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STUDIES ON ITK-SYK SIGNALING PATHWAYS

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“All the world began with a yes. One molecule said yes to another molecule and life was born.”

Clarice Lispector, The Hour of the Star

"*If you thought that science was certain - well, that is just an error on your part.*"

- Richard P. Feynman (1918-1988), Theoretical physicist

To
Martin Luther King, Jr.,
(1929-1968)

ABSTRACT

Chromosomal alterations are frequent causes of cancer. Until now, SYK is reported in two different chromosomal translocation events generating the ITK-SYK-fusion protein in a subset of peripheral T cell lymphomas and the TEL-SYK fusion protein in a case of myelodysplastic syndrome. T lymphocyte-expressed ITK is the only member of the TEC-family of tyrosine kinases reported as a fusion partner in transforming translocations and here we have studied this fusion. The comparison of ITK-SYK with SYK revealed that related tyrosines of ITK-SYK are phosphorylated at the linker-region and at the activation-loop and that the fusion protein localizes to the plasma membrane and potently phosphorylates the adapter proteins SLP-76 and BLNK. Moreover, membrane localization and phosphorylation of adapter substrates are blocked with PI3K inhibitors. SYK, on the other hand, showed phosphorylation at the linker-region, but not at the activation-loop tyrosines and failed to phosphorylate SLP-76 or BLNK under the same conditions.

Since BTK is the predominantly expressed TEC family kinase in B lymphocytes, we engineered the corresponding fusion kinase, BTK-SYK. We then investigated the role of the N-terminal region in the regulation of fusion kinases ITK-SYK, BTK-SYK and TEL-SYK. Unlike ITK-SYK, BTK-SYK showed more nuclear and cytoplasmic localization and PI3K inhibitors, unexpectedly, did not block its capacity to phosphorylate the adapter substrate SLP-76. Interestingly, non-membrane-tethering PH-TH domain-mutants ITK-SYK-R29C and BTK-SYK-R28C potently phosphorylated SLP-76. On the same ground, a TEL-SYK mutant, lacking the dimerization domain, was equally phosphorylated as the full-length fusion protein, but induced highly compromised CD69 upregulation compared with TEL-SYK or ITK-SYK.

Further investigations revealed that ITK-SYK-mediated activation of T cells was dependent on the adapter function of SYK-family kinases (SYK or ZAP-70), but independent of their kinase activity. Moreover, SLP-76 adapter function was not only indispensible for ITK-SYK-mediated CD69 upregulation and IL-2 secretion, but also for the phosphorylation of activation-loop tyrosines of SYK. Mutagenesis revealed a hierarchical phosphorylation pattern in the activation of ITK-SYK. In spite of loss of phosphorylation of the tyrosines, known to act as targets in SYK, the fusion protein potently retained phosphorylation capacity for substrate adapter proteins. Phosphorylation-independent constitutive activation was further confirmed by ITK-SYK expression in SYF cells (cells lacking SRC-family kinases), since there was no detectable phosphorylation on target tyrosines, yet the substrate SLP-76 was potently phosphorylated. Altogether, our studies indicate that lack of auto-inhibition renders fusion kinase constitutive activation suggesting that many of the tyrosine phosphorylations known to be critical in the activation of SYK are dispensable for ITK-SYK activation.

LIST OF PUBLICATIONS

This thesis is based on two published articles and a manuscript, which will be referred to in the text by Roman numerals.

I. **Hussain, A.**, Faryal, R., Nore, B. F., Mohamed, A. J., and Smith, C. I., Phosphatidylinositol-3-kinase-dependent phosphorylation of SLP-76 by the lymphoma-associated ITK-SYK fusion-protein. (2009) *Biochemical and Biophysical Research Communications* **390**, 892-896

II. **Hussain, A.,** Hamasy, A., Mohammad, D. K., Gustafsson, M. O., Nore, B. F., Mohamed, A. J., and Smith, C. I., Role of N-terminal region in the regulation of SYK-fusion kinases ITK-SYK, BTK-SYK and TEL-SYK. (Manuscript)

III. **Hussain, A.,** Mohammad, D. K., Gustafsson, M. O., Uslu, M., Hamasy, A., Nore, B. F., Mohamed, A. J., and Smith, C. I., Signaling of the ITK (interleukin 2-inducible T cell kinase)-SYK (spleen tyrosine kinase) fusion kinase is dependent on adapter SLP-76 and on the adapter function of the kinases SYK and ZAP70. (2013) *The Journal of Biological Chemistry*

Publications by author not included in the thesis.

IV. **Hussain, A.,** Yu, L., Faryal, R., Mohammad, D. K., Mohamed, A. J., and Smith, C. I., TEC family kinases in health and disease--loss-of-function of BTK and ITK and the gain-of-function fusions ITK-SYK and BTK-SYK. (2011) *The FEBS Journal* **278**, 2001-2010

V. Genead, R., Fischer, H., **Hussain, A.,** Jaksch, M., Andersson, A. B., Ljung, K., Bulatovic, I., Franco-Cereceda, A., Elsheikh, E., Corbascio, M., Smith, C. I., Sylven, C., and Grinnemo, K. H., Ischemia-reperfusion injury and pregnancy initiate time-dependent and robust signs of up-regulation of cardiac progenitor cells (2012) *PLoS One* **7**, e36804

VI. Gustafsson, M. O., **Hussain, A.,** Mohammad, D. K., Mohamed, A. J., Nguyen, V., Metalnikov, P., Colwill, K., Pawson, T., Smith, C. I., and Nore, B. F., Regulation of nucleocytoplasmic shuttling of Bruton's tyrosine kinase (Btk) through a novel SH3-dependent interaction with ankyrin repeat domain 54 (ANKRD54). (2012) *Molecular and Cellular Biology* **32**, 2440-2453

VII. Mohammad, D. K., Nore, B. F., **Hussain, A.,** Gustafsson, M. O., Mohamed, A. J., and Smith, C. I., Dual phosphorylation of Btk by Akt/PKB Provides Docking For 14-3-3ζ, Regulates Shuttling and Attenuates both Tonic and Induced Signaling in B Cells. *Molecular and Cellular Biology* (In press)

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LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
ABL	Abelson murine leukemia viral oncogene homolog
ALL	Acute lymphoblastic leukemia
BCL	B cell lymphoma protein
BCR	B cell receptor
BCR	Breakpoint cluster region
BH	BTK homology
BLNK	B cell linker protein
BMX/ETK	Bone marrow tyrosine kinase/ Epithelial and endothelial tyrosine kinase
BTK	Bruton tyrosine kinase
CD69	Cluster of differentiation 69
CDK	Cyclin-dependent protein kinase
DAG	Diacylglycerol
DIC	Differential interference contrast
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
ETV	E 26 transforming-specific translocation variant gene 6
FcεRI	High affinity IgE receptor
GFP	Green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
HLH	Helix-loop-Helix
IDA	Interdomain-A
IDB	Interdomain-B
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
IP3	Inositol (1,4,5)-trisphosphate
ITAM	Immunoreceptor tyrosine based activation motif
ITK	IL-2 inducible T cell kinase
LAT	The linker for activation of T cells
NFκB	Nuclear factor kappa B

NFAT	Nuclear factor of activated T cells
PE	Phycoerythrin
PEI	Polyethylenimine
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3 kinase
PIN-1	Peptidyl-prolyl cis/trans isomerases-1
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PRR	Proline rich region
PKB	Protein kinase B (AKT)
PLCγ	Phospholipase Cγ
PNT	Predicted pointed
PTEN	Phosphatase and tensin homolog deleted on chromosomes 10
PVDF	Polyvinylidene difluoride
RAS	Rat sarcoma
RLK/TXK	Resting lymphocyte kiase/ T and X cell expressed kinase
SAM	Sterile alpha motif
SDS	Sodium dodecyle sulphate
SH2	Src homology 2
SH3	Src homology3
SHIP	SH2 containing inositol phosphatase
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
STAT	Signal Transducer and Activator of Transcription
SYK	Spleen tyrosine kinase
T-BET	T-box expressed in T cells
TCR	T cell receptor
TFII-I	Transcription factor II-I
TH	Tec homology
TIM-3	T-cell immunoglobulin domain and mucin domain-3
VEGF	Vascular endothelial growth factor
WT	Wild type
XID	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
ZAP-70	ζ-chain associated protein tyrosine kinase of 70 kDa

1 INTRODUCTION

With an increasing number of cases from 11.3 million in 2007 to the estimated, 15.5 million in 2030, cancer remains a leading killer worldwide (http://www.who.int/features/qa/15/en/index.html). Abnormal and unchecked division of cells causes this pathology with a possible capability of invading other organs of the body. A plethora of protein molecules orchestrates various cell functions including cell division and cell death. Disarray in the concert of these molecules often leads to diseases including cancer. Causative agents for these pathologies largely remained elusive over a long period of time. In 1914, Theodor Boveri suggested chromosomal abnormalities as the responsible causative elements of cancer (Balmain, 2001; Rowley, 2001). In 1960, Peter C. Nowell and David Hungerford were the first to report a *bona fide* genetic alteration in chronic myelogenous leukemia, which was named as the Philadelphia chromosome (Balmain, 2001; Nowell and Hungerford, 1960; Rowley, 2001). In 1970, improved cytogenetic techniques enabled the mapping of the Philadelphia chromosome as a translocation between chromosome 9 and 22 (Balmain, 2001; Rowley, 2001). Further developments in molecular biology techniques led to the identification of the fusion genes and their product as BCR-ABL. This was followed by another translocation report in Burkitt's lymphoma between chromosomes 8 and 14 that resulted in juxtaposition of C-MYC oncogene under control of Immunoglobulin promoter. The association of chromosomal abnormality with cancer started a whole new era of cancer biology (Drexler et al., 1995; Rabbitts, 1994).

Seminal work of Edmond H. Fischer and Edwin G. Krebs led to the discovery of reversible serine-phosphorylation of the glycogen phosphorylase by the phosphorylase kinase (Fischer and Krebs, 1955). Isolation and purification of protein kinase A (PKA/c-AMP-dependent kinase) suggested a wider role of phosphorylation than thought (DeLange et al., 1968; Walsh et al., 1968). Threonine and serine phosphorylations were suggested to be of physiological relevance (Shoji et al., 1979). In 1979, Tony Hunter discovered first tyrosine phosphorylation on the SRC proto-oncogene (Eckhart et al., 1979; Hunter and Sefton, 1980). Although tyrosine phosphorylation is the least frequent (2%), compared with threonine (12%) or serine (86%), it is more dynamic and crucial in signaling networks (Ding et al., 2007; Hunter, 1996; Zhang et al., 2007). The role of phosphorylation in protein regulation was mostly

revealed by the studies conducted on serine/threonine kinases PKA, cyclin-dependent protein kinase 2 (CDK2) and extracellular regulated kinase (ERK) and tyrosine kinase insulin receptor kinase (IRK) (Dissmeyer and Schnittger, 2011; Johnson, 2009; Johnson and Lewis, 2001).

More importantly, the publication of the human-kinome generated a wealth of information that has kept providing us with the much needed foundations for understanding protein kinases in health and diseases (Manning et al., 2002). The human genome contains a total of 518 kinases which make up 2% of the total gene number. 90 tyrosine kinases and 5 pseudo-tyrosine kinases have been reported in humans (Manning et al., 2002). Out of these, 58 are receptor tyrosine kinases that have been distributed in 20 families, while the rest are non receptor tyrosine kinases grouped into 10 subfamilies; ABL, ACK, CSK, FAK, FES, FRK, JAK, SRC, TEC and SYK (Manning et al., 2002). To counter the action of tyrosine kinases, the genome is also equipped with 107 protein tyrosine phosphatases (Alonso et al., 2004).

A major advancement has been achieved in understanding the mechanism of activation of the kinases and their role in health and malignancies (Ghigo et al., 2012; Hussain et al., 2011; Masson and Ronnstrand, 2009; Pyne et al., 2009). Many of the kinases have been cloned and characterized *in vivo* and *in vitro*. More than half (51 of 90) of the tyrosine kinases have been implicated in pathologies due to deregulated activity or expression (Blume-Jensen and Hunter, 2001). Recently, a non-random reciprocal chromosomal translocation t(5;9)(q33;q22) was discovered in a subset of non-specific peripheral T cell lymphomas (Streubel et al., 2006). The consequence of this translocation event is a constitutively active chimeric kinase, ITK-SYK; containing the PH-TH domain doublet of ITK and the kinase domain and most of the linker-B region of SYK (Hussain et al., 2009; Rigby et al., 2009). ITK is member of the TEC family kinases (TFKs) while SYK belongs to the SYK family of kinases. To our knowledge, this is the only example of the involvement of a TFK in chromosomal translocation resulting in cancer, although SYK has also been reported earlier in another translocation event, TEL-SYK (Kanie et al., 2004; Kuno et al., 2001).

1.1 TEC Family Kinases

TEC family of kinases consists of five members: TEC, BTK, ITK, RLK/TXK, and BMX. With the exception of RLK, all Tec family kinases (TFKs) have PH-TH domain doublet at the N-terminus, followed by SH3, SH2, and a kinase domain (Figure 1) (Smith et al., 2001). PH-TH domain doublet of TFKs binds to the phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is the product of Phosphatidylinositol3-kinase (PI3K), and tethers the PH-TH domain doublet bearing proteins on to the plasma membrane (Nore et al., 2000; Salim et al., 1996; Scharenberg et al., 1998). In RLK, the PH domain is replaced by a cysteine string motif, which serves the same membrane-binding function for this kinase (Debnath et al., 1999). Loss-of-function mutations in BTK lead to a block in B cell development between pro-B and pre-B cell stages and result in the complete lack of immunoglobulins in man, while similar mutations in mice result in a milder phenotype (Rawlings et al., 1993; Thomas et al., 1993). Simultaneous loss-of-function mutations in BTK and TEC show a severe phenotype in mice and TEC is considered to compensate for the BTK loss in mice but not in humans (Ellmeier et al., 2000). BTK plays an important role in the B cell proximal signaling (Nore et al., 2000; Salim et al., 1996; Scharenberg et al., 1998). ITK is mainly expressed in T cells, which also express TEC and RLK kinases (Berg et al., 2005; Tomlinson et al., 2004b). ITK seems to have similar regulation and function in T cells, as BTK has in B cells, yet there are fundamental differences between the two (Tomlinson et al., 2004b).

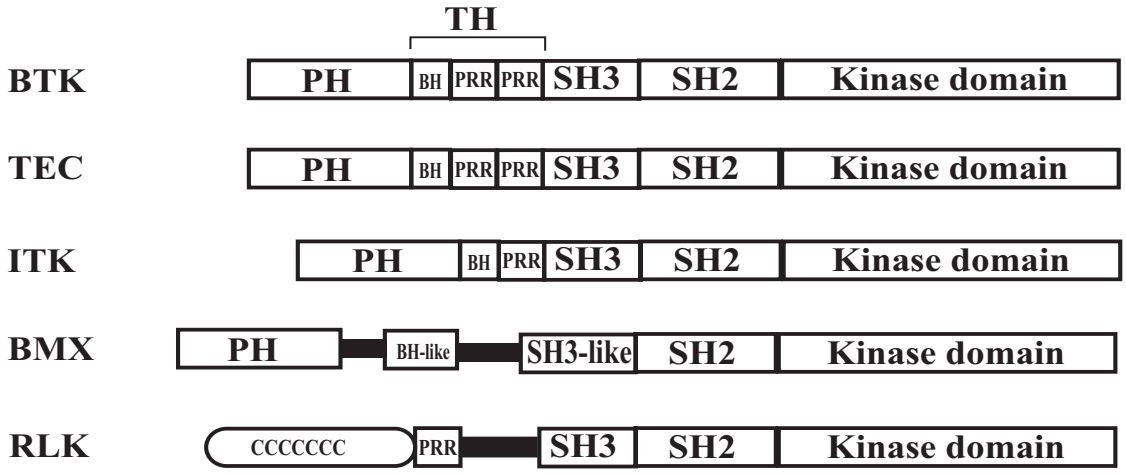


Figure 1. Schematic representation of TEC family kinases.

PH-TH domain doublet mediated membrane translocation is an essential step for placing the TFKs in close contact with other activating and/or interacting signaling partners like SRC family kinases (SFKs) and adapter proteins like SLP-65/BLNK and SLP-76 (August et al., 1997; Kersseboom et al., 2006; Rawlings et al., 1996). A loss-of-function PH-TH domain BTK mutant (R28C) fails to translocate to the plasma membrane and causes the primary immunodeficiency X-linked agammaglobulinemia (XLA) in human and X-linked immunodeficiency (Xid) in mice (Rawlings et al., 1993). Studies have shown that inhibition of PI3K has similar inhibitory effects on TFKs activation like the loss-of-function PH-TH domain mutation (R28C in BTK and R29C in ITK) (August et al., 1997; Fukuda et al., 1996; Yang et al., 2001). Random mutagenesis revealed few negatively charged surface residues that play important roles in the membrane binding and activation of TFKs (Li et al., 1995). Mutation of a glutamic acid in the PH-TH domain of BTK (E41K) results in enhanced membrane translocation and transformed phenotype in NIH3T3 cells. In similar studies, negatively charged amino acids D43 and E45 have been replaced with positive residues D43R and E45K/E45R, respectively. D43R caused a rounded phenotype while E45K/E45R showed milder cytoskeletal changes (Mohamed et al., 2009). Two highly conserved serines in the PH-TH domain doublet of the Tec family kinases are believed to be important. Thus, S21 and S115 in the BTK have been shown to regulate BTK stability/half-life in a cell-cycle dependent manner via its modification with Peptidyl-prolyl cis/trans isomerases-1 (PIN-1) (Yu et al., 2006).

TFKs play important roles in lymphocytes proximal signaling where receptor ligation results in the activation of SRC family members that phosphorylate ITAMs and create docking sites for SYK family kinases. SYK family kinases bind to the phosphorylated ITAMs and in turn are activated (Keshvara et al., 1998; Kulathu et al., 2009; Mocsai et al., 2010; Smith-Garvin et al., 2009; Turner et al., 2000). Activated SYK family kinases phosphorylate the adapter protein BLNK in B cells, and SLP-76 in T cells and both adapters couple SYK function with other interacting molecules like GRB2 and PLCγ (Gross et al., 1999; Koretzky et al., 2006; Kulathu et al., 2009). At the same time, receptor ligation and activation recruits TFKs to the cell membrane where SFKs phosphorylate the TFKs at their activation-loop tyrosines. Membrane binding and phosphorylation at the activation-loop tyrosines results in activation of the TFKs. TFKs further phosphorylate their own SH3 domains and other substrates such as PLCγ or

SLP-76 (Kurosaki and Hikida, 2009; Nore et al., 2003; Perez-Villar and Kanner, 1999; Sela et al., 2011).

1.1.1 ITK

IL-2-inducible T cell kinase (ITK) was discovered as a T cell kinase inducible by IL-2 (Heyeck and Berg, 1993; Siliciano et al., 1992). ITK is expressed mainly in T cells along with mast cells (Yamada et al., 1993). ITK, like other TEC family kinases has an N-terminal PH domain followed by the TEC homology (TH) domain containing a proline rich region (PRR). The TH domain is followed by the SH3, SH2 and kinase domains in the C-terminus (Figure 1). These domains regulate various functions of the ITK like autoinhibtion, activation, subcellular localization, protein-protein interactions and substrate phosphorylation.

ITK has been suggested to maintain an autoinhibtory conformation in resting cells through SH3 domain binding to PRR in TH domain (Andreotti et al., 1997; Bradshaw, 2010). Disruption of the autoinhibitory conformation through mutation or competition with a peptide results in elevated ITK activation; deletion of PRR, on the other hand results in dampened activity (Andreotti et al., 1997; Hao and August, 2002). Similarly, intramolecular interactions between SH3 domain and SH2 domain have also been shown to result in head-to-tail dimerization of ITK in inactive state (Andreotti et al., 1997; Brazin et al., 2000). Yet fluorescence complementation assays have revealed that ITK monomer adopts a folded conformation in an inactive state (Qi and August, 2009). On the contrary, isolated ITK kinase domain, lacking autoinhibitory domains, also shows compromised catalytic activity, suggesting a positive regulatory function of domains outside the kinase domain along with autoinhibitory function (Joseph and Andreotti, 2009; Joseph et al., 2007).

PH domain doublet binding to the membrane phospholipids facilitates membrane translocation of the ITK (Ching et al., 1999). This phospholipid binding is mediated by a unique FYF motif present in the PH domain of only TEC family kinases and is absent in other PH domain containing proteins. Although the ITK PH domain can also bind to phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 4-phosphate (PI4P), it shows highest affinity binding with the phosphorylation product of PI3K, PIP3. PIP3-bound, membrane-localized ITK is phosphorylated by SRC family kinases

and gets activated. Soluble inositol 1,3,4,5-tetrakisphosphate (IP4) has also been implicated in ITK activation and regulation of feedback loop of PLCγ1 activation in T cells (Huang et al., 2007). ITK membrane translocation and thus activation is reversed by the phosphatase and tensin homolog deleted on chromosomes 10 (PTEN) that removes D3 phosphorylation and converts PIP3 into PIP2. Cells lacking PTEN not only show constitutive membrane localization of ITK but also enhanced response to CD3 mediated T cell activation (Hirve et al., 2012; Shan et al., 2000). A similar negative regulatory function has also been suggested for inositol-5 phosphatase SHIP1, that blocks NFAT activation in T cells through TEC kinase inhibition (Tomlinson et al., 2004a). SHIP1 has also been suggested to negatively regulate BTK activation through PIP3 dephosphorylation (Bolland et al., 1998).

SH3 domains modulate protein function or cellular localization through PRR interactions. ITK SH3 domain interacts with a number of proteins including c-CBL, VAV-1, and SLP-76 (Bunnell et al., 1996). ITK SH3 domain binds to PRR of SLP-76 and then SH2 domain of ITK binds to the phosphorylated tyrosine 145 of SLP-76 (Bunnell et al., 2000). A recent report has shown that ITK phosphorylates tyrosine 173 of SLP-76, which is required for antigen receptor mediated activation of PLCγ1 in mast and T cells (Sela et al., 2011). The SH3 domain can also modulate nuclear transport of ITK through its interaction with the nuclear import chaperone karyopherin α (Rag cohort 1α, Rch1α). Disruption of this interaction not only leads to reduced nuclear localization of ITK, but also highly compromised IL-2 production (Perez-Villar et al., 2001). SH2 domain binding to phosphorylated tyrosine provides yet another mechanism of protein-protein interaction. The SH2 domain of ITK interacts with LAT, VAV-1, SLP-76 and PLCγ1, although whether the interaction with PLCγ1 is direct or through SLP-76 is not resolved yet (Bogin et al., 2007; Ching et al., 2000; Dombroski et al., 2005; Perez-Villar and Kanner, 1999; Shan et al., 2000).

ITK phosphorylates a number of targets including SLP-76 (Y173), PLCγ1 (Y775, Y783), T-BET (Y525), TIM-3 (Y265) and TFII-I (248) (Bogin et al., 2007; Hwang et al., 2005; Sacristan et al., 2009; Sela et al., 2011; van de Weyer et al., 2006). Interaction of ITK with SLP-76 needs both the SH3 and the SH2 domains. Disruption of this interaction via a competitive peptide abrogates TCR induced plasma membrane recruitment of ITK (Grasis et al., 2010). Consistently, ITK phosphorylation at Y511,

actin polymerization, and Th2 cytokine production are blocked following TCR activation in the presence of a competitive peptide that disrupts the SLP-76/ITK interaction (Grasis et al., 2010). ITK SH2 and SH3 domains bind to phosphorylated tyrosine 145, and PRR of SLP-76, respectively (Bunnell et al., 2000; Su et al., 1999). Similarly, the SH3 domain of PLCγ1 also interacts with the PRR of SLP-76 and is pivotal for PLCγ1 activation (Deng et al., 2005; Gonen et al., 2005; Singer et al., 2004). ITK, SLP-76 and PLCγ1 interactions are essential for functionally critical phosphorylation of PLCγ1(Y783) (Bogin et al., 2007; Serrano et al., 2005). Activated PLCγ1 catalyses PIP2 that generates inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). IP3 mediated elevation of Ca^{2+} causes NFAT and NFκB pathways activation, while DAG translates in RAS activation. These activated signaling pathways result in transcriptional modulation of various genes (Andreotti et al., 2010; August and Ragin, 2012).

1.2 SYK Family Kinases

1.2.1 Structure

SYK family kinases consist of two related non-receptor tyrosine kinases SYK and ZAP-70. Following receptor ligation, SYK family kinases play key roles in signal transduction (Fischer et al., 2010; Kulathu et al., 2009; Mocsai et al., 2010; Palacios and Weiss, 2007; van Oers and Weiss, 1995). SYK was purified as a protein tyrosine kinase from bovine thymus by Geahlen, in 1986 (Zioncheck et al., 1986). Yamamura, in 1990, purified a cytosolic protein tyrosine kinase of 40 kDa from porcine spleen and named it “spleen tyrosine kinase” (SYK) (Kobayashi et al., 1990). Later, molecular cloning of the gene revealed SYK as a 72 kDa non-receptor protein tyrosine kinase (Taniguchi et al., 1991). In 1992, Chan et al. cloned cDNA of a 70 kDa protein tyrosine kinase. This kinase was phosphorylated following TCR activation, and associated with the ζ-chain, thus named as Zeta-chain associated protein kinase of 70 kDa, ZAP-70 (Chan et al., 1992). Mice lacking SYK show perinatal lethal phenotype and blocked B cell development at the pro-B to pre-B cell transition (Cheng et al., 1995; Turner et al., 1995). Similarly, severe defects in T cell development have been observed in humans and mice lacking ZAP-70 (Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995).

Both SYK and ZAP-70 have a similar architecture consisting of tandem SH2 domains and a C-terminus kinase domain. "linker-A" bridges N-terminal SH2 (N-SH2) domain to C-terminal SH2 (C-SH2) domain and C-SH2 is joined to kinase domain via "linker-B". SYK is widely expressed while ZAP-70 is mainly expressed in T cells and also in early B cells as well as in some B cell lymphomas (Palacios and Weiss, 2007). SYK also expresses a shorter, alternatively spliced form SYK-B that lacks 23 amino acids in the N-terminus of linker-B region (Latour et al., 1996). ZAP-70 linker-B region is also shorter compared with the SYK and resembles more to SYK-B (Latour et al., 1996). Following receptor ligation, SYK family kinases are activated and bind to the phosphorylated ITAMs through their SH2 domains. SYK/ITAMs binding results in BCR signalling amplification through a positive feedback loop (Rolli et al., 2002). Inactive SYK family kinases are kept in an "autoinhibited" conformation through interactions among residues in the linker-regions and kinase domain by adopting a so-called "linker-kinase-sandwich" (El-Hillal et al., 1997; Kulathu et al., 2009). Upon ITAMs binding or through phosphorylation of the functional-tyrosines, SYK family kinases are released from the autoinhibition and get activated (Au-Yeung et al., 2009; Bradshaw, 2010).

SYK has a number of tyrosines that play critical roles in the activation, receptor-detachment, inhibition and interaction of the kinases with other signaling partners (Geahlen, 2009; Kulathu et al., 2009). Figure 2, shows the structure of ITK-SYK, SYK, and ZAP-70 along with their corresponding tyrosines. Table 1 describes the corresponding tyrosines of ITK-SYK, SYK and ZAP-70 and their suggested functions for SYK or ZAP-70.

ITK-SYK	SYK	ZAP70	Motif	Function*
NA	Y131	Y126	IDA	BCR-dissociation/increase kinase activity
Y183	Y323	Y292	IDB	binds Cbl-SH2/p85(PI3K)
Y208	Y348	Y315	IDB	binds Vav, PLC-γ-SH2, + kinase activity
Y212	Y352	Y319	IDB	binds PLC-γ-SH2, +regulates kinase activity
Y385/Y386	Y525/Y526	Y492/Y493	AL	Activation-loop
Y489-91	Y629-31	Y597/Y598	C-tail	SLP-65-binding, autoinhibition

Table 1. Corresponding tyrosines (numbered as in humans) of ITK-SYK, SYK, and ZAP-70. *Functions have only been described for SYK or ZAP-70.

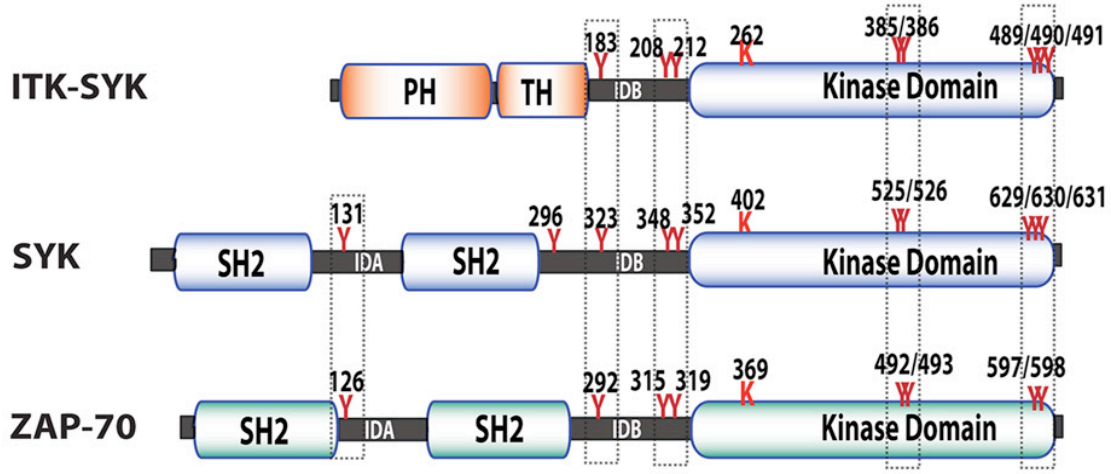


Figure 2. Schematic representation of ITK-SYK, SYK and ZAP-70 (Hussain et al. III)

1.2.2 Linker-Region Tyrosines

Y131 of SYK corresponds to Y126 of ZAP-70. These tyrosines are located in the linker-A or interdomain-A (IDA) region of SYK family kinases, while a related tyrosine in ITK-SYK is missing (Table 1). Phosphorylation of Y131 results in conformational changes that dissociate SYK from ITAMs. Thus, in spite of increased basal kinase activity, BCR dependent phosphorylation of downstream cellular proteins is compromised in SYK-Y131E mutant (Keshvara et al., 1997; Zhang et al., 2008). In ZAP-70, loss of Y126 results in significantly reduced Ca^{2+} signal following CD3 activation in a T cell line (Szabo et al., 2012). In interdomain-B (IDB) or linker-B region, SYK Y323, Y348 and Y352 correspond to Y292, Y315, and Y319 of ZAP-70. c-CBL binding to Y323 leads to downregulation of SYK activity by proteasomal degradation, while the same tyrosine also binds to the p85 subunit of PI3K (Lupher et al., 1998; Moon et al., 2005). Negative regulatory function is also observed for ZAP-70 where loss of the corresponding tyrosine, 292 leads to enhanced downstream signaling with upregulated IL-2 secretion compared with wild type ZAP-70 (Kong et al., 1996).

Y348 and Y352 are present on the same tryptic peptide of SYK and correspond to Y315 and Y319 of ZAP-70 (Geahlen, 2009). Following BCR engagement, phosphorylation of either one or both of these two tyrosines can occur. In one report, both of these sites have been demonstrated to be phosphorylated in cells lacking the SRC family kinase LCK, suggesting autophosphorylation as the possible mechanism responsible for this phosphorylation (Geahlen, 2009; Keshvara et al., 1998; Tsang et al., 2008). Interestingly, kinase-inactive SYK can also get phosphorylated on these tyrosines in the presence of LCK proposing a transphosphorylation mechanism (Geahlen, 2009; Keshvara et al., 1998; Tsang et al., 2008). Phosphorylation at Y348 and Y352 positively regulates SYK function in signal transduction. Phenylalanine replacement of Y348 in mast cells results in dampened phosphorylation of PLCγ, LAT, SLP-76 and VAV-1. In contrast, in primary mast cells, ERK and AKT phosphorylation is affected upon loss of Y352 (Groesch et al., 2006; Simon et al., 2005; Zhang et al., 2002). Simultaneous loss or replacement of both tyrosines with phenylalanine has profound negative effects on the kinase activity and downstream signaling, which is greater than loss of either single one. Interestingly, loss of Y323, along with dual-loss of Y348 and Y352 rather recoups downstream signaling in B cells or mast cells (Hong et al., 2002; Simon et al., 2005).

Also, corresponding tyrosines in ZAP-70 (Y315 and Y319) are believed to play similar roles. Phenylalanine replacement of Y315 inhibits phosphorylation of ZAP-70 itself, VAV, SLP-76 and SHC and abrogates VAV-1 binding (Wu et al., 1997). In T cells, although, replacement of Y319 with phenylalanine does not affect the kinase activity of ZAP-70, the downstream signaling involving Ca^{2+} mobilization, RAS activation and CD69 expression are highly compromised (Williams et al., 1999). In another study, replacement of Y315 with phenylalanine had no impact on downstream signaling. However, Y319 has been shown to be indispensible for IL-2 secretion or NFAT activation following anti-TCR induced T cell activation (Di Bartolo et al., 1999). Mouse model studies have revealed indispensible role of Y315 and Y319 in mediating positive and negative T cell selection in thymus (Gong et al., 2001).

1.2.3 Activation-Loop Tyrosines

The paired tyrosines 525/526 are present in the activation-loop of SYK kinase domain and correspond to the paired tyrosines 492/493 of ZAP-70. The activation-loop tyrosines of the SYK are phosphorylated following BCR or FcεRI receptor engagement in B and mast cells, respectively (Carsetti et al., 2009; El-Hillal et al., 1997; Furlong et al., 1997; Keshvara et al., 1998; Zhang et al., 2000). Although loss of the activation-loop tyrosines in SYK does not show any effect on catalysis, downstream signaling events are dampened (Carsetti et al., 2009; Kulathu et al., 2009; Mocsai et al., 2010; Zhang et al., 2000). Moreover, biochemical studies show that in the presence or absence of the activation-loop phosphorylation, catalysis rate of SYK kinase remains the same (Papp et al., 2007). Activation-loop tyrosines of ZAP-70 are phosphorylated by LCK in response to TCR engagement (Au-Yeung et al., 2009; Watts et al., 1994). In ZAP-70, Y492 replacement with phenylalanine (Y492F) results in elevated catalysis. In spite of no effect on basal catalysis, Y493 replacement with phenylalanine (Y493F) abrogates pervanadate or LCK-mediated activation of ZAP-70 (Wange et al., 1995). Similarly, mutating Y492 (Y492F) led to the enhanced activation of Ca^{2+}. Whereas mutation of the adjacent tyrosine Y493 (Y493F) resulted in compromised Ca^{2+} activation, following CD3 activation of a T cell line.

Activation-loop tyrosines are known to play key roles in switching the kinase activity from low basal levels to elevated catalysis (Adams, 2003). Crystal structures have been critical in elucidating the phosphorylation mediated activation-loop conformational changes. Protein kinase A (PKA) and Insulin receptor kinase (IRK) are two such examples (Hubbard, 1997; Zheng et al., 1993). Following phosphorylation at activation-loop residues, activation-loop swings from inhibitory "loop-in" conformation to activating "loop-out" conformation. Crystal structures of isolated kinase domains of SYK and ZAP-70 showed unusual loop-out conformation in the non-activated state (Adams, 2003; Atwell et al., 2004; Jin et al., 2004). Thus, the activation-loop, phosphorylated or not, does not seem to impact catalysis rates in SYK family kinases. This is also supported by biochemical studies, where replacement of both activation-loop tyrosines to phenylalanine shows kinetics comparable with pre-phosphorylated activation-loop tyrosines in SYK (Papp et al., 2007). Thus, it was thought that the activation-loop does not regulate substrate binding or ATP access

(Geahlen, 2009). Role of activation-loop tyrosines in SYK remained intriguing. A recently published high resolution crystal structure has revealed that inhibited full length SYK adopts a different conformation as compared with "loop-out" conformation of the isolated kinase domain (Gradler et al., 2013).

1.2.4 C-Terminus Tyrosines

SYK has three tyrosines 629, 630 and 631 at the C-terminus that correspond to two tyrosines 597 and 598 of ZAP-70 at the C-terminus. In insect cells, Y630 was phosphorylated following BCR reconstitution, providing a binding motif for BLNK (Kulathu et al., 2008). In mast cells, phosphorylation was detected on Y629 and Y630 (Cao et al., 2007; Kulathu et al., 2008). Replacement of C-terminal tyrosines with phenylalanine resulted in elevated phosphorylation of activation-loop tyrosines – yet, the downstream signaling events including NFκB were diminished (de Castro et al., 2010). Recently, a number of molecules including NCK, SHIP1, GRB2, SLP-76 and TULA2 were shown to interact with the SYK C-terminal phosphoprylated tyrosines 629 and 630 in mast cells. SHIP1 and TULA2 negatively regulate SYK function downstream of high affinity IgE binding receptor FcεRI (de Castro et al., 2012). In ZAP-70, phenylalanine replacement of C-terminus tyrosines 597 and 598 results in activation of the kinase (Deindl et al., 2007; Zeitlmann et al., 1998). Similarly, the C-terminus tyrosines of ZAP-70 are involved in autoinhibition and regulation but lack SLP-76 binding motif (Deindl et al., 2007; Deindl et al., 2009; Kulathu et al., 2009; Zeitlmann et al., 1998).

1.2.5 Autoinhibition

Expression levels, half-life, phosphorylation, dephosphorylation, subcellular localization and interaction with other proteins, tightly regulate the signaling molecules in time and space. To maintain a low basal state of activity in resting cells, most kinases including SYK, are generally autoinhibited (Kulathu et al., 2009). Unphosphorylated, isolated kinase domain of SYK adopts an "open" conformation and possesses most of the features of active, phosphorylated form of LCK (Ablooglu et al., 2001; Atwell et al., 2004; Yamaguchi and Hendrickson, 1996). 3D structure determined by the single-

particle electron microscopy for a full-length SYK suggested an inhibited conformation (Arias-Palomo et al., 2007). Similar to the isolated kinase domain of SYK, ZAP-70 kinase domain also adopted an active, "loop-out" conformation despite lack of phosphorylation at the activation-loop tyrosines (Ablooglu et al., 2001; Jin et al., 2004). Crystal structure of full-length ZAP-70 showed a distinct autoinhibitory conformation (Deindl et al., 2007). Autoinhibited conformation of ZAP-70 is similar to inactive conformation, which was first observed in CDK2 and later in c-SRC and HCK (De Bondt et al., 1993; Schindler et al., 1999; Sicheri et al., 1997; Xu et al., 1999). Considering the similarity between the SYK family kinases and lack of a full-length high resolution crystal structure, an autoinhibitory model for SYK was developed on the basis of available crystal structure of full-length ZAP-70 (Kulathu et al., 2009). ZAP-70 conforms an autoinhibited state of linker-kinase sandwich, so called because interdomain-B tyrosines 315 and 319 sandwich the conserved tryptophan (W131) in interdomain-A and make hydrophobic interactions with the proline P396, in the kinase domain (Brdicka et al., 2005; Deindl et al., 2007; Jin et al., 2004). Moreover, to further stabilize this autoihibitory conformation, C-terminal tyrosines 597 and 598 interact with P147 in the interdomain-A (Deindl et al., 2009; Jin et al., 2004). A recent publication of a full-length SYK crystal structure, in spite of similar overall topologies, presented a number of conformational differences compared with ZAP-70 (Gradler et al., 2013). Autoinhibition "hot-spot region" interdomain-B tyrosines 348 and 352 do not superimpose on the corresponding Y315 and Y319 of ZAP-70. Instead of aligning with Y315 of ZAP-70, Y348 of SYK aligns with Y319 of ZAP-70, while Y315 of ZAP-70 aligns with M343 of SYK (Gradler et al., 2013).

1.2.6 SYK Inhibitors

Achievements made in the field of molecular biology and biochemistry have led to the development of small molecular inhibitors for kinases. 300 recombinant protein kinases have been used to test 178 commercially available kinase inhibitors (Anastassiadis et al., 2011; Davis et al., 2011). Natural pigment curcumin that was isolated from *Curcuma longa* induced apoptosis in leukemia and malignant cancer cell lines (Jiang et al., 1996; Kuo et al., 1996). A number of inhibitors that target the conserved ATP binding site of SYK have been developed (Ruzza et al., 2009; Uckun and Qazi, 2010). Recent studies on small molecular inhibitor R406 and its prodrug R788 (fostamatinib disodium) showed that SYK inhibition resulted in B lymphocyte

depletion (Barr et al., 2012). Another study has shown therapeutic potential of this drug in non-Hodgkin lymphoma and chronic lymphocytic leukemia (Friedberg et al., 2010). Along with SYK inhibition, fostamatinib also showed side effects that have been attributed to VEGF receptor inhibition (Friedberg et al., 2010). Recently a pentapeptide inhibitor (mimic C-16) has been developed that blocks substrate-binding (P-) site of the SYK and thus abrogates its kinase activity (Uckun et al., 2010b). C-16 showed no toxicity or side effects in cynomolgus monkeys but destroyed more than 99.9% Acute Lymphoblastic leukemia (ALL) cells *in vivo* (Uckun et al., 2010a). Another mechanism of inhibition for SYK has been achieved through allosteric regulation (Hall et al., 2012). This allosteric inhibitor X1 is noncompetitive against ATP or substrate binding (P-) site and inhibits the kinase by reinforcing the regulatory interactions between the SH2 and kinase domains (Hall et al., 2012). Whether it shows safe functionality *in vivo* remains to be elucidated.

1.3 SLP-76 And SLP-65

SLP-65/BLNK and SLP-76 are adapter proteins lacking intrinsic catalytic activity. However, since they are multidomain molecules, are able to efficiently act as scaffolds and can readily modulate signal transduction by nucleating signaling complexes (Koretzky et al., 2006; Tomlinson et al., 2000; Yablonski and Weiss, 2001). B cells and macrophages express SLP-65 adapter protein that couples the B-cell receptor to downstream signaling by establishing a so called signalosome (Yablonski and Weiss, 2001). Loss of SLP-65 results in the partial block of B cell development at the pre-B cell stage and subsequently, loss of peripheral B1 cells and decreased number of B2 cells. Moreover, 5-15% of the mice lacking SLP-65 show pre-B cell leukemia (Herzog et al., 2006). In macrophages, both adapters SLP-65 and SLP-76 are expressed, yet their deficiency does not manifest any phenotype (Nichols et al., 2004). SLP-76 is expressed in a variety of cells, including T cells, B cells, mast cells, platelets, neutrophils, macrophages and NK cells (Clements et al., 1998). T cell development is blocked at double-negative 3 (DN3) stage upon SLP-76 loss and is required for transition from the double-positive to the single-positive stage (Koretzky, 2009; Koretzky et al., 2006). Mast cells lacking SLP-76 not only show reduced degranulation and cytokine production, but also loss of systemic anaphylaxis (Kettner et al., 2003; Pivniouk et al., 1999). Neutrophils show SLP-76 dependent production of reactive

oxygen intermediates upon Fcγ receptor ligation or adhesion. Similarly, integrin-mediated spreading of neutrophils is also SLP-76 dependent (Newbrough et al., 2003). Absence of SLP-76 causes *in utero* anastomosis, a lethal phenotype where blood and lymphatic systems fail to separate (Abtahian et al., 2003). Interestingly, this condition is phenocopied in SYK or PLCγ deficient mice (Cheng et al., 1995; Turner et al., 1995).

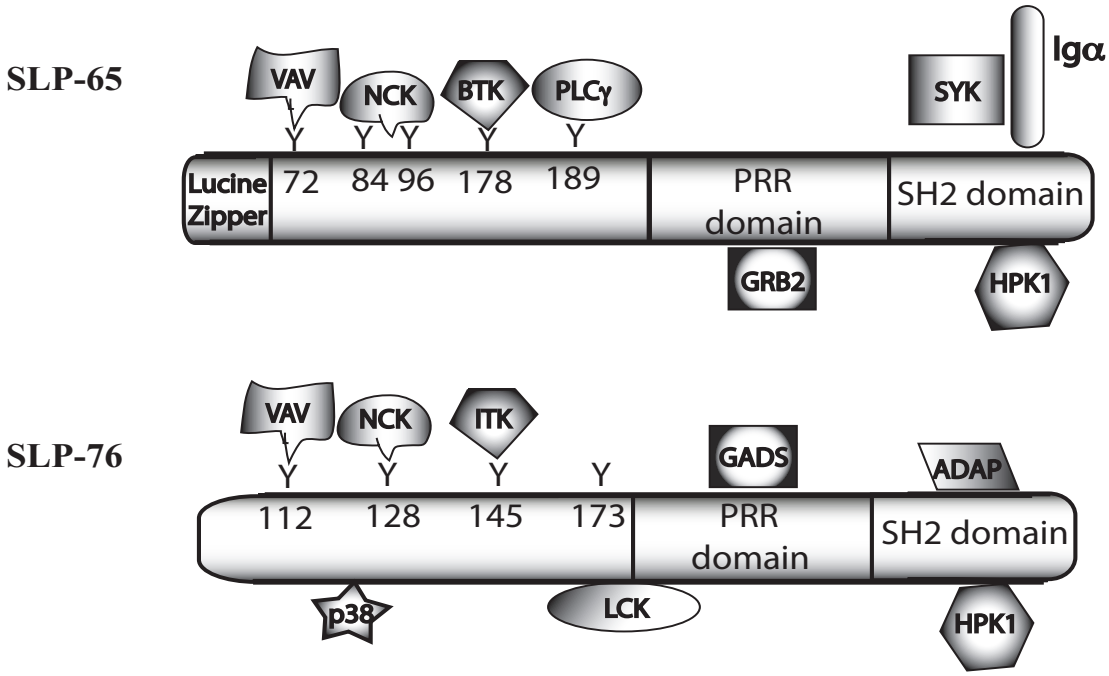


Figure 3. Schematic representation of SLP-65 and SLP-76 showing the domain structures and interacting signaling partners.

SLP-65 consists of a leucine-zipper motif responsible for its membrane targeting (Figure 3) (Kohler et al., 2005). Yet another study showed SH2 domain-mediated membrane translocation, which additionally needs adapter function of SYK for downstream signaling (Abudula et al., 2007). SLP-76 contains a similar N-terminal region harboring leucines and isoleucines. However, it is not a *bona fide* leucine-zipper and fails to do the same function (Abraham and Weiss, 2004; Koretzky et al., 2006). In T cells, it is LAT protein that mediates the membrane tethering of phosphorylated SLP-76 already bound to p85 subunit of PI3K (Shim et al., 2011). Following, leucine-zipper, SLP-65 has an N-terminal region with phosphorylatable tyrosines, a proline-rich domain and a C-terminus SH2 domain. Despite only 33% sequence homology, remarkable similarity exists between the domain structures of SLP-65 and SLP-76

(Koretzky et al., 2006). These conserved tyrosines in SLP-65 and SLP-76 are mostly SYK family phosphorylated and create binding sites for other interacting partners like VAV, NCK, ITK and GADS (Bubeck Wardenburg et al., 1996). A recent report suggested another tyrosine phosphorylation (Y173) of SLP-76 by ITK (Sela et al., 2011). Interestingly, adapter proteins not only play a role in the temporal and spatial availability of the partner proteins, but may also take part in the activation/opening of the molecules. Such a phenomenon is known for SYK, where SLP-65 binds a C-terminus tyrosine and maintains its open/active conformation (Kulathu et al., 2009). SYK and SLP-65 association mediates BTK phosphorylation, which plays an important role for BCR signaling and B cell development (Baba et al., 2001). Although such physical binding has not been shown between SYK and SLP-76, it will not be surprising if they also physically interact and modulate signaling. SYK and SLP-76 association is also supported by the fact that SYK or SLP-76 deficient mice show a similar phenotype (Abtahian et al., 2003).

1.4 ITK-SYK

Recurrent chromosomal translocations are often associated with haematological and non-haematological malignancies. Novel t(5;9)(q33;q22) was discovered in a subset of non-specific peripheral T cell lymphomas (Huang et al., 2009; Streubel et al., 2006). Sequence analysis revealed a previously undescribed chimera caused by the in-frame fusion of the N-terminal region of ITK with the C-terminal region of SYK. The resulting chimeric kinase ITK-SYK inherits PH-TH domain doublet of ITK and much of the linker-B region and the kinase domain of SYK (Hussain et al., 2009; Rigby et al., 2009; Streubel et al., 2006). Immunophenotypic analysis showed that lymphoma cells were $CD3^+$ $CD2^+$ $CD5^+$ $CD4^+$ $BCL6^+$ $CD10^+$ (Streubel et al., 2006). One patient was exception as it was also negative for CD4 (Streubel et al., 2006). Moreover, these cells were negative for CD8 and SYK. Quantitative RT-PCR (qRT-PCR) analysis showed overexpression of ITK-SYK that was comparable to the patient with 5 copies of SYK (Streubel et al., 2006).

ITK-SYK shows constitutive kinase activity *in vitro* and demonstrates a potent T cell specific oncogenic activity *in vivo* (Dierks et al., 2010; Hussain et al., 2009; Hussain et al., 2013; Pechloff et al., 2010; Rigby et al., 2009). ITK-SYK retains 8 putative functional tyrosines of SYK; three in the linker-region, a pair in the activation-loop, and

a triplet in the C-terminal tail. ITK-SYK was strongly phosphorylated at the linker-region and the activation-loop tyrosines, although under the same conditions, SYK showed no detectable phosphorylation at the activation-loop tyrosines and only residual phosphorylation at a linker-region tyrosine (Dierks et al., 2010; Hussain et al., 2009). Accordingly, ITK-SYK efficiently phosphorylated the adapter proteins SLP-76 or BLNK, whereas SYK did not (Hussain et al., 2009).

Presence of the PH-TH domain doublet, like TEC family kinases, suggested a PI3K dependent PIP3-tethered plasma membrane localization of the ITK-SYK (Hussain et al., 2009; Hussain et al., 2011; Rigby et al., 2009). Indeed, ITK-SYK showed membrane translocation in COS7 and HEK-293T cells and was fractionated with lipid rafts in Jurkat cells (Hussain et al., 2009; Pechloff et al., 2010; Rigby et al., 2009). Consistently, PI3K inhibition not only led to cytoplasmic localization of the fusion kinase but also abrogated ITK-SYK mediated phosphorylation of SLP-76 (Hussain et al., 2009). This observation was further supported upon introduction of a PH-TH domain point mutation (R29C) in the fusion kinase, which was no longer able to maintain PIP3-bound membrane-translocation and lost *in vitro* transformation capacity (Rigby et al., 2009). On the same ground, ITK-SYK-R29C was isolated in non-lipid raft fractions and failed to phosphorylate PLCγ1 or to produce IL-2, where ITK-SYK with intact PH-TH domain doublet was not only found in lipid rafts fraction and phosphorylated PLCγ1, but also led to profound IL-2 secretion (Pechloff et al., 2010). Conflicting results were obtained when ITK-SYK-R29C was co-expressed with the SLP-76 and led to equal phosphorylation of the substrate, compared with the intact ITK-SYK (Hussain et. al. II). ITK-SYK mediated activation of T cells as measured by IL-2 secretion or CD69 expression upregulation, was abrogated upon expression of kinase-inactive fusion protein or application of the SYK inhibitor R406 (Pechloff et al., 2010). Thus, kinase-inactive ITK-SYK did not show phosphorylation on the linker-region and the activation-loop tyrosines, except one linker-region tyrosine that also showed residual phosphorylation in ITK-SYK-KD and SYK (Dierks et al., 2010; Hussain et al., 2009). Consistently, increasing concentrations of SYK inhibitor curcumin inhibited not only ITK-SYK phosphorylation, but also ITK-SYK mediated activation of STAT5 and PLCγ1 *in vitro* (Dierks et al., 2010). Similarly, *in vitro* incubation of ITK-SYK positive cells with increasing concentrations of various SYK inhibitors resulted in inversely proportional survival of lymphoma cells (Dierks et al.,

2010).

ITK-SYK potently induces T cell lymphoproliferative disorder in animals mimicking human disease (Dierks et al., 2010; Pechloff et al., 2010). Similar to human disease, 50% to 60% of lymphoma cells were $CD3^+CD5^+CD4CD8^-$ and 40% were $CD3^+CD5^+CD4^+CD8^-$ in mice expressing ITK-SYK (Dierks et al., 2010). In contrast to human disease, mouse lymphoma cells induced by ITK-SYK were negative for BCL6 expression (Dierks et al., 2010). In accordance with the *in vitro* transformation capacity, fusion protein driven oncogenesis was kinase activity-dependent in mouse models (Dierks et al., 2010; Pechloff et al., 2010). Interestingly, CD19-driven over expression of ITK-SYK failed to induce B cell lymphoma; instead it resulted in a delayed T cell lymphoproliferative disorder (Pechloff et al., 2010). It was suggested that probably it is lack of ITK expression in B cells that does not support ITK-SYK mediated oncogenesis in these cells (Mulloy, 2010). Not withstanding the PH-TH domain doublet mediated activation of ITK-SYK *in vitro*, expression of ITK-SYK-R29C in mouse models resulted in perhaps augmented lymphomagenesis (Dierks et al., 2010). These observations suggest that in mouse models, ITK-SYK is a T cell specific potent oncogene that is dependent on its kinase activity, but independent of PH-TH domain doublet mediated membrane translocation.

Conflicting results about the role of the PH-TH domain doublet in ITK-SYK mediated activation demanded another explanation for constitutive activation of the fusion protein. Lack of tandem SH2 domains that maintain the autoinhibitory "closed" conformation of the SYK in the inactive state, may result in an "open" conformation causing constitutive kinase activity of ITK-SYK (Hussain et al., 2013). An open conformation, following the loss of tandem SH2 domains is supported by the isolated kinase domain of SYK that shows 10-fold higher kinase activity, compared with the full length molecule (Papp et al., 2007). An "open" conformation of ITK-SYK is also supported by the biochemical studies where the fusion kinase is shown to phosphorylate substrates in resting cells (Hussain et al., 2009). Mutagenesis studies, where putative functional tyrosines were replaced with phenylalanine, showed that kinase activity of the fusion protein is independent of phosphorylation on these tyrosines (Hussain et al., 2013). Mutagenesis results were further supported by the expression of ITK-SYK in SYF cells (lacking SRC family kinases), where in spite of lack of phosphorylation on tyrosine residues, it robustly phosphorylated SLP-76

(Hussain et al., 2013). Moreover, the fusion kinase is expressed under the control of an inducible promoter, which is a rare example of the use of inducible promoter by an oncogene. Unexpectedly, ITK-SYK harboring a PH-TH domain mutation that disables its translocation to the membrane has been shown to be more oncogenic compared with wild type. Additionally, while it has been a very potent oncogene for T cells, its forced expression under the control of a B cell promoter did not lead to B cell lymphoma.

Tightly regulated signaling pathways are not only controlled through posttranslational modifications of molecules, but also through expression level control. Promoters mediated expression ensures the availability of proteins not only at the right time but also in right quantity. Abnormal expression of proteins has also been documented in malignancies. For example, Burkitt's lymphoma and some subsets of diffuse large B-cell lymphoma are induced due to over expression of the c-MYC oncogene under control of Immunoglobulin promoter (Klapproth and Wirth, 2010). Similarly, not only SYK over expression is reported in T cell lymphomas but also loss of expression in breast cancer (Coopman et al., 2000; Feldman et al., 2008). ITK-SYK is an interesting example where translocation results in expression of the fusion kinase through an inducible promoter of IL-2 inducible tyrosine kinase ITK (Gibson et al., 1993; Siliciano et al., 1992; Streubel et al., 2006).

The stability of proteins is yet another level of regulation of the signaling molecules. c-CBL is known as negative regulator of SYK function (Zou et al., 2009). ITK-SYK was found to interact with c-CBL through the linker-region tyrosine corresponding to Y323 of SYK (Dierks et al., 2010). ITK-SYK-R29C that shows cytoplasmic localization, showed reduced c-CBL binding and shorter survival of the transgenic animals. Accordingly, membrane localized ITK-SYK-E42K not only showed enhanced Y323 phosphorylation and increased c-CBL binding, but also resulted in prolonged survival of the animals. This study shows that ITK-SYK half-life is controlled through Y323 phosphorylation and thus binding of c-CBL, ultimately regulating the oncogenesis and survival of the animals (Dierks et al., 2010).

1.5 TEL-SYK

TEL, frequently involved in different human leukemias, is a nuclear protein expressed in a wide variety of cells (De Braekeleer et al., 2012). TEL, also known as ETV6 (E26

transforming-specific translocation variant gene 6) is a member of a large transcription family ETS (E-twenty six). ETV6 protein consists of a Helix-loop-Helix (HLH) oligomerization domain and an ETS domain. HLH domain is also known as predicted pointed (PNT) or Sterile alpha motif (SAM) domain. HLH domain-mediated dimerization results in the activation of the ETV-fusion proteins including TEL-PDGFRβ, TEL-ABL, TEL-JAK2 and TEL-SYK (Cools et al., 1999; Eguchi et al., 1999; Kuno et al., 2001; Lacronique et al., 1997; Papadopoulos et al., 1995; Suto et al., 1997).

TEL-SYK was identified in one patient with myelodysplastic syndrome with t(9;12)(q22;p12). Expression of TEL-SYK in IL-3 dependent murine leukemia cells BAF3 resulted in growth factor independent transformation and proliferation of the cells. A deletion mutant lacking the dimerization domain of TEL, Δ-TEL-SYK, failed to transform these cells (Kuno et al., 2001). Analysis of BAF3 cells stably expressing TEL-SYK showed interaction with the p85 subunit of PI3K. This interaction was abrogated in cells expressing the Δ-TEL-SYK mutant (Kanie et al., 2004). Moreover, TEL-SYK constitutively activated PI3K, MAPK and JAK2-independent STAT5 signaling pathways. Also, the BAF3 cell line showed inverse proliferation rates with increasing doses of the PI3K inhibitor LY294002 (Kanie et al., 2004). TEL-SYK transformed pre-B cells and induced leukemia in mice due to deregulated, constitutive activity of the SYK kinase domain (Wossning et al., 2006).

1.6 SYK-Fusion Kinases Mechanism Of Activation Switch

Activation mechanisms of TEC, SYK and SRC family kinases are well studied (Bradshaw, 2010). Kinases, in general, are kept “autoinhibited” through various means including posttranslational modifications such as dephosphorylation, phosphorylation, and placement in a certain subcellular compartment (Au-Yeung et al., 2009; August et al., 1997; Brdicka et al., 2005; Debnath et al., 1999; Deindl et al., 2007; El-Hillal et al., 1997; Kulathu et al., 2009; Roskoski, 2005). Various modules within the proteins, allosteric ligands, and other kinases or phosphatases regulate autoinhibition/activation of these molecules (Adams, 2003; Au-Yeung et al., 2009; Brdicka et al., 2005; Deindl et al., 2007; El-Hillal et al., 1997; Jin et al., 2004). Thus, kinases, depending on their modular structure, are released from autoinhibitory state to activated state through

different mechanisms (Au-Yeung et al., 2009; Brdicka et al., 2005; Deindl et al., 2007; Jin et al., 2004).

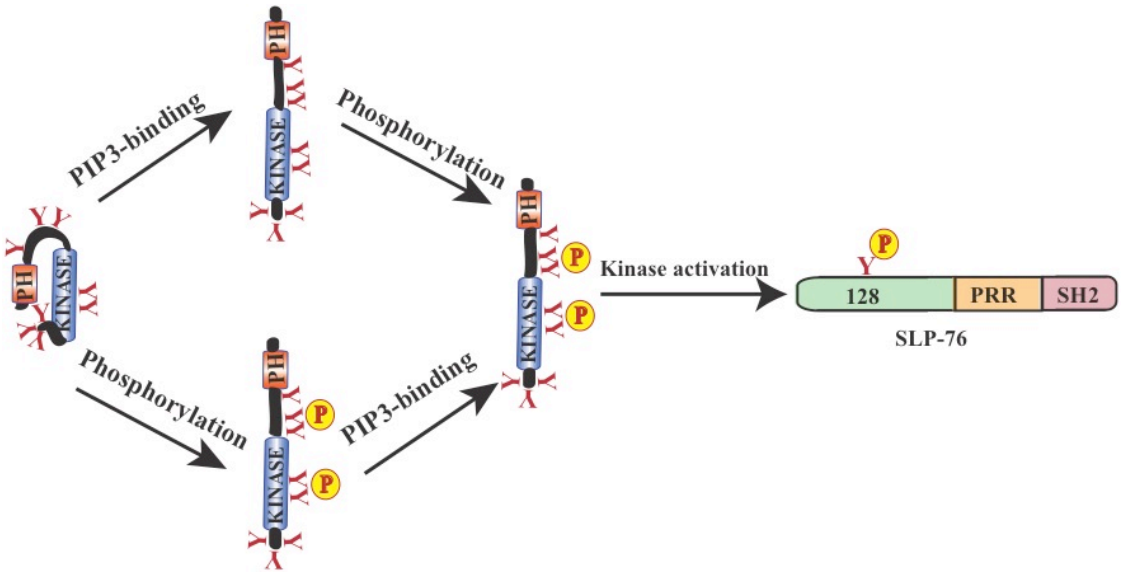
These mechanisms of activation result in signaling switches. Herein, two types of switches are described; "GRADED" or "ALL-or-NONE" switch (Bradshaw, 2010). SRC family kinases are called "GRADED" switches as they are activated step-wise, each step leading to elevated kinase activity. "ALL-or-NONE" switches that are either in activated, "ON" state, or autoinhibited, "OFF" state, are further divided into "AND" or "OR" switches. TEC family kinases are called "AND" switches as they are activated in two steps; i) membrane binding through PH-TH domain doublet and ii) phosphorylation by SRC family kinases. SYK family kinases can be activated either by phosphorylation at key regulatory tyrosines or through ITAMs binding; thus are called "OR" switch (Brdicka et al., 2005; Gradler et al., 2013; Tsang et al., 2008; Visco et al., 2000).

SYK-fusion kinases are constitutively active and do not obey standard regulatory rules due to aberrant modular structures lacking autoinhibitory function (Hussain et al., 2009; Hussain et al., 2013; Kanie et al., 2004; Kuno et al., 2001). Instead of tandem SH2 domains, TEL-SYK has an N-terminal SAM-PNT domain, while ITK-SYK has a PH-TH domain doublet. TEL-SYK is activated through dimerization mediated by the SAM-PNT domain (Kanie et al., 2004; Kuno et al., 2001). ITK-SYK was thought to be activated via membrane tethering mediated by its PH-TH domain doublet (Figure 4A) (Hussain et al., 2009; Rigby et al., 2009). Also, this membrane translocation was aborted in ITK-SYK-KD, suggesting equal role of kinase activity (Pechloff et al., 2010). Later studies have revealed that ITK-SYK robustly phosphorylated SLP-76 in the absence of phosphorylation on key tyrosines (Hussain et al., 2013). Similarly, ITK-SYK-R29C, also potently phosphorylated SLP-76 (Hussain et al. II). These observations suggest a model of activation that is independent of membrane translocation and phosphorylation (Figure 4B). SYK-fusion kinases do not require any stimulus for activation and are constitutively "ON". ITK-SYK constitutive kinase activation is accomplished by "open" conformation, independent of tyrosine phosphorylation.

Higher kinase activity of isolated kinase domain of SYK, compared with the activated full-length molecule suggests that SH2 domains and linker regions result in suppression

of catalytic capacity (Papp et al., 2007). This is in accordance with our hypothesis that it is lack of inhibitory regions in ITK-SYK that results in constitutive catalytic activity (Hussain et al., 2013).

A.



B.

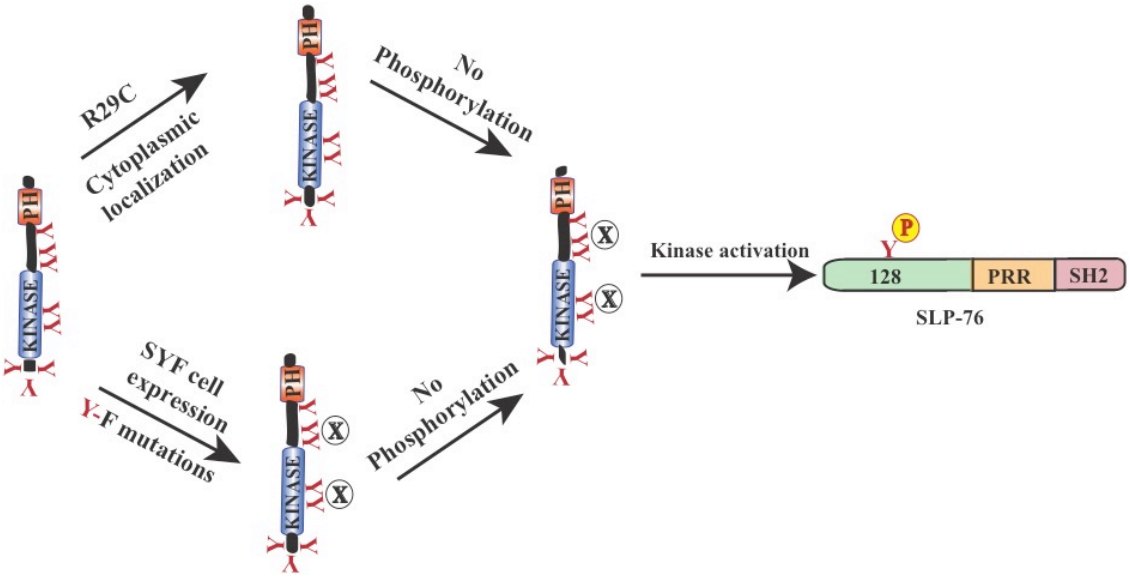


Figure 4. A) Hypothetical "closed" conformation model based on ITK and SYK activation, describing membrane translocation and/or phosphorylation dependent activation of ITK-SYK. **B)** "Open" conformation model with the corresponding observations showing that activation of ITK-SYK is independent of membrane translocation and/or phosphorylation.

2 AIMS

This study was primarily undertaken to understand the activation and signaling mechanisms of the ITK-SYK fusion oncogene. Along that line, we decided to also investigate the corresponding fusion kinase, BTK-SYK. Moreover, TEL-SYK was also used in this study to compare the regulation of SYK-fusion kinases.

The specific aims were:

(I) To study potential differences between SYK and ITK-SYK in terms of phosphorylation, activation, and localization in heterologous system.

(II) To investigate the roles of N-terminal regions in the stability, activation and subcellular localization of the SYK-fusion kinases ITK-SYK, BTK-SYK and TEL-SYK.

(III) To delineate the role of putative functional tyrosines in the activation of ITK-SYK and determine the role of proximal TCR signaling molecules in the ITK-SYK mediated activation of T cells.

3 MATERIALS AND METHODS

3.1 Western Blot And Immunoprecipitation

Western blot or immunoblot is a frequently used procedure for the detection of proteins in phosphorylated or native states. Electrophoretic transfer of proteins, resolved on acrylamide gels, to nitrocellulose membrane was developed by Towbin et al. al in 1979, and its modified form was named as Western blot (Towbin et al., 1979). Protein samples were solubilized using sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). The buffer was further supplemented with a cocktail of protease and phosphatase inhibitors to block degradation and dephosphorylation of the proteins.

Proteins were subsequently transferred either using wet method in a tank or through iBlot transfer, onto nitrocellulose or PVDF membrane. A battery of primary antibodies was used to detect specific proteins. Following incubation with secondary antibodies, results were captured either on X-ray films or membranes were scanned on LI-COR imager, depending on the type of secondary antibodies employed.

Immunoprecipitation (IP) is used to precipitate a specific antigen based on antigen-antibody interaction. This method is also frequently used to detect protein-protein interaction. In our studies, we immunoprecipitated all phosphorylated proteins using a general phosphotyrosine antibody, followed by the detection of proteins.

3.2 Transfection And Electroporation

F. L. Graham and A. J. van der Eb, in 1973, developed calcium phosphate method for infectivity of adenovirus 5 DNA (Graham and van der Eb, 1973). C. Chen and H. Okayama, used this method for ectopic expression of plasmid DNA in 1987 (Chen and Okayama, 1987). We frequently used cation polyethylenimine (PEI), or cationic lipid FuGene6 (Roche) for adherent cell lines like COS7, HEK-293T and NIH3T3. For suspension cell lines like Jurkat or BAF3, Nucleofection (Lonza) or Neon Transfection System (Life Technologies), 100 uL was used.

3.3 Flow Cytometry

Wallace H. Coulter patented first impedance based flow cytometry in 1953. The flow cytometry method was reported by M. J. Fulwyler in 1965 (Fulwyler, 1965). In 1972, L. A. Herzenberg sorted functional viable lymphocytes (Julius et al., 1972). Using this technique, cells can not only be counted and separated on the basis of cell size but also using antibody tags or fluorescent dyes. FACSCalibur (BD) was used to study the expression levels of CD69, using a PE-coupled antibody.

3.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) was developed by Engvall and Perlmann in 1971 as a method for quantitative detection of proteins (Engvall et al., 1971; Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971). In this work, ELISA was employed for determining IL-2 secretion in Jurkat cells expressing ITK-SYK. Briefly, microtiter plate was coated with capture antibody and medium from cells expressing ITK-SYK was added into the wells. Secondary antibody was added and read out was taken using SpectraMax 250 (Molecular Devices), and SOFTmaxPro 3.1.2 software.

3.5 Microscopy

Although, Dutch spectacle-maker Hans Jansen and his son Zacharias invented the first compound microscope in 1595, it is Robert Hooke and Antonie van Leeuwenhoek who used it for scientific investigations in 1665 and 1673, respectively. Nomarski differential interference contrast microscopy (DIC), combined with immunofluorescence studies have been used for cell and molecular studies (Khalaf and Bainbridge, 1981). Later advancements allowed high resolution imaging, either by laser based pinhole confocal microscopy or digital deconvolution (Sheppard and Shotton, 1997). Confocal microscopy (Leica DMRXA) was used to capture high resolution optical images of cells. The fusion proteins were tagged with green fluorescent protein (GFP-ITK-SYK) and subsequently transfected into cells. In certain images where SYK antibody was used for the detection of ITK-SYK, cells were fixed, permeabilized and stained with the primary antibodies, followed by the secondary fluorescent antibodies. Images captured on confocal microscope were processed and analysed using “Slide

Book" (Intelligent imaging Innovations Inc. Denver, Colorado, USA). Olympus microscope (Olympus-IX81) was used to capture nomarski images of the NIH3T3 cells. Images were processed using CellSense Dimension, provided by the Olympus, Tokyo, Japan

4 RESULTS AND DISCUSSIONS

4.1 Paper I

ITK-SYK was discovered in a subset of unspecified T cell lymphoma as a result of t(5;9)(q33;q22) (Streubel et al., 2006). The fusion protein comprises of the PH-TH domain doublet of IL-2 inducible T cell kinase (ITK), and the kinase domain of spleen tyrosine kinase (SYK). The PH-TH domain doublet in Tec family kinases is responsible for the membrane translocation of the proteins through PIP3-binding (August et al., 1997; Hirve et al., 2012; Kane and Watkins, 2005). To compare ITK-SYK with SYK, we cloned the fusion oncogene into a mammalian expression vector and expressed it in heterologous cell lines. When expressed in COS7 cells, ITK-SYK was phosphorylated on the linker-region and activation-loop tyrosines, compared with SYK that showed only residual phosphorylation at the linker-region and none at the activation-loop tyrosines.

In spite of only residual phosphorylation at the linker-region tyrosine, a large number of phosphorylated proteins were immunoprecipitated in cells expressing SYK, than ITK-SYK. On the contrary, ITK-SYK led to robust phosphorylation of ectopically expressed adapter proteins SLP-76 and BLNK, while under the same conditions, SYK did not.

ITK-SYK localized to the cell membrane following serum activation. However, treatment of cells with the PI3K inhibitors Wortmannin or LY294002 reversed membrane localization to cytoplasmic distribution. Consistently, PI3K inhibitors abrogated ITK-SYK kinase activation and thus phosphorylation of adapter protein SLP-76.

4.2 Paper II

Spleen tyrosine kinase (SYK) is involved in two translocation events leading to TEL-SYK in a case of myelodysplastic syndrome and ITK-SYK in a subset of patients with unspecified peripheral T cell lymphoma (Kuno et al., 2001; Streubel et al., 2006). Unlike, PH-TH domain doublet of ITK-SYK, TEL-SYK has N-terminal SAM-PNT dimerizing domain. We cloned BTK-SYK as ITK-SYK counterpart for B cells and compared various attributes of the fusion kinases including activation, stability,

localization and their response to various inhibitors. Figure 5 shows the structure of various kinases and SYK-fusion kinases used in this study.

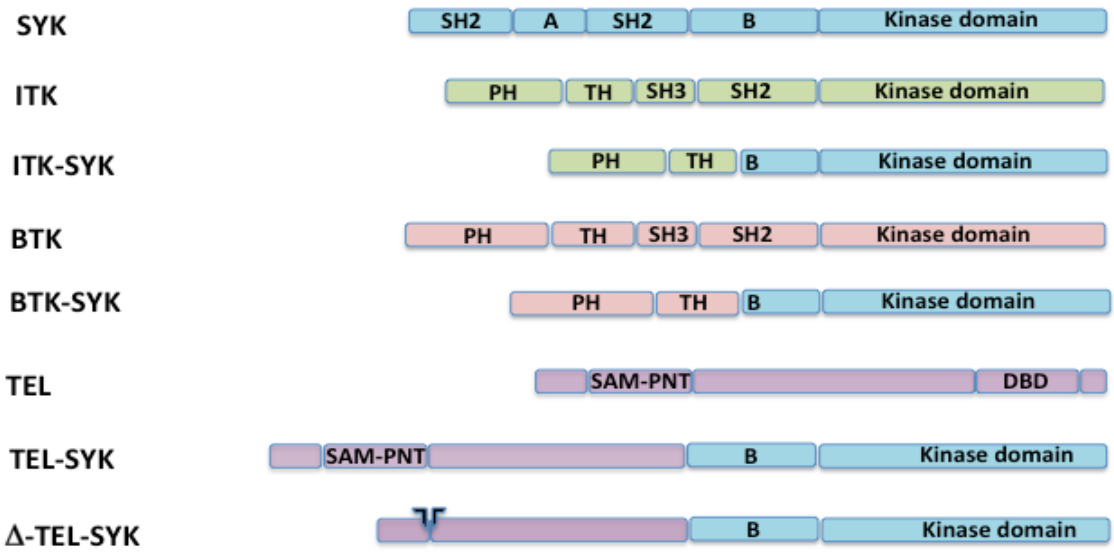


Figure 5. Schematic representation of SYK-fusion constructs along with SYK, ITK, BTK and TEL. (Hussain et al. II)

In COS7 cells, ITK-SYK and BTK-SYK were equally expressed and phosphorylated. In contrast, in HEK-293T cells, steady-state levels of BTK-SYK were lower than ITK-SYK. Under serum deprived growth conditions, phosphorylation was compromised only in cells expressing BTK-SYK, but not in ITK-SYK.

SYK inhibitor significantly blocked ITK-SYK phosphorylation but not BTK-SYK under the same conditions in COS7 cells. Similarly, PI3K inhibitors abrogated ITK-SYK activation, although under the same conditions, BTK-SYK showed only modest inhibition. Consistent with the PI3K inhibition results, BTK-SYK localized to the cytoplasm following serum activation. Interestingly, the PH-TH domain loss-of-function mutants ITK-SYK-R29C and BTK-SYK-R28C robustly phosphorylated SLP-76 in COS7 cells.

Expression of ITK-SYK and BTK-SYK resulted in loss of contact inhibition in NIH-3T3 cells. This transformed phenotype of NIH-3T3 cells was modest in those expressing BTK-SYK, compared with ITK-SYK. Control cells expressing pEGFP,

were contact inhibited. In BAF3 cells, expression of ITK-SYK or BTK-SYK induced IL-3 independent growth that was again milder in BTK-SYK transfected cells.

The degree of tyrosine phosphorylation was found to be much stronger in TEL-SYK than in ITK-SYK. Surprisingly, Δ-TEL-SYK was also equally phosphorylated as TEL-SYK. Furthermore, a SYK inhibitor blocked ITK-SYK but not TEL-SYK or Δ-TEL-SYK phosphorylation. Although, Δ-TEL-SYK was equally tyrosine phosphorylated in COS7 cells, it failed to induce CD69 expression upregulation in Jurkat cells as compared with TEL-SYK or ITK-SYK.

4.3 Paper III

ITK-SYK harbours a number of tyrosines in the linker-B, kinase domain and C-terminus tail of SYK (Hussain et al., 2009; Rigby et al., 2009; Streubel et al., 2006). In SYK, these tyrosines are known to regulate the kinase activation and interaction with other proteins. Deregulation of SYK has been implicated in various malignancies (Sada et al., 2001; Weil et al., 1999; Wossning et al., 2006). Thus, overexpression of SYK has been detected in the B and T cell lymphomas (Buchner et al., 2009; Feldman et al., 2008). On the contrary, in breast cancer SYK expression was abrogated (Coopman et al., 2000; Kanwal et al., 2012; Stewart and Pietenpol, 2001).

In resting cells, SYK family kinases are known to adopt a “closed”, autoinhibitory conformation that changes to “open” active conformation following phosphorylation of the regulatory tyrosines in the linker-B region or SH2-domain mediated binding of SYK family kinases on to the phosphorylated ITAM (Kulathu et al., 2009). ITK-SYK retains three tyrosines in the linker-region, a pair at the activation-loop, and a triplet in the C-terminus tail (Rigby et al., 2009). In this study, we have investigated the role of the regulatory tyrosines in the activation of ITK-SYK. Moreover, the role of proximal TCR signaling molecules SYK, ZAP-70 and SLP-76 was examined.

Linker-region tyrosines of ITK-SYK showed a hierarchical phosphorylation pattern. Loss-of-function mutant of Y208, (ITK-SYK-Y208F) resulted in the loss of phosphorylation at the linker-region Y212 and the activation-loop Y385/Y386. Consistently, replacement of Y208 with glutamic acid augmented phosphorylation at

the linker-region Y212 and the activation-loop Y385/Y386. Similar results were observed when Y212 was mutated. Thus ITK-SYK-Y212F resulted in loss of phosphorylation at the activation-loop tyrosines 385/386 and ITK-SYK-Y212E, augmented this phosphorylation. Interestingly, these mutants (ITK-SYK-Y208F, ITK-SYK-Y208E, ITK-SYK-Y212F, and ITK-SYK-Y212E), retained catalytic activity and equally phosphorylated the substrates SLP-76 or SYK. Phosphorylation-independent kinase activity of ITK-SYK was further confirmed in SYF cells, a fibroblast cell line deficient in the SRC family kinase members, SRC, YES and FYN. As expected, ITK-SYK was not phosphorylated at the linker-region or activation-loop tyrosines, even in the presence of pervanadate. Surprisingly, in spite of the lack of detectable tyrosine phosphorylation, ITK-SYK potently phosphorylated SLP-76, strongly suggesting a tyrosine phosphorylation-independent kinase activity.

Mutation of the activation-loop tyrosines to phenylalanine (ITK-SYK-Y385F, ITK-SYK-Y386F, ITK-SYK-Y385/Y386FF) did not significantly affect ITK-SYK phosphorylation at the linker-region tyrosines or substrate phosphorylation. However, replacement of the activation-loop tyrosine(s) with glutamic acid(s) showed interesting results. Whereas, ITK-SYK-Y385E did not alter phosphorylation or catalytic activity of the fusion protein, ITK-SYK-Y386E completely abrogated phosphorylation and kinase activity. Similar results were observed for ITK-SYK-Y385/Y386EE, where phosphorylation and the kinase activity were highly affected. C-terminus triplet loss-of-function mutations (ITK-SYK-Y489F, ITK-SYK-Y490F, ITK-SYK-Y491F, ITK-SYK-Y489-91FFF) did not affect phosphorylation at the linker-region and activation-loop tyrosines or catalytic activity of the fusion protein. Interestingly, ITK-SYK-Y489-91EEE, not only showed highly compromised phosphorylation levels at the linker-region and the activation-loop tyrosines, but also impaired catalytic activity.

To investigate the role of proximal TCR signaling molecules in the ITK-SYK mediated activation of T cells, Jurkat cell lines deficient in SYK (E6-1), SYK and ZAP-70 (P116) and SLP-76 (J14) were used. Expression of either of the SYK family kinases augmented ITK-SYK mediated T cell activation. Interestingly, kinase-inactive mutants of SYK family kinases showed equal activation of T cells expressing ITK-SYK, suggesting that adapter function of SYK family kinases is essential in fusion kinase signaling and that their kinase activity is dispensable. By the same token, the adapter protein SLP-76 was indispensible for the ITK-SYK mediated T cell activation. Thus, in

the absence of SLP-76, or following co-expression of a mutant of SLP-76 lacking three functional tyrosines in the N-terminal, ITK-SYK driven T cell activation was highly dampened.

5 CONCLUDING REMARKS

The chimeric oncogene, ITK-SYK is the main focus of this study. Different aspects of this fusion protein like subcellular localization, phosphorylation and capacity to phosphorylate adapter proteins were studied. ITK-SYK is a constitutively active oncogene with a seemingly tyrosine phosphorylation-independent kinase activity. In this study we found that:

1. ITK-SYK is constitutively active and localizes to the cell membrane following serum activation. Plasma membrane translocation and phosphorylation is abrogated when cells are treated with PI3K inhibitors (LY294002 and Wortmannin). ITK-SYK displays robust phosphorylation on the linker-region and activation-loop tyrosines. In contrast, SYK is modestly phosphorylated at the linker-region but not at the activation-loop tyrosines. Moreover, ITK-SYK but not SYK potently phosphorylates the adapter proteins SLP-76 and BLNK.

2. SYK-fusion kinases TEL-SYK and ITK-SYK differ mainly in their N-terminal regions, which are believed to be critical in regulating these oncogenes. The corresponding fusion kinase BTK-SYK showed not only cytoplasmic localization but, surprisingly, its catalytic activity was also independent of PI3K signaling. Like its wild type counterpart, the Δ–TEL-SYK mutant was phosphorylated in COS7 cells, but different in that it failed to induce CD69 upregulation in Jurkat cells.

3. ITK-SYK retains a number of putative functional tyrosines in the linker-region, activation-loop and C-terminal tail. To abolish phosphorylation, these tyrosines were mutated to phenylalanine. To create a permanent negative charge, these residues were replaced with glutamic acid. Unexpectedly, all non-phosphorylatable, phenylalanine mutants showed, equal kinase activity as compared with ITK-SYK, suggesting a tyrosine phosphorylation independent function. In the absence of detectable tyrosine phosphorylation, ITK-SYK robustly phosphorylated SLP-76 in SYF cells (lacking SRC family kinases). In addition, the adapter protein SLP-76 was not only required for activation of T cells but also ITK-SYK mediated phosphorylation of SYK activation-loop tyrosines. Similarly, adapter function of SYK family kinases, independent of their kinase activity, was required for ITK-SYK mediated activation of T cells.

This thesis presents a number of potentially interesting findings that will hopefully contribute to our understanding of the molecular mechanism(s) underlying activation and signaling of these oncogenes. Our studies on ITK-SYK showed a constitutive kinase activity, which is independent of phosphorylation of known putative functional tyrosines. Similarly, role of intact SLP-76 is indispensible for the ITK-SYK mediated activation. Interestingly, SYK family kinases adapter function is required which is independent of their kinase activity. N-terminal regions play key roles in the stability, activation and localization of SYK-fusion kinases.

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