express ZAP-70 (35, 36). Syk is localized mostly in the cytosol, but a fraction of Syk is also found in the nucleus (37). Upon BCR activation, Syk translocates to the plasma membrane where it associates with the BCR. However, Syk is excluded from the nucleus at later time points following BCR triggering (37). How exactly Syk shuttles between the nucleus and the cytoplasm is not known but appears to be regulated by interdomain B (38).

### Autoinhibition of Syk family kinases

The activity of signaling proteins inside the cell is tightly regulated in time and space. Most of these proteins are maintained in an inactive state in resting cells and only become active for a short time at the right subcellular location in activated cells. This tight regulation of signal activity is often achieved by autoinhibition. In the autoinhibited state, the different domains of a signaling protein inhibit each others' function by a network of intermolecular contacts (39). A recently published crystal structure of the Syk family kinase ZAP-70 revealed, in molecular detail, the autoinhibitory interactions of the ZAP-70 domains (40). In this structure, a conserved tryptophan (W131) in interdomain A is sandwiched between two tyrosines (Y315 and Y319) of interdomain B that also makes a hydrophobic contact to a proline residue (P396) in the αC-β4 loop of the kinase domain. These amino acids form an interface called the 'linker–kinase sandwich' that is stabilized through aromatic–aromatic residue interactions. This inhibitory sandwich is further stabilized by an interaction of P147 of interdomain A with two tyrosines (Y597 and Y598) at the C-terminal tail of the ZAP-70 kinase domain. Importantly, in the autoinhibitory structure of ZAP-70, the tandem SH2 domains are not properly aligned for ITAM binding and the kinase domain is distorted in a way that does not favor the phospho-transfer reaction. Furthermore, several tyrosines that become phosphorylated upon ZAP-70 activation and play an important role for the adapter function of this kinase are part of, or close to, the linker–kinase sandwich and are not available for phosphorylation in this conformation. Thus, all three functions, SH2 domain binding, kinase, and adapter function of ZAP-70, are inhibited by the autoinhibitory structure.

Although the complete structure of Syk has not yet been determined, it is very likely that the structure is similar to that of ZAP-70. All the amino acids involved in autoinhibition of ZAP-70 are found at identical positions in Syk, indicating that the two kinases may be regulated in a similar manner. In the S2 system, it was found that either the engagement of the SH2 domains or the deletion of the tandem SH2 domains increases the kinase activity of Syk (18, 41). Interestingly, in the autoinhibitory structure, the N- and C-terminus of ZAP-70 are both situated in close proximity to the linker–kinase sandwich. A similar structural arrangement of Syk may explain why fusion proteins of Syk carrying either a green fluorescence protein (GFP) domain or an affinity tag at the N- or C-terminus are more active than wildtype Syk (42). The notion that a linker–kinase sandwich also regulates the activity of Syk is further supported by a mutational analysis of Syk (see below). However, it is feasible that Syk can form alternative autoinhibitory structures. For example, a Syk deletion mutant that lacks the first SH2 domain and the interdomain A is still autoinhibited, although this mutant should not be able to form the linker–kinase sandwich in a way described for full-length ZAP-70 (41).

Autoinhibitory structures are known from several kinases including the cyclin-dependent kinases, Btk, the Abelson kinase, and several Src family kinases (43, 44). Similar inhibitory structures are also found in the case of protein tyrosine


 

phosphatases such as SHP-1 (SH2 domain-containing phosphatase 1) and SHP-2 and thus seem to be a general mechanism to regulate activity of signaling enzymes (45, 46). The same maybe true for adapter proteins that only have binding capability but no catalytic activity. However, very few structures of adapter proteins exist, and this may indicate that these proteins are more flexible and can adopt many different conformations. Furthermore, the state of inhibition and activation of a signaling protein may be determined by post-translational modifications and interaction partners rather than by intramolecular interactions, as has been found for the regulation of B-Raf (47).

### Activation of Syk at the ITAM sequence

As discussed above, most Syk molecules in the cytosol of resting cells are likely to assume a closed autoinhibited structure, thus ensuring that Syk does not phosphorylate proteins at random. The equilibrium between closed and open Syk is thus shifted toward autoinhibition (*Fig. 1B*). Indeed, Syk is not a very active kinase when expressed in S2 cells together with only its substrate, the adapter protein SLP-65. A shift in equilibrium toward the activated state of Syk is an essential event during the initiation of BCR signaling. To reach a stable open conformation, Syk requires an allosteric activator, and this is the ITAM sequence. When a Syk molecule adopts an open conformation, its kinase and SH2 domain-binding function are simultaneously active for a few milliseconds (*Fig. 2*). If this happens in the proximity of an accessible ITAM sequence, Syk can phosphorylate the two ITAM tyrosines and bind to the dual phosphorylated ITAM (ppITAM). This first Syk/ppITAM complex that is formed at antigen-bound BCR has the role of an initiator. In this complex, the tandem SH2 domains in Syk bind to the ppITAM sequence in a head-to-tail fashion, such that the C-terminal SH2 domain of Syk binds to the first ITAM tyrosine. This places the kinase domain of Syk in the active conformation, close to the membrane. Active Syk then rapidly phosphorylates neighboring ITAM sequences resulting in more Syk binding and ITAM phosphorylation and thus the amplification of the BCR signal. Syk is a potent ITAM kinase, as has been shown by an *in vitro* kinase assay which also demonstrated that not only the kinase but also the SH2 domains are required for this function (18). It has been shown that Syk phosphorylates both ITAM tyrosines. The Src family kinase Lyn, in contrast, phosphorylates predominantly only the first ITAM tyrosine (18, 48, 49). In this way, Lyn could help more in the initiation rather than in the amplification process of the Syk/ITAM-signaling. However, it is unknown how BCR engagement results in Lyn activation, as this process requires dephosphorylation rather than the phosphorylation of Lyn.

The initiation and amplification process at the BCR is under the tight control of tyrosine phosphatases such as SHP-1. An active SHP-1 molecule can dephosphorylate a ppITAM more rapidly than Syk can phosphorylate the ITAM sequence, and thus neither initiation nor amplification of a BCR signal can occur. BCR activation requires not only the activation of kinases but also the inhibition of phosphatases at the BCR, and this

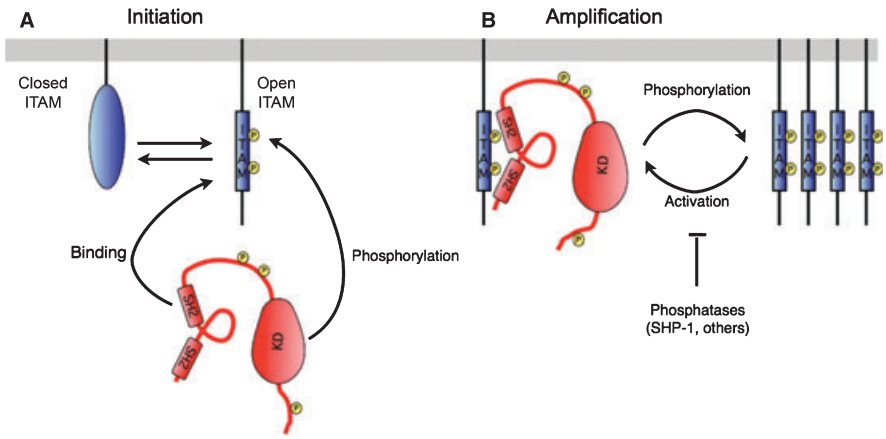


***Fig. 2.* Initiation and amplification of BCR signaling.** (A) The cytoplasmic tails of Igα and Igβ in the resting BCR exist as a compact structure where the immunoreceptor tyrosine-based activation motif (ITAM) tyrosines are not accessible. Conformational changes upon antigen binding are transmitted to the cytoplasmic tails of Igα and Igβ, resulting in a relaxed conformation where the ITAM tyrosines are accessible. The Src homology 2 (SH2) domains of Syk bind to the ITAM tyrosines and in turn get activated. (B) Active Syk phosphorylates neighboring ITAMs and laterally propagates amplification of BCR signals. Phosphatases such as SHP-1 negatively regulate BCR activation by dephosphorylating the BCR and associated molecules. This sets a threshold and amplification of the BCR signal by Syk is a crucial step to counter the negative effect of SHP-1 and cross the threshold to result in BCR activation.

could be achieved via the production of radical oxygen species in activated B cells (50, 51).

The duration of the Syk/ppITAM complex at the active BCR could also be limited by means other than an SHP-1-mediated dephosphorylation of the ppITAM tyrosines. For example, the interdomain A of Syk contains a regulatory tyrosine (Y131) that is autophosphorylated upon Syk activation at the BCR. When Y131 is phosphorylated, this disrupts the binding of the SH2 domains to the ppITAM (52). Substituting Y131 with an acidic amino acid (i.e. E131) also reduces the binding affinity of the tandem SH2 domains of Syk for ppITAMs. A systematic study employing biophysical methods revealed that the Y131E mutant of Syk has a disordered interdomain A structure that is not compatible with pITAM binding by its SH2 domains (53). Thus, the phosphorylation of Y131 is a mechanism to switch off Syk activity at the BCR. Furthermore, the phosphorylation of Y131 also results in enhanced kinase activity of Syk, probably by interfering with the formation of the proper linker–kinase sandwich in the autoinhibitory structure of Syk. When phosphorylated on Y131, Syk can remain active in an ITAM-independent manner. Indeed, the SykY131E mutant has been shown to upregulate integrin signaling in B cells independent of BCR engagement (54) and to localize to centrosomes in breast cancer cells (55). Another way to limit the duration of the Syk/ppITAM complex at the active BCR is through the binding of the SH2 domain of SLP-65 to phosphorylated Y630 at the C-terminal tail of Syk (42). Interestingly, this binding also interferes with the autoinhibition of Syk and ensures that, in the Syk–SLP65 complex, Syk remains active. The phosphorylation of Y630 may thus initiate the switch from a Syk/ppITAM complex to a Syk–SLP65 complex with different signaling functions (see below).

### ITAM-independent activation of Syk by membrane targeting

Syk loses from the autoinhibited conformation when it binds to the ppITAM sequence, as described above. Thus, membrane localization and activation of Syk occur in a single process. The Src family kinase Lyn, in contrast, is stably bound to the plasma membrane via myristoylation and palmitoylation anchors that are appended to the N-terminus of this kinase. In the S2 cell reconstitution system, Syk is active only in the presence of the BCR, whereas Lyn is constitutively active and is strongly autophosphorylated (18). To study the signaling function of a Syk mutant that is constitutively bound to the plasma membrane, we expressed an nLyn–Syk fusion protein that carries the N-terminal membrane anchor of Lyn in S2 cells and compared its activity to wildtype Syk (*Fig. 3A*). In comparison to Syk, the nLyn–Syk fusion protein is expressed at higher levels, indicating that the N-terminal membrane anchor protects Syk from degradation (*Fig. 3B*, panel 3). Furthermore, nLyn–Syk is constitutively active in S2 cells, and its activity is not increased much in the presence of the BCR. This is indicated by a strong autophosphorylation as well as by the constitutive phosphorylation of the Syk sub-

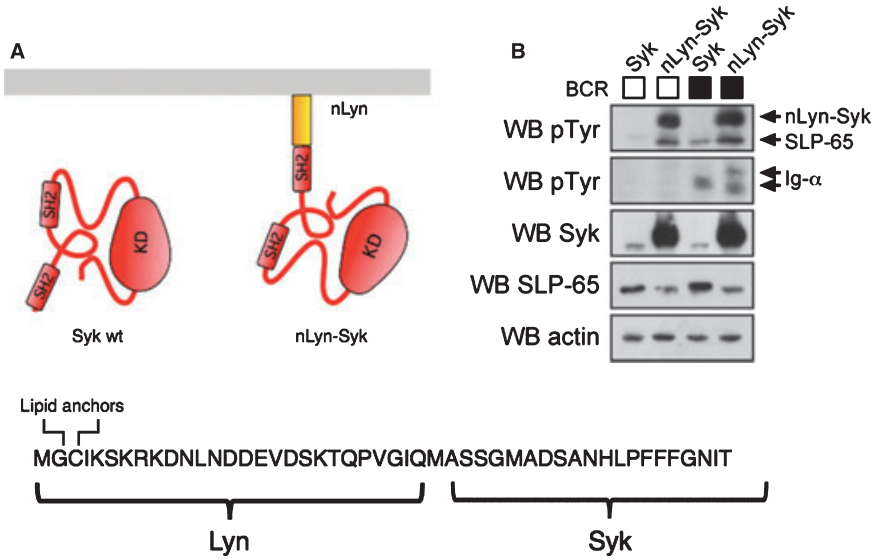


**Fig. 3. Membrane targeting activates Syk independent of the B-cell antigen receptor (BCR).** (A) Targeting Syk to the membrane by fusing the first 24 amino acids of Lyn containing the myristoylation and palmitoylation signals to the N-terminus of Syk. (B) Western blot analysis of tyrosine phosphorylation in S2 cell lysates expressing Syk or nLyn–Syk in combination with SLP-65 either in the presence or absence of the BCR. Overall tyrosine phosphorylation (upper panel) and expression levels of Syk and Ig-α in the total cell lysates is shown.


 

strate protein SLP-65 (*Fig.* 3B, panel 1). Wildtype Syk, in contrast, phosphorylates SLP-65 only in the presence of the BCR (*Fig.* 3B, panel 1). Both Syk and nLyn–Syk phosphorylate the tyrosines of Ig-α (*Fig.* 3B, panel 2). However, if one considers the different expression levels of the two proteins, nLyn–Syk is not a very active ITAM kinase, which could indicate that most of the nLyn–Syk is not located in the proximity of the BCR but may reside in a raft-like membrane microdomain where Src family kinases are also found (56).

### Regulation of Syk kinase activity by interdomain B tyrosines

Many of the phosphorylated tyrosines in Syk are sites of protein interactions that are important to connect Syk to downstream signaling. Interestingly, some of these tyrosines are situated in regions that regulate the activity of Syk. Phosphorylation of tyrosines disrupts autoinhibitory interactions and results in an open active conformation of Syk. Using single-particle electron microscopy, it was shown that autophosphorylation results in conformational changes in regulatory regions of Syk (57). Indeed, in an *in vitro* assay system comparing kinase activities, autophosphorylated Syk shows higher kinase activity and an increased initial rate of activity as compared to dephosphorylated Syk (58). The binding of proteins to these phosphorylated tyrosines in Syk can also disrupt inhibitory intermolecular contacts within Syk. Moreover, these phosphorylated tyrosines are now shielded from phosphatases and cannot be dephosphorylated, thus stabilizing the kinase in a signaling competent active conformation.

The constitutive and ITAM-independent activity of membrane-targeted nLyn–Syk allowed us to study the amino acids involved in the regulation of Syk activity. As described above, two tyrosines of the interdomain B play an essential role in the formation of the autoinhibitory linker–kinase sandwich of ZAP-70. Mutation of these tyrosines to phenylalanine increases the autoinhibition state of ZAP-70 (59). We have mutated the two regulatory tyrosines (Y348 and Y352) of the interdomain B of human Syk (2Y) to either phenylalanine (2F) or to glutamic acid (2E) and compared the activity of mutated and unmutated nLyn–Syk in the S2 system (*Fig.* 4*A*,*B*). The nLyn–Syk2F mutant lost constitutive activity but can still be activated by the BCR (*Fig.* 4C, panel 1, lanes 2 and 5). On the contrary, nLyn–Syk2E is more active in phosphorylating the adapter protein SLP-65 than unmutated nLyn–Syk. However, similar to nLyn–Syk2F, the nLyn–Syk2E mutant is less phosphorylated, suggesting that Y348 and Y352 of the interdomain B are dominant autophosphorylation sites of this

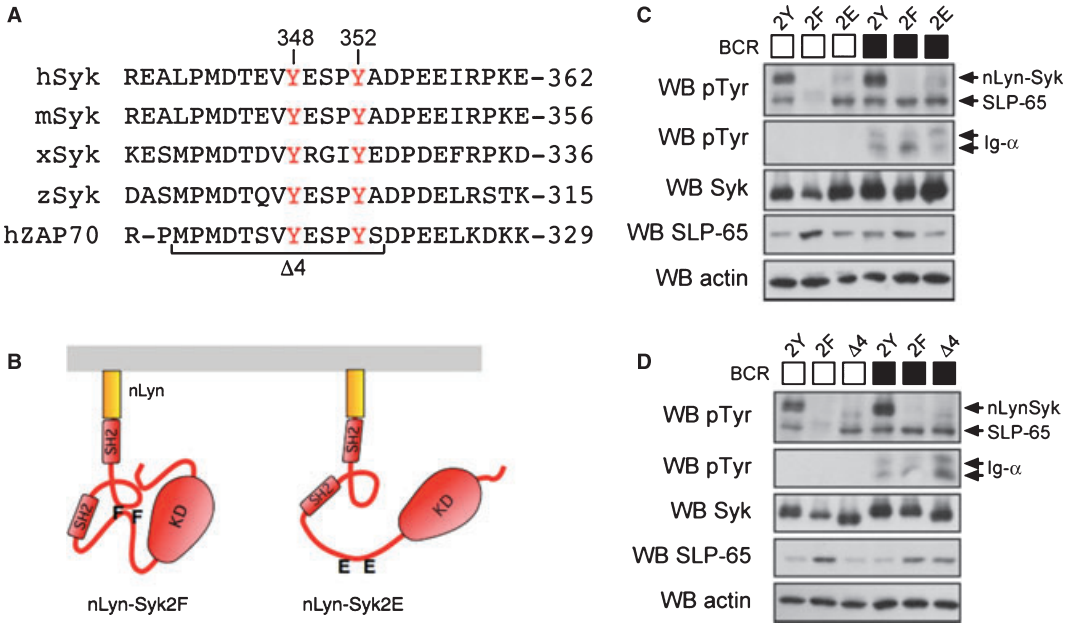


***Fig. 4.* Regulation of Syk kinase activity by conserved interdomain B tyrosines.** (A) Sequence alignment of region of interdomain B containing the tyrosines 348 and 352 (highlighted in red) of Syk and its homologue ZAP-70, from human, mouse, frog, and fish is shown. (B) Membrane targeted Syk as a tool to study allosteric regulation of Syk. Schematic representation depicting nLyn–Syk with interdomain B tyrosines Y348 and 352 mutated to phenylalanine (2F) or glutamate (2E). (C) Tyrosine phosphorylation was analyzed in lysates of S2 cells transiently transfected with SLP-65 and nLyn Sykwt or 2F or 2E mutants, either in the absence or presence of the BCR (Igα, Igβ, scδm). (D) Western blot analysis of tyrosine phosphorylation in S2 cell lysates expressing SLP-65 and nLyn–Syk or nLyn–SykD4 carrying a deletion of amino acids 341–353, in the absence or presence of the BCR. Western blot against Syk and SLP-65 is shown in the bottom panels.

kinase. In their unphosphorylated state, these tyrosines play a role in inhibiting Syk activity. This conclusion is supported by the analysis of a deletion mutant (Δ4) of nLyn–Syk lacking 13 amino acids in interdomain B that contain the two regulatory tyrosines (*Fig. 4A*). The nLyn–SykΔ4 mutant behaves similarly to the nLyn–Syk2E mutant, indicating that this part of interdomain B is involved in the formation of an autoinhibitory linker–kinase sandwich (*Fig. 4D*).

To study the regulation of Syk in B cells, we started with an Ig-$\alpha^{-/-}$Syk$^{-/-}$ double KO pre-B-cell line and generated an IgM–BCR-expressing B-cell line. This line was further transduced with either an IRES–GFP (pMIG as control) or Syk–IRES–GFP-containing retroviral vectors (42). We then compared the function of wildtype Syk with Syk mutants carrying alterations (F or E) of the two regulatory tyrosines (Y348 and Y352) of the interdomain B of Syk. All transfectants expressed the same amount of IgM–BCR except for the Syk2E transfectant that expressed reduced levels of IgM on their surface (*Fig. 5A*, green line). Western blot analysis shows that the Syk2E mutant is expressed at lower levels compared to Syk or Syk2F in the transfected B-cell lines (*Fig. 5B*, panel 2). Furthermore, the Syk2E transfectant already displays some Akt phosphorylation in unstimulated cells (*Fig. 5B*, lane 10), whereas the other two Syk transfectants [Syk wildtype (Sykwt) and Syk2F] only phosphorylate Akt upon the stimulation of the B cell with anti-IgM antibodies (*Fig. 5B*). In the absence of Syk expression (pMIG control), these B cells display only a marginal $Ca^{2+}$ influx in response to anti-IgM antibodies (*Fig. 5C*). This background response maybe due to the presence of ZAP-70, which is weakly expressed in these pre-B cells. Transfection of these cells with the Syk vector restores the $Ca^{2+}$ response (*Fig. 5C*, upper panel). In comparison to Sykwt, the Syk2F mutant displays a delayed and impaired $Ca^{2+}$ response (*Fig. 5C*, lower panel). The delayed response can be explained by increased autoinhibition in the Syk2F mutant. Surprisingly, the Syk2E mutant, which has higher kinase activity, also produces an impaired $Ca^{2+}$ response with lower amplitude in comparison to Sykwt. The reduced $Ca^{2+}$ response can be explained by the lower expression level of the BCR and the Syk2E protein and/or by the anergic state of the transfected B cells. Indeed, anergic B cells are known to have reduced surface IgM expression levels and a reduced $Ca^{2+}$

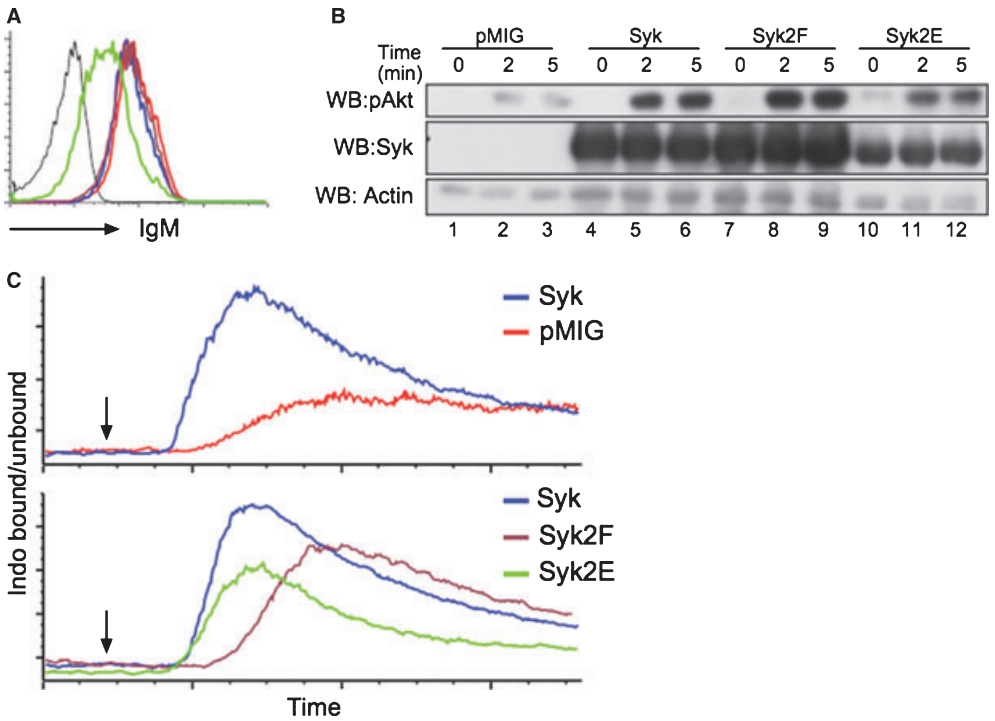


***Fig. 5. Interdomain B regulates kinase activity and B-cell signaling responses.*** (A) Ig$\alpha^{-/-}$Syk$^{-/-}$ pro-B cells were reconstituted with μHC, λLC, and Igα to express a B-cell antigen receptor (BCR) in the cells. These cells were transduced with IRES–GFP-containing retroviral vectors encoding for Syk or Syk2F or Syk2E and enriched for GFP expression. Expression of BCR on the surface of pMIG (red curve), Syk (blue curve), Syk2F (brown curve), and Syk2E (green curve) expressing cells are shown. The black curve represents parental cells that do not express a BCR. (B) Reconstituted BCR-expressing cells were stimulated with 10 μg/ml of anti-IgM for 0, 2, and 5 min. Total cell lysates were separated by 10% SDS gels, and Western blot analysis was performed for Akt phosphorylation at S473, Syk expression, and actin. (C) BCR crosslinking-mediated intracellular $Ca^{2+}$ mobilization as indicated by the Indo-1 ratio as a function of time is shown. The stimulus was added after 1 min (indicated by arrow) and measured for 5 min.


 

response (60). Clearly, in B cells, the Syk2E mutant is hyperactive and the Syk2E-transfectants display an anergic phenotype. The Syk2E-transfected B cells could thus be used as a model system to study signaling in anergic B cells.

Syk not only plays an important role in transmitting maintenance signals and activation of the BCR, but it is also required for the function of the pre-BCR. The pre-BCR comprises a membrane-bound μ chain, the surrogate light chain (LC) proteins (λ5 and VpreB), and the Igα and Igβ proteins (61, 62). Expression of the pre-BCR is an important checkpoint in the transition from the pro-B to the pre-B-cell stage, which requires a productive Ig heavy chain gene rearrangement (63–65). The newly expressed pre-BCR induces proliferation as well as differentiation and the kinase activity of Syk is involved in the two opposing signal programs (66). Indeed, in the absence of Syk, B-cell expansion and development are blocked at the pre-B-cell stage (15).

To study the function of Syk interdomain B in pre-B cells, we introduced different retroviral IRES–GFP vectors in Syk$^{-/-}$ pre-B cells growing in culture with interleukin-7 (IL-7). In particular, we wanted to compare the interaction of the Syk2F and Syk2E mutant with the pre-BCR (*Fig. 6A*). The expression of Sykwt or Syk2E resulted in the downregulation of pre-BCR from the cell surface (*Fig. 6B*, 2nd column). This downregulation is not seen in Syk2F-expressing cells, suggesting that this mutant interacts less well with the pre-BCR. Withdrawal of IL-7 from the pre-B-cell culture results in differentiation, as measured by the expression of κ light chain on the surface of these cells. In comparison to GFP, only pre-B cells expressing Syk show an enhanced capacity to differentiate and the same was true to a lower extent for the Syk2F-expressing cells (*Fig. 6B*, 3rd column). The Syk2E-expressing pre-B cells, however, do not differentiate at all upon IL-7 withdrawal but continue to proliferate (*Fig. 6B*, 3rd column). This important finding suggests that the switch from proliferation to differentiation requires a downregulation of Syk kinase activity. Syk mutants with increased kinase activity strongly promote proliferation, and this could lead to oncogenic transformation of pre-B cells or other Syk-expressing cells. Indeed, Syk fusion proteins such as translocated ETS leukemia (TEL)–Syk have been discovered

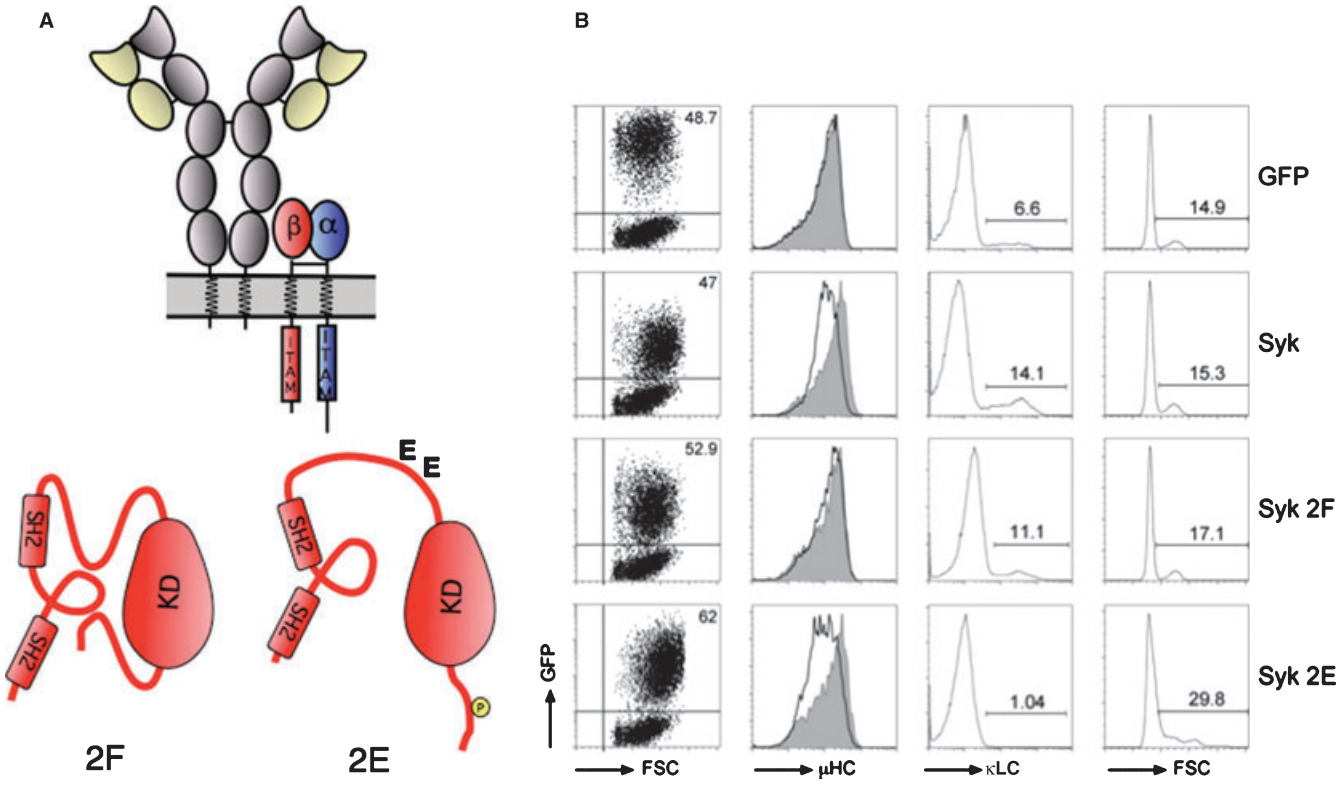


***Fig. 6.* Kinase activity and interactions of Syk with SLP-65 and PLC-γ2 regulate pre-B-cell proliferation and differentiation.** (A) Kinase activity of Syk regulates differentiation of pre-B cells. (B) Syk$^{-/-}$ pre-B cells were retrovirally transduced with IRES–GFP vectors encoding either Syk, Syk2F, or Syk2E. FACS profile of forward scatter (FSC) versus GFP is shown in the left panel. In the second panel, pre-BCR (μHC) surface expression of transduced cells (GFP$^+$; open curves) were compared to untransduced cells (GFP$^-$; filled curves) within the same culture, 48 h after transduction. In the third panel, expression of κ light chain (κLC) on the surface of transduced cells (GFP$^+$) measured 3 days after withdrawal of IL-7 from culture is shown. In panel 4, FSC profile of the same cells is shown.

in tumor cells (67). These oncogenic forms of Syk can drive proliferation and allow the expansion of pre-B cells, even in the absence of IL-7 (68).

### Adapter function of interdomain B tyrosines of Syk

Upon activation, Syk becomes phosphorylated at several tyrosines that are crucial for the function of Syk in signaling. Tyrosines 296, 323, 348, and 352 in the interdomain B are phosphorylated either by autophosphorylation or by Src family kinases. Tyrosine 323 (Y317 in murine Syk) is one of the major sites of phosphorylation detected in B cells after BCR activation and this negatively regulates Syk activity (69). Reduced levels of Y323 phosphorylation are detected in Lyn-deficient B cells, implying that Y323 phosphorylation is mainly catalyzed by Src family kinases (31). Indeed, a kinase dead version of Syk is phosphorylated on Y323 when expressed in Syk-deficient cells. A mutation of Y323 to phenylalanine results in enhanced signaling and $Ca^{2+}$ responses upon BCR triggering (69, 70). The negative role of Y323 is mediated by the binding of the tyrosine kinase-binding (TKB) domain of the Ring-finger E3 ligases c-Cbl and Cbl-b to phosphorylated Y323 (*Fig. 7A*). This results in the attachment of polyubiquitin chains and proteasomal degradation of Syk (71, 72). However, experimental evidence also suggests that there are non-degradative functions for ubiquitylated Syk, since prolonged phosphorylation of Syk is observed in platelets deficient for c-Cbl (73). Y323 is also the docking site for the C-terminal SH2 domain of the p85 subunit of PI3K (74). Competition between the TKB domain of Cbl proteins and the SH2 domain of p85 for binding to Y323 is predicted to determine the role that this tyrosine plays in Syk function.

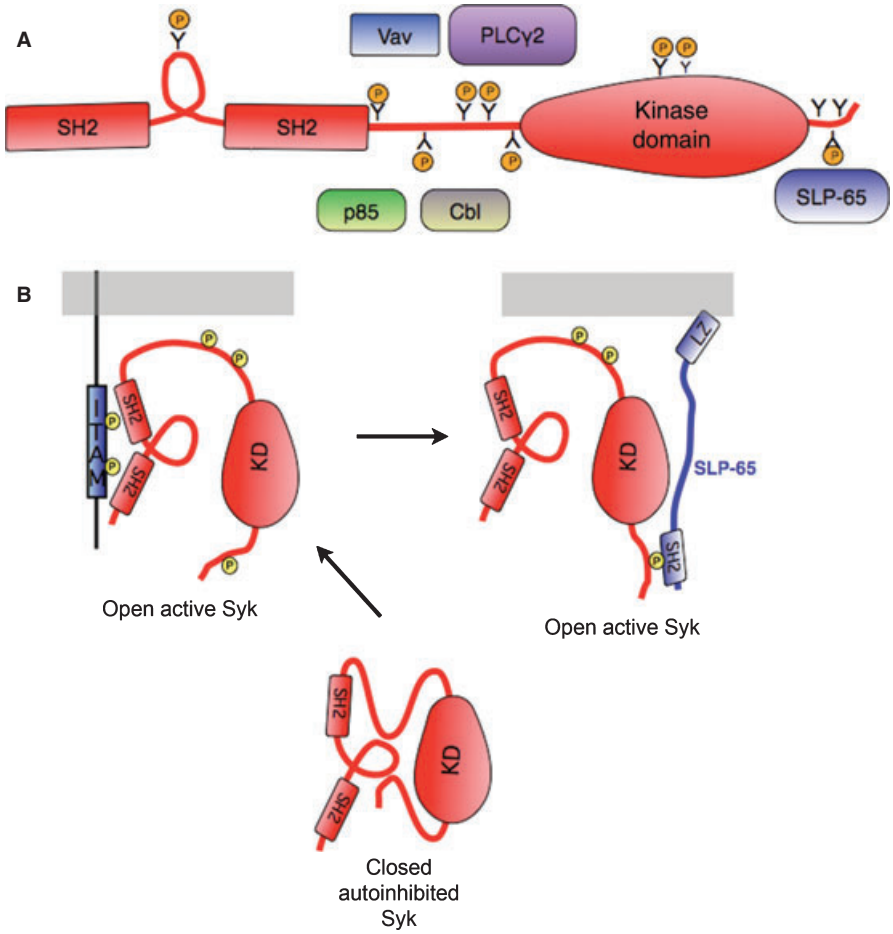


***Fig. 7.* Adapter function of Syk and the allosteric regulation of Syk by the BCR and SLP-65.** (A) Schematic representation showing sites of interaction of some of the signaling proteins binding to phosphorylated tyrsosines in interdomain B and the C-terminus of Syk. (B) In resting cells, Syk is in the cytosol in a closed autoinhibited conformation in a manner analogous to ZAP-70. In the presence of a phosphorylated ITAM, the Src homology 2 (SH2) domains bind to the ITAM tyrosines and Syk transitions to an open active conformation. When Y630 of Syk is autophosphorylated, the SH2 domain of SLP-65 can bind to it. This prevents the C-terminus of Syk from mediating inhibitory interactions and stabilizes Syk in an open active conformation.


 

The two regulatory tyrosines, Y348 and Y352, implicated in autoinhibition of Syk (see above), are primarily autophosphorylated (31). Phosphorylation of Y348 and Y352 creates binding sites for the SH2 domains of many signaling proteins including Vav, Grb2, p85 subunit of PI3K, and PLCγ (19, 75) (*Fig. 7A*). Of the two SH2 domains in PLC-γ2, only the C-terminal one binds to phosphorylated Y348 and Y352 (76). A structural study of the C-terminal SH2 domain of PLC-γ2 in complex with a Syk peptide including phosphorylated Y348 and 352 revealed that this SH2 domain has the unique ability to bind to two tyrosines at the same time (77). Mutation of Y348 and/or Y352 of Syk to phenylalanine greatly impairs receptor-mediated signaling (78). The Syk mutants are defective in coupling activation of various receptors to phosphorylation of PLCγ and to $Ca^{2+}$ mobilization (69, 78, 79). However, it is not always clear whether the phenotype of these mutations is caused by an alteration of autoinhibition or of the adapter function of Syk. The analogous tyrosines in the interdomain B of ZAP-70 are also important for TCR signaling and T-cell development (80–83). The notion that Syk and ZAP-70 can act as adapters is also supported by the finding that kinase negative mutants of these kinases are not inert but can exercise several signaling functions (84, 85).

### Adapter function of the C-terminal tyrosines of Syk

The C-terminal tail of ZAP-70 contacts interdomain A and plays an important role in stabilizing the autoinhibited conformation of the kinase. The two tyrosine residues (Y597 and Y598) in this region of ZAP-70 form a cleft in which a proline residue of interdomain A is inserted. The C-terminal tail of Syk carries three analogous tyrosines (Y629-Y631) of which Y630 and 631 are autophosphorylated. Using an antibody that recognizes phosphorylated Y630, we have shown, in S2 and in B cells (unpublished observations), that upon Syk activation this tyrosine becomes autophosphorylated (42). Phosphorylation of Y630 is likely to destabilize the inhibitory linker–kinase sandwich and disrupt autoinhibition of Syk. Indeed, a Y630F mutation of Syk that can no longer be phosphorylated at this site shows increased autoinhibition (86).

The Y630 is part of an YXDV sequence motif that is the preferred binding site for the SH2 domain of the SLP family of adapter proteins. We have shown, *in vitro* and in S2 cells, that the SH2 domain of SLP-65 binds to phosphorylated Y630 of Syk. This binding is not observed when a catalytically dead mutant of Syk is expressed in S2 cells, suggesting that this tyrosine is a site of autophosphorylation. When the SH2 domain of SLP-65 binds to Y630, it stabilizes Syk in an open, active conformation (*Fig. 7B*). Thus, SLP-65 is not only a major downstream substrate of Syk but also an activator that, via a positive feedback loop, increases the activity of Syk. However, in S2 cells, the co-expression of Syk and SLP-65 does not result in Syk activation. This activation requires the presence of a BCR carrying the allosteric activator of Syk, namely an ITAM sequence. Thus, SLP-65 alone cannot overcome the autoinhibited state of Syk and only binds to Syk when the kinase becomes active at the BCR. This is different in the case of a GFP–Syk fusion protein. Due to the N-terminally attached GFP, the fusion protein is less well autoinhibited than wildtype Syk. Therefore, the presence of SLP-65 alone is sufficient to counteract the weaker autoinhibition of GFP–Syk, as long as SLP-65 has a functional SH2 domain binding to pY630 (42). The C-terminus of ZAP-70 does not carry a YXDV motif and cannot bind to the T-cell adapter SLP-76. The SLP adapter–kinase complex is thus a feature specific for Syk.

### Modulation of pre-BCR signaling by the adapter function of Syk

The expression of a pre-BCR is an important checkpoint in the transition from the pro-B to the pre-B-cell stage. The pre-BCR displays a continuous and cell autonomous signaling behavior (66). Two alternative and opposing signaling pathways are emitted by the pre-BCR resulting either in the proliferation or the differentiation of pre-B cells (*Fig. 8*). Upon *de novo* expres-

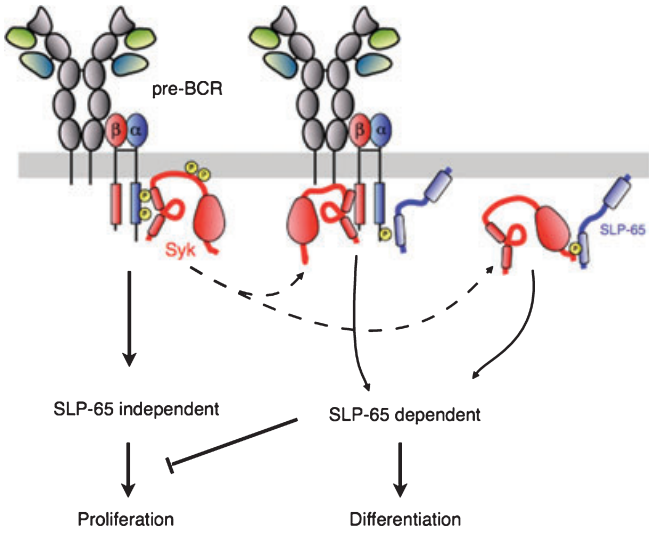


***Fig. 8.* Regulation of pre-B cell proliferation and differentiation by opposing signals from the pre-BCR.** Different signaling modes of the pre-BCR: Syk-dependent signals from the pre-BCR promote proliferation of pre-B cells. Syk that does not signal via SLP-65 emits these proliferation signals. When the Src homology 2 (SH2) domain of SLP-65 binds to Y204 of Igα and Y630 of Syk, Syk-mediated signals are now transmitted over SLP-65 (SLP-65-dependent signaling). The SH2 domain of SLP-65 controls activation of differentiation.

sion of their receptor, the pre-B cells first undergo several rounds of proliferation and then switch on the differentiation program. Recent studies have shown that the proliferation and survival of pre-B cells are controlled by PI3K signaling and that Syk is involved in the activation of this signaling pathway (6, 8, 87, 88). The co-receptor CD19 plays an important role in the activation of PI3K in B cells (89, 90). The kinases Syk and Lyn, activated at the pre-BCR, mediate phosphorylation of CD19 and thus synergize in the activation of PI3K. The adapter protein BCAP also supports the recruitment and activation of PI3K in pre-B cells (7). Complete loss of PI3K activation is observed in double ($CD19^{-/-}BCAP^{-/-}$) KO DT40 B cells, and double KO mice display a severe block of B-cell development (91). The phosphorylation of tyrosines in the cytoplasmic tail of CD19 generates binding sites for the SH2 domains of signaling molecules such as Lyn, Grb2, Vav, PLC-γ2, and the p85 subunit of PI3K (92, 93). Interestingly, several of these proteins can also bind to the interdomain B of Syk. It is thus feasible that Syk cooperates in the formation of a CD19–Vav/PLCγ–Syk adapter complex that helps in the recruitment of Syk to the CD19 molecule and the phosphorylation and activation of PI3K (*Fig.* 9). The adapter function of Syk thus could play an important role in the selection and activation of those Syk substrates involved in the proliferation signal of the pre-BCR. Furthermore, as part of a CD19–Vav/PLCγ–Syk complex, the kinase could remain active at the plasma membrane even after the internalization of the pre-BCR. The postulated CD19-signaling complex may also play an important role for the activation of mature B cells, particularly when they are exposed to membrane-bound antigens. Here, it has been shown that CD19 and the BCR co-cluster in complexes that also contain Syk, PLC-γ2, and Vav. Furthermore, CD19 and several of its binding partners are required for cytoskeletal rearrangements and spreading behavior of activated B cells (94, 95).

The switch from proliferation to differentiation in pre-B cells is associated with the adapter protein SLP-65. In the absence of SLP-65, pre-B cells continue to proliferate in IL-7 cultures and are blocked in their further differentiation to the B-cell stage (13, 87). This increased proliferation can lead to leukemia in mouse and human (13, 96). The exact mechanism by which SLP-65 counteracts the proliferation of pre-B cells it not known. At one point during the expansion phase of pre-B cells, the adapter function of SLP-65 has to be activated to induce the differentiation signal. We have shown that the N-terminus and the SH2 domain of SLP-65 are required for the signaling function of the adapter (42, 97). The N-terminus targets the adapter to a yet unknown membrane compartment, and the SH2 domain of SLP-65 is required for the binding of the adapter to the pY204 in the cytoplasmic tail of Igα as well as to the pY630 at the Syk tail. One scenario is that the activation of Syk at the pre-BCR not only results in increased ITAM but also in Y204 phosphorylation. This allows SLP-65 to bind to the pre-BCR in the vicinity of the ppITAM/Syk complex. The phosphorylation of SLP-65 by Syk then allows BTK and PLC-γ2 to bind to the adapter. The resulting SLP-65/BTK/PLC-γ2 signalosome controls the $Ca^{2+}$ responses in activated B cells. Furthermore, via the binding of the SH2 domain of SLP-65 to pY630 at the Syk tail, the adapter could recruit Syk to the SLP-65/BTK/PLC-γ2 signalosome.

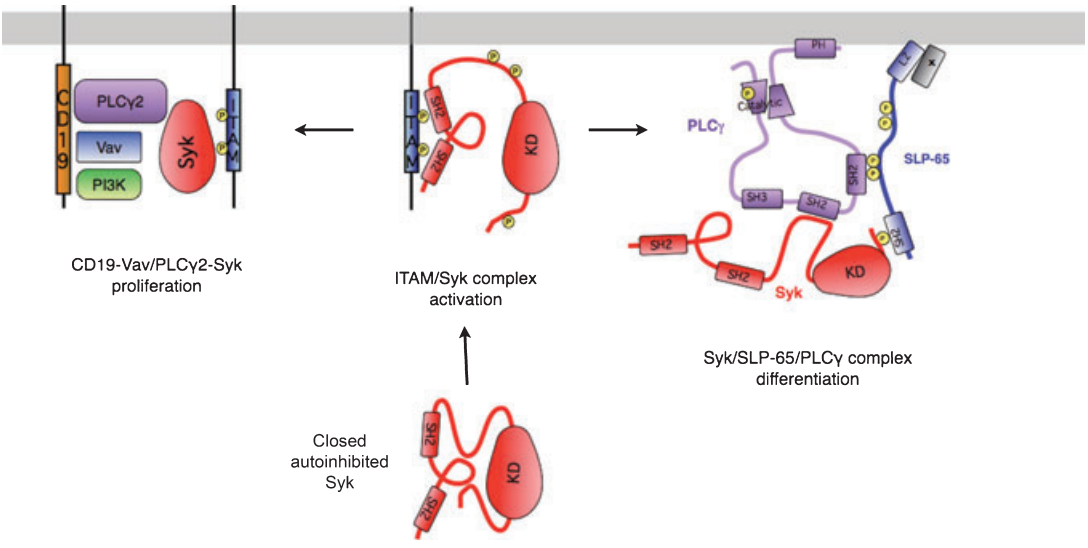


***Fig.* 9. Remodeling of Syk-dependent complexes determines cell fate choice.** Activation of Syk at the pre-BCR and signaling from the pre-BCR and CD19–Vav/PLC-γ2/PI3K–Syk complex drives the proliferation of pre-B cells. Remodeling of CD19–Syk-signaling complexes to SLP-65-dependent signaling complexes containing Syk and PLC-γ2 is required for the switch from proliferation to differentiation of pre-B cells.


 

This would lead to prolonged Syk activity, even in the absence of an ITAM and to the efficient phosphorylation and activation of BTK and PLC-γ2.

In our mutational analysis, we found that the binding of SLP-65 to the C-terminal tail of Syk is required for sustained $Ca^{2+}$ signaling and NFAT activation in B cells (42). Indeed, it has been found previously that it is the sustained elevation of cytosolic $Ca^{2+}$ levels rather than the sudden increase that promotes dephosphorylation and nuclear translocation of NFAT (98, 99). By recruiting Syk to the SLP-65/BTK/PLC-γ2 signalosome, SLP-65 could inhibit the formation of the CD19–Vav/PLCγ–Syk complex and in this manner counteract the proliferation signal. In line with this scenario is our finding that pre-B cells with a Y204F mutation of Igα and a Y630F mutation of Syk display an enhanced proliferation in limiting IL-7 conditions as compared to their wildtype counterparts and are blocked in their further differentiation (Y. Kulathu, unpublished observations). In summary, the switch from proliferation to differentiation of pre-B cells could be the result of a transition of Syk from the CD19 complex to the SLP-65 complex (*Fig.* 9).

A deregulation of Syk that prevents the transition from proliferation to differentiation could result in continuous proliferation and tumors, as has been found to be the case with oncogenic forms of Syk. For example, a patient with myelodysplastic syndrome was identified carrying a t(9;12)(q22;p12) chromosomal translocation that results in the expression of a TEL–Syk fusion protein. TEL–Syk consists of the dimerization domain of transcription factor TEL and the C-terminal half of Syk starting from amino acid 266 in the interdomain B (67). TEL-Syk has a constitutive signaling behavior that, via the activation of PLC-γ2 and PI3K signaling, results in oncogenic transformation of pre-B cells (100). Other fusion proteins such as Itk–Syk have been identified, and Syk is increasingly implicated in cancer (101). Understanding the exact molecular mechanism by which Syk is regulated and how the kinase activity is modulated in cells would make it possible to design drugs to inhibit the increased activity of these oncogenic Syk forms.

## Concluding remarks

Considering the discovery of the ITAMs 20 years ago, we made good progress in understanding of how antigen binding to the BCR results in B-cell activation. This understanding has been made possible by the identification of key proteins such as the kinases, adapters, and phosphatases that mediate this signaling. Understanding how each of these molecules interact with each other and regulate each other's function has been the focus of research in many laboratories. Further, the identification of properties of signaling networks such as feedback loops and bistability and the role of adapters as specificity determinants and signal integrators have helped us understand several aspects of BCR signaling. However, several important questions need to be addressed. We still do not know exactly how the BCR is activated and the initial steps leading to ITAM phosphorylation and initiation of signaling are. Many of the signaling complexes that are formed during signal transduction are too transient to be detected and analyzed by standard biochemical techniques. Future work in understanding these dynamics and mechanistic details of BCR signaling will require novel approaches.

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