Tec Family Kinases: Transcriptional and Posttranslational Regulation

Liang Yu
From Department of Laboratory Medicine
Clinical Research Center
Karolinska Institutet, Stockholm, Sweden

Tec Family Kinases: Transcriptional and Posttranslational Regulation

Liang Yu

Stockholm 2009
Cover: PH domain of Btk. Reproduced from Mohamed AJ and Yu L et al (1)
To my family
ABSTRACT

Tec family tyrosine kinases consist of five members: Btk, Itk, Tec, Rlk/Txk and Bmx. These kinases are highly expressed in hematopoietic cells, including B cells, T cells and mast cells. Tec kinases execute important functions in signaling pathways downstream of the antigen receptors, and the activation of receptor and non-receptor protein tyrosine kinases is one of the first steps in the signal transduction cascades that lead to activation of hematopoietic cells. Following the identification of mutations in the Btk gene as the cause of human X-linked Agammaglobulinemia (XLA) and mice X-linked Immunodeficiency (xid) in 1993, Tec family kinases have received widespread research interest. Although much progress has been made in elucidating the signalling of Tec kinases, the molecular mechanisms that underlie the biology of these enzymes are largely unknown. This study was undertaken to address the transcriptional and posttranslational regulation of Tec kinases (Btk, Itk and Tec).

In the first paper, we show that the peptidyl-prolyl cis/trans isomerase Pin1 is a negative regulator of Btk. The negative regulatory effect of Pin1 was observed both in B cell lines and in cells from Pin1−/− mice and was found to be dependent on a functionally intact Btk. Our results indicate that Pin1 functionally interacts with Btk in a cell cycle dependent manner. Accordingly, Pin1 was found to interact with serine 21 (S21) during mitosis and serine 115 (S115) in interphase cells.

In the second study, we found that NF-κB is required for the transcription of the Btk gene. Previously, it has been reported that NF-κB signals downstream of Btk. In this work, we found that proteasome and NF-κB inhibitors suppress Btk transcription. Second, two functionally active NF-κB binding sites were identified in the Btk promoter. Third, using hydrodynamic transfection technology, we show that Bortezomib can block Btk transcription in mice. Collectively, we show that Btk uses a positive auto-regulatory feedback mechanism to stimulate transcription from its own promoter via NF-κB.

Paper III: We demonstrated that proteasome inhibitors effectively block HIV-1 replication by suppressing viral transcription and depleting a key cellular component Itk, a Tec-family kinase critical for HIV replication. First, we showed that proteasome inhibitors shut down transcription from the HIV-1 LTR-promoter. Second, replication of HIV-1 in PBMC was severely compromised following treatment with proteasome inhibitors. Finally, these drugs led to depletion of the steady state levels of Itk in PBMC. Altogether, these findings suggest that proteasome
inhibitors not only function as *bone fide* antiretroviral drugs, but may also boost resistance of host cells by affecting a key endogenous component (Itk).

Paper IV: We found that NF-κB is required for the optimal expression of the *Tec* gene, and identified a highly conserved and functionally active NF-κB binding site in the Tec promoter. Moreover, the NF-κB subunit p65/RelA was found to induce the Tec promoter. Finally, we showed that proteasome inhibitors repress Tec transcription by blocking the NF-κB signaling pathway.

In conclusion, the present work shows that NF-κB is an important transcription factor for the expression of Tec family kinases (Btk, Tec and Itk), and proteasome inhibitors repress their transcription by disabling the NF-κB signaling pathway. This work also provides important insights into the molecular mechanisms underlying the functional regulation of Tec family kinases by NF-κB. Second, we demonstrate that Pin1 functionally interacts with Btk and regulates tyrosine phosphorylation and steady-state levels of Btk.
LIST OF PUBLICATIONS


# TABLE OF CONTENTS

1. Introduction 1
  1.1 Overview of the Tec family tyrosine kinases 1
  1.2 Expression of Tec family kinases 2
  1.3 Subcellular localization of Tec Kinases 3
    1.3.1 Subcellular localization of Btk 3
    1.3.2 Subcellular localization of other Tec members 4
  1.4 Functional interaction of Tec kinases with signaling proteins 6
  1.5 Tec kinases in antigen receptor signaling pathways 8
  1.6 Transcriptional regulation of Tec kinases 10
  1.7 Btk, XLA and xid 11
  1.8 Itk 12
  1.9 Tec 13
  1.10 NF-κB 14
  1.11 Peptidyl-prolyl cis/trans isomerase Pin1 17

2. Aims of the thesis 19

3. Methodological considerations 21
  3.1 Western blot and Immunoprecipitation 21
  3.2 Chromatin Immunoprecipitation (ChIP) assay 22
  3.3 Electrophoretic Mobility Shift Assay (EMSA) 22
  3.4 RNA interference (RNAi) 23
  3.5 Hydrodynamic transfection 23
  3.6 Site-Directed mutagenesis 24
  3.7 Promoter luciferase reporter constructs and luciferase assay 24

4. Ethics 25

5. Results and Discussion 27
  5.1 Paper I 27
  5.2 Paper II 28
  5.3 Paper III 29
  5.4 Paper IV 31

6. Concluding Remarks 33

7. Acknowledgements 35

8. References 37
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>Azidothymidine</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activation factor belonging to the TNF family</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BH</td>
<td>Btk homology</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker protein</td>
</tr>
<tr>
<td>Bmx/Etk</td>
<td>Bone marrow tyrosine kinase / epithelial and endothelial tyrosine kinase</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent protein kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation assay</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>Emt</td>
<td>Expressed in mast cells and T cells (also known as Itk)</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FceRI</td>
<td>High-affinity IgE receptor</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
</tr>
<tr>
<td>GADS</td>
<td>Grb2-related adaptor downstream of Shc</td>
</tr>
<tr>
<td>IBtk</td>
<td>Inhibitor of Btk</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitory protein of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IND</td>
<td>Indinavir</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol (1,4,5) trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine activation motif</td>
</tr>
<tr>
<td>Itk</td>
<td>IL-2 inducible T cell kinase (also known as Emt and Tsk)</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker of activated T cells</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LTβ</td>
<td>Lymphotoxin β</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
</tbody>
</table>
NFAT  Nuclear factor of activated T cells
NF-κB  Nuclear factor kappa B
NIK   NF-κB inducing kinase
PH    Pleckstrin homology
PHA   Phytohemagglutinin
Pin1  Protein interacting with NIMA (never in mitosis A)-1
PI3K  Phosphoinositide 3 kinase
PIP   Phosphatidylinositol phosphate
PIP2  Phosphatidylinositol (4,5)-bisphosphate
PIP3  Phosphatidylinositol (3,4,5)-trisphosphate
PIP5K Phosphatidylinositol 4-phosphate 5-kinase
PKC   Protein kinase C
PLCγ  Phospholipase Cγ
PMA   Phorbol myristate acetate
PPIase Peptidyl-prolyl cis/trans isomerase
Pre-BCR Pre-B cell receptor
PRR: Proline-rich region
PTEN: Phosphatase and tensin homolog
PTK   Protein tyrosine kinase
Rlk   Resting lymphocyte kinase (also known as Txk)
RNAi  RNA interference
SH    Src homology
SHIP  SH2-containing inositol phosphatase
SLP-65 SH2-containing linker protein of 65 kDa
SLP-76 SH2-domain-containing leukocyte protein of 76 kDa
TCR   T cell receptor
Tec   Tyrosine kinase expressed in hepatocellular carcinoma
TH    Tec homology
TNFα  Tumor necrosis factor α
Tsk   T cell specific kinase (also known as Itk)
WASP  Wiskott-Aldrich syndrome protein
Xid   X-linked immunodeficiency
XLA   X-linked agammaglobulinemia
1. INTRODUCTION

1.1 Overview of the Tec family tyrosine kinases

Tec family of tyrosine kinases is the second largest family of cytoplasmic protein tyrosine kinases (PTK) (2) and consists of five members: Btk, Itk, Tec, Rlk/Txk and Bmx. In mammalians, these kinases are predominately expressed in hematopoietic cells, including B cells, T cells and mast cells. Btk and Tec are expressed in B cells while Itk, Tec and Rlk/Txk are expressed in T cells (2). Tec kinases are also expressed in other species, including skate, Drosophila melanogaster, Zebrafish, sponge and choanoflagellate Monosiga brevicollis (3-9). In immune cells, Tec kinases have important functions in lymphocyte development and activation. Following antigen engagement, activation of non-receptor and receptor PTKs is one of the first steps in the signal transduction cascades that lead to full activation of B and T lymphocytes.

In 1993, when mutations of the Btk gene were shown to be associated with the genetic disorder X-linked agammaglobulinemia (XLA) in humans and the X-linked immunodeficiency (xid) in mice (10-13), Tec family kinases immediately received widespread research interest. Thus, the identification of the molecular basis of the primary immunodeficiency XLA highlighted the importance of tyrosine kinases in lymphocyte development and differentiation (2, 14, 15).

Structurally, each Tec family member has a C-terminal kinase domain followed by an SH2 (Src homology 2) and SH3 domains (Figure 1). At the N-terminal side of the SH3 domain, Tec family kinases, with the exception of Bmx, possess a proline-rich region (PRR). Btk and Tec have two PRR regions, whereas Itk and Rlk/Txk have only one. At the N-terminal side of the PRR in Btk, Tec and Itk, there is a Zn\(^{2+}\)-binding region known as the BH (Btk homology) motif. The BH and the PRR are combined together to form TH (Tec homology) domain. Finally, at the amino terminus, all Tec family kinases, except Rlk/Txk, possess a PH (pleckstrin homology) domain. In contrast, the N-terminal region of Rlk/Txk contains a unique cysteine-string motif (2, 14).
Figure 1. Domain structure of Tec family kinases.

1.2 Expression of Tec family kinases

Generally, Tec family kinases are predominantly expressed in hematopoietic cells; however, each individual Tec kinase has a distinct cell type-specific expression pattern. Furthermore, each particular cell type has a hierarchy of expression levels and specific functions for the Tec kinases expressed. Of the three Tec kinases (Itk, Tec and Rlk/Txk) expressed in T cells, Itk appears to play a predominant role in T cell receptor (TCR) signaling. Although Itk is expressed in all stages of T cell development, the expression is highest in the mature adult thymus. Moreover, its expression dramatically increases following TCR engagement (16-20). Rlk/Txk, on the other hand, is expressed in thymocytes and mature resting T cells; however, Rlk/Txk mRNA levels are 3- to 10-fold lower than the levels of Itk mRNA in resting T cells (21-24). In contrast to Itk, Rlk/Txk is downregulated at both mRNA and protein levels following TCR stimulation (22, 24). Tec is expressed at much lower levels in resting T cells, with mRNA levels 100-fold lower than that of Itk (14). Interestingly, Tec is upregulated in T cells 2–3 days following their stimulation, suggesting a more important role for Tec in T cell effector function and restimulation, rather than in T cell development or initial activation (25).

The expression levels of Tec kinases are also individually regulated during T helper cell differentiation. When naive CD4+ T cells differentiate into T helper (Th) cells, Itk levels increase approximately two- to threefold in Th2 cells versus Th1 cells, consistent with the known role of Itk in Th2 responses (21, 23). Similar to Itk, Tec is expressed at two-fold higher levels in Th2 cells than in Th1 cells; however, in this latter case, the functional significance of this differential
expression is not known, since Tec-deficient mice have no reported T cell signaling defects (25). In contrast to Itk and Tec, Rlk/Txk is downregulated following naive CD4+ T cell activation, and is induced in Th1 cells, but not in Th2 cells; these data suggest a specific role for Rlk/Txk in Th1 responses (22, 23, 26).

1.3 Subcellular Localization of Tec Kinases

1.3.1 Subcellular localization of Btk

Although predominantly cytosolic, plasma membrane recruitment is a critical step in the activation of Btk. Following BCR engagement, phosphatidylinositol-3-kinase (PI3K) is activated leading to the generation of the phosphoinositol phosphatidylinositol-3,4,5-trisphosphate (PIP3). Accumulation of PIP3 in the inner surface of the plasma membrane results in the recruitment of Btk, which via its PH domain binds to PIP3 (27, 28). Thus, deletion of the PH domain renders Btk mainly cytoplasmic, although a substantial amount of the protein translocates to the nucleus (29). In spite of the fact that a PH domain-less Btk is highly phosphorylated, it fails to functionally activate downstream signaling molecules. Notably, a subtle alteration in the PH domain could have disastrous effects on the overall function of the Btk protein. This is substantiated by the fact that the common R28C mutation in the PH domain leads to classical XLA. Moreover, other missense mutations that occur in the PH domain have been shown to cause disease (30, 31). In contrast, when the glutamic acid at position 41 in the PH domain of Btk is substituted with lysine (E41K), Btk achieves a gain-of-function state with increased membrane targeting and tyrosine phosphorylation levels (32). In addition, this gain-of-function mutation has been shown to display high transformation capacity in the anchorage-independent soft agar assay. According to the structure of the PH domain, the E41K mutant binds to two PtdIns(3,4,5)P3 molecules compared to the wild type that binds to only one (33). Therefore, it is possible that this type of mutation enables Btk to spend more time at the inner surface of the plasma membrane leading to a sustained activation of the protein.

An interesting report indicates that in stimulated B cells, Btk can promote its own activation by a positive feedback loop mechanism involving PI3K (34). Following BCR stimulation, Btk binds to PI5K which translocates to the plasma membrane. At the plasma membrane, PI5K converts PI(4)P into PI(4,5)P2, thereby providing a renewable source of substrate for PI3K, and prolonging the activation signal.
Although predominantly cytoplasmic, Btk is also continuously shuttling between the nucleus and cytoplasm (29). The molecular mechanism(s) responsible for the nuclear localization of Btk is not fully understood. In contrast, the nuclear export of Btk has been shown to be mediated by the nuclear export receptor CRM1 (exportin), since leptomycin B treatment of B lymphocyte leads to nuclear arrest of Btk (29).

Btk has been implicated in NF-κB activation in B cells, indirectly linking Btk to nuclear signaling (35, 36). In addition, TFII-I, a versatile transcription initiation factor, has been shown to be tyrosine phosphorylated by Btk after B cell receptor (BCR) stimulation (37, 38). Consistent with this, overexpression of wild-type Btk induced TFII-I-dependent transcriptional activation in COS7 cells (39). In addition, TFII-I was also found to associate with the PH and kinase domains of Btk. These data suggest that Btk phosphorylation of BAP/TFII-I provides a link between BCR engagement and the modulation of gene expression (38). In addition, the chromatin remodeling protein, Bam11, has been shown to bind to the Btk PH domain and inhibits Btk kinase activity (40). These data suggest a model in which Btk activates BAM11 and the SWI/SNF transcriptional complex via TFII-I activation in B cells (41).

1.3.2 Subcellular localization of other Tec members

Each Tec family kinase shows a distinct pattern of subcellular localization. At steady state, Itk and Tec are found in the cytoplasm. Following activation of PI3K and the subsequent generation of PIP3 at the plasma membrane, Itk and Tec are recruited to the membrane via their PH domains (42-44). On the other hand, Rlk/Txk, which lacks a PH domain, remains associated with the plasma membrane via its palmitoylated cysteine-string motif. Thus, unlike Itk and Tec, membrane recruitment of Rlk/Txk is PI3K-independent (45). Following TCR engagement and activation of PI3K, Itk recruitment to the membrane is mediated by its PH domain and is independent of other domain interactions (42, 46). Thus, deletion of the PH domain abolishes the activation-induced colocalization of Itk with the TCR complex at the plasma membrane and also prevents the subsequent tyrosine phosphorylation and activation of Itk. Interestingly, when the PH domain was replaced by a membrane localization sequence from Lck, Itk could successfully translocate to the plasma membrane. Yet, this plasma membrane resident form of Itk was deficient in its ability to undergo tyrosine phosphorylation following TCR stimulation (42).
Recruitment of Tec to the plasma membrane following TCR stimulation has some distinct features. First, Tec, like other members of this family, is recruited to the membrane through the interaction of its PH domain with PIP3. However, interaction of the Tec PH domain with PIP3 seem to be different from other Tec kinases. To this end, substitution of the glutamic acid residue at position 41 with lysine (E41K) in the Btk PH domain has been shown to increase Btk binding to PIP3. In sharp contrast, the corresponding mutation (E42K) in the PH domain of Tec reduces Tec binding to PIP3 (44). Second, membrane recruitment of Tec could also be mediated by the SH2 domain (44). Finally, the PH domain of Tec is dispensable for Tec accumulation at the plasma membrane, and instead the SH3 domain is essential for accumulation of Tec at the immunological synapse (47).

Since membrane localization is a prerequisite for the function of Tec family kinases in antigen receptor signaling pathways, these signals can be terminated by inhibition of membrane recruitment. Itk recruitment to the plasma membrane is negatively regulated by the lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), which removes phosphates from the D3 position of phosphoinositides, and thereby reduces the levels of PIP3 at the membrane (43). In PTEN−deficient cells, Itk is constitutively localized to the plasma membrane and is therefore hyperresponsive to TCR signaling (43). Membrane localization of Tec is regulated by the Src homology 2−containing inositol−5−phosphatase (SHIP) family of inositol phosphatases (48). SHIP dephosphorylates PIP3 leading to decreased PH domain−mediated recruitment of Tec to the plasma membrane. Unlike the indirect regulation of Itk by PTEN, Tec seems to directly interact with SHIP1 and SHIP2, an interaction that is dependent on the Tec SH3 domain (48). It is possible that interaction of SHIP with the SH3 domain might be sufficient to preclude PI3K−independent Tec membrane localization.

Apart from Btk, other Tec kinases have also been shown to translocate to the nucleus. The data demonstrating the ability of Rlk/Txk to traffic to the nucleus is compelling. Two isoforms of Rlk/Txk, which arise from alternative sites of translation initiation on the same mRNA, have been described (49). The larger, a 58−kDa isoform is cytoplasmic and localizes to lipid rafts through the cysteine−string motif upon palmitoylation. In contrast, the shorter 52−kDa isoform, lacks the cysteine−string motif and localizes to the nucleus when expressed in the absence of the larger form. Consistent with these data, a mutation that abolishes palmitoylation in the cysteine−string motif of the larger isoform allows this protein to migrate to the nucleus (49). In spite of the fact that the two isoforms contain a nuclear localization sequence (residues 57−71), both proteins are
found only in the cytoplasm when coexpressed, suggesting a direct physical interaction between the two isoforms. Moreover, it was reported that Rlk/Txk could bind to the IFNγ-promoter region, and its expression was upregulated in Th1 cells, indicating the Rlk/Txk may play critical roles in Th1 cell development and signaling (49).

Itk may also traffic to the nucleus. In CD3-stimulated Jurkat T cells, a small proportion of the Itk was found in the nucleus. In this case, the nuclear localization was mediated by karyopherin alpha (Rch1a), a nuclear transporter which binds to the Itk SH3 domain via its PRR (proline 242 is the key residue). Expression of a mutant Rch1α (P242A) in Jurkat cells abolishes the nuclear translocation of Itk as well as the mitogen–induced IL–2 production. These data suggest that the nuclear form of Itk may play a role during T cell activation (50). Consistent with this notion, Itk might directly interact with and phosphorylate T–bet, a constitutively active nuclear transcription factor that regulates IFNγ transcription (51).

### 1.4 Functional interaction of Tec kinases with signaling proteins

Like other signaling pathways, protein-protein interaction is critical in regulating and activating Tec family kinases. A plethora of the Tec family interacting/regulating partners has been identified (Table 1).

Under physiological conditions, the activity of many signaling pathways is tightly controlled. Establishment of positive and negative feedback loops is one of the major mechanisms that regulate cellular signaling. Inhibitory molecules that bind signaling proteins is a common mechanism for regulating the activity of several kinases. Activated Tec tyrosine kinases lead to dramatic changes in cell physiology, cell proliferation, protein-protein interaction and gene expression. One way to control these signaling pathways is through the negative interacting/regulating partners that bind to the kinases. For instance, the phosphatase SHIP represses PI3K activity by facilitating recruitment of signaling molecules, which are dependent on PH domain–mediated interactions with PIP3, to the plasma membrane. It has been shown that SHIP–1 directly interacts with Tec and represses its activity (48). Accordingly, interaction and formation of SHIP-Dok-Tec complex can suppress Tec kinase activity (48, 52, 53). Moreover, BCR-induced activation can be suppressed by yet another phosphatase PTP20 in the B cell line Ramos (54).
Table 1. Tec family kinase domains and their interacting/regulating partners:

<table>
<thead>
<tr>
<th>Kinase</th>
<th>PH</th>
<th>TH</th>
<th>SH3</th>
<th>SH2</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btk</td>
<td>BtM (11) (40)</td>
<td>BAP-135 (TFII-I) (37, 38)</td>
<td>F-actin (55)</td>
<td>Gα12 and Gβγ (57, 58)</td>
<td>IBtk (59)</td>
</tr>
<tr>
<td></td>
<td>BAP-135 (TFII-I) (37, 38)</td>
<td>F-actin (55)</td>
<td>Gα12 and Gβγ (57, 58)</td>
<td>IBtk (59)</td>
<td></td>
</tr>
<tr>
<td>Tec</td>
<td>PIP3 (62)</td>
<td>PI5K (34)</td>
<td>C-kit (77)</td>
<td>Dok-1 (52, 53)</td>
<td>BRDG1 (82)</td>
</tr>
<tr>
<td>Itk</td>
<td>PIP3 (85)</td>
<td>PIP3 (86)</td>
<td>PLCγ1 (87)</td>
<td>Fyn (67)</td>
<td>Cyclophilin A (90)</td>
</tr>
<tr>
<td>Rlk/Txk</td>
<td>Fyn (45)</td>
<td>Fyn (45)</td>
<td>Pim-1 (96)</td>
<td>p130Cas (Cas) (97)</td>
<td>Caveolin-1 (76)</td>
</tr>
<tr>
<td>Bmx</td>
<td>FAK (92)</td>
<td>Pak1 (93)</td>
<td>PTPD1 (94)</td>
<td>STAT3 (95)</td>
<td>Caveolin-1 (76)</td>
</tr>
</tbody>
</table>

With respect to Btk, BAM11 and PKC have been shown to interact with the PH-TH domain of Btk, and inhibit Btk activity (40, 65, 98). In paper I of this thesis, we demonstrated that the peptidyl-prolyl isomerase, Pin1, interacts with the PH domain of Btk in a cell cycle dependent manner and negatively regulates the activity of Btk. Also, IBtk, a small 203 amino acid protein, negatively regulates Btk following interaction with Btk PH domain (59, 99). Sab (SH3BP5), a SH3-domain binding protein, is also a negative regulator of Btk. Overexpression of Sab in B cells
reduces BCR–induced Btk tyrosine phosphorylation, calcium mobilization, and PIP3 generation (8, 72, 100), and in the fruitfly the corresponding protein, parcas, inhibits Btk29A (101).

The cis–trans proline isomerization of the Itk SH2 domain plays important roles in regulating Itk activity. The peptidyl–prolyl cis–trans proline isomerase, cyclophilin A, binds to the SH2 domain of Itk and inhibits its kinase activity. In contrast, the cyclophilin A inhibitor, cyclosporine A, increases Itk activation. Furthermore, T cells from cyclophilin A–deficient mice are hypersensitive to TCR stimulation due to the increased Itk activity (21, 90, 102).

1.5 Tec kinases in antigen receptor signaling pathways

Since the discovery of the Btk gene, much progress has been made in elucidating the molecular mechanism of Btk signaling. There is credible evidence indicating that Btk is involved in the B cell receptor (BCR) signalling pathway (2, 103-105). Following BCR stimulation, Src family kinases such as Lyn are activated, leading to the phosphorylation of the ITAM (immunoreceptor tyrosine activation motif) of the Igα and Igβ chains. Similarly, PI3K is activated to catalyze the conversion of membrane-associated PIP2 to PIP3 leading to Btk recruitment to the plasma membrane through the interaction of its PH domain with the PIP3 (105). Concomitantly, the phosphorylation of ITAM Igα and Igβ leads to the recruitment of the Syk kinase to the Ig chains via the Syk SH2 domains. Later, BLNK/SLP-65 is recruited to a phosphorylated tyrosine on Igα where it is further phosphorylated by Syk, thereby providing binding sites for Btk and PLC-γ2. Following binding of Btk to BLNK/SLP-65 through its SH2 domain, Btk is phosphorylated by a Src kinase at Y551. Phosphorylation at Y551 triggers conformational change in Btk followed by autophosphorylation of Y223 potentially leading to the full activation of the protein (Figure 2). Following activation, Btk ignites multiple downstream signals generating pleiotropic effects: 1) PLC-γ2 activation, generation of second messengers, such as inositol(1,4,5)-triphosphate (IP3), diacylglycerol (DAG) and calcium; 2) Cytoskeletal remodeling; 3) Cell proliferation, differentiation, apoptosis and survival, either by direct effects on these proteins or indirectly through an influence on transcription and gene expression (106-110). However, the molecular basis of many of these pathways is not fully understood.
Figure 2. Schematical diagram of Btk activation upon BCR engagement.

Although differing in details, the TCR signaling pathways in T cells share a similar overall scheme with that of BCR in B cells. Briefly, following TCR stimulation, Src family kinases such as Lck, are activated. Lck phosphorylates the ITAMs, which in turn recruits the Syk family kinase, ZAP-70, leading to ZAP-70 phosphorylation and activation by Lck. Activated ZAP-70 phosphorylates the adaptor molecules SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) and LAT (linker of activated T cells), which constitutively associate with lipid rafts (46, 111). At the same time, TCR stimulation leads to a PI3K dependent increase of PIP3 levels within the plasma membrane. The enrichment of PIP3 leads to recruitment of Tec kinases, such as Itk or Tec, to the plasma membrane in a PH-domain-dependent manner. Following this recruitment, a multimolecular signalosome complex consisting of a Tec family member, PLC-γ1, SLP-76, Gads and Grb-2 forms around LAT within lipid rafts. The Tec kinases are then transphosphorylated by a Src kinase, followed by an autophosphorylation event results in activation of PLC-γ1. Activation of PLC-γ1 leads to hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2) into inositol (3,4,5)-trisphosphate (IP3) and diacylglycerol (DAG). IP3 is required for intracellular Ca\(^{2+}\) release, which triggers sustained calcium influx that activates downstream effectors like the NFAT transcription factors (15, 112). DAG activates the PKC/Ras/Raf/MEK/ERK (extracellular–signal–regulated kinase) pathway, as well as the RAS-GRP (RAS guanyl–releasing protein) pathway leading to the activation of JNK (Jun amino–terminal kinase), thereby regulating
the heterodimeric transcription factor AP-1 (2, 105, 113, 114).

1.6 Transcriptional regulation of Tec kinases

In multicellular eukaryotic organisms, all cells harbour the same information encoded in their genes. Gene regulation is essential for eukaryotes as it increases the versatility and adaptability of an organism by allowing the cell to express a certain protein when needed. Furthermore, gene regulation drives the processes of cellular differentiation and morphogenesis, leading to the creation of different cell types in multicellular organisms where the different types of cells possess different gene expression profiles, though they all carry the same genome sequence.

Any step in gene expression can be modulated, from the transcription step to post-translational modification of a protein. These consist of: chromatin structure, transcription, post-transcriptional modification (RNA capping, splicing, poly A tail adding, mRNA degradation and transport), translation, and post-translational modifications (such as palmitoylation, phosphorylation and ubiquitination). Among all of these, transcriptional regulation has been shown to play critical roles in controlling gene expression. Gene transcription is affected by many factors: RNA polymerase, general transcription factors and co-factors, enhancers and repressors.

Tec kinases perform many important physiological functions in immune cells, and much progress has been achieved in elucidating their biology and signaling in the past decade. The structure of the Btk and Tec promoters has been described and some transcription factors that bind to them have been identified. Nevertheless, the transcriptional regulatory mechanisms of Tec family kinases have not yet been fully investigated.

The transcriptional regulation of the Btk gene has been studied quite extensively, and a number of transcription factors including Sp1, Sp3, Spi-B, PU.1 (115-117) and the OCT1/OBF1 co-activator (118) have been shown to bind and activate the Btk promoter. However, the detailed transcriptional regulation of the Btk gene is unknown. In PU.1-deficient fetal liver cells as well as in Sp1<sup>−/−</sup> embryonic stem cells, expression of Btk was shown to be reduced, but not completely abolished (116, 117) indicating that additional factors may also contribute to Btk promoter activity.

With respect to Tec, molecular cloning of the murine Tec promoter has been reported (119, 120) and the transcription factors SP1 and PU.1 were shown to be required for its transcription.
However, additional regulatory factors remain to be identified.

1.7 Btk, XLA and xid

1.7.1 Bruton's tyrosine kinase (Btk)
Btk is a nonreceptor tyrosine kinase belonging to the Tec family kinase of Src-related protein tyrosine kinases (PTKs). Btk is expressed in all hematopoietic cells except T-lymphocytes and plasma cells (121). Btk consists of five domains, all of which are necessary for its proper function (Figure 1). The PH domain is important for membrane translocation during B cell receptor (BCR) signaling (122), while the BH Zn$^{2+}$ binding motif of the TH domain is essential for the stability of the protein. The SH2 domain has a pocket that binds phosphotyrosine-containing peptide motifs, while the SH3 domain binds to proline-rich segments having the consensus sequence PXXP (X is any amino acid). Two key tyrosine phosphorylation sites (pY551 and pY223) have been identified in Btk. Following B-cell antigen receptor (BCR) engagement, Btk translocates to the cell membrane and becomes sequentially phosphorylated on these two tyrosine residues. Notably, Src family kinases (e.g. Lyn) phosphorylate tyrosine 551 in the activation loop of the catalytic domain of Btk. This transphosphorylation is followed by an additional autophosphorylation event involving tyrosine 223 in the SH3 domain leading to activation of the protein (2, 123-125).

1.7.2 X-linked agammaglobulinemia (XLA)

First described by Dr. Ogden C. Bruton in 1952 (126), X-linked Agammaglobulinemia (XLA) is a primary immunodeficiency disease, the hallmark of which is the complete absence of circulating B lymphocytes. XLA, which has a frequency of about 1 in 200,000 males, is caused by mutations (>600 known to date) in the gene encoding Btk. Although mutations occur all over the gene, the severity of the disease is often independent of the nature of the mutation (107, 127). Due to the nature of X-chromosome linked inheritance, only males are affected. In contrast, female carriers are healthy [with a single exception, a Japapanese female XLA patient (128)], and display non-random X-chromosome inactivation that is limited to B cells resulting in the selection of cells using the unmutated X-chromosome. The disease ensues after a developmental block in the maturation of B cells starting with a partial block at the transition from pro-B cell to pre-B cell stage followed by an essentially complete block at the transition from pre-B cell to B lymphocyte stage. B lineage cells in all organs are affected, resulting in a reduced size of lymph nodes and tonsils. Current treatment regimens are still the same as those that Dr. Bruton used to prescribe his patients – a combination of antibiotics to treat infections and gamma globulin substitution as a
prophylaxis against infections. This treatment is not a cure and patients are still highly prone to infections. Recently, gene therapy-based approaches were developed and could represent an alternative strategy for the management of XLA patients (129, 130). However, particular attention should be paid to the improvement of safety before gene therapy becomes an acceptable option for XLA patients.

1.7.3 X-linked immunodeficiency (xid)

X-linked Immunodeficiency (xid) is caused by a naturally occurring single point mutation in the PH domain of murine Btk, whereby a highly conserved arginine in position 28 is replaced by a cysteine residue (R28C) (131). In comparison with XLA, xid is a relatively mild disease and affected mice have still 50% of the normal value of B lymphocytes in their spleen. Detailed analysis of the immune defects in xid mice shows that only the peritoneal CD5 positive cells and the IgM and IgG3 subfractions of antibodies are absent or markedly reduced, respectively. Moreover, transgenic mice with a targeted deletion of the Btk gene (Btk−/−) also present a milder phenotype (132), suggesting that there are species-specific differences with regard to the function of Btk in humans and mice.

1.8 Itk

Itk (Interleukin-2 inducible T-cell kinase) is a member of the Tec family of non-receptor tyrosine kinases. Itk is expressed primarily in T-cells, NK cells, and mast cells. It has been shown that Itk plays important roles in T-cell development, proliferation, activation and function. In T cells, Itk is required for Ca\(^{2+}\) mobilization, activation of PLCγ, MAPK, and NFAT family of transcription factors (14, 133, 134). Upon stimulation of the T-cell receptor (TCR), Itk is activated and serves to amplify the TCR signaling cascade (135-137). Murine CD4+ T-cells lacking Itk showed reduced IL-2, IL-4, IL-5, and IL-13 production upon TCR stimulation (138-140).

Itk is functionally active in mast cells. These cells play critical roles in the initiation of allergic reactions. Following cross-linking of the high-affinity IgE receptor (FcεRI), PLCγ is activated followed by calcium release, degranulation (release of histamines, leukotrienes and other biological mediators) and secretion of cytokines, leading to the activation of inflammatory effector cells (141-143). In FcεRI-stimulated mast cells, Itk is phosphorylated inducing its interaction with multiple PKC members (144). Itk-deficient mice have profound defects in mast cell degranulation (145). A recent study shows that the impaired mast cell degranulation in Itk−/− mice is due to the
high IgE levels and reduced IgE/allergen-induced airway hyperresponsiveness and histamine release to systemic anaphylaxis (146). In the same study, it was shown that although the degranulation level of the Itk<sup>−/−</sup> BMMCs (bone marrow-derived mast cells) was not changed, the levels of IL-13 and TNFα were elevated, indicating that Itk may differentially regulate FcεRI signals in mast cells.

Itk plays also important roles in TCR induced actin polymerization. Actin polymerization results from effective T cell activation (147). Thus, Itk mutants in the PH, SH2 and kinase domain of Itk have been shown to inhibit TCR stimulated actin polymerization (148). In addition, T cells from Itk-deficient mice show impaired actin polymerization upon TCR stimulation (149, 150). An intact actin cytoskeleton is required for the TCR-mediated integrin adhesion and Itk is also involved in this process. Accordingly, a Jurkat cell line stably expressing a kinase-defective form of Itk was shown to be impaired in β1 integrin adhesion upon anti-CD3 stimulation (110, 148).

Itk is also critical in T lymphocyte gene expression. Thus, Itk-deficient T cells show decreased activation of the Ca<sup>2+</sup>-sensitive NFAT family transcription factors, such as NFATc1 and NFATc2. NFATc1 is important for Th2 cytokine production while NFATc2 regulates transcription of the Egr family of transcription factors (138, 140). Moreover, the phenotypes of Itk-deficient mice resemble those of mice harboring NFAT mutants (151). Finally, AP-1 activation is impaired in Itk-deficient mice (14, 138).

1.9 Tec

Tec is a nonreceptor tyrosine kinase belonging to the Tec family of protein tyrosine kinases (PTK). It was originally cloned from a mouse liver cDNA library (152). Initially, it was thought that Tec expression was tissue specific and could only be detected in hepatocytes and liver tumor cells. However, it was later shown that Tec is also expressed in hematopoietic cells, such as B and T lymphocytes, myeloid lineage cells and human neutrophils and is particularly important for hematopoietic cell development (153-160). Moreover, Tec and other members of this group show a high degree of conservation across species (2, 161, 162).

Tec regulates the function of B and T lymphocytes as well as other types of cells. Kitanaka and colleagues showed that Tec is activated in both mature and immature B-lymphoid cells, and is therefore implicated in B cell development and activation (153). In primary T cells, downregulation of Tec expression by an antisense strategy caused reduction in IL-2 production in
response to TCR stimulation (44). In contrast, overexpression of Tec in the T-cell line Jurkat synergized with PMA (phorbol myristate acetate) to induce NFAT activation, whereas Itk overexpression had no effect (163). These data suggest that Tec is important for optimal TCR signaling. Tec-deficient mice do not display any overt phenotype in the form of disease; however, knockout mice lacking both Btk and Tec (Btk<sup>−/−</sup>/Tec<sup>−/−</sup>) display a severe phenotype resembling XLA (160). In addition, Btk-deficient cell lines can be functionally reconstituted with Tec (164, 165). Tec activation and phosphorylation also play critical roles in IL-1 and IL-8 secretion, and generation of the chemotactic activity in supernatants from stimulated neutrophils (166). Recently, it was reported that Tec is involved in the Fcγ receptor-induced signaling and phagocytosis as well as the regulation of osteoclast differentiation (161, 162). Moreover, Tec kinase has also been shown to be involved in the intracellular signaling of a number of cytokines, such as interleukin IL-3, IL-6, erythropoietin (EPO), and granulocyte colony stimulating factor (G-CSF) (80, 157, 167, 168).

1.10 NF-κB

NF-κB (Nuclear factor-κB) is a nuclear transcription factor that was first identified in 1986 by Sen and Baltimore. It was so named, because it was found in the nucleus bound to an enhancer element of the immunoglobulin kappa light chain gene in B cells (169, 170). It was initially considered to be a B-cell–specific transcription factor, but was later shown to be a ubiquitous transcription factor present in every cell type. The NF-κB transcription factor family is composed of several structurally related proteins that exist in organisms ranging from insects to humans. The vertebrate NF-κB family includes five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel and RelB (171, 172). These proteins can form homodimers or heterodimers that give diverse combinations of dimeric complexes, which bind to target DNA in κB sites and regulate target gene expression. The prototype NF-κB dimer is the p50/RelA heterodimer. The different NF-κB proteins have unique ability to form dimers, distinct preferences for different κB sites, and distinct abilities to bind to inhibitory subunits known as IκBs (173). Thus, following stimulation, NF-κB complexes can be induced in different cell types and interact in distinct ways with other transcription factors and regulatory proteins to control gene expression.

NF-κB plays a key role in regulating genes responsible for both innate and adaptive immune response. Upon activation, NF-κB translocates to the nucleus to upregulate genes involved in B and T-cell development, maturation, and proliferation. Consistent with this, aberrant regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral
infection, and improper immune development. The role of NF-κB in the acute and innate immune response is evolutionarily conserved. NF-κB has also been shown to be crucial for the development of several mammalian hematopoietic cell lineages as well as formation of secondary lymphoid-organ structures (174).

Genes encoding NF-κB family members have been disrupted in mice leading to a variety of phenotypes in these animals. For instance, NF-κB1−/− mice show immune response defects due to abnormal B cell response, reduced marginal zone B cells, decreased CD5+ peritoneal B cell population and diminished response to LPS stimulation (175-178). On the other hand, RelA deficiency has been shown to be embryonically lethal as a result of extensive TNFα (tumor necrosis factor-α)-mediated fetal hepatocyte apoptosis (179). RelA can protect B cells, T cells and macrophages from the TNFα-mediated apoptosis (180-182). Moreover, RelA plays an important role in generating secondary lymphoid organs (183). In CD40, TLR4, and IgM receptor pathways-mediated B cell proliferation, NF-κB is also critical. Accordingly, B cells from mice that are deficient in c-Rel or p65 display decreased responses to antigen cross-linking and failed to respond to CD40 ligation (184, 185). Finally, B cells in mice expressing a transdominant form of IκBα presented an xid-like phenotype (186).

Activation of NF-κB is a tightly controlled event. In normal cells, NF-κB becomes activate only after cells receive the appropriate signal leading to regulating the transcription of its target genes. There are two major activation pathways of NF-κB, classical, or canonical, and the alternative, or non-canonical, pathway (Figure 3). In the canonical pathway, NF-κB dimers are, in steady state, kept in the cytosol by inhibitory proteins IκBs. Various agents including proinflammatory cytokines TNFα, IL-1β (interleukin-1β), EGF (epidermal growth factor), T- and B-cell mitogens (antigen receptors), LPS (lipopolysaccharide), bacteria, viruses, viral proteins, double-stranded RNA, physical and chemical stresses, as well as the cellular stresses such as ionizing radiation and chemotherapeutic agents activate NF-κB (187). Following stimulation, the IKK (IκB kinase) complex is activated. IKK complexes contain the catalytic subunits IKKα and IKKβ, and the regulatory subunit IKKγ (also known as NEMO). IKK activation induces the NEMO ubiquitination, and the subsequent phosphorylation of IKKβ at two serine residues (S177 and S181) in its activation loop. Activated IKK leads to the phosphorylation of IκBα at conserved serine residues (S32 and S36) in its N-terminal regulatory domain. Once phosphorylated, IκBs almost immediately undergo a second post-translational modification event, polyubiquitination. The major ubiquitination sites in human IκBα are lysines 21 and 22. Following ubiquitination,
IκBs are degraded by the 26S proteasome leading to the release of NF-κB. Consequently, free NF-κB dimers translocate to the nucleus and, following binding to cognate regulatory elements on target genes, modulate gene expression (188, 189). This pathway plays a major role in the control of innate immunity and inflammation (190, 191).

**Figure 3.** Canonical and non-canonical pathway of NF-κB activation (for simplicity, not all components involved are depicted).

NF-κB activation and signaling is known to be strictly dependent on the activity of the 26S proteasome. Protein ubiquitination is a critical posttranslational modification event regulating fundamental cellular processes, such as cell cycle, endocytosis, antigen presentation and apoptosis (192, 193). Ubiquitin is a small 76-amino acid protein, which, following activation, is conjugated to a lysine residue of a target protein. The covalent attachment of a single ubiquitin to a target protein triggers further incorporation of additional ubiquitin molecules onto the monoubiquitinated substrate leading to the formation of a growing chain of polyubiquitin. Consequently, the polyubiquitinated protein will be targeted for degradation by the UPP (Ubiquitination-Proteasome-
Pathway). The fact that UPP is involved in key biological processes including cell cycle and apoptosis validates it as a novel and legitimate therapeutic target.

The non-canonical pathway is stimulated by cytokines that belong to the TNF super-family, such as BAFF (B cell activation factor belonging to the TNF family, also known as TNFSF13B or BLyS), LTβ (lymphotoxin β), and CD40L (CD40 ligand). This pathway involves the upstream kinase NIK (NF-κB inducing kinase), which activates IKKα, thereby inducing the phosphorylation and proteasome-dependent processing of p100, the main RelB inhibitor, resulting in RelB–p52 and RelB–p50 nuclear translocation and DNA Binding (194-198). This pathway has a crucial role in controlling the development, organization and function of secondary lymphoid organs, and in B cell maturation and survival (191, 199).

The activation of the canonical as well as the non-canonical NF-κB pathways relies on the inducible phosphorylation of IκB inhibitory proteins (IκBα for the classical pathway and p100 for the alternative pathway) by the IKK complex. Disruption of genes encoding individual subunits has shown that IKKβ and IKKγ are critical for the activation of the canonical pathway. However, RelB–p50 and RelB–p52 activation is dependent on IKKα, but not on IKKβ or IKKγ (194, 200). It is worth noting that in order to adequately control the activity of NF-κB signaling, NF-κB also induces the transcription of its own inhibitory proteins (IκBα and NF-κB2), which form a negative feedback loop to control NF-κB signaling pathway (187, 201).

1.11 Peptidyl-prolyl cis/trans isomerase Pin1

Protein phosphorylation is a key cellular signaling mechanism that induces changes in protein conformation (202, 203). Phosphorylation of tyrosine residues and some serine/threonine residues acts as a signal to recruit proteins into signalling networks or to place enzymes close to their substrates. Serine or threonine residues that precede Proline (Ser/Thr-Pro) are major regulatory phosphorylation motifs in cells. Enzymes that are responsible for such phosphorylations belong to a large superfamily of Proline-directed protein kinases, and include cyclin-dependent protein kinases (CDKs), extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun-N-terminal kinases (SAPKs/ JNKs), p38 kinases, glycogen synthase kinase~3 (GSK3) and Polo-like kinases (PLKs) (204-206). The proline residues in this milieu exist in two completely distinct cis and trans conformations and provide a backbone switch in proteins controlled by peptidyl-prolyl isomerization (PPlases). There are three families of PPlases, cyclophilins, FKBP5s (FK506 binding proteins), and the parvulin and Pin1 (207-210). The discovery of the unique and
conserved PPIase Pin1 [protein interacting with NIMA (never in mitosis A)-1] was a major breakthrough in understanding the mechanism of proline-directed phosphorylation and conformational changes (207, 211, 212).

Figure 4. Schematic representation of the conformational changes induced by Pin1. Pin1 recognizes phosphorylated Serine or Threonine followed by a Proline motif (pSer/Thr-Pro) and catalyzes the prolyl isomerization and conformation change.

Pin1 has emerged as an important regulator of cell proliferation and the DNA-replication checkpoint (207, 213). Pin1 interacts with a number of phosphoproteins through recognition of phosphorylated Serine-Proline and/or phosphorylated Threonine-Proline (pSer/Thr-Pro) motifs by its amino-terminal WW domain (214, 215). Protein phosphorylation on Ser/Thr-Pro motifs is a principal regulatory mechanism in cellular response to a variety of signals (216, 217). The peptidyl-prolyl bond, which exists in two completely distinct cis/trans conformations, is important in determining protein’s structure and activity. By promoting the cis-trans isomerization of this peptide bond (Figure 4), Pin1 regulates the function [such as: protein-protein interaction, protein dephosphorylation, protein expression, stability and localization (206, 218-222)] of substrates through its carboxy-terminal prolyl isomerase (PPIase) domain (207).
2. AIMS OF THE THESIS

To further broaden our understanding of the signalling of Tec family kinases, this study was undertaken to address the transcriptional and posttranslational regulation of this family.

The specific aims were:

(1) To investigate the potential role of the Peptidyl-prolyl cis/trans isomerase, Pin1, in the regulation of Btk expression and activation.

(2) To explore the potential role of NF-κB signaling in the transcriptional regulation of the Tec family tyrosine kinases.

(3) Based on the fact that Itk transcription was NF-κB dependent, a further aim was to explore the therapeutic potential of proteasome inhibitors in HIV/AIDS.
3. METHODOLOGICAL CONSIDERATIONS

3.1 Western blot and Immunoprecipitation

**Western blot** (protein blot or immunoblot) is a powerful and important procedure for the immunodetection of proteins. Since the inception of the protocol for protein transfer from an electrophoresed gel to a membrane in 1979 (223), protein blotting has greatly evolved. Western blotting (WB) evolved from DNA (Southern) blotting (224) and RNA (Northern) blotting (225) techniques. The term WB was coined to describe a procedure that was slightly modified from that of Towbin et al to retain the ‘geographic’ naming tradition initiated by Southern. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE. The antigens are then electrophoretically transferred in a tank or a semidry transfer apparatus to a nitrocellulose, PVDF, or nylon membrane. The subsequent employment of antibody probes are used to detect specific proteins that are bound to the nitrocellulose membrane (226).

In the present study, WB was used to detect Btk, Pin1, pSer/Thr-Pro proteins, tyrosine phosphorylated proteins and polyubiquitinated proteins (paper I), Btk, Tec, Itk, Bmx, Syk, Lyn and ERK (paper II), NF-κB p65, Itk and GFP (paper III).

**Immunoprecipitation (IP)** is the technique of precipitating an antigen out of solution by specific antigen-antibody interaction. This method can be used to identify protein complexes present in cell extracts by targeting any one of the proteins assumed to be in the complex. IP can be used to screen protein-protein interactions, purify proteins from a given extract and for determining posttranslational modification of proteins (for example, phosphorylation and ubiquitination). A similar mechanism can be used to identify protein-DNA interactions (see details of ChIP assay later). IP consists of multiple ordered steps: lysing the cell. Binding of a specific antigen to an antibody, antibodies can be bound non-covalently to immunoabsorbents such as protein A or protein G agarose. Precipitating the antibody-antigen complex. Washing the precipitate, and dissociating the antigen from the immune complex. The dissociated antigen is then analyzed by electrophoretic methods (227, 228)

By IP, we found that Btk contains the pSer/Thr-Pro motif and functionally interacts with the peptidyl-prolyl cis/trans isomerase Pin1 (paper I).
3.2 Chromatin Immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) is a widely used method to explore *in vivo* interactions between proteins and DNA. Chromatin is composed of DNA, proteins and RNA. Chromatin structure is dynamic, responds to extracellular signals, and controls gene expression, cell division and DNA repair. Chromatin is one of the most intensely studied structures in biology and ChIP assays have proven to be a powerful means to investigate a host of DNA-dependent processes. ChIP is successfully used to identify transcription factors in a given promoter. It uses a process of formaldehyde fixation, which preserves protein modifications and protein-DNA interactions, followed by specific DNA enrichment using antibodies direct against the protein of interest (229). In this method, intact cells are fixed using formaldehyde which by cross-linking preserve protein-DNA interaction. The DNA is then sheared into small, uniform fragments (usually 200-1000 bp) by sonication. Next, the protein-DNA complexes are immunoprecipitated by an antibody against a desired transcription factor. After immunoprecipitation, the protein-DNA cross-linking is reversed, proteins are removed by proteinase K treatment and the DNA is purified. The DNA is then amplified by PCR using the specific primers corresponding to a region in the promoter.

By ChIP assay, we demonstrated that NF-κB is an essential transcription factor for the adequate expression of Btk (paper II) and Tec (paper IV).

3.3 Electrophoretic Mobility Shift Assay (EMSA)

Interaction of proteins and DNA is central for controlling of many cellular processes including DNA replication, recombination and repair, transcription, and viral assembly. One critical technique used for studying gene regulation and determining protein-DNA interactions is the Electrophoretic Mobility Shift Assay (EMSA).

The EMSA technique is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay. An advantage of studying DNA-protein interactions by EMSA is the ability to resolve complexes of different stoichiometry or conformation. Another advantage is that the source of the DNA-binding protein may be a crude nuclear or whole cell extract rather than a purified preparation. EMSA can be used qualitatively to identify sequence-specific DNA-binding proteins (such as transcription factors) in crude lysates.
and, in conjunction with mutagenesis, to identify the important binding sequences within a given gene’s upstream regulatory region (230, 231).

Using EMSA, we found that the NF-κB subunits p65 and p50 bind to the Btk promoter (paper II).

### 3.4 RNA interference (RNAi)

Small interfering RNAs (siRNAs), also known as short interfering RNAs, is a class of 21-25 nucleotide long double-strand RNA molecules that play a variety of roles in biology. Most notably, siRNAs induce effective posttranscriptional silencing of specific target genes (232, 233).

RNA interference (RNAi) is the phenomenon by which double-stranded RNA (dsRNA) induces potent and specific inhibition of gene expression via degradation of complementary messenger RNA (mRNA). In eukaryotic organisms, dsRNA produced in vivo is processed into 21-25 nucleotide double-stranded siRNA by an enzyme called Dicer, a member of the RNase III family of dsRNA-specific endonucleases. Synthetically produced siRNAs mimic the products of this process. siRNA is incorporated into a large protein complex called RNA-Induced Silencing Complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation.

Using RNAi technology, we successfully knocked down the Pin1 gene in cultured B cells (A20) and NIH 3T3 cells (paper I).

### 3.5 Hydrodynamic transfection

Hydrodynamic gene delivery to the liver was discovered a decade ago when large volumes of DNA solution was rapidly infused into the livers of rodents via the portal vein or retrograde via the hepatic veins. Subsequently, it was found that rapid injection of large volumes of DNA solution (corresponding roughly to 8-10% of body weight) into the tail veins of mice also resulted in high levels of gene delivery to the liver. This technique is now in widespread experimental use in rodents (234, 235).

By hydrodynamic transfection technique, we got a very efficient expression of the Btk promoter luciferase reporter constructs (paper II), HIV-1 promoter luciferase reporter constructs (paper III), and Tec promoter luciferase reporter constructs (paper IV) into the liver of mice.
3.6 Site-Directed mutagenesis

Site-directed mutagenesis (also known as oligonucleotide-directed mutagenesis) is the term used to describe when changes in DNA are made at a desired position. Site specific mutagenesis was invented in 1978 by Clyde Hutchison and Michael Smith. Michael Smith was given the Nobel Prize for discovering it in 1993 (236). The basic procedure starts with synthesizing a short DNA primer, containing the desired base change. Next, this synthetic primer has to hybridize with a single-stranded DNA containing the gene of interest. Third, the single stranded fragment is extended using DNA polymerase, which copies the rest of the gene. Fourth, the obtained double stranded molecule is introduced into a host cell and cloned. Fifth, mutants are selected for further study.

By site-directed mutagenesis, we generated mutant Btk plasmids (paper I), mutant Btk promoter luciferase reporter constructs (paper II), and mutant Tec promoter luciferase reporter constructs (paper III).

3.7 Promoter luciferase reporter constructs and luciferase assay

The luciferase reporter system is a powerful and widely used tool in gene expression studies. Using this system, one can easily and effectively analyze the transcriptional activity of the regulatory region of a particular gene. Functional characterization of the specific promoter can be initiated using deletion constructs. The promoter luciferase reporter constructs can be used for identifying the transcription factors in a given gene promoter.

In this study, using the promoter-less pGL3-basic vector as backbone, we cloned Btk promoter luciferase reporter constructs and mutants (paper II), HIV-1 promoter luciferase reporter construct (paper III), and Tec promoter luciferase reporter constructs and mutants (paper IV), for the study of the transcriptional regulation of Btk, HIV-1 and Tec genes.
4. ETHICS

All experiments were performed in accordance with approved ethical permissions. Harvard Medical (USA) protocol number 03036 (paper I), Dnr: S59-03 and S198-07 (paper II, III and IV).
5. RESULTS AND DISCUSSION

5.1 Paper I

The peptidyl-prolyl cis/trans isomerase Pin1 is an important regulator of cell proliferation and the DNA-replication checkpoint. Pin1 interacts with a number of phosphoproteins through recognition of phosphorylated serine-proline and/or phosphorylated threonine-proline (pSer/Thr-Pro) motifs by its amino-terminal WW domain (207, 213-215).

In this study, we found that Pin1 is a negative regulator of Btk. Overexpression of Pin1 induced tyrosine dephosphorylation of Btk, and led to a decrease in its steady state levels by reducing the half-life. The negative regulatory effect of Pin1 was observed both in cell lines and in Pin1<sup>−/−</sup> mice and was found to be dependent on a functionally intact Btk. This may constitute a feed back loop for the regulation of Btk.

![Diagram](image_url)

**Figure 5.** Cell cycle dependent negative regulation of Btk by Pin1 (a schematic model). Pin1 binds to phosphorylated Serine-115 in Btk (during interphase), or phosphorylated Serine-21 (during mitosis), thereby negatively regulating Btk by exerting its isomerase activity.
The Pin1 target region in Btk localizes in the pleckstrin homology domain suggesting that during interphase phosphorylation of serine 115 (S115) was required, whereas in mitosis phosphorylation of serine 21 (S21) was critical. Accordingly, Pin1 was shown to associate with Btk through binding to S21 and S115, respectively, both of which lie in a classical Pin1-binding pocket (Figure 5). Using a phospho-mitotic antibody, we found that Btk harbors a *bona fide* MPM2 epitope corresponding to a phosphorylated serine or threonine residue followed by a proline. Our results indicate that the peptidyl-prolyl isomerase Pin1 interacts with Btk in a cell cycle-dependent manner and regulates its expression.

**5.2 Paper II**

Both Btk and NF-κB are important for B cell development (10, 11, 13, 237). Btk regulates a plethora of signaling proteins including NF-κB. Btk and its substrate, phospholipase Cγ2 (PLC-γ2), are essential for the activation of NF-κB in response to BCR engagement. Thus, NF-κB signaling is severely compromised in cells that are deficient in Btk (35, 36, 238).

In this study, we presented strong evidence indicating that NF-κB is required for the transcription of the *Btk* gene. First, we found that both proteasome and NF-κB inhibitors could suppress Btk transcription and intracellular expression levels (Figure 6B and not shown data). Second, two functionally active NF-κB binding sites were identified in the Btk promoter. We found that NF-κB binds to these sites and induces Btk transcription (Figure 6A and C). Third, in live mice, using hydrodynamic transfection technology, we demonstrate that Bortezomib (an inhibitor of the proteasome and NF-κB signaling pathway), as well as NF-κB binding sequence-oligonucleotide decoys can block Btk transcription (Figure 6D and data not shown). Furthermore, we demonstrated that Btk could induce NF-κB signaling in mice. Collectively, we have shown that Btk uses a positive auto-regulatory feedback mechanism to stimulate transcription from its own promoter via NF-κB.
Figure 6. NF-κB is an essential transcription factor for adequate Btk expression. A. Structure of the Btk promoter. Transcription factors and co-activators (OCT1, PU.1 and sp1/sp3) that have been shown to bind to the Btk promoter. Btk promoter reporter constructs (500-Btk and 1000-Btk) and the two putative NF-κB binding sites. B. The 500- and 1000-Btk were introduced into A20 cells and cells were treated with MG132 (10 µM) for 16 hours. C. Wild type and mutant Btk promoter reporter constructs were cotransfected with p65 plasmid into A20 cells. 48 hours later, luciferase activity was measured. D. NMRI mice were transfected with 1000-Btk using the hydrodynamic procedure. The mice were treated with 1 mg/kg Bortezomib or 1 mg/kg LPS as indicated. In vivo biophotonic imaging was performed using the IVIS imaging system. Reproduced from Yu L et al (239).

5.3 Paper III

HIV-1 has proved to be notoriously difficult to tackle despite the availability of more than 20 clinically approved drugs. The existing antiretroviral (ARV) therapy induces severe side effects, in particular, when administered in combination and over prolonged periods (240-242). Moreover, during treatment, the emergence of highly drug-resistant viral strains remains a real challenge (243). The HIV-1 long terminal repeat (LTR)-promoter harbors two tandem NF-κB binding sites (244, 245). Moreover, it was recently reported that inhibition of ItK severely interfered with HIV
infection (246). To establish whether NF-κB signaling is critical for viral replication, we set out to investigate the effect of proteasome inhibitors on viral gene expression.

We demonstrated that proteasome and NF-κB inhibitors effectively shut down transcription from the HIV-1 LTR-promoter (Figure 7A). Conversely, expression of the NF-κB subunit p65 dramatically induced the LTR-promoter. We showed that replication of HIV-1 in PBMC was severely compromised following treatment with proteasome inhibitors alone or in combination with other antiretroviral drugs (Figure 7C). Finally, incubation of PBMC with these drugs reduced

Figure 7. Proteasome inhibitors inhibit HIV-1 replication by suppress the LTR promoter and deplete a key cellular component Itk. A. Plasmid pLTR-Luc was transfected into Jurkat cells. 32 hours posttransfection, cells were grown for 16 hours in the presence of proteasome or NF-κB inhibitors as indicated. B. PBMC were stimulated with PHA (2.5 µg/ml) and IL-2 (75 U/ml) and then treated with MG132 over night (* Cells were treated with MG132 for 1 hour prior to the addition of PHA and IL-2). Itk level was measured by western blot. C. PBMC from 12 health donors were stimulated with PHA (2.5 µg/ml) and IL-2 (75 U/ml) for 2 days. Cells were either left uninfected or infected with HIV-1(Bal) in the presence of drugs (Bortezomib 2.5 nM, MG132 200 nM, IND 1 µM, AZT 1 µM). Box plots (range and median) represent level of active RT in supernatants at day 7 following infection (as pg/well). *** (P <0.0001).
expression of Itk (Figure 7B), a Tec-family kinase recently was shown to be required for HIV-1 replication (246). Altogether, our findings strongly suggest that proteasome inhibitors not only function as bone fide antiretroviral drugs, but that they also boost resistance of host cells by affecting key endogenous components.

5.4 Paper IV

Tec, a nonreceptor protein tyrosine kinase (PTK), was originally cloned from a mouse liver cDNA library (152), and was later shown to be important for hematopoietic cell development (156, 157, 160, 247). It is expressed in hematopoietic cells, such as B and T lymphocytes, myeloid lineage cells and neutrophils (153-159). Tec has many important physiological functions, including regulation of B and T lymphocytes, fine-tuning of TCR and BCR signaling, NFAT activation, as well as a non-redundant role upon defective Btk signaling. Previous work has shown that the transcription factors Sp1 and PU.1 can bind and regulate the Tec promoter (119, 120).

![A. Schematic representation showing the functionally active NF-κB binding site is conserved in human and mouse Tec promoters. B. Tec promoter reporter construct was cotransfected with pcDNA1-p65 into HepG2 cells. 32 hours later, cells were treated with MG132 (10 µM), or Bay-117085 (5 µM), or Bortezomib (20 nM) (C), for another 16 hours as indicated.](image)
In this study, we demonstrate that NF-κB is an essential transcription factor for adequate expression of the Tec gene and identified a highly conserved and functionally active NF-κB binding site in the corresponding promoter (Figure 8A). The NF-κB subunit p65/RelA induced transcriptional activity of the Tec promoter. Moreover, proteasome inhibitors repressed Tec transcription through inactivation of the NF-κB signaling pathway (Figure 8B). This study together with our previous findings that proteasome inhibitors repress Btk transcription via NF-κB (paper 2)(239), provide important insight into the mechanisms underlying the role of NF-κB in Tec family kinase signaling and lymphocyte development.
6. CONCLUDING REMARKS

This study focused on the transcriptional and posttranslational regulation of Tec family kinases. Therefore, the role of NF-κB in the regulation of Tec kinases transcription and expression, as well as the Peptidyl-prolyl cis/trans isomerase Pin1 in the regulation of Btk expression and activation has been investigated. In this thesis we found that:

1. The peptidyl-prolyl cis/trans isomerase Pin1 functionally interacts with Btk in a cell cycle-dependent manner, and negatively regulates Btk signaling (paper I). This work together with the previous findings that the peptidyl–prolyl cis–trans proline isomerase cyclophilin A binds to Itk and inhibit its kinase activity (21, 90, 102), indicate that the peptidyl-prolyl cis/trans isomerases play critical roles in the regulation of the functions of Tec family kinases.

2. NF-κB is crucial for Tec family kinase gene expression. First, Btk uses a positive auto-regulatory feedback mechanism to stimulate transcription from its own promoter via NF-κB (paper II). Second, NF-κB is an essential transcription factor for Tec and Itk genes (Paper III & IV, and author’s unpublished data). Altogether, these results provide important insights into the molecular mechanisms underlying the functional regulation of Tec family kinases by NF-κB.

3. By blocking at least one cellular component as well as viral gene expression, proteasome inhibitors effectively suppress HIV replication (paper III).

This thesis presents a number of findings that hopefully will contribute to further broaden our understanding of the signaling and regulation of Tec family kinases. It also brings up new questions that warrant future studies. For example, the role of NF-κB signaling in the transcriptional regulation of Tec kinases has not been fully examined (role in the regulation of Rlk/Txk and Bmx); although we showed that proteasome inhibitors, including the clinical drug Bortezomib, effectively block HIV-1 replication, it remains uncertain whether these inhibitors can successfully suppress viral replication in HIV patients. Therefore, additional experimental and clinical studies are needed for addressing these questions. The future prospects are unclear, but we are getting closer…
7. ACKNOWLEDGEMENTS

This work has been performed at the Department of Laboratory Medicine, Clinical Research Center at Novum. I would like to express my sincere gratitude and appreciation to all people for their concern, encouragement and support, which inspired me to accomplish this thesis, especially:

Professor **Edvard Smith**, my supervisor with an extremely broad scientific knowledge, for giving me a chance to work in your group, for sharing your expertise in signaling transduction and beyond, for your support, encouragement, trust and constructive advice during the past years.

Associate professor **Abdalla J. Mohamed**, my co-supervisor, for guiding me into the field of molecular biology, for your kindness, encouragement and the innumerable discussions.

**Dr. Leonardo Vargas**, my co-supervisor, for sharing your experience in confocal microscopy, for your advice and suggestion on writing, for the help of preparing presentations, room booking and many things.

All co-authors: **Beston Nore, Anna Berglöf, Emelie Blomberg, Oscar Simonson and Jose Arteaga, Venkatramanan Mohanram** and **Anna-Lena Spetz** (CIM, KI), **Greg Finn** and **Kun Ping Lu** (Harvard, USA), for good collaboration and contribution to the papers in this thesis.

Colleagues in and outside Edvard Smith’s group: **Karin Lundin, Lotta Asplund, Jessical Lindvall, Pedro Moreno, Eman Zaghloul, maria cardona, Alamdar Hussain, Joana Viola, Sofia Stenler, Iulian Oprea, Hossain nawaz, Maroof Hasan, Juhana Heinonen, Rongbin Ge, Mathias Svahn, Rani Faryal, Manuela Gustafsson** and **lenis Alvarez** for scientific support, discussions, the nice working atmosphere and all the good time outside the lab.

Thanks to: **Anna Berglöf** for the well organized animal experiments. **Beston Nore, Pedro Moreno and Mathias Svahn** for the MAC soft ware support.

The administrative staff at Clinical Research Center and Department of Laboratory Medicine, for providing all the needed help.

My Chinese friends in Sweden: **Wenfeng Yu and Wei Ren, Ruisheng Duan and Yinchun Dou, Jianguang Ji** and **Xiao Wang, Hai Dong** and **Ailing Xu, Yu Huang** and your family members,
Jianjun Xie, Hairong Song, Jin Xiu, Xiang Hua and Jun Su, Zheng Ge, Feng Wang, Lili Mo, Zhizhong Guan, Qingyang Wu, Yan Li, Xiaojin Hao, Rong Liu, Xinwen Zhou and Guanghui Li for the merry time we have spent in Sweden. Now, many of you have moved to other countries, I wish you all the best in your life.

Yuhui and Hongmei, my brother and sister and your lovely kids, Amanda and Amelie, for all your support and encouragement, we spent lots of fun time and travel to many interesting place together, you really make our (me and my family) life bright and colourful in Sweden.

My parents, for your love, understanding and strong support. Mother left me forever during the time when I working on this thesis, you will stay in my heart all the time. Ming and Yan, my brother and sister and your family, for being behind me and taking care of parents over the years when I was away.

My beloved family, Xiumei, my dear wife, for your love, understanding, enormous support, encouragement and sharing all the happiness and sadness with me, Zhihan and Kevin, my lovely sons, you make our family life colourful and full of funs, you are my future.

This work was supported by Swedish Cancer Fund, Wallenberg Foundation, Swedish Research Council, Swedish International Development Cooperation Agency/Department for Research Cooperation, the European Union Grant EURO-POLICY-PID, EU FP6 EUROPRISE, Swedish Foundation for Strategic Research, Swedish Hemophilia Society, Stockholm County Council (research grant ALF Projektmedel Medicine), the Goljes/Lindströms Foundations, and the National Institutes of Health Grant GM58556.
REFERENCES


Dolinski, K., Muir, S., Cardenas, M., and Heitman, J. 1997. All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae.


