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RECENT ADVANCES IN LYMPHOCYTE SIGNALING AND REGULATION

Chun Kung[1] and Matthew L. Thomas[2]

Department of Pathology, HHMI, Washington University, 660 S. Euclid Avenue, St. Louis, MO 63110

TABLE OF CONTENTS

1. ABSTRACT

The antigen receptor signaling pathway in lymphocytes is vital to their development and biological function. Recent studies have shown that protein tyrosine kinases and phosphatases are essential components in this receptor signaling pathway and therefore, are critical for the development of mature and functionally competent lymphocytes. The Src kinase family of protein tyrosine kinases coordinates the early signaling events in antigen receptor signaling via phosphorylation of tyrosine-based substrates. These kinases are regulated by the concerted action of the Csk family of non-receptor protein tyrosine kinases and the protein tyrosine phosphatase, CD45. A complex set of phosphorylation and dephosphorylation events regulate protein tyrosine kinase activity. Upon antigen stimulation, Src protein tyrosine kinases in conjunction with the tyrosine kinases, ZAP-70 and Syk initiate downstream effectors leading to Ca^{2+} mobilization, the activation of the Ras pathway and transcriptional activation. The roles of the various adapter proteins in these pathways are now being elucidated. It is apparent that a network of phosphorylation events connect the antigen receptor to intracellular signaling pathways.

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[1] To whom correspondence should be addressed, at: Department of Pathology, Washington University School of Medicine, Box 8118, 660 South Euclid Avenue, St. Louis, MO 63110 Phone: 314-362-8721, Fax: 314-362-8888, E-mail: chunkung@pathbox. wustl.edu

2. INTRODUCTION

Current model for antigen receptor signaling in lymphocytes

The T cell receptor (TCR) is a multisubunit complex of eight transmembrane proteins (1-3) (Fig. 1). The antigen recognition heterodimer consists of alpha-beta subunits that are non-covalently attached to CD3 components, gamma-epsilon, delta-epsilon and a homo- or heterodimer consisting of zeta or eta chains. Zeta chain homodimers are the most prevalent. The alpha-beta dimer confers efficient receptor recognition, while the CD3 dimers and zeta chains are critical for receptor expression and signal transduction. Expression of the TCR requires a fully assembled receptor, supporting the notion that the subunits are necessary for normal T-cell function. A functionally similar receptor complex appears in B cells (Fig. 2). The B cell receptor (BCR) consists of the surface Ig (sIg), which is the antigen recognition structure, in a non-covalent complex with two disulfide bonded heterodimers, containing an Igalpha subunit and an Igbeta subunit (1-4).

T- and B-cell activation requires stimulation of antigen receptors to initiate signal transduction via tyrosine phosphorylation (1-3). In addition, co-stimulatory proteins such as CD4, CD8 and CD28 for T cells, and CD19 and CD21 for B cells participate in the antigen recognition process. TCR engagement through either MHC antigen coupling or anti-TCR antibody crosslinking results in tyrosine phosphorylation by the Src kinases, Lck and Fyn of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of

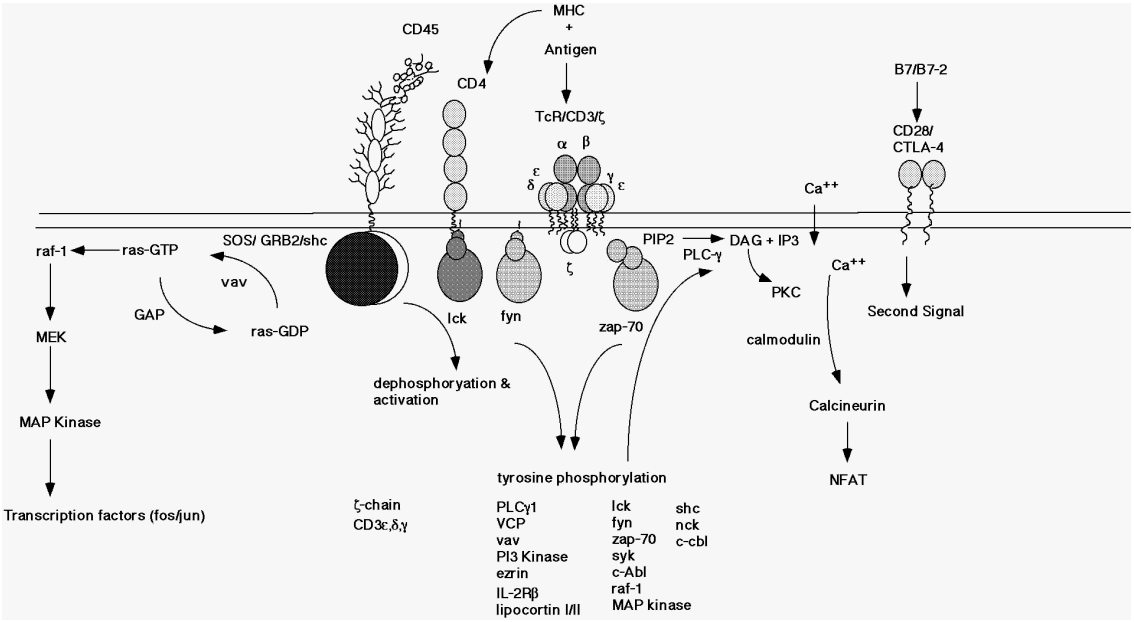

Figure 1. Regulation of antigen receptor-mediated signal transduction in T cells.
Prior to TCR activation, CD45 dephosphorylates Lck and preserves it in an active state, ready for coupling to the TCR upon antigen stimulation. After TCR activation, Lck and Fyn phosphorylate the zeta chains, enabling ZAP-70 interaction and initiating the signal transduction. Noted are the signaling pathways leading to IP_3 and Ras. In addition, substrates which are tyrosine phosphorylated after TCR activation are listed.

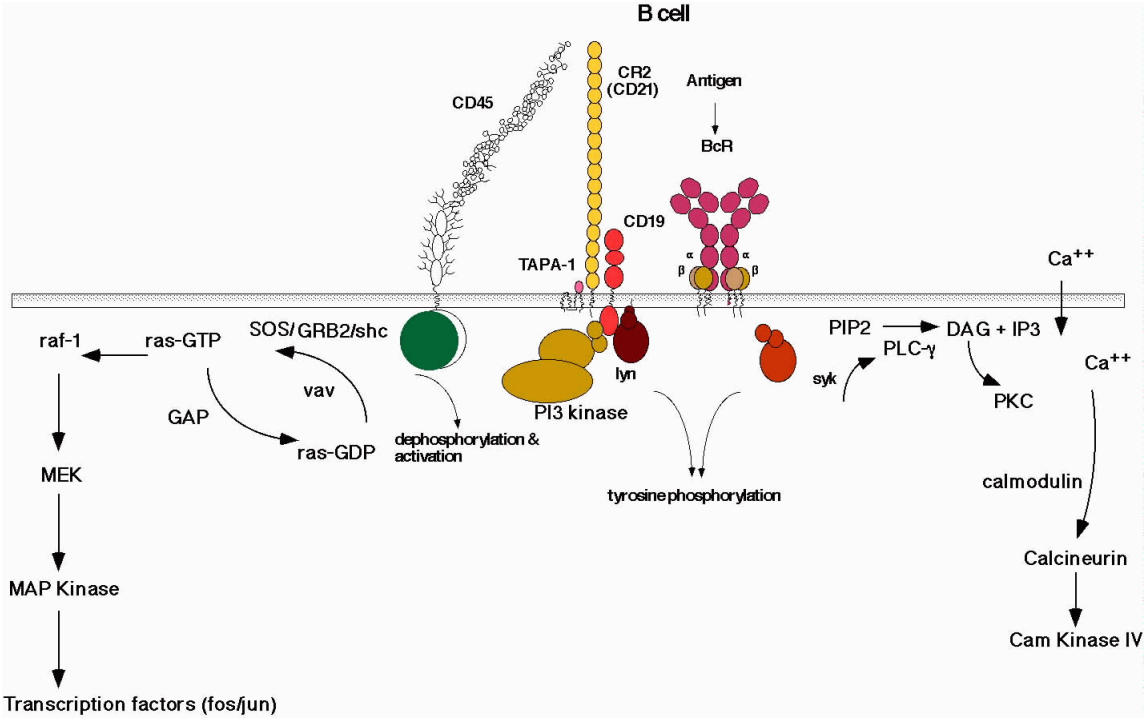

Figure 2. Regulation of antigen receptor-mediated signal transduction in B cells.
The early events of BCR signal transduction are postulated to follow a pattern similar to those for TCR signal transduction. CD45 dephosphorylates Lyn and maintains it in an active state for coupling to the BCR upon antigen stimulation. After BCR activation, Lyn phosphorylates BCR, enabling Syk interaction to initiate signal transduction. Noted are the signaling pathways leading to IP_3 and Ras.

CD3 and zeta chains. The ITAM consensus sequence, $D/EX_7D/EX_2YX_2L/IX_7YX_2L/I$ (where X is any amino acid), is present three times in the zeta chain and once in each CD3 component. Phosphorylation of the ITAMs leads to rapid recruitment and phosphorylation of the protein tyrosine kinase, ZAP-70 which in turn interacts with signaling molecules to initiate downstream effectors for inositol 1,4,5-triphosphate (IP_3) and Ras. In B cells, a similar process occurs. BCR stimulation results in the phosphorylation by Lyn, Fyn and Blk, and of the Igalpha and Igbeta subunits, followed by rapid recruitment of Syk and other downstream effectors, thereby initiating IP_3 and the Ras pathway (1-4).

Recent studies have further clarified the lymphocyte signaling pathway and characterized important components governing signal transduction. In addition, significant gains have been made in describing the interactions of these components with associated molecules and their effects on cell proliferation, differentiation, function and apoptosis. This review focuses on the current roles elucidated for the Src and Syk/ZAP-70 protein tyrosine kinases and the protein tyrosine phosphatase CD45.

3. PROTEIN TYROSINE KINASES

The Src family of protein kinases contains nine family members: Src, Blk, Yes, Yrk, Fgr, Hck, Fyn, Lyn and Lck (1). The primary structure of the Src kinases can be subdivided into several interaction domains (Fig. 3). The amino-terminal region contains a Src homology 4 (SH4) domain for myristylation, palmitylation and interactions with acidic phospholipids. Adjoining the SH4 domain is a 50-80 amino acid stretch which acts as a putative cell surface protein binding region. This is followed in order by an SH3 domain and an SH2 domain, which bind to proline-rich sequences and tyrosine phosphorylated proteins, respectively. The kinase domain adjacent to the SH2 domain contains a tyrosine autophosphorylation site, that potentiates enzymatic activation. Another tyrosine phosphorylation site, located in the carboxyl-terminus, serves a negative regulatory function. Phosphorylation of these tyrosines regulates Src kinase activity.

T cells express three Src family members: Lck, Fyn and Yes. Lck interacts with the cytoplasmic tails of CD4 and CD8alpha, whereas Fyn associates with the cytoplasmic tail of CD3 chains and zeta chain (2,5,6). Upon TCR activation, Lck and Fyn initiate the phosphorylation of ITAMs on zeta chains, CD3eta, CD3gamma and CD3delta. In addition they are implicated in the phosphorylation and activation of ZAP-70 and/or Syk (6-8).

B cells express Lyn, Lck, Fyn and Blk (1, 4). Similar to TCR activation, a cascade of phosphorylation events occurs upon BCR stimulation. The Src kinases are activated and presumably phosphorylate the ITAMs on Igalpha and Igbeta, which leads to the phosphorylation of Syk.

Syk and ZAP-70 have similar primary structures that consist of two amino-terminal SH2 domains and a kinase domain linked by hinge regions (1,6,9). Antigen activation results in the recruitment of ZAP-70 and Syk to the TCR and BCR, respectively by interacting via their SH2 domains with the doubly phosphorylated ITAMs. Trans- and auto-phosphorylation by Src and/or ZAP-70/Syk increases kinase activity (6).

3.1 Signaling pathways involving protein tyrosine kinases

Activation of the immune response in lymphocytes induces various cellular events such as cytoskeletal rearrangement, gene transcription and cell proliferation. The Ras/Rho family of GTPases are important in initiating these events. These GTPases convert extracellular stimuli into intracellular signals by regulating the activities of serine/threonine kinases, known as mitogen activated protein kinases (MAPK) (10). MAPK in turn controls gene expression important for many cellular functions, including cell growth and differentiation. The MAPK family can be subdivided into three subfamilies: extracellular signal regulated kinases (ERK), stress activated protein kinases (SAPK) or c-Jun N-terminal kinases (JNK) and p38 kinase (11). The Ras family controls ERK through the serine/threonine protein kinase, Raf. The Rho family of small GTPases is responsible for regulating stress activated protein kinases, SAPK or JNK. Along with the Ras and Rho pathways, mobilization of intracellular Ca^{2+} is also activated during the activation of the immune response. This is achieved through the activation of phospholipase C (PLC)-gamma1 and PLC-gamma2. IP_3 and diacylglycerol are formed from the hydrolysis of phosphoinositol bisphosphate (PIP_2) by PLCgamma. In due course, intracellular Ca^{2+} mobilization results from IP_3 stimulation. Concurrently, diacylglycerol activates various isoforms of protein kinase C, which gives rise to serine/threonine phosphorylation of selective substrates (3).

To transduce the signal from the membrane receptors to any of the downstream pathways requires intermediary molecules known as adapter proteins. Many of these adapter proteins contain SH2 and/or SH3 domains making them fully capable of binding and recruiting numerous proteins (2). Upon stimulation of the immune response, adapter proteins are tyrosine phosphorylated conceivably by Src kinases or ZAP-70/Syk (1,2). In the Ras pathway, putative SH2 adapter proteins such as Vav and Shc are tyrosine phosphorylated. The 46-52kDa protein, Shc binds to the adapter protein Grb2, which is constitutively associated to Sos, the guanine

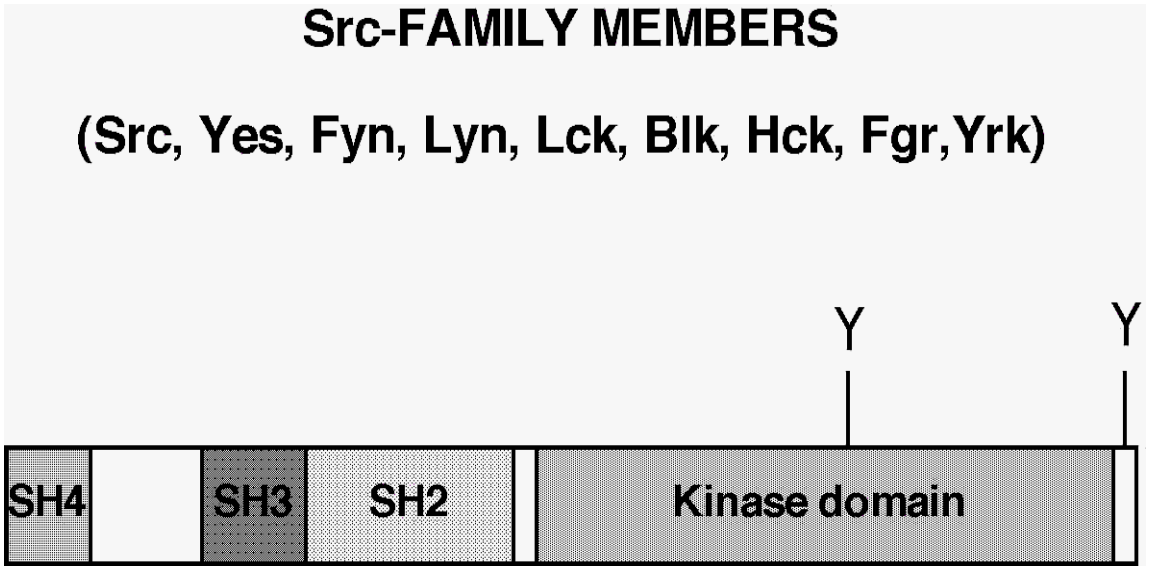

Figure 3. The domain structure of the Src protein tyrosine kinase family.
The domain structure of the Src kinase family is shown, with the tyrosines in the autophosphorylation site and carboxyl-terminal domain indicated. The SH2, SH3, SH4 and kinase domains are shown. Nine members of the Src family are listed.

nucleotide exchange factor for Ras. Shc can also interact directly with the phosphorylated zeta chains of the TCR, thereby coupling TCR stimulation to the Ras pathway (12). Vav, a 95kDa guanine nucleotide exchange factor, is implicated in Rho and Ras pathways. Moreover in the Ca^{2+} pathway, tyrosine phosphorylation of PLC-gamma1 and PLC-gamma2 leads to enhanced lipase activity, resulting in increased formation of IP_3 and diacylglycerol from PIP_2, and consequently elevated intracellular Ca^{2+} concentration.

ZAP-70 and Syk are involved in phosphorylating components necessary in initiating the Ras signaling pathway (1,2). Vav interacts with and is phosphorylated by Syk and ZAP-70 in activated B and T cells, respectively (13). Syk interaction with Vav is dependent upon a catalytically active Syk, the SH2 domain of Vav and the phosphorylated tyrosine residues in the linker region of Syk. In addition, T cells transfected with Syk and Vav results in increased activation of the nuclear factor of activated T cells (NFAT). Hence Syk and ZAP-70 via Vav couple the antigen receptor to the Ras signaling pathway.

Additional evidence associates Vav phosphorylation in COS-7 cells with the activation of JNK and the engagement of Rac-1 activity (14,15). Rac-1 is a member of the Rho family of GTPases, which are responsible for regulating JNK. Furthermore Vav signaling through JNK is down regulated in Rac-1 dominant negative mutants, supporting a relationship between Rac-1 and Vav (15). Other studies show Vav and Rac-1 involvement in mitogenesis, the Ras pathway and NFAT related T cell responses (16-19). Since Vav has SH2 and SH3 domains, it is possible that it can recruit proteins involved in both Ras and Rho pathways and couple them to antigen receptor-associated tyrosine kinases. Further clarification of the Vav signaling pathway is required.

As noted above, Shc and Grb2 are linked in the Ras pathway. Upon BCR stimulation, Shc is tyrosine phosphorylated. B cells deficient in Lyn or Syk display a decrease in Shc phosphorylation and Grb2-Shc association is reduced (20). These findings suggest that Shc phosphorylation is dependent on Lyn and/or Syk. Immunoprecipitation studies of Shc in B cells and co-transfected COS-1 cells show that Syk associates with and phosphorylates Shc. Altogether these results point to an involvement for Syk and Lyn in coupling the antigen receptor to the Ras pathway via Shc and Grb2.

SLP-76, a 76kDa SH2 adapter protein that is tyrosine phosphorylated during T cell activation, is preferentially phosphorylated by ZAP-70, and interacts with Grb2 and PLC-gamma1 (21). Over expression of SLP-76 in T cells results in a hyperactive receptor, whereas expression of a mutant SLP-76 that cannot be phosphorylated diminishes receptor function. Furthermore, decreased phosphorylation of SLP-76 is found in T cells expressing a catalytically inactive ZAP-70. These findings imply a role for SLP-76 in antigen receptor signaling which appears to require ZAP-70, and may involve the Ras and Ca^{2+} pathways.

Fyn and Lyn phosphorylate c-Cbl, a 116 kDa product of a proto-oncogene, which binds to Grb2 and the p85 subunit of phosphatidylinositol 3'-

kinase (PI 3-kinase) (22-25). Fyn, through its SH2 domain, associates with and tyrosine phosphorylates c-Cbl in activated T cells and IL-3 stimulated murine myeloid cells (23-25). Also in activated B cells, the phosphorylation of c-Cbl is dependent on interactions with Lyn but not Syk (25). However binding assays using GST-fusion proteins demonstrate that the SH2 domain of Fyn can bind to non-phosphorylated c-Cbl, suggesting that Fyn may also interact with c-Cbl in a phosphotyrosine-independent manner (24). These results indicate that Fyn probably binds to c-Cbl regardless of it phosphorylation state.

Although the biological function of c-Cbl is not well defined, recent findings suggest that it may play a role in the Ras signaling pathway. Antigen stimulation causes Crk, a SH2/SH3 adapter protein, to bind to tyrosine phosphorylated c-Cbl, and results in the presence of c-Cbl-Crk complexes in the membrane particulate fraction (26). These Crk complexes are formed via interactions with SH2 domains of Crk. The SH3 domains of Crk preferentially bind C3G, a nucleotide exchange factor involved in the activation of Rap, the negative regulator of the Ras pathway. These results imply that Crk proteins may be responsible for co-localizing C3G to Rap at the membrane surface. As a result, Lyn or Fyn phosphorylates c-Cbl which in turn interacts with Crk, thereby engaging Rap mediated down regulation of Ras.

Ca^{2+} mobilization requires PLC-gamma action on PIP_2 to form IP_3 However, studies on B cells deficient in tyrosine kinase show that there are possibly two pathways leading to Ca^{2+} mobilization (27). With activated B cells lacking Syk, PLC-gamma2 is not phosphorylated and, IP_3 production and Ca^{2+} mobilization are absent (27). Correspondingly, activated B cells that lack Bruton's tyrosine kinase (Btk) exhibit similar characteristics (28). In contrast, for BCR activation in B cells that lack Lyn, IP_3 generation remains unaffected and a slow Ca^{2+} mobilization occurs (27). Immuno-precipitation studies on activated B cells show that Syk is associated with PLC-gamma1 (29). Collectively, these findings point to tyrosine kinases directing two mechanisms for intracellular Ca^{2+} production. Syk regulates Ca^{2+} mobilization through PLC-gamma and IP_3 production, whereas Lyn affects Ca^{2+} mobilization through a different route.

3.2 Regulation of protein tyrosine kinase activity

The activity of Src kinases is regulated by phosphorylation and dephosphorylation of specific tyrosines (1). Src kinases contain an autophosphorylation site within the kinase domain that serves to potentiate kinase activation. The carboxyl-terminal negative regulatory tyrosine when phosphorylated, interacts intramolecularly with the SH2 domain thereby decreasing kinase activity (30-32). The crystal structures of c-Src and Hck indicate that Src kinase inactivation resulting from these intramolecular interactions arises from a conformational change in the molecule (31,32). The family of non-receptor protein tyrosine kinases, which consist of Csk and Ntk, have been shown to phosphorylate the negative regulatory domain tyrosine, thereby decreasing Src kinase activity (33,34). Studies using Csk/Ntk deficient cells suggest that Csk/Ntk are required for inactivating Src kinases. Fyn and Lyn in Csk-deficient mice are constitutively activated and exhibit increased phosphorylation, suggesting that Csk is required to repress tyrosine kinase activity (35,36). Csk cannot only phosphorylate Lck and Fyn but has also been shown to phosphorylate CD45, and thus increase phosphatase activity (37).

Apart from the negative regulation by Csk/Ntk, Src kinases are positively regulated by the protein tyrosine phosphatase, CD45 which has been shown to dephosphorylate the negative regulatory domain, thus increasing the kinase activity necessary for TCR activation (1). However, CD45 may also negatively regulate Src kinases by dephosphorylating the autophosphorylation site on Src kinases (38). Yac-1 T cells deficient in CD45 exhibit Lck hyperphosphorylated at both the autophosphorylation site and negative regulatory domain, but to a higher degree at the latter. In addition, the phosphatase domain of CD45 can dephosphorylate the autophosphorylation site of active Lck *in vitro*. All in all, these results point to a role for CD45 in negatively and positively regulating Lck activity.

It is also possible that Lck may be regulated by sequestration as an inactivated pool within a glycolipid enriched membrane domain (39). Lck within the glycolipid enriched membrane domain has its negative regulatory domain in a hyperphosphorylated state. It is postulated that this results from the absence of CD45 which is excluded by the glycolipid enriched membrane domain.

Another protein tyrosine phosphatase that is important in the negative regulation of protein tyrosine kinase activity is SHP-1. In T cells, SHP-1 interacts through its SH2 domains with ZAP-70 and dephosphorylates ZAP-70 (40). Thymocytes from motheaten (me) mice, which have a deficiency in SHP-1, exhibit elevated tyrosine phosphorylation after TCR stimulation due to increased activation of Src kinases (41). This implies that SHP-1 may also dephosphorylate members of the Src kinase family. In B cells, SHP-1 binds to CD22 and FcgammaRIIB to negatively regulate BCR signaling (42,43). Altogether, SHP-1 is a vital negative regulator of antigen receptor mediated signaling in both B cells and T cells.

3.3 Lymphocyte development

Thymocyte development occurs as a series of selection stages, where only those meeting a defined criteria pass into mature lymphocytes (44).

Immature thymocytes begin as multipotent cells which do not express CD4 or CD8, and as such are designated double negative (DN) ($CD4^-$ $CD8^-$). Upon expansion, alphabeta gene rearrangement and expression of the TCR, the thymocytes express TCR, CD4 and CD8, and enter the double positive (DP) stage. At this point they are designated $CD4^+$ $CD8^+$ TCR^{low}. The thymocytes then undergo selection for self recognition which is dependent on the avidity of the TCR for antigen presented by MHC molecules within the thymus. During the final development stage, DP thymocytes become single positive (SP) in either CD4 or CD8 with specificity for MHC class II or class I, respectively. Those that survive have undergone positive selection and are ready for maturation.

In the bone marrow, the process of B cell development comprises several stages where the preB cells are selected to develop into long-lived mature B cells (45). At the same time the B cells are screened for tolerance against autoantigens. Initially, the pro-B/preB cells undergo rearrangement in their Heavy-chain gene loci. Those that contain the proper in frame gene rearrangement undergo Light-chain gene rearrangement to form immature B cells. After further secondary Light-chain gene rearrangement and selection against autoantigens, immature B cells exit the bone marrow to become mature B cells.

To examine the biological relevance of the Src protein tyrosine kinases in lymphocyte signaling and maturation, mice expressing mutations in a Src kinase or a deficiency in Src kinases are analysed. In mice deficient in Lck, thymocytes are generally blocked at the DP stage, but a small number of single positives are found (46). However, those that do develop exhibit only partial signaling in response to TCR stimulation. In contrast, thymocytes from mice deficient in Fyn are able to mature but SP thymocytes are hyporesponsive to TCR ligation (47,48). Thus unlike Lck, Fyn contributes to TCR signaling but is not critical for thymopoiesis. For mice deficient in both Lck and Fyn, thymocyte development is blocked at the DN stage and no mature alphabeta T cells are observed in the peripheral lymphoid organs (49,50). However, there are normal numbers of natural killer cells which have normal cytolytic activity (50). In all, these results point to a possible redundancy effect of Fyn for Lck in T cell development.

Expression of a gain of function Fyn (Y528F) transgene in *lck*$^{-/-}$ mice restores DP thymocyte development and enhances the DP to SP transition of thymocytes, further supporting a redundancy between Lck and Fyn (49). However, the Fyn transgene only marginally affects *RAG1*$^{-/-}$ mice whereas expression of a constitutively active Lck restores normal DP thymocyte development (49,51). In addition, a dominant negative Fyn does not affect T cell development while a dominant negative Lck abrogates DP thymocyte development (52-54). Taken together, these results show that Fyn and Lck do not have identical functions. Nonetheless, Fyn can transduce signals required for positive selection of DP thymocytes and can subserve Lck in some aspects of T cell development.

It is apparent that Lck is required for positive selection of thymocytes. In support of this, a catalytically inactive Lck expressed in DP thymocytes unambiguously blocks positive selection (55). However, other defects present in DP thymocytes deficient in Lck can also influence positive selection. It is noted that thymocytes deficient in Lck are blocked at the DP stage and have decreased CD4 dependent signaling but relatively unaffected TCR signaling (41). In addition, these thymocytes display decreased CD5 expression and increased TCR expression. CD5, an accessory signaling molecule, is important since it negatively regulates TCR and BCR signaling, and is required for the positive selection of thymocytes (56,57). Similarly, CD4/TCR co-aggregation is essential for ZAP-70 activation in DP thymocytes (58). Hence, altered CD5 or TCR expression can affect the positive selection of DP thymocytes deficient in Lck. Nevertheless expression of the Fyn transgene (Y528F) normalizes CD5 and TCR expression (49).

Lyn is critical for BCR signal transduction and this is supported by the findings from mice deficient in Lyn (59). These mice exhibit decreased numbers of B cells, which may result from a failure in B cell expansion. It is noteworthy that the lymph nodes of mice deficient in Lyn have deformed germinal centers. B cell function is also impaired as shown by their poor response to lipopolysaccharide stimulation. However, their response to CD40 stimulation is normal. In addition, these mice exhibit elevated levels of serum IgM due to increased numbers of plasma cells producing IgM, circulating autoreactive antibodies and symptoms characteristic of an autoimmune disease. Therefore, these results demonstrate that Lyn is vital for proficient B cell signaling and establish Lyn dependence in B cell selection.

ZAP-70 and Syk are also important for lymphocyte development. A portion of humans or mice with severe combined immunodeficiency (SCID), display a defective ZAP-70 or a deficiency in ZAP-70 (60-62). The normal number of $CD4^+$ cells are present but they are non-functional. No $CD8^+$ thymocytes are present in the periphery. Mice deficient in ZAP-70 have a thymocyte block at $CD4^+$ $CD8^+$ TCR^{low} stage but can be rescued with human ZAP-70 (63). Deficient mice exhibit elevated numbers of normal DP thymocytes. Mice deficient in Syk exhibit normal thymopoiesis (64,65). Therefore ZAP-70 but not Syk is vital for thymocyte development. However, mice deficient in Syk have decreased numbers of mature B cells and signaling through the BCR is impaired (64-66). Furthermore,

they display impaired gammadelta T cell development (67).

ZAP-70 and Syk have similar structural and functional properties, which could imply a redundancy between these two kinases. However the different expression patterns of ZAP-70 and Syk may affect their roles (68). ZAP-70 is not expressed in peripheral B cells but is restricted to T cells, natural killer cells and thymocytes. However, Syk is expressed in thymocytes and predominantly in peripheral B cells but down regulated in peripheral T cells. Recent findings attempt to address the functional overlap between ZAP-70 and Syk. In B cells deficient in Syk, the BCR is non-functional. Nonetheless, expression of ZAP-70 in these cells reconstitutes BCR signaling (69). Functionally competent SH2 and catalytic domains of ZAP-70 are essential for full BCR activity. ZAP-70, like Syk, binds to the phosphorylated Igalpha and Igbeta subunits, with affinities similar to their interactions with the CD3eta subunit. Therefore under these conditions, ZAP-70 can substitute for Syk in its role in BCR signal transduction.

4. THE PROTEIN TYROSINE PHOSPHATASE, CD45

CD45 is a single chain transmembrane glycoprotein with two cytoplasmic phosphatase domains, of which the second domain appears to be inactive. This protein exists in various isoforms of molecular weights 180-220kDa, as a result of alternative splicing between exons 4, 5 and 6 (or A, B and C). These exons encode for the amino-terminal extracellular O-linked glycosylated region (70-73). Thus, these alternatively spliced isoforms differ in the lengths of their extracellular domains. Furthermore, they are differentially expressed on T cell subsets and resting or activated T cells, and their expression is dependent on cell differentiation and activation. CD45 is expressed by all hematopoietic cells except mature erythrocytes and platelets.

4.1 CD45 regulates protein tyrosine kinases

CD45 functions to regulate Src kinase activity. Evidence for this has been obtained from T and B cells deficient in CD45. In these cells, the negative regulatory domains of Lck and Fyn are hyperphosphorylated. As a consequence antigen-mediated signal transduction is compromised (74-76). CD45 does not regulate Lck and Fyn equally. (75,77). In T cells deficient in CD45, Lck tyrosine phosphorylation increases 8-10 fold over wild type compared to a 2-3 fold increase for Fyn, despite equal expression of the Src kinases. Deletion of the SH4 domain from Lck or replacement of it with the analogous domain from Fyn results in a 5 fold increase in tyrosine phosphorylation of the negative regulatory domain (78). This suggests that there are mechanisms that mediate CD45 interaction with specific Src kinases.

In B cells, Src kinases are also regulated by CD45. Lyn is hyperphosphorylated at its negative regulatory domain and autophosphorylation site in chicken DT40 B cells deficient in CD45 (79). Accordingly, BCR signaling in these cells is severely compromised. In comparison, DT40 B cells deficient in Csk and in the resting state exhibit a constitutively activated Lyn, whose autophosphorylation site is hyperphosphorylated but its negative regulatory domain is unphosphorylated (80). This implies that dephosphorylation of the carboxyl-terminal negative regulatory domain tyrosine by CD45 is a prerequisite for Lyn activity during BCR signaling. Consequently CD45 is an important positive regulator of Lyn activity and may also participate in dephosphorylating the autophosphorylation site of Lyn.

Lck and Fyn are hyperphosphorylated at the negative regulatory domain, and exhibit hyperactivity in Yac-1 T cells deficient in CD45 (74). Phosphopeptide studies on Lck from these cells demonstrate that Lck is hyperphosphorylated at both the autophosphorylation site and the negative regulatory domain, although to a greater degree at the latter site (38). Tyrosine to phenylalanine mutations of Lck at the autophosphorylation site (Y394) and negative regulatory domain (Y505) establish that the autophosphorylation site is more dominant in affecting Lck activity. In cells expressing mutations at both Y505F and Y394F, no kinase activity is observed. Furthermore, *in vitro* assays using the phosphatase region of CD45 and active Lck in its native conformation demonstrates that CD45 can dephosphorylate the autophosphorylation site of Lck. Thus CD45 is responsible for dephosphorylating both regulatory phosphorylation sites on Lck (38). These findings point to CD45 as a negative and positive regulator of Src kinase activity.

4.2 The extracellular domain of CD45 affects antigen-mediated signal transduction

The extracellular region of CD45 is modified by alternative splicing of exons 4, 5 and 6 (or A, B and C), which code for O-linked glycosylation and thus govern the amount of O-linked glycosylation present (70-73). As a result, CD45 isoforms are highly regulated and differentially expressed on the various lymphocyte subsets. Results from CD45 chimeric experiments indicate that the cytoplasmic domain of CD45 is sufficient for supporting TCR signaling (81,82). However, CD45 isoform expression has been associated with lymphocyte maturation and activation. This argues that CD45 isoforms, and in particular the extracellular domain of CD45, may influence lymphocyte function. Studies expressing the individual CD45 isoforms in transgenic mice or T cell lines demonstrate that each CD45 isoform affects TCR signaling differently (83-85). In fact, cells expressing the low molecular weight isoform of

CD45 appear to be the most effective in TCR signaling.

Not only is TCR signaling affected by which CD45 isoforms are present but also the tyrosine phosphorylation pattern of intracellular proteins (86,87). Adapter proteins such as Vav and SLP-76 show differential phosphorylation and varying degrees of physical association, with higher levels of each in the presence of the largest CD45 isoform (86). The regulatory effect of the CD45 isoforms may result from selective interactions of the particular isoform with cell surface molecules.

The extracellular domain of CD45 is important in mediating interactions with other membrane surface proteins which are involved in T cell activation. CD2, a 55-60 kDa glycoprotein, is involved in T cell activation. However, signal transduction via CD2 stimulation requires CD2 interactions with other signaling molecules, such as CD3eta and zeta chain (88-90). Moreover, the protein tyrosine kinases, Lck and Fyn associate with the signaling complex formed by CD2 and zeta chain (91,92). In addition, CD45 interacts with CD2 to modulate its activation of T cells (93). Studies using CD2 chimeras with CD4, CD28 and CD58 show that CD45/CD2 complexes are primarily governed by extracellular domain interactions and to a lesser extent by cytoplasmic associations. Apparently, the cytoplasmic domain of CD2 associates mainly with the zeta chain of the TCR complex. These findings point to CD45 involvement in regulating CD2 activation of T cells.

CD4, a co-stimulatory protein, associates with Lck and is involved in the antigen recognition process. CD45 isoform studies demonstrate that low molecular weight and not high molecular weight isoforms of CD45 preferentially interact with CD4 and TCR, and this association affects antigen recognition (94). Furthermore, the interaction between CD4 and CD45 is dependent upon the external domains of the CD45 isoforms but independent of the cytoplasmic domains. These results point to CD45's role in regulating antigen receptor signaling and also CD4 function in antigen recognition. In addition, CD45 interaction with CD4 may regulate Lck function and activity.

4.3 Effects of CD45 on lymphocyte development

Mice deficient in CD45 exhibit defects in T cell development and impaired B cell signaling (95-98). Two separate gene targeted mice have been described in which either exon 6 or exon 9 was replaced with a neomycin cassette (95,98). The phenotype of deficient mice developed from either targeted exon is similar. T cell development is severely inhibited at two distinct stages: development of DP thymocytes from DN thymocytes is reduced twofold and the maturation of DP in to SP is decreased fivefold. In addition, TCR induced apoptosis of thymocytes is impaired whereas non-TCR stimulated apoptosis is unaffected. Altogether, these results demonstrate that CD45 is required for T cell development and is consistent with the observation that CD45 is necessary for efficient signaling through the TCR.

Exon 6 targeted mice have normal numbers of B cells, which are responsive to lipopolysaccharrides (95,96). However, IgM stimulation fails to induce B cell proliferation. Furthermore while extracellular Ca^{2+} influx is abrogated, intracellular Ca^{2+} mobilization is normal upon anti-Ig induction. In mice with the exon 9 mutation, B cell development is unaffected but no BCR signaling is observed when stimulated by anti-IgM or anti-IgD (98). However stimulation through CD40 (anti-CD40) is unaffected compared to reduced signaling through CD38 (anti-CD38). Altogether, CD45 plays an important role in Ig mediated-BCR signaling and in some aspects of CD38 signaling. It also may be important for extracellular Ca^{2+} influx.

As noted above, immature B cells undergo selection during maturation to determine the competency of the BCR. In mice deficient in CD45, this selection process is altered due to changes in antigen receptor signaling (97). The threshold signal required for selection is abnormally lowered compared to wild type, eliminating B cells which normally would be selected. Clearly, the signal generated here from antigen receptor stimulation is recognized by the deficient B cell as being improper for B cell maturation. These results demonstrate that antigen signaling is a requirement for normal mature B cell accumulation and the degree of signaling regulates proper selection. Accordingly, CD45 appears to act as a positive regulator of the signaling threshold required for B cell maturation.

During lymphocyte development, the level of CD45 expression is important for antigen receptor signaling. CD45 expression is up regulated during T cell maturation particularly during the positive selection of SP thymocytes (99). CD45 levels are low on DP thymocytes but increase when cells differentiate to $CD4^+$ or $CD8^+$ SP, in conjunction with increased levels of TCR-CD3 complex. Consequently greater than 90% of the positively selected thymocytes display a $CD45^{high}$ phenotype in contrast to a $CD45^{low}$ phenotype for non-selected thymocytes. Similarly CD45 expression is drastically increased during the developmental period which correlates BCR up regulation with B cell maturation (99). As a result, CD45 expression during lymphocyte development is tightly regulated with those for TCR and BCR complexes.

4.4 Regulation of CD45 activity

Currently, only a limited amount of information is known about the mechanism and participants involved in the regulation of CD45

activity. Recent studies propose that CD45 activation and function could be regulated through phosphorylation by Csk, a negative regulator of Src kinase activity (37). Cotransfection of Csk and CD45 into COS-1 cells reveal that CD45 is phosphorylated on two tyrosines, and upon phosphorylation, CD45 exhibits increased phosphatase activity. Therefore, Csk may up regulate CD45 activity and down regulate Src kinase activity.

CD45 dimerization may also be involved in regulating CD45 activity. EGF receptor chimeras containing phosphatase domain of CD45 dimerize in the presence of EGF (100). Dimer formation neutralizes CD45 function and TCR activity. However, the addition of a EGF receptor with its cytoplasmic tail truncated restores both activities. These findings suggest that CD45 dimer formation can potentially regulate CD45 activity via inactivation of phosphatase activity. Moreover CD45 is related to receptor protein tyrosine phosphatase alpha (RPTPalpha). The crystal structure of the RPTPalpha membrane-proximal catalytic domain has been solved (101). The deduced structure shows that a dimer is formed from two catalytic domains, with the N-terminal region of one monomer wedged into the active site of the other monomer. This association blocks the active site of one catalytic domain, making it inaccessible to substrate. As a result dimer formation by RPTPalpha could play a role in regulating phosphatase function, and as such a similar event may also be important in regulating CD45 function.

4.5 CD45 is involved in cell adhesion

T cell activation requires both antigen presentation and cellular adhesion with the antigen presenting cell. TCR activation concomitantly stimulates integrin-mediated cell adhesion (102). In general, cell adhesion is mediated by integrin stimulation and the formation of focal adhesions. During this process tyrosine phosphorylation of intracellular proteins occurs. Focal adhesion kinase (FAK), a protein tyrosine kinase, is phosphorylated in response to integrin cross-linking and has been implicated in the Ras-MAPK pathway (1,2). Phosphorylation of FAK provides binding sites for Src kinases, Grb2 and paxillin. Integrin stimulation and cell adhesion induce the phosphorylation of paxillin, a cytoskeletal protein involved in transducing signals to the nucleus (1,2). Both of these proteins are major components of focal adhesions.

Apart from CD45's involvement in signal transduction, CD45 is involved in regulating the phosphorylation of paxillin and FAK in B cells (87). The phosphorylation of FAK and paxillin is dependent on the presence of CD45 in both stimulated and unstimulated B cells. Apparently, stimulation of B cells decreases FAK phosphorylation. B cells deficient in CD45 exhibit no phosphorylation of either FAK or paxillin, regardless of stimulation. These results suggest an involvement for CD45 in regulating cytoskeletal functions and cell adhesion.

CD45 influences homotypic cell adhesion of T and B cells (103-105). For T cells, only activated T cells can be induced via CD45 ligation to aggregate. Antibodies to the extracellular domain of certain CD45 isoforms are able to induce homotypic adhesion, whereas others inhibit adhesion. This adhesion can be blocked using antibodies against LFA-1, ICAM-1 and ICAM-3, suggesting that LFA-1/ICAM-1 and LFA-1/ICAM-3 pathways are involved. Typically, CD45 is found to co-localize with LFA-1 at the cell-cell contacts after induction of cell aggregation via CD45 ligation. Antibodies to CD45 which block adhesion alter tyrosine phosphorylation of intracellular proteins induced by adhesion-activating antibodies to ICAM-3 or LFA-1. These results indicate that CD45 is an important component in mediating LFA-1 induced cell-cell aggregation.

CD45 also associates with CD100, a disulfide-linked dimer involved in T cell proliferation and this interaction increases during T cell activation (106). The expression pattern of CD100 is similar to that for CD45. Epitope-dependent antibody coupling of CD45 down regulates CD100 expression at the cell surface and induces shedding of a soluble 120kDa form of CD100. Homotypic adhesion of T cells stimulated by antibodies against CD45 is enhanced by antibodies against CD100. However the CD100 antibody does not induce homotypic adhesion. Therefore CD45 modulates CD100 function in cell aggregation and proliferation.

The association of CD45 with other membrane-associated proteins during cell adhesion may be mediated through CD45AP. Monomeric and dimeric forms of CD45 interact with the putative adapter protein CD45AP, a 36kDa phosphoprotein also known as the lymphocyte phosphatase associated protein (LPAP) (107-110). CD45AP expression in T and B cells correlates with that for CD45 (111). Cells deficient in CD45 show no surface expression of CD45AP although normal levels of its mRNA are present. Transfection of CD45 into these cells restores CD45AP expression. Therefore complex formation between CD45 and CD45AP prevents CD45AP from proteolytic degradation. Both molecules interact mainly through their transmembrane domains. Interestingly, the cytoplasmic domain of CD45AP is marked by a putative WW domain, which functionally resembles SH3 domains and may bind proline rich sequences (112). As a result, CD45AP can potentially act as an adapter protein for CD45 substrates.

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