Regulation of activation of NF-κB by Calmodulin in T-lymphocytes

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To My Family and Friends
"Take up one idea. Make that one idea your life - think of it, dream of it, live on that idea. Let the brain, muscles, nerves, every part of your body, be full of that idea, and just leave every other idea alone. This is the way to success."
— Swami Vivekananda (1863-1902)
# TABLE OF CONTENTS

**ABSTRACT** ................................................................. 1

**ABBREVIATIONS** ......................................................... 2

**PAPERS IN THIS THESIS** .............................................. 3

**INTRODUCTION** ........................................................... 5

**Eukaryotic Gene Regulation** ........................................ 5

* Protein modifications by phosphorylation and dephosphorylation .......................... 6
* Protein modification by ubiquitination ............................................ 7

**The Immune system** .......................................................... 9

* Innate immunity ................................................................. 9
* Adaptive immunity .............................................................. 10
* Antigen receptor activation and the functional outcomes .................................. 11

**NF-κB Transcription Factors and their Features** .................. 13

* Ways of activation and the regulated genes .................................... 14
* T-Cell Receptor signalling to NF-κB ........................................ 17
* The CBM proteins in TCR signalling ......................................... 18
* Down-regulation of NF-κB signalling ......................................... 21
* NF-κB and diseases ............................................................ 21

**Calcium Signalling and its Regulation** .............................. 23

* Ca\(^{2+}\) homeostasis ......................................................... 24
* Specificity of Ca\(^{2+}\) signalling ........................................... 24
* Ca\(^{2+}\) signalling in lymphocytes ........................................ 25
* Ca\(^{2+}\)-binding proteins ..................................................... 28
* Calmodulin ................................................................. 28
* Target binding by Calmodulin ................................................ 29
* Calmodulin-regulated phosphatases and kinases ................................ 30
* Regulation of CaMKII ......................................................... 30
* Calmodulin regulation of transcription in lymphocytes .......................... 32

**AIMS** ........................................................................... 34

**RESULTS AND DISCUSSION** ........................................ 35

* Interaction of calmodulin with Bcl10 modulates NF-κB activation
  *(Paper I)* ................................................................. 35
* CaM interacts with Bcl10 ...................................................... 35
Interaction of CaM with Bcl10 modulates the binding of Bcl10 to Carmal and consequently activation of NF-κB.

CaMKII targets Bcl10 in TCR-induced activation of NF-κB (Paper II)

CaMKII is recruited to the immunological synapse and modulates interactions within the CBM complex.

Phosphorylations of Bcl10 by CaMKII modulate NF-κB activation.

Bcl10 sites phosphorylated by CaMKII regulate phosphorylation of IKKα/β and JNK2.

Defect CaMKII-induced phosphorylation of Bcl10 prevents K63-linked ubiquitination of Bcl10 and IKKγ.

CaMKII regulates the phosphorylation of IKK by TAK1.

Concluding Remarks

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REFERENCES
Nuclear factor kappa B (NF-κB) is a widely expressed family of transcription factors that are involved in a diverse number of processes. These include inflammation or differentiation, survival or apoptosis, and proliferation or cell cycle arrest. NF-κB is usually associated with inhibitory κB proteins (IκB), which mask the nuclear localisation sequence (NLS) of NF-κB and renders it in the cytoplasm. Various stimuli result in the activation of the I kappa B kinase (IKK) protein complex, which phosphorylates IκB proteins and thereby marks them for degradation by the ubiquitin-proteasome pathway. Thereby NF-κB enters the nucleus and acts on its target genes. The study of T- and B-lymphocyte antigen receptor signalling to NF-κB is a field of intense investigation, with much attention being focused on the molecular scaffolding proteins Carma1, Bcl10 and MALT1 and their post-translational modifications. These have been shown to be crucial for the organization of the immunological synapse structure under the activated receptor, to which IKK is recruited and becomes activated, which subsequently leads to the activation of NF-κB.

T cell receptor (TCR) activation results in a rapid increase in the intracellular Ca²⁺ level and NF-κB activation is known to be regulated by those increases, but the mechanisms have remained unclear. Calmodulin (CaM) is a calcium sensory protein that responds to increases in intracellular Ca²⁺ levels. When CaM binds Ca²⁺ ions, it leads to structural changes that directly as well as indirectly, through CaM dependent kinases (CaMKs), phosphatases and other enzymes, alters a variety of cellular processes, among them transcriptional regulation. Here CaM is shown to interact directly with Bcl10 in a Ca²⁺ dependent manner. Increases in the intracellular Ca²⁺ level are shown to induce the proximity of Bcl10 and CaM in vivo. Carma1 associates with Bcl10 through a CARD-CARD domain interaction that is known to be crucial for TCR signalling to NF-κB. The interaction of CaM with Bcl10 was mapped to the CARD domain and was shown to be a negative regulator for the Bcl10-Carman1 interaction. Inhibition of the CaM interaction by a point mutation within the CaM binding site of Bcl10 results in decreased binding of CaM to Bcl10 in vivo, as well as an increased ability of Bcl10 to induce NF-κB transcriptional activity, which is further enhanced by TCR activating stimuli.

NF-κB activation is also shown here to be regulated by CaM indirectly through actions of CaMKII. The CaMKII is recruited to the immunological synapse where it interacts with Bcl10 in an inducible fashion and phosphorylates Bcl10. Phosphorylations of Bcl10 by CaMKII are shown to be important for the ability of Bcl10 to induce NF-κB transcriptional activity. Upon mutation of its most important CaMKII site, Bcl10 fails to activate an NF-κB reporter and an NF-κB target gene (IL-2). This mutated Bcl10 also fails to induce activating phosphorylations of IκKα/β and the kinase JNK2 but not JNK1. Furthermore, phosphorylation of Bcl10 by CaMKII regulates the interactions within the important Carman1, Bcl10, Malt1 signaling complex and the essential signal induced ubiquitinations of Bcl10 and IKKγ. Phosphorylation of IκK by TAK1 is also regulated by CaMKII, and serine 82 is a putative CaMKII target site of TAK1 that appears to be important for IκBα degradation.

In summary, this thesis explores that not only NF-κB but also CaM is a double-edged sword, since the multi-functional NF-κB family of transcription factors is regulated by CaM both negatively and positively.
**ABBREVIATIONS**

Antigen-presenting cell (APC)  
B-cell receptor (BCR)  
Calcium (Ca$^{2+}$)  
Calcineurin (CaN)  
Calcium release-activated calcium channel (CRAC)  
Calmodulin (CaM)  
Calmodulin-dependent kinase (CaMK)  
Calmodulin-dependent kinase kinase (CaMKK)  
Caspase recruitment domain (CARD)  
De-ubiquitinating enzyme (DUB)  
Diacylglycerol (DAG)  
Endoplasmic reticulum (ER)  
IkB kinase (IKK)  
Inositol 1, 4, 5 triphosphate (IP$_3$)  
Major histocompatibility complex (MHC)  
NF-κB essential modulator (NEMO)  
Nuclear export sequence (NES)  
Nuclear factor κB (NF-κB)  
Nuclear localisation sequence (NLS)  
Nuclear pore complex (NPC)  
Pattern recognition receptor (PRR)  
Phospholipase C-γ (PLC-γ)  
Protein kinase A (PKA)  
Protein kinase C (PKC)  
Rel-homology domain (RHD)  
Store-operated calcium channel (SOC)  
T-cell receptor (TCR)  
Toll-like receptor (TLR)  
Transactivation domain (TAD)  
Tumour necrosis factor-α (TNF-α)
PAPERS IN THIS THESIS

I  Edin S, Oruganti SR, Grundström C and Grundström T (2010)
Interaction of calmodulin with Bcl10 modulates NF-κB activation.
Molecular Immunology 47: 2057-2064

II  Oruganti SR*, Edin S*, Grundström C and Grundström T (2011)
CaMKII targets Bcl10 in T-cell receptor induced activation of NF-κB.
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INTRODUCTION

Eukaryotic Gene Regulation

The main difference between eukaryotic and prokaryotic cells is that the former do not always manifest their genes. The regulation of cells of eukaryotes is complex in that these organisms comprise many different types of cells that each has its own unique function (Brown, 1981). As expected from these complex characteristics of eukaryotic cells, their regulation is managed at many levels. The initiation of transcription is one focal point of control (Shapiro, 1982). Transcription factors bind to specific DNA sequences and work together with co-activators and co-repressors, chromatin remodeling enzymes and the machinery of general transcription at the gene promoters. The organization of all these contributors together acts as the regulator of the initiation of transcription. They all act together in order to ensure that gene transcription is well managed and regulated. Putting together all transcription factors to their specified sequences at the proximal and distal promoter and enhancer elements of the DNA verifies the consolidation of the general transcription machinery with the promoter components of the gene. The expression of a certain gene can either be activated or repressed by the transcription factors that thereby are of great importance in regulating transcription. The control of such regulatory processes is basically accomplished through cell signalling events.

Gene promoters and enhancers have DNA binding sites for a variety of transcription factors. These transcription factors specifically bind their DNA recognition sites via their DNA-binding domains, and they usually bind DNA as dimers. In most cases, transcription factors have also transcriptional activation domains that bind to other important components in the initiation process (Gaston and Jayaraman, 2003; Näär et al., 2001). The human body has about 2,000 expressed transcription factors (Brivanlou and Darnell, 2002). This fact manifests the significance of transcription factors in regulating the transcription of the genome. Many processes and methods whereby transcription factors impact gene transcription have been uncovered (Kadonaga, 2004). Most transcription factors are activated by different signals and belong to multi-protein families, such as members of the NF-κB family. As exemplified by the NF-κB family, multiple members of a transcription factor family can also serve as dimers in different combinations, thereby increasing the number of functional transcription factors. Modifications of the transcription factors or proteins signalling to them by different post-translational processes are evident and include acetylation,
phosphorylation, ubiquitination and nitrosylation. These events affect in large part how transcription factors are activated. The NF-κB family provides a good example of such regulation via post-translational modifications (Perkins, 2006). Aside from modifications, there can be cross-coordination between transcriptional activators and repressors. A particular cell, with its gene expression profile, may depend upon the kind of signalling mediators, the transcription factor expression and their co-operation. It may likewise depend on the co-activators and co-repressors of the promotors and enhancers of the target genes.

Cells have to adapt to environmental changes. Owing to this, they develop the capability to transmit signals or other indications from the outside to the nucleus. At the appropriate times, the signalling proteins are altered. This is called signal transduction. The alterations can be effected through various modifications that result in changes in activities that transmit signals. Some of the changes take place outside the cell and are detected by receptors found at the surface of the cell. If these receptors are activated, signalling is produced in a cascading manner. The signals are relayed from one protein to the next. In the course of this process, signal transduction pathways are developed that serve in various ways to control the activity of the transcription factors. As a consequence, gene transcription is aptly regulated. Changes in conformation, subcellular localization and catalytic activities transform into signals between proteins. Such changes can be driven by direct interactions of proteins or indirectly via certain protein modifications such as phosphorylation, glycosylation or ubiquitination or via the binding of ligands such as Ca^{2+}. The latter is an effective mediator of signals within cells. It will be further taken up in the section Calcium signalling and its regulation.

**Protein modifications by phosphorylation and dephosphorylation**

A very well-known mechanism to regulate protein function is by phosphorylation. It has also been very widely investigated. Protein kinases that add phosphorylations and protein phosphatases that dephosphorylate them are both involved in this mechanism. Phosphorylation of proteins and its reversal is an essential regulatory mechanism that is found in prokaryotic as well as eukaryotic organisms (Barford et al., 1998; Chang and Stewart, 1998; Cozzone, 1988; Olsen et al., 2006). The action of phosphorylation pertains to the increment of phosphate groups to those of hydroxyl present in serine, threonine and tyrosine residues in eukaryotic proteins. However, in prokaryotic proteins, phosphorylation happens on the amino acid residues histidine, arginine and lysine in addition to serine, threonine and tyrosine residues (Barford et al., 1998; Cozzone, 1988). Conformational changes in the
protein can be an outcome of the phosphorylation. Proteins can thus be activated or deactivated in this process. One classic illustration of how phosphorylation can regulate protein function is the phosphorylation of the p53 tumor suppressor protein. P53 is a protein that has over eighteen different phosphorylation sites where its activity undergoes tight regulation (Ashcroft et al., 1999). If p53 is activated by phosphorylation, it can cause cell cycle arrest, which can be reversed. It can also result in apoptotic cell death (Bates and Vousden, 1996). Such dramatic effects occur by signalling at conditions when there is cell damage or when there is a disturbance of cell physiology. Another manifestation of such modifications is the phosphorylation of the inhibitory kappa B proteins (IκB) by IκB kinase (IKK), which can lead to the degradation of IκB proteins (Häcker and Karin, 2006). Thereby the NF-κB transcription factor becomes free to activate its targeted genes.

**Protein modification by ubiquitination**

Modification by ubiquitin is implicated in the degradation of proteins by the proteasome, and the modifications by ubiquitin have more recently gained an even broader significance. This process has been found to manage and control cell signalling also via proteasomal-independent mechanisms. As a response mechanism to diverse stimuli, ubiquitination effectively regulates various classes of physiological and pathological processes, for example in the NF-κB pathway (Adhikari and Chen, 2009; Chen and Sun, 2009; Finley et al., 1987; Hurley et al., 2006; Johnson, 2002; Kirisako et al., 2006; Liu and Chen, 2011; Makarova and Koonin, 2010; Pickart, 2001). Ubiquitin is very conserved and ubiquitously expressed in eukaryotes. It is a small protein that contains 76 amino acids. Ubiquitin is encoded by four separate genes either as a complete linear polyubiquitin or as a fusion protein between a ubiquitin and a ribosomal subunit (Finley et al., 1987; Liu and Chen, 2011). Ubiquitin can be connected to other proteins by way of its C-terminal tail. The attachment mechanism has been determined as a stepwise enzymatic reaction using three types of enzymes, E1, E2, and E3, as illustrated in Figure 1. First the C-terminus of the ubiquitin is coupled to an E1 enzyme by an ATP dependent process. Then the ubiquitin is transferred from an E1 to an E2 enzyme. Finally, with the help of an E3 enzyme, the ubiquitin is coupled to a target protein through an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin (Liu and Chen, 2011; Pickart, 2001). Humans have two E1s, approximately 50 E2s, and 700 or more E3s (Liu and Chen, 2011). This illustrates how complex modification by ubiquitin and its regulation are. Proteins having a ubiquitin binding domain (UBD) can recognize ubiquitinated substrates. This can result in distinct functional outcomes as responses to varying signals. One type of ubiquitination makes
the ubiquitinated protein recognized by the proteasome. The latter in turn leads to the degradation of the protein. There are seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) in ubiquitin, and each of these can be used to attach to another ubiquitin to form a chain of polyubiquitin (Kirisako et al., 2006; Liu and Chen, 2011).

Figure 1. Schematic view of the three-step ubiquitination cascade.  
(Based on (Liu and Chen, 2011))

Several specific functions in the cell are served by various polyubiquitins that are linked at different lysines (Adhikari and Chen, 2009). For instance, K48- and K11-linked polyubiquitin interlinks normally aim at directing proteins for proteasomal degradation. K63-linked polyubiquitin chains and monoubiquitination on the other hand manage and control various cellular functions like protein kinase activation, repair of DNA, trafficking of
membrane proteins and remodeling of chromatin. These processes are controlled mostly by proteasomal-independent mechanisms (Chen and Sun, 2009; Johnson, 2002; Liu and Chen, 2011). Ubiquitination is like phosphorylation a process that can be reversed. Deubiquitination is attained by a large group of proteins that are known as deubiquitination enzymes (DUBs) (Hurley et al., 2006).

**The Immune System**

There are two major subdivisions of the immune system. The first is the innate immune system and the other one is the adaptive immune system. These two immune systems have developed over time for the purpose of giving protection from infections caused by microbes and other health hazards in the surroundings. The distinctions between the two systems lie on the receptors they utilize to identify pathogens (Chaplin, 2006; Janeway, 2005; Medzhitov, 2007).

The front-liner in defending and protecting the body from potential threats from the environment is the innate immune system. It effectively manifests reactions to a wide array of microorganisms. It thus delivers a quick response although it is not specific. On the other hand, the adaptive immune system responds specifically to the aggressor but this development happens during several days of infection. This feature is related to the requisites of affinity maturation and clonal expansion of the responding cells. Aside from acting to clear the infection, the adaptive immune system may likewise provide lifetime protective immunity to a repeat infection by the same pathogen.

**Innate immunity**

As already mentioned, the primary shield of the human body against pathogens is the innate immune system. Comprising anatomical barriers such as epithelial and mucosal surfaces, it is in the forefront to defend us from infections. It consists also of physiological barriers like areas with a hostile pH. On the whole, the innate immune system complements the other defense features of the body (Chaplin, 2006; Janeway, 2005; Medzhitov, 2007). Phagocytic cells such as monocytes, macrophages and neutrophils are the initial cells in the immune system that identify and effectively act to pathogenic attacks using their pattern recognition receptors (PRRs). This involves Toll like receptors (TLRs) that have a major part in this process (Akira et al., 2006). In the engagement with pathogens, phagocytes in addition to being antimicrobial also secrete out cytokines and chemokines that function during the inflammatory process and gather other cells of the immune system.
Adaptive immunity
The adaptive immune system consists of the T and B lymphocytes that become active as they recognize foreign antigens via antigen receptors on their surfaces (Chaplin, 2006; Janeway, 2005; Medzhitov, 2007). With the aid of somatically rearranged genes, the individual lymphocyte develops antigen receptors with unique specificity. Once a foreign antigen is bound to the antigen receptors of a lymphocyte, the activation, clonal expansion and differentiation of the lymphocyte develops. This results in a large repertoire of adaptive immune responses (Chaplin, 2006; Janeway, 2005; Medzhitov, 2007). These responses are subdivided into two different groups depending on the type of activated lymphocyte. When T lymphocytes (T cells) are presented with an antigen through the antigen-presenting cells (APCs), they elicit immune responses called cell-mediated immunity. On the other hand, when antigen receptors of B lymphocytes recognize the antigen, they produce antibodies and the process is called humoral immunity (Janeway, 2005).

There are two major subsets of T lymphocytes. They differ in the expression of either CD4 or CD8 co-receptors on their surface and they differ in their functions. The T helper cells are CD4+ and are pertinent coordinators of the immune regulation. The T helper cell has the major responsibility of enabling or augmenting the immune responses. This it does by secreting specialized factors by which other white blood cells are activated to battle off infections. Another kind of T cell that plays an important role in the immune process is called the T killer/suppressor subset or CD8+ T cell. These cells suppress specific types of tumor cells and virus-infected cells and certain parasites. The body has an abundant supply of both types of T cells that are usually present in secondary lymphoid organs, including the lymph nodes, tonsils, and spleen. These organs are the venues where the cells are activated. These cells are also found in other tissues and organs such as the liver, lungs, blood and the intestinal and reproductive tracts (Chaplin, 2006; Janeway, 2005; Medzhitov, 2007).

The key role of B lymphocytes is to produce the antibodies that respond to and combat foreign antigens. Antibodies are proteins that have special functions in the defence system of the body. They specifically recognize and bind to a particular target, often a protein, in the immune process. The produced antibody also functions by alarming other immune cells to engulf, kill, demolish or discard such harmful substances from the body (Janeway, 2005). To prevent a similar health hazard in the future, certain activated B lymphocytes differentiate into memory B cells. These help as a protective immune mechanism to avoid repeated infection.
There are also other types of lymphocytes known as natural killer cells or NK cells that have similarities to the T killer/suppressor subset or CD8+ T cell. They are effective in directly killing special types of tumors like melanomas, lymphomas and virus-infected cells such as herpes virus or cytomegalovirus-infected cells. NK cells, unlike the CD8+ T cells, can destroy their target cells without any previous coordination in the lymphoid organs. Nevertheless, NK cells that are activated by CD4+ T cells are more effective in killing their tumor or virus-infected targets (Janeway, 2005).

**Antigen receptor activation and the functional outcomes**

Antigen receptors, once activated by foreign antigens and not by self antigens, produce effective immune responses and avoid auto-immune responses. This is accomplished by lymphocyte coordination and cooperation with co-receptor activation as well as with the presence of growth factors and cytokines in the surroundings (González et al., 2007; Jun and Goodnow, 2003). The fate and survival of the lymphocyte is determined by the activation of various transcription factors like NFAT, AP-1 and the NF-κB family that are activated by the signalling cascades from the antigen receptors. Acting in coordination with each other, transcription factors determine the immune responses. These responses however depend on the signals received from the antigen receptor (T cell receptor (TCR) or B cell receptor (BCR)). After the receptor is activated, the lymphocyte has three main options to select from as illustrated in **Figure 2.** Efficient binding of antigen to antigen receptors along with co-receptor stimulation results in the formation of an immunological synapse and subsequent activation, proliferation and differentiation of the lymphocyte. In case the co-receptor activation or the binding strength of the antigen is not good enough, then the lymphocyte ends up in a state of unresponsiveness called T cell anergy, or it can induce apoptosis (suicide). Antigen receptor activation results in an increase in the intra-cellular calcium level (described in detail later in this thesis), which plays an important role when the lymphocytes choose which road to go. Activation of the NF-κB family of transcription factors after the receptor activation is crucial for proper adaptive immune responses. Despite enormous efforts, the mechanisms regarding how antigen receptor-induced activation of NF-κB is governed have not yet been clearly determined (Baltimore, 2011; Bonizzi and Karin, 2004; Gerondakis et al., 1999; Noelle and Erickson, 2005; Sun and Liu, 2011).
Figure 2. Functional outcomes of lymphocyte activation.
**NF-κB Transcription Factors and their Features**

NF-κB transcription factor was first discovered in 1986 by Ranjan Sen and David Baltimore. NF-κB was initially found to be a protein that binds to the immunoglobulin kappa-light-chain enhancer in the nucleus of the B cells, hence the name nuclear factor kappa-light-chain-enhancer of activated B cells, referred to as "NF-κB" (Sen and Baltimore, 1986). Later it was however found in almost every cell. It participates in the regulation of the physiological processes in response to stimuli such as cytokines, free radicals, stress, UV, and components of bacteria and viruses (Gilmore, 2006). As a matter of fact, NF-κB is reputed to be the master regulator of the immune system. Many studies have shown that cancer, inflammatory and autoimmune diseases, septic shock, several infections by viruses and improper development of the immune system are all linked to deregulated NF-κB (Li and Verma, 2002). It has also roles in synaptic plasticity and memory processes (Albensi and Mattson, 2000).

![Diagram of NF-κB and IκB families](image)

**Figure 3. The mammalian NF-κB and IκB families.**
Every NF-κB family protein has a DNA binding Rel homology domain in its N-terminus. There are five different members in the mammalian NF-κB family. These include the RelA, RelB, and c-Rel proteins that all possess a trans-activation domain in their C-terminus. The NF-κB1 and NF-κB2 proteins are large precursors, p105, and p100, respectively. They undergo proteolytic cleavage and result in generation of the mature NF-κB subunits p50 and p52, respectively, as shown in Figure 3 (Gilmore, 2006; Karin and Ben-Neriah, 2000; Senftleben et al., 2001). On their own, the p50 and p52 NF-κB subunits cannot activate transcription because they both lack a trans-activation domain. They are only functional as transcriptional activators when they form dimers with other subunits (Li and Verma, 2002).

The inhibitory IκB proteins (IκBs) are a family of proteins that are built of inhibitory ankyrin repeats (Jacobs and Harrison, 1998). The IκBs cover the nuclear localization signal (NLS) of NF-κB proteins and maintain them sequestered and inactive in the cytoplasm (Jacobs and Harrison, 1998). Aside from their inhibitory properties due to their six or more ankyrin repeats, the IκBs contain an N-terminal regulatory domain and also a PEST domain. The IκBα, IκBβ, IκBγ, IκBε, and Bel-3 are the members of the IκB family. The most well studied of them is the IκBα. p105 and p100 likewise act and serve as IκB proteins by the ankyrin repeats found in their C-terminus (Basak et al., 2007; Dobrzanski et al., 1995). This half of p100 acts as an inhibitor, known as IκBδ. IκBδ is subjected to degradation in non-canonical activation pathways in response to developmental stimuli including signals induced by LTβR (Basak et al., 2007; Lo et al., 2006).

NF-κB is also present in other species including for example sea anemones, corals, sponges, and insects (Ghosh et al., 1998). The sequencing of the genomes of these animals has given opportunities for comparative genetic and evolutionary researches on NF-κB (Waterhouse et al., 2007). In the case of insects, the activation of NF-κB is driven by the Toll pathway and by the Imd (immune deficiency) pathway (Tian and Brasier, 2003).

**Ways of NF-κB activation and the regulated genes**

NF-κB belongs to the group of rapid-responding transcription factors, and it has important roles in the regulation of a broad range of cellular responses. Rapid-acting primary transcription factors can be found present in an inactive state in cells and new protein synthesis is therefore not required to get the activate protein. Among other families of transcription factors, this kind of
regulation is found for example for Jun/Fos, STATs, and nuclear hormone receptors (Kumar and Thompson, 1999; Udou et al., 2004; Vinkemeier et al., 1998). NF-κB quickly responds to dreadful and hazardous cellular stimuli (Höffmann and Baltimore, 2006; Takemoto et al., 1999). For additional sources of information on molecules, conditions, inducers, genes, and proteins related to NF-κB regulation, the website www.nf-kb.org under management of Dr. Tom Gilmore is of tremendous assistance. NF-κB is, as illustrated in Figure 4, activated by various substances and physiological inducers (Hayden et al., 2006; Höffmann and Baltimore, 2006) including:

a) Fungal, viral or bacterial products usually recognized by Toll like receptors (TLRs).

b) Intercellular mediators that act as signalling molecules and are identified with the TNF receptor super-family.

c) Immunoglobulin receptors containing domains that are responsible for regulation of adaptive immune responses via the recognition of free antigen or antigen on antigen-presenting cells or through intercellular signalling.

d) Genotoxic or metabolic stress signalling molecules usually signalling upon environmental risks or potential dangers.

Degradation of IκB proteins is signal-induced by a kinase called the IκB kinase (IKK). The inducible IKK contains a heterodimer of the catalytic IKKα and IKKβ subunits together with a "master" regulatory protein called IKKγ, also known as NF-κB essential modulator (NEMO). Upon activation caused by a variety of signals (Figure 4), IKK phosphorylates two serine residues in the regulatory domain of IκB (Ser 32 and Ser 36 in the case of human IκBα) (Häcker and Karin, 2006). After the phosphorylation, IκBs are modified by ubiquitination, which finally leads to the degradation of the IκB by the proteasome (Nelson et al., 2004). After the IκB is degraded, the NF-κB dimers enter the nucleus and regulate the expression of the target genes. Depending on which genes are activated by a particular NF-κB stimulation of a certain cell type, that particular kind of physiological response is induced. For instance, it may be an inflammatory or immune response or it may be a cell survival response or cellular proliferation. NF-κB also regulates the expression of its own repressor IκBα (Höffmann and Baltimore, 2006). Thereby, the newly synthesized IκBα re-inhibits NF-κB. Therefore it establishes a feedback loop that creates oscillating levels of NF-κB activity. This is illustrated in the bottom of Figure 4. Several viruses including the AIDS virus, HIV, have also binding sites for NF-κB. The
NF-κB controls the expression of viral genes that help in viral replication or viral pathogenicity (Hiscott et al., 2001). For HIV-1, activation of NF-κB may be involved in activating the virus from a latent, inactive state. Infectious agents have to cope with NF-κB activation or block it, since NF-κB is a key regulator of immune responses. For example, the bacterium Yersinia pestis, which is the causative agent of plague, secretes the protein YopP that prevents the ubiquitination of IκB. By this process, the pathogen effectively inhibits the NF-κB pathway and therefore restrains the immune response of a body that is infected with Yersinia (Adkins et al., 2008).

Figure 4. Schematic view of NF-κB regulation
(Modified from (Hoffmann and Baltimore, 2006))
**T-Cell Receptor signalling to NF-κB**

NF-κB is a vital transcription factor family that has essential roles in the responses to T-cell receptor (TCR) activation. TCR-induced NF-κB is indispensible for the proliferation, differentiation and survival of T cells (Schulze-Luehrmann and Ghosh, 2006; Shambharkar et al., 2007). The initiation of TCR signalling is carried out by the T cell interaction with antigen together with the major histocompatibility complex (MHC) on the surface of APCs. The area of contact between the T cell and APC is referred to as an immunological synapse, which typically congregates the TCR complex and signalling molecules at lipid raft areas of the membrane of T cells (Bromley et al., 2001; Shambharkar et al., 2007; Xavier and Seed, 1999). The activation of NF-κB is facilitated by the T cell stimulation through the CD3 protein complex, and the co-activation of CD28 by its ligands further strengthens this activation. The stimulation results in the activation of Src tyrosine kinase family members, Lck and Fyn, that results in phosphorylation of conserved amino acid sequence motifs on CD3. This in turn leads to the recruitment of Zap-70 that phosphorylates the adapter molecules LAT and SLP-76γ. These events are regarded as the proximal signalling events in the schematic illustration of Figure 5. These signalling events trigger the activation of protein kinase C, PKC (Lobry and Weil, 2007; Weil and Israël, 2006). Numerous studies infer PKC0, which is the predominant isoform of PKC in T cells, to be of great significance in these cells. Over-expression of an active form of PKC0 (A148E) and several genetic studies of effects of lack of PKC0 have ascertained the specific need of PKC0 in the activation of NF-κB upon TCR stimulation (Lobry and Weil, 2007; Weil and Israël, 2006). PKC0 is the sole isoform found at the immune synapse after TCR activation (Lobry and Weil, 2007; Weil and Israël, 2006). The functionality of PKC0 is controlled by its conformational changes and membrane recruitment. The location of PKC0 to the membrane is to “lipid raft” microdomains and eventually leads to NF-κB activation (Bi et al., 2001; Lobry and Weil, 2007). Immense research is currently carried out on the molecular scaffold proteins that control NF-κB activation downstream of antigen receptor engagement (Lin and Wang, 2004; Lobry and Weil, 2007). Genetic and biochemical analyses showed that T cells from mice deficient in any of the scaffold/adaptor proteins CARMA1, Bcl10, and MALT1, collectively known as the CBM protein complex, fail to activate NF-κB following antigen receptor engagement and hence these scaffolds are essential in TCR-induced NF-κB activation (Thome, 2004).
Figure 5. Schematic view of NF-κB activation from the T cell receptor in T lymphocytes

**The CBM proteins in TCR signalling**

CARMA1 is a prominent member of the MAGUK kinases that has various characteristic domains such as CARD (caspase recruitment domain), SH3 (Src homology 3 domain), PDZ domain, a coiled-coil domain and a C-terminal guanylate kinase (GUK) domain (Blonska and Lin, 2011; Gaide et al., 2002). During TCR engagement, CARMA1 features in the immune synapse where it recruits Bcl10 via its CARD domain. The coiled-coil domain is a protein interaction domain and it is essential since substitution of Leu 298 to Gln in it leads to impaired antigen receptor-mediated NF-κB activation. Mutations in the SH3 domain block membrane recruitment of the CARMA1-dependent trimolecular CBM protein complex. The PDZ domain has also a role in NF-κB activation. The protein kinase PDK1 is pivotal in the aligning of the proximal TCR activation complex to PKCθ and the CBM complex, and
the GUK domain of CARMA1 interacts specifically with PDK1. TCR-mediated NF-κB activation is attenuated by mutations in the CARD domain (L39R mutation), the coiled-coil domain (L298Q mutation) and the SH3 domain (L808P mutation) of CARMA1 (Gaide et al., 2002). The activation of CARMA1 in T and B lymphocytes inevitably needs PKCθ and PKCβ, respectively, which phosphorylate a serine within the linker region of CARMA1 (serine 552 in the human protein; serine 564 in the mouse) that eventually leads to the activation of IKK and NF-κB activation (Thome and Weil, 2007). Ishiguro et al. have put forth that CaMKII phosphorylates human CARMA1 at serine 109 in the CARD domain (serine 116 in mice) and that this potentiates the interaction between CARMA1 and Bcl10, resulting in enhanced NF-κB activation (Ishiguro et al., 2006).

The Bcl10 protein was first characterized in MALT lymphomas. Bcl10 contains an N-terminal CARD domain and a C-terminal Ser/Thr rich domain, and it can readily interact with CARMA1 via its CARD domain as illustrated in Figure 5 (Blonska and Lin, 2011). The regulatory mechanisms involved in the molecular interactions between CARMA1 and Bcl10 after receptor triggering are yet to be known. The C-terminal Ser/Thr-rich domain of Bcl10 binds to immunoglobulin motifs of MALT1 (Blonska and Lin, 2011), and Bcl10-mediated oligomerisation of MALT1 has been shown to activate NF-κB (Lin and Wang, 2004; Thome and Tschopp, 2003). Zhou and co-workers (Zhou et al., 2004) have described Bcl10-assisted NF-κB activation by means of MALT1- and UBC13-dependent ubiquitination of IKKγ (NEMO). Bcl10 oligomers can aid in organizing signalling complexes composed of kinases as well as their substrates and thereby act as scaffolds for the IKK and Jun N-terminal kinase (JNK) pathways (Blonska and Lin, 2011; Blonska et al., 2007). In such cases, Bcl10 oligomers occur in stimulated Jurkat T cells by aligning with JNK2 and its upstream kinases, MKK7 and TAK1 (Blonska and Lin, 2011; Blonska et al., 2007). This mode of oligomerised Bcl10 can control the TCR-induced actin polymerization. During TCR stimulation, Serine 138 of Bcl10 has been shown to be a phosphorylation site for IKK and directly or indirectly also for CaMKII. This phosphorylation does not trigger NF-κB activation but it appears to serve as a regulatory check leading to Bcl10 degradation (Ishiguro et al., 2007; Thome and Weil, 2007).

DNA cloning from MALT lymphoma patient samples led to the recognition of MALT1 (Akagi et al., 1999; Blonska and Lin, 2011; Uren et al., 2000), which is as important as Bcl10. The Malt1 gene is at a point of chromosome breakage in MALT lymphomas localized at 18q21. While t(11;18)(q21;q21) generates an API2-MALT1 fusion protein, t(14;18)(q32;q21) puts the Malt1 gene adjacent to the immunoglobulin locus,
thereby up-regulating the expression (Akagi et al., 1999; Blonska and Lin, 2011; Du, 2007; Ye et al., 2005). The protease activity of MALT1 was obscure in the beginning and the protein was presumed to only be an E3 ligase for the regulatory subunit of the IKK complex (Lucas et al., 2001; Uren et al., 2000; Zhou et al., 2004). Later however, MALT1 was found to also regulate caspase-8 (CASP8) activation through the Paracaspase domain in a protease-independent manner and this subsequently results in the activation of NF-κB and production of IL-2 (Kawadler et al., 2008). The MALT1-dependent CASP8 activation is involved in positive regulation of TCR-induced NF-κB activity (Bidère et al., 2006; Su et al., 2005).

IKK is activated by the CBM complex by means of K63-linked polyubiquitinations as illustrated in Figure 5. MALT1 possesses binding sites for two TRAF proteins, TRAF2 and TRAF6, out of which TRAF6 gets oligomerised by BCL10 and MALT1, which activates the E3 ligase activity of TRAF6 (Liu and Chen, 2011; Sun et al., 2004). TRAF6 is regarded as indispensable for NF-κB activation by the IL-1 and Toll-like receptors (IL-1R/TLR) and the TCR/BCR pathways (Cao et al., 1996; Deng et al., 2000; Ishida et al., 1996; Liu and Chen, 2011). Binding of MALT1 to TRAF6 followed by catalytic K63-linked polyubiquitinations results in activation of a kinase complex that consists of TGF-β activated kinase 1 (TAK1, also known as mitogen-activated protein kinase kinase kinase 7) along with the adaptor proteins TAB2 and TAB3 (Liu and Chen, 2011; Wang et al., 2001; Yamaguchi et al., 1995). A ZnF type UBD of TAB2 and TAB3 binds to the K63-linked polyubiquitin chains, causing auto-phosphorylation and activation of TAK1 (Kanayama et al., 2004). TAK1 phosphorylates both IKKβ and MAP kinase kinases (MKKs). While the former activate NF-κB, the latter activate JNK and p38 kinase cascades. A recent study categorizes the phosphorylation and the ubiquitination of IKK as distinct processes. While the latter is based on CARMA1 and mediated by the downstream BCL10–MALT1–TRAF6 complex, PKC regulates TAK1 through a CARMA1-independent pathway (Shambharkar et al., 2007).

K63-linked polyubiquitination of NEMO is catalyzed by the E3 ligase MALT1 and Ubc13/Uev1A (Zhou et al., 2004). Lysine 399 of IKKγ has been identified as the ubiquitination site. However, “knockin” experiments did not show any significant evidence of a key importance of this site, suggesting that this site may not be essential for NF-κB activation from the TCR (Ni et al., 2008). BCL10 and MALT1 may also be polyubiquitinated by TRAF6 (Oechkinghaus et al., 2007; Wu and Ashwell, 2008). Nevertheless, NF-κB activation by TCR is not interrupted by T-cell-specific deletion of TRAF6 in mice because of the redundancy of TRAF proteins in T lymphocytes.
However RNAi of both the TRAF2 and TRAF6 genes together ceases the NF-κB activation by TCR stimulation (King et al., 2006; Sun et al., 2004). Extensive research is going on to find out the mechanisms of activation of TAK1 and IKK by TRAF6-mediated polyubiquitinations (Liu and Chen, 2011).

**Down-regulation of NF-κB signalling**

After NF-κB activation, the NF-κB activation is stopped by several mechanisms of negative feedback. One mechanism of negative regulation of NF-κB is by re-synthesis of IκB as illustrated in Figure 4. The expression of IκB is regulated by NF-κB, and activation of NF-κB causes increased expression of the inhibitor. IκBα has both nuclear localisation signal (NLS) and nuclear export signal (NES) sequences. Newly synthesised IκBα enters the nucleus, binds to NF-κB, and exports it to the cytoplasm. At several levels, the DUBs CYLD and A20 negatively regulate signalling to NF-κB. CYLD interacts with NEMO and TRAFs, selectively degrading the K63-linked ubiquitin chains of NEMO, TRAF-2 and TRAF-6 (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). The expression of A20 is regulated by NF-κB and A20 subjects TRAF6 and RIP1 to signal-dependent negative regulation (Boone et al., 2004; Wertz et al., 2004). Another way the signalling to NF-κB is negatively regulated is through induced degradation of signalling intermediates. For example, Bcl10 is subjected to negative regulation through phosphorylations by IKK that induce ubiquitin-mediated proteasomal degradation of Bcl10 and changes in the properties of the CBM complex after TCR stimulation (Scharschmidt et al., 2004; Wegener et al., 2006). IKKα is presumed to contribute to facilitating nuclear degradation of RelA, thus suggesting that signal-dependent alterations in the stability of NF-κB proteins also regulate termination of the NF-κB response (Lawrence et al., 2005). Due to the critical role of NF-κB as a regulator of immune responses, inflammation and cell proliferation, it is important to control the activity of NF-κB to prevent autoimmune reactions, chronic inflammation and tumour growth. Thus, the mechanisms of negative regulation of NF-κB may be diverse and complex.

**NF-κB and diseases**

Despite safety mechanisms being put in place to secure a balanced NF-κB response, there are situations when such systems fail and consequently cause severe diseases. Irregular response of NF-κB has been linked with many...
diseases, including allergies, asthma, inflammatory bowel disease and rheumatoid arthritis (Barnes, 2006; Simmonds and Foxwell, 2008; Tak and Firestein, 2001). Furthermore, it has been noted that such conditions as ischemia and atherosclerosis, which are pathological states of the cardiovascular system, have boosted inflammatory activities through increased activity of NF-κB (Collins and Cybulsky, 2001; Hall et al., 2006). Parkinson’s and Huntington’s diseases have also been associated with NF-κB responses (Mattson and Camandola, 2001; Mémet, 2006). Some anti-inflammatory drugs have been found to partially slow down NF-κB responses. These drugs include glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin and sodium salicylate) (Baldwin, 2001; Courtois and Gilmore, 2006; Karin et al., 2002; Naugler and Karin, 2008). NF-κB has the capacity to trigger growth of tumours through multiple mechanisms including cell proliferation and inhibition of apoptosis. Tumorigenesis is also boosted by NF-κB through the production of factors that advance metastasis and angiogenesis; such factors have been noted to include cytokines, chemokines and adhesion factors (Escárcega et al., 2007). Mutations in signalling to NF-κB in several human diseases have been identified and are correlated to the disease (Courtois and Gilmore, 2006).
**Calcium Signalling and its Regulation**

The ability of Calcium ions (Ca\(^{2+}\)) to have varying intracellular concentration levels that differ from the extracellular concentration creates a great potential for the Ca\(^{2+}\) ion to act as a messenger. Cellular stimulations alter the intracellular concentration of the Ca\(^{2+}\) and this can be sensed by proteins that change properties when binding Ca\(^{2+}\). Ca\(^{2+}\) has concentrations that differ with up to four orders of magnitude between the intracellular and the extracellular milieu: inside the eukaryotic cell the Ca\(^{2+}\) concentration is approximately 100 nM whereas in the extracellular milieu the concentration is at levels of 1 to 2 mM. Ca\(^{2+}\) has been found to be extremely versatile and broadly used as an intracellular messenger. It has been shown to appropriately regulate for example muscle contraction (Bers, 2008; Csernoch, 2007; Floyd and Wray, 2007), neural transmission (Augustine et al., 2003), cell proliferation and cell death (Roderick and Cook, 2008), fertilization and development (Jones, 2007), and transcription (Ikura et al., 2002; Savignac et al., 2007).

The process of Ca\(^{2+}\) signalling starts when the level of free Ca\(^{2+}\) increases in the cells. Ca\(^{2+}\) can access the cells through the specific Ca\(^{2+}\) channels in the plasma membrane (Parekh and Putney, 2005). The process can also be initiated when the Ca\(^{2+}\) is released within the cell from intracellular stores. The Ca\(^{2+}\) signalling process has elaborate mechanisms. There are specific channels that allow passage of Ca\(^{2+}\) into the cells. Excitable cells, for instance muscle and neuron cells, have voltage-operated channels (VOCs) (Parekh and Putney, 2005). Neurons generate rapid Ca\(^{2+}\) fluxes that control synaptic endings and generate rapid Ca\(^{2+}\) fluxes that in this case can control muscle contraction. In the case of non-excitable cells, the process of Ca\(^{2+}\) signalling is usually initiated by activation of tyrosine kinase receptors or G protein-coupled receptors (Parekh and Putney, 2005). These receptors are found at the plasma membrane and when activated can stimulate the release of Ca\(^{2+}\) from intracellular stores of the cells. An example of intracellular Ca\(^{2+}\) store is the endoplasmic reticulum (ER) (Parekh and Putney, 2005). When the ER is depleted of the stored Ca\(^{2+}\), a process is initiated to regain the Ca\(^{2+}\) balance. This process specifically allows the entry of Ca\(^{2+}\) through the store operated Ca\(^{2+}\) channels (SOCs) of the plasma membrane (Parekh and Putney, 2005). The calcium release-activated calcium channel (CRAC) is a very significant SOC as it is the main source of Ca\(^{2+}\) that is used in the message transmission in lymphocytes.

The Ca\(^{2+}\) signalling process leads to big changes in the concentration of free cytoplasmic Ca\(^{2+}\) in the cells. The concentration can change from approximately 100 nM to approximately 1μM. The concentration change can
take place in a time duration of between milliseconds to many seconds or in some cases minutes (Berridge et al., 2003; Clapham, 2007). The variations in Ca\(^{2+}\) concentration are very significant as they for example make it possible for the rapid Ca\(^{2+}\) pulses to be effective in the process of muscle contraction of muscle cells as well as in the process of synaptic transmission in the case of neuronal cells. The variations in Ca\(^{2+}\) concentration are also effective in regulation of transcriptional responses where it often is prolonged Ca\(^{2+}\) signals that are effective.

**Ca\(^{2+}\) homeostasis**

The concentration of Ca\(^{2+}\) in the cytoplasm is maintained at a low level by uptake and buffering systems as well as by removal of excess Ca\(^{2+}\). Ca\(^{2+}\) is extruded across the plasma membrane via plasma membrane Ca\(^{2+}\)-ATPases (PMCas) or Na\(^+\)/Ca\(^{2+}\) and Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchangers (NCXs and NCKXs), and Ca\(^{2+}\) is taken up into intracellular stores, by transport into the endoplasmic, or sarcoplasmic, reticulum (ER/SR) via sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs) or by transport into mitochondria via the Ca\(^{2+}\) uniporter (Rizzuto and Pozzan, 2006). The Golgi, endosomes, and lysosomes are also considered to be potential Ca\(^{2+}\) storage sites (Rizzuto and Pozzan, 2006). Ca\(^{2+}\) also binds to Ca\(^{2+}\) buffering and Ca\(^{2+}\) sensory proteins. Prolonged strong Ca\(^{2+}\) stimulation as well as high concentration of Ca\(^{2+}\) in the cell for any other reason are toxic. To counter the toxic potential of Ca\(^{2+}\) after it has executed its duty, the Ca\(^{2+}\) ions are not allowed to stay free for long. The ions are immediately extruded, taken and bound. These processes are quick in removing free Ca\(^{2+}\) that otherwise would block the ability to re-stimulation and poison the cell after passing the signal.

**Specificity of Ca\(^{2+}\) signalling**

The issue of specificity with respect to the functioning of Ca\(^{2+}\) has been quite challenging. Since Ca\(^{2+}\) ions control numerous cellular processes, it is evident that there must be ways through which specificity of the signalling is achieved. The response of a cell to the increase in Ca\(^{2+}\) has been tied down to the signal mechanisms used. Specificity is to a large extent achieved by determining the shape of the Ca\(^{2+}\) signal used: “the cellular pattern of Ca\(^{2+}\) channels, pumps, buffers and sensors with different affinities for Ca\(^{2+}\) and with different abilities to buffer or transport Ca\(^{2+}\) determines the shape of the Ca\(^{2+}\) signal” (Graier et al., 2007). The mitochondria also play a role in the signalling process of Ca\(^{2+}\) and therefore can be used as well in the determination of the shape of the Ca\(^{2+}\) signal. Mitochondria play a vital role in lengthening the duration of Ca\(^{2+}\) signals by first holding back Ca\(^{2+}\) from the cytoplasm but later releasing the Ca\(^{2+}\) to the cytoplasm again (Graier et al.,
Different cell types do not have the same sets of Ca$^{2+}$ signalling proteins. The Ca$^{2+}$ signalling proteins can differ in reference to duration, frequency and amplitude and their location can be in different sub-cellular compartments or parts of these in the cells (Berridge, 2006). The responses to Ca$^{2+}$ signals will therefore be specific to the cell type, as expected since the set of distinct Ca$^{2+}$ signalling proteins is specific to the type of cell. The specificity of the signals makes it possible for Ca$^{2+}$ ions to control various processes in highly specific manners. Based on the unique cellular patterns, the Ca$^{2+}$ signals have been classified into different types. Sparklets are signals formed when the VOCs opens briefly during cardiac cell excitation and exocytosis at the synaptic endings (Berridge, 2006). Puffs are signals formed when intracellular stores are depleted of Ca$^{2+}$. Puffs are the building blocks of Ca$^{2+}$ waves in the cells (Berridge, 2006). Ca$^{2+}$ waves are common in prolonged stimulations. It has been noted that the transients in prolonged stimulations are “repeated into regular Ca$^{2+}$ oscillations that have been shown to be important for the regulation of many different Ca$^{2+}$ targets, including transcription factors of the NF-AT, AP1 and NF-κB families” (Quintana et al., 2005).

**Ca$^{2+}$ signalling in lymphocytes**

The process of Ca$^{2+}$ signalling in lymphocytes has been reviewed recently (Feske, 2007; Luik and Lewis, 2007; Oh-hora and Rao, 2008; Scharenberg et al., 2007). Immuno-receptors, for instance antigen receptors, co-stimulatory receptors, and chemokine receptors, have all been identified to play roles in the process of Ca$^{2+}$ signalling in lymphocytes. The entry of Ca$^{2+}$ through the CRAC channels in the plasma membrane of the lymphocytes supplies a significant source of Ca$^{2+}$ signalling in the cells. The Ca$^{2+}$ signals act in harmony with the other signalling events of lymphocyte receptors to bring about the regulation of lymphocyte activation, proliferation and differentiation. The Ca$^{2+}$ signals therefore are significant players in the adaptive immune responses of lymphocytes. **Figure 6** below illustrates the Ca$^{2+}$ signalling of lymphocytes in summary.
When the antigen receptors are engaged, they lead to the activation of phospholipase C-γ (PLC-γ) and the production of diacyl glycerol (DAG) and IP₃ by cleavage of PIP₂. DAG in turn binds to and activates PKC, and IP₃ will bind to IP₃ receptors, which are Ca²⁺ channels located in the membrane of the endoplasmic reticulum (ER) intracellular Ca²⁺ store, leading to the liberation of Ca²⁺ from the intracellular store (ER) to the cytoplasm. Consequently, the liberation of Ca²⁺ in turn causes transient Ca²⁺ puffs. ER occupies about 100 fold smaller volume than the cytoplasm (Hogan and Rao, 2007) so the Ca²⁺ signal produced has to be reinforced by other Ca²⁺ signals events. The liberation of Ca²⁺ from the ER depletes the ER of the Ca²⁺ and this stimulates the inflow of Ca²⁺ through the CRAC channels of the plasma membrane (Hogan and Rao, 2007). This inflow of Ca²⁺ enables sustained Ca²⁺ signals necessary for changes in gene expression and proliferation. The fact that the inflow of Ca²⁺ through the CRAC channels sustains the Ca²⁺ signals makes it...
a very significant mechanism for Ca\(^{2+}\) signal generation in lymphocytes. The ER depletion and Ca\(^{2+}\) influx based mechanism remained a mystery until recently when STIM1 was singled out as a sensor of Ca\(^{2+}\) in the depletion process (Hogan and Rao, 2007). STIM1 is a protein of the ER membrane. The STIM1 has an EF-hand Ca\(^{2+}\) binding site that faces the lumen of the ER, and in that position it senses the concentration of Ca\(^{2+}\) in the lumen. Low concentration of Ca\(^{2+}\) leads to the oligomerisation and relocation of STIM1 to specific regions of junctional ER. Regions of junctional ER are structures of ER that are situated within a range of 10-25 nm of the plasma membrane (Hogan and Rao, 2007). The ORAI1 protein has also been singled out to play some role in activating the formation of the pores of the CRAC channels. It has been assumed that STIM1 and ORAI1 interact to trigger the influx of Ca\(^{2+}\) through the CRAC channels. This assumption is backed up by the fact that STIM1 and ORAI1 have been found at the junctional ER in close contact (Hogan and Rao, 2007).

There are suggestions that transient receptor potential channels (TRPs) (Islam, 2011) and ryanodine receptors (RyRs) also play a role in the Ca\(^{2+}\) modulation mechanism of lymphocytes though their function is yet to be clearly understood with the exception that they appear to have different roles in B and T lymphocytes. The modulation of Ca\(^{2+}\) currents through the CRAC channels is by the general Ca\(^{2+}\) homeostasis mechanisms discussed above.

Most of the processes in lymphocytes that depend on Ca\(^{2+}\) have been shown to require Ca\(^{2+}\) signals with prolonged duration as well as gene transcription. A few processes are however regulated by short duration Ca\(^{2+}\) signals, including regulation of motility. Examples of processes that are regulated by the long duration Ca\(^{2+}\) signals are proliferation and differentiation. The influx of Ca\(^{2+}\) through the CRAC channels causes prolonged Ca\(^{2+}\) signals. These prolonged signals have been deemed to be very significant in the context of lymphocytes. The signals have been tied down to transcription activation processes that play significant roles in cell proliferation, differentiation and cytokine production of lymphocytes. Some of the genes that are regulated by Ca\(^{2+}\) are cytokine genes, including granulocyte macrophage colony stimulating factor (GM-CSF), TNF-\(\alpha\) and IFN-\(\gamma\). These play important roles in signalling in the immune system, illustrating that Ca\(^{2+}\) signals play important roles in the immune system. Much regulation of the transcription process is accomplished by Ca\(^{2+}\) signals. The signals can act on the transcriptional repressor DREAM or on transcription factors of the NFAT, MEF-2, E-protein, Jun/Fos (AP-1) and NF-\(\kappa\)B families (Oh-hora and Rao, 2009; Quintana et al., 2005; Saarikettu et al., 2004; Youn et al., 2000). NF-AT, AP-1 and NF-\(\kappa\)B are some of the most
significant transcription factor families under regulation by Ca\(^{2+}\) signalling (Quintana et al., 2005). AP-1 and NF-κB are activated by transient high amplitude Ca\(^{2+}\) signals whereas NFAT is activated by more prolonged Ca\(^{2+}\) signals of lower amplitudes (Dolmetsch et al., 1997; Dolmetsch et al., 1998). Amplitude and frequency of oscillations have been found to modulate the sensitivity and response of transcription factors to Ca\(^{2+}\) signals. Ca\(^{2+}\) signals can thereby be decoded to allow differential activation of transcription factors and this avails great opportunities for the modulation of immune responses.

**Ca\(^{2+}\)-binding proteins**

Ca\(^{2+}\) binding proteins are classified in accordance to their main function. Thus, they are either Ca\(^{2+}\) buffers or Ca\(^{2+}\) sensors. Ca\(^{2+}\) buffers include parvalbumin (Ulfig, 2002), calbindin D\(_{0K}\) and calreticulin (Michalak et al., 2002), and their main purpose is to chelate Ca\(^{2+}\). The Ca\(^{2+}\) buffers function in shaping the Ca\(^{2+}\) signals by holding back Ca\(^{3+}\) at local spots. The Ca\(^{2+}\) buffers can also transport Ca\(^{2+}\) between different locations in the cell. Ca\(^{2+}\) sensors translate transient Ca\(^{2+}\) signals into prolonged signals by capturing them. The binding of Ca\(^{2+}\) to Ca\(^{2+}\) sensory proteins has a 2-fold effect of changing the shape and consequently the function of the protein, which makes transmission of the Ca\(^{2+}\) signal possible. Ca\(^{2+}\) directly regulates some Ca\(^{2+}\) sensors with enzymatic functions such as PI3K and some PKC family members, whereas other Ca\(^{2+}\) sensors such as calmodulin, S100 proteins, troponin C and others play the role of intermediaries that bind to and change the properties of target proteins. The Ca\(^{2+}\) sensor protein calmodulin has been greatly studied (Bhattacharya et al., 2004).

**Calmodulin**

Calmodulin (CaM) is a small (17 kD) protein that is considered to be the major Ca\(^{2+}\) sensor in mammals. CaM has been found to be ubiquitously present in animals, plants, fungi and protozoa. CaM has been found to be extremely highly conserved in evolution (Schaub and Heizmann, 2008). From the analysis of higher vertebrate species it has been found that there is an exact amino acid sequence identity for this protein suggesting that all CaM residues are indispensable. CaM is known to bind to numerous different proteins and control the activities carried out by these proteins. Some of the proteins whose activities have been identified to be controlled by CaM are “ion channels and pumps, various enzymes, cell cycle regulators, cell motility and contraction proteins, and transcription factors” (Davis et al., 1986). Deletion of CaM has been demonstrated to be lethal in yeast (Davis et al., 1986), which in contrast to mammals possesses a single CaM gene, and the participation of CaM in so many vital processes strongly argues that its
deletion would be lethal in mammals also. CaM is a member of the EF-hand family of Ca\(^{2+}\)-binding proteins that is also called the Calmodulin Superfamily. CaM binds Ca\(^{2+}\) by use of its EF-hands that are helix-loop-helix domains where Ca\(^{2+}\) ions are coordinated in the loops. CaM binds up to four Ca\(^{2+}\) ions since it has two pairs of such EF-hands, and the two pairs of EF-hands are separated by a flexible linker. The EF-hands of CaM have different affinities and co-operability enabling CaM to efficiently sense Ca\(^{2+}\) signals over a wide range of concentrations. With Ca\(^{2+}\) binding constants in the range of intracellular Ca\(^{2+}\) concentrations, most CaM molecules are Ca\(^{2+}\) free at basal Ca\(^{2+}\) concentrations whereas most CaM molecules are Ca\(^{2+}\) loaded at the high Ca\(^{2+}\) concentrations during strong Ca\(^{2+}\) signalling (Carafoli, 1986).

**Target binding by Calmodulin**

Most of the interactions of CaM have been shown to be through 'wrap-around' binding. In this interaction, CaM binds to its target with its two Ca\(^{2+}\)-binding domains by contacting a small binding region of the target (Ishida and Vogel, 2006). CaM transmits the Ca\(^{2+}\) signals in two steps. When Ca\(^{2+}\) is at a resting level, CaM exists in its Ca\(^{2+}\)-free form (apo-CaM). The first step in the transmission of Ca\(^{2+}\) signals is the binding of Ca\(^{2+}\) to CaM. This binding leads to exposure of a hydrophobic surface located on each of the Ca\(^{2+}\)-binding domains, thus enabling the binding of the CaM to its targets. The second step starts when CaM interacts with the target and collapses around the binding domain of the target protein. The collapsing of the CaM leads to alteration of properties of the target protein hence completing the Ca\(^{2+}\) signal process.

The structural alterations brought about by Ca\(^{2+}\) binding are instrumental in enabling CaM to bind to and regulate many target proteins. CaM easily adapts to the differences in the surface of its target proteins because of the presence of flexible methionine residues and the connection of the highly flexible central linker (Ishida and Vogel, 2006; Yamniuk and Vogel, 2004). This effectively makes it possible for CaM to bind to a number of targets without necessarily much sequence homology. In most cases, the binding of CaM is at \(\alpha\)-helical segments of the target, and targets have been classified based on their different type of CaM-binding \(\alpha\)-helix (Yamniuk and Vogel, 2004). Though the wrap-around binding is the classical and most common way that CaM interacts with its targets, there are other interaction modes that have been identified. There are also some targets that have been shown to bind CaM in its Ca\(^{2+}\)-free form. Examples of such targets have been shown to include the RyR (Buratti et al., 1995), neuromodulin, adenylate cyclase and some VOCs (Jurado et al., 1999). The specificity and versatility of CaM
transmission of Ca\(^{2+}\) signals become enhanced by the different modes of target regulation.

**Calmodulin-regulated phosphatases and kinases**

The binding of CaM changes the properties of the target protein and usually it gains new functions. CaM binding is therefore mostly used to activate the protein targets. Protein targets that are activated by these means include the CaM-dependent phosphatase calcineurin (CaN) and the CaM-dependent kinases (CaMKs). The CaMKs are members of a large family of Ser/Thr kinases and are activated by Ca\(^{2+}/CaM\). Different members have different levels of activation. For instance, CaMKII achieves full activation when bound by Ca\(^{2+}/CaM\), whereas others such as CaMKI and CaMKIV require further activation by phosphorylation. This further activation by phosphorylation takes place in the activation loop of the kinase by an upstream kinase that is CaMK kinase. In some cases, targets are inhibited by binding of Ca\(^{2+}/CaM\) (Chin and Means, 2000), thus further diversifying the target regulation by CaM. Such targets include some G-protein-receptor kinases, IP3R type 1 and E-protein transcription factors (Chin and Means, 2000; Cornelissen et al., 1994; Saarikettu et al., 2004).

**Regulation of CaMKII**

CaMKII is expressed from four genes in mammals: CaMKII\(\alpha\), CaMKII\(\beta\), CaMKII\(\gamma\), and CaMKII\(\delta\). A very large family of multi-functional protein kinases is produced from these genes since they are subjected to extensive alternative splicing of the transcripts. The \(\beta\), \(\gamma\), and \(\delta\) isoforms have a wide expression as opposed to the \(\alpha\) form that is localised to the brain tissue (Tombes et al., 2003). The structure and regulation of the various isoforms display a lot of similarities. CaMKII\(\alpha\) was cloned first and has been much studied. CaMKII\(\alpha\) has been shown to play a very significant role in the brain by enhancing the processes of memory and learning. Mice with their CaMKII\(\alpha\) gene knocked out showed learning and memory defects (Frankland et al., 2001)

CaMKII has a unique ability to decode the frequency of intracellular Ca\(^{2+}\) oscillations due to its sophisticated structure that makes the activity of this kinase highly sensitive to Ca\(^{2+}\) oscillations (Dupont and Goldbeter, 1998). In the inactive state of CaMKII, the catalytic domain is sequestered by an interaction with an auto-inhibitory domain as schematically illustrated in [Figure 7](#). The interaction is released when Ca\(^{2+}\)-loaded CaM is bound to CaMKII, and thereby the kinase is activated and an auto-phosphorylation of Thr\(^{286}\) (Thr\(^{287}\) in CaMKII\(\beta\), \(\gamma\) and \(\delta\)) takes place in the autoinhibitory domain.
(Figure 7). The auto-phosphorylation makes it possible for CaMKII to remain active even for some time after the Ca\(^{2+}\) signal is removed. By CaM trapping, the affinity for Ca\(^{2+}\)/CaM is increased by more than 1000-fold, and the auto-phosphorylation also prevents the auto-inhibitory domain from re-sequestering the catalytic domain. Through these properties, CaMKII gains the unique ability to store previous Ca\(^{2+}\) messages for a short time in the form of auto-phosphorylations and thus enables a memory of recent previous Ca\(^{2+}\) signals. Furthermore, a flower-like structure is formed since CaMKII forms holoenzymes of two layers of six CaMKII subunits each. The catalytic ends of the kinase subunits spread outwards to form the flower-like structure. Inside the holoenzymes, Ca\(^{2+}\)/CaM binding is spread to adjacent subunits by inter-molecular auto-phosphorylations, implying that holoenzyme formation increases the sensitivity of the kinase to Ca\(^{2+}\) (reviewed in (Griffith, 2004; Hudmon and Schulman, 2002; Hunter and Schulman, 2005)).

![Figure 7](image.png)

**Figure 7. A) Structure and regulation of CaMKII activity  
B) The CaMKII holoenzyme**

The removal of a Ca\(^{2+}\) signal leads to gradual inactivation of CaMKII in two steps. In the first step, there is a dissociation of Ca\(^{2+}\)/CaM from the autoinhibitory domain leading to revelation of two more auto-phosphorylation sites, Thr\(^{305}\) and Thr\(^{306}\) (Thr\(^{306/307}\) in CaMKII\(\beta\), \(\gamma\) and \(\delta\)). When these sites undergo phosphorylation, Ca\(^{2+}\)/CaM is prevented from re-associating with the
kinase and thus no more activation takes place. The second step involves the removal of the auto-phosphorylation at Thr\textsuperscript{286} by protein phosphatase (PP1, 2A or 2C) or by the dedicated CaMK phosphatase (CaMKP) (Reviewed in (Ishida et al., 2003)).

**Calmodulin regulation of transcription in lymphocytes**

CaM is instrumental in the interpretation of intracellular Ca\textsuperscript{2+} signals into changes in gene transcription in lymphocytes. CaM accomplishes this interpretation in two ways; one is through direct action on some transcriptional regulators and the other is by indirect action through activation of CaN, CaMKII, CaMKIV or other proteins. MEF2 is a family of transcription factors directly regulated by binding of Ca\textsuperscript{2+}/CaM. MEF2 is constitutively bound to DNA, but its interactions with the transcriptional repressors Cabin1 and HDC 4, 5 and 7 keep it inactive in the absence of Ca\textsuperscript{2+} signals. Ca\textsuperscript{2+}/CaM has dual functions in the regulation of MEF2-dependent transcription. First, Ca\textsuperscript{2+}/CaM is bound to the repressor and disrupts the interaction of MEF2 with the repressor (Youn et al., 2000; Youn et al., 1999), and in addition, activation of CaMKIV leads to phosphorylation and nuclear export of both Cabin1 and the HDACs, leading to reduced repression of MEF2-dependent transcription (Lu et al., 2000; McKinsey et al., 2000; Pan et al., 2005). Ca\textsuperscript{2+}/CaM inhibit the DNA binding of the transcriptional repressor DREAM. Since DREAM binds to sites in the promoters of many Ca\textsuperscript{2+}-regulated genes, the binding of Ca\textsuperscript{2+}/CaM to DREAM proteins will liberate DREAM from DNA and consequently relieve repression of gene transcription (Carrión et al., 1999).

NFAT is a family of transcription factors that take part in the activation and/or repression of approximately 75% of Ca\textsuperscript{2+}-dependent genes in T lymphocytes (Feske et al., 2000). CaM indirectly activates NFAT by Ca\textsuperscript{2+}/CaM-dependent activation of CaN. When cells are not activated (resting), NFAT proteins are phosphorylated and reside in the cytoplasm. Activation of CaN is triggered by a raise in intracellular Ca\textsuperscript{2+} concentration, and after activation of CaN it rapidly dephosphorylates NFAT. This leads to the revelation of the nuclear localization sequence (NLS) of NFAT and consequently there is a rapid nuclear translocation of NFAT and transcription of target genes. Other transcription factors such as AP-1, which is a dimer of proteins of the jun and fos family, collaborate with NFAT in gene transcription. AP-1 is activated through the MAPK pathway and it is viewed that the cooperation of NFAT with AP-1 allows communication between the two pathways. Ca\textsuperscript{2+}/CaM is also involved in the regulation of gene transcription in an indirect manner through other mechanisms, for example by
CaMKII-dependent phosphorylation of the Ets1 transcription factor (Liu and Grundström, 2002).
AIMS

The aim of this thesis was to find out the functional roles of CaM in signalling to NF-κB activation in lymphocytes. Specifically the aim was to find the role of CaM in the pathway from the antigen receptor to NF-κB activation and how Ca$^{2+}$ signals can be integrated in the immune response. To achieve this, the more specific aims were:

- To search for direct interactions between CaM and proteins in the signalling pathway from the antigen receptor to NF-κB activation, and to define the functional roles of these interactions.

- To define functional roles of CaMKII in the pathway from the T cell receptor to NF-κB activation.
RESULTS AND DISCUSSION

This section presents and discusses the main findings of the two papers and unpublished results. The figures of the papers are referred to by the use of the Roman numeral of the article in which they are located, followed by the figure number in the article.

Interaction of calmodulin with Bcl10 modulates NF-κB activation (Paper I)

CaM interacts with Bcl10

CaM takes part in the regulation of IKK and thereby NF-κB activation after TCR engagement or after mimicking of this by activation of protein kinase C (PKC) with phorbol ester (Hughes et al., 1998). The activation of CaM-dependent kinase II (CaMKII) has been found to play a role in triggering of NF-κB signalling from PKC (Hughes et al., 2001) though this was an insufficient explanation of the full effect of CaM in the pathway. The above findings prompted an investigation to find out whether CaM somehow regulates TCR signalling to NF-κB through direct interaction with a signalling protein in the pathway from PKC. In an attempt to identify a possible direct interaction partner for CaM, the ability of recombinant full-length Bcl10 and a Carma1 (1–160) derivative (amino acids 1–160) to bind to CaM Sepharose was examined. Bcl10 was found to bind to CaM Sepharose in a Ca²⁺-dependent manner but no interaction was witnessed for Carma1 (1–160) (I, Figure 1A). Using spectrofluorimetric analysis, the binding of Bcl10 to dansyl-labeled CaM was found to be of high affinity with a $K_d$ of approximately 160 nM (I, Figure 1B). This binding strength is of high biological significance taking into consideration that many targets of CaM have binding strengths that range in that level and that in vivo concentrations of CaM can be in the μM range. Further, it was found that the binding occurred at a 1:1 ratio and was highly cooperative in nature giving the impression of a typical wrap-around binding interaction by CaM.

To find out whether there was a functional significance of the interaction of CaM with Bcl10, a mutant with reduced binding to CaM was created. The first step was the identification of the interaction site by deletion mapping. CaM was found to bind to the CARD domain of Bcl10 (I, Figure 2). The probability that an α-helix in the CARD domain of N-terminal Bcl10 was the target for CaM was very high considering that CaM has a preference for α-
helices and that CARD domains have several $\alpha$-helixes (Chou et al., 1998; Ishida and Vogel, 2006; Yamniuk and Vogel, 2004). A sequence including the sixth $\alpha$-helix of the CARD domain was of particular interest since it is rich in basic and hydrophobic amino acids that have important roles in target recognition by CaM (Ishida and Vogel, 2006; Yamniuk and Vogel, 2004) (I, Figure 3 A and B). Mutation of a basic amino acid within the possible CaM binding sequence in the CARD domain of Bcl10 reduced binding of Bcl10 to CaM Sepharose by approximately 65%, strongly suggesting that the mutated segment was indeed the one to which CaM binds (I, Figure 3C). The CaM binding mutant of Bcl10 retained binding to Carma1 (I, Figure 4B), suggesting that the CARD domain of the mutant is without any change in general structure. The finding of a proline, which has $\alpha$-helix-destabilizing properties, in the corresponding region of Carma1 could, together with fewer basic and hydrophobic amino acids, explain the failure by CaM to bind to the corresponding CARD domain of Carma1 (I, Figure 3 A and B).

The strong Ca$^{2+}$-dependent binding between CaM and Bcl10 in vitro gives an indication of a possible in vivo binding. To determine if Ca$^{2+}$ signalling induces proximity between CaM and Bcl10 in vivo, in situ proximity ligation assays (PLA) were performed. To generate fluorescence signals when the two proteins are in close proximity (≤40 nm), Jurkat T cells were stained using antibodies against CaM and Bcl10 and secondary antibodies conjugated with oligonucleotides (PLA probes). A distinct individual red dot (Jarvius et al., 2007; Söderberg et al., 2006; Söderberg et al., 2008; Söderberg et al., 2007) was given by the fluorescence signal. The number of dots was significantly higher than in controls where either the $\alpha$-Bcl10 or $\alpha$-CaM primary antibody was omitted (I, Figure 5). To determine if stimulation played a role in the proximity, the Jurkat cells were treated for 10 min with PMA, with ionomycin - which increases the intracellular Ca$^{2+}$ level - or with PMA plus ionomycin (Figure 5). Treatment with PMA or PMA plus ionomycin created a small effect on the number of PLA dots while the stimulation with ionomycin led to a 3.5 fold increase in the number of dots. Thus, proximity between CaM and Bcl10 increased by treatment that increases the intracellular Ca$^{2+}$ level but the increase was smaller when the Ca$^{2+}$ signal was combined with a PKC activating stimulus. To find out whether the arginine 88 to alanine (R88A) mutant of Bcl10 with defect CaM interaction in vitro was defect also in vivo, the proximities of the wild-type and mutant Bcl10 to CaM were compared. To make the comparison, PLA was performed in a derivative of the Jurkat T cell line with strongly down-regulated expression of endogenous Bcl10. After transient transfection of expression vector for wild-type Bcl10, the R88A mutant of Bcl10 or empty vector control together with GFP expression vector
to detect transfected cells, the cells were either left unstimulated or stimulated with ionomycin for 10 min. It was observed that the stimulation with ionomycin increased the number of proximities by approximately fourfold in the cells transfected with wild-type Bcl10, but there was no increase in the cells transfected with the R88A mutant or with empty vector (Figure 6).

Interaction of CaM with Bcl10 modulates the binding of Bcl10 to Carma1 and consequently activation of NF-κB

The recruitment of Bcl10-Malt1 to Carma1 after the PKCθ-induced phosphorylation of Carma1 is considered one of the major events in the signalling from the TCR to IKK (Matsumoto et al., 2005; Rueda and Thome, 2005; Sommer et al., 2005). The conserved CARD domains of Carma1 and Bcl10 are necessary for this interaction and for the activation of NF-κB (Rawlings et al., 2006). Binding of CaM to Bcl10 was likely to affect the interaction between Bcl10 and Carma1, since the CARD domain of Bcl10 - to which CaM binds - also mediates the important interaction with Carma1. When recombinant Bcl10 protein was pre-incubated with Ca²⁺/CaM, it was found that the binding of Bcl10 to Carma1 (1–160) Sepharose reduced in a dose-dependent manner (I, Figure 4A). The CaM-binding defective mutant of Bcl10, R88A, was found not to be inhibited by CaM, suggesting that Ca²⁺ signalling through CaM regulates the interaction between Bcl10 and Carma1 (I, Figure 4B).

To investigate the functional significance of CaM regulating Bcl10, the effect of mutating the CaM binding site of Bcl10 on the ability of Bcl10 to activate NF-κB was examined. Jurkat T cells with down-regulated endogenous level of Bcl10 were transiently transfected with expression vector for the wild-type or the CaM binding mutant of Bcl10, together with an NF-κB-dependent reporter. The CaM binding mutant of Bcl10 was found to activate the NF-κB reporter 1.5–2-fold more potently than wild-type Bcl10, both in the absence and the presence of cellular stimulation (I, Figure 7), implying a negative effect of the binding of CaM for NF-κB activation in vivo.

The results implied a negative role for the binding of CaM to Bcl10 in regulation of NF-κB activation that is likely to occur through inhibition of the interaction between Bcl10 and Carma1. Further studies on the in vivo relevance of this interaction would be of high importance. To investigate the full negative effect of CaM binding on NF-κB activation, it would be necessary to use a mutant of Bcl10 where CaM binding is completely disrupted. To construct such a novel mutant in this domain may be quite
challenging considering the facts that CaM is very flexible in its target interactions, (Ishida and Vogel, 2006; Yamniuk and Vogel, 2004) and that such a mutation can easily affect the structure of the CARD domain or its interaction with Carma1 or another component of the large protein complex. A recent study showed that the CARD domain of Bcl10 mediates an interaction with Malt1 (Langel et al., 2008), and one of the mutations used in that study was at a direct neighbor of R88 mutated in the study of this thesis. That mutant, R87A, displayed similarities to the mutant used in this study: the mutant could bind to both Carmal and Malt11 and in the same way as the CaM-binding mutant R88A of Bcl10, it also increased the transcriptional activation of NF-κB by Bcl10. This reinforces the notion that the mutant used in this thesis probably is functional in the binding to both Carma1 and Malt1. It is also a confirmation that this domain of Bcl10 is subject to negative regulation, which is likely to occur by the identified CaM-mediated inhibition of the interaction between Bcl10 and Carma1.

**CaMKII targets Bcl10 in TCR-induced activation of NF-κB**

*(Paper II)*

**CaMKII is recruited to the immunological synapse and modulates interactions within the CBM complex**

Previous findings have shown that CaMKII is needed for TCR-induced activation of IKK and that this activation is PKC-dependent (Hughes et al., 2001). We therefore decided to investigate the functional mechanism(s) behind this role of CaMKII. TCR stimulation has been shown to trigger a rapid translocation of PKCθ to the immunological synapse (Monks et al., 1997). To investigate whether CaMKII plays a role at the immunological synapse, studies by immunohistochemistry were first carried out on the localization of endogenous PKCθ and CaMKII after the TCR and the coreceptor CD28 of Jurkat T cells were stimulated. It was observed that CaMKII relocated together with PKCθ to the site of the cross-linked receptors *(II, Figure 1A)*. NF-κB activation was confirmed by the induced degradation of IκBα observed in cellular extracts prepared from the cross-linked cells *(II, Figure 1B)*. Thus, CaMKII is recruited to the immunological synapse.

There was a possibility that CaMKII modulates interactions within the CBM complex at the immunological synapse. This possibility was supported by the findings that inhibition by CaM and CaMKII inhibitors was over-came by over-expression of either Bcl10 or Carma1 in Jurkat T cells *(II, Figure 2)*. Phorbol ester together with Ca²⁺ ionophore induced NF-κB activation that
was readily inhibited by both inhibitors (II, Figure 2), and since it is known
that PKC is activated directly by phorbol ester and that PKC directly activates
Carma1 (Matsumoto et al., 2005; Rueda and Thome, 2005; Sommer et al.,
2005), these results therefore place the functional role of CaMKII at the level
of Carma1 and Bcl10. Co-immunoprecipitation further revealed an inducible
interaction between over-expressed CaMKII and endogenous Bcl10 in Jurkat
T cells (II, Figure 3A). Over-expression of CaMKII also changed the
interactions of Bcl10 with its known partners Carma1 and Malt1. The over-
expression of CaMKII potentiated the Bcl10:Carma1 interaction, but the
interaction of Bcl10 with Malt1 was instead disrupted by CaMKII (II, Figure
3B and C). The Bcl10:Malt1 interaction is regulated by IKK through a
negative-feedback (Lobry et al., 2007; Wegener et al., 2006). Therefore, the
dissociation of Bcl10 and Malt1 could be by a negative feedback regulation
induced by activation through over-expressed CaMKII. Together, these
results evidently suggest that CaMKII modifies interactions within the CBM
complex at the immunological synapse.

The above results are reinforced by a study of Ishiguro and co-workers,
where they demonstrated a redistribution of CaMKII to the immunological
synapse of T cells (Ishiguro et al., 2006). In the study, they observed that the
interaction between Carma1 and Bcl10 and the activation of NF-κB are
enhanced by phosphorylation of serine 109 of Carma1 by CaMKII (Ishiguro
et al., 2006). Their study supported a potential role for CaMKII in the
pathway from the TCR to NF-κB by modulation of the interaction between
Carma1 and Bcl10, but their study did not answer the question if
phosphorylation of this site was the only mechanism or even the main
mechanism by which CaMKII regulated NF-κB activation. In Paper II, NF-
κB activation that was induced by Carma1 with a serine 109 to aspartate
mutation, mimicking a phosphorylation, was still inducible by phorbol ester
and ionomycin and was still readily inhibited by a CaMKII inhibitor (II,
Figure 2C). Thus, phosphorylation of serine 109 of Carma1 is presumably
not the dominant CaMKII regulatory event in this pathway.

**Phosphorylations of Bcl10 by CaMKII modulate NF-κB activation**

There was a possibility that Bcl10 could also be a target of CaMKII activity,
considering the finding that CaMKII inducibly interacts with Bcl10 in Jurkat
T cells (II, Figure 3A). By analyzing the Bcl10 sequence, there was a
revelation of putative CaMKII target sites at serine 48 (S48) and threonine 91
(T91) within the CARD domain (II, Figure 4A). In vitro phosphorylation of
recombinant Bcl10 and Carma1 showed that Bcl10 was much more potently
phosphorylated by CaMKII than Carma1 (II, Figure 4B). This identified
Bcl10 as a phosphorylation target of CaMKII. To find out the functional significance of phosphorylation of Bcl10 by CaMKII for NF-κB activation, defect Bcl10 was needed. This was accomplished by mutating S48 and T91 of Bcl10 to alanine, which cannot be phosphorylated, or to aspartate, mimicking a phosphorylation. Combined mutation of putative CaMKII phosphorylation sites to alanines reduced in vitro phosphorylation of Bcl10 by CaMKII, supporting that S48 and T91 of Bcl10 are target sites of CaMKII (II, Figure 4C and D). Alanine mutants of S48 and T91 of Bcl10 were found to be defective in activating an NF-κB reporter in a Jurkat-derived T cell line where endogenous expression of Bcl10 had been down-regulated (II, Figure 5A). This suggested CaMKII-dependent phosphorylation of Bcl10 as a regulator of NF-κB activation. Either with or without Ca²⁺ ionophore, the alanine mutation at S48 (S48A) was found to reduce the activation of NF-κB induced by phorbol ester. The S48 aspartate mutant was close to wild-type implying that NF-κB activation might be promoted by a phosphorylation of Bcl10 at S48.

There was a strong inhibition of NF-κB activation when mutating T91 to either alanine or aspartate, and T91 mutations were observed to be dominant when combined with mutation of S48. It was, however, observed that mutants of both S48 and T91 were effective in co-operating with Carma1 in activating NF-κB and in interacting with Carma1 in vitro (II, Figure 5B and C), implying that the mutation effects on NF-κB activation are not as a result of unspecific general structural changes. It was also found, against early presumptions, that the functional role of phosphorylation by CaMKII was apparently not to regulate the interaction between Bcl10 and Carma1, since the S48 and T91 aspartate mutants of Bcl10 were not affected in their ability to bind Carma1 in vitro. To make a more biologically relevant analysis of the significance of mutations of Bcl10 for NF-κB signalling, the TCR and the co-receptor CD28 were co-stimulated using anti-CD3 and anti-CD28 antibodies. The attention was focused on the threonine 91 site as it was deemed to be the most important site (II, Figure 5A). The Jurkat T-cell derivative where endogenous Bcl10 has been down-regulated was used. NF-κB activation by anti-CD3 plus anti-CD28 was restored to approximately the level of the parental Jurkat cells when the cells were reconstituted by transfection with expression vector for wild-type mouse Bcl10 (II, Figure 5D and E). This was the case both for activation of an NF-κB reporter (II, Figure 5D) and for activation of expression of mRNA from the endogenous IL-2 gene (II, Figure 5E), an important natural target of NF-κB after TCR stimulation (Hoyos et al., 1989; Lenardo et al., 1989). The restoration of the activation of NF-κB after α-CD3 plus α-CD28 stimulation did not succeed when expressing either
the T91A or the T91D mutant of Bcl10 (II, Figure 5D and E). The threonine 91 mutants were confirmed with western blot to be expressed at comparable levels to wild-type Bcl10 (II, Figure 5F).

To analyze the phosphorylation of Bcl10 in vivo, expression vector for mouse Bcl10 was first transfected into the Jurkat human T-cells that have down-regulated endogenous Bcl10. There was an appearance of a mouse Bcl10 band in the α-Bcl10 immunoprecipitates as well as slower migrating Bcl10 (II, Figure 6A). The slower migrating Bcl10 was assumed to be phosphorylated Bcl10 for the following reasons: first; it appeared upon transfection with Bcl10 expression plasmid, second, it was immunoprecipitated and detected in western blot with α-Bcl10 antibody, and thirdly it disappeared when the T-cell extract was treated with a calf intestine phosphatase.

To determine whether CaMKII made the phosphorylation(s), parental Jurkat T-cells were used. There was a rapid phosphorylation of Bcl10 (indicated with an arrow in II, Figure 6B) induced by stimulation with PMA plus ionomycin, and this phosphorylation of Bcl10 was blocked in cells treated with the CaMKII inhibitor KN93. This gives a strong indication that CaMKII is significant for the main phosphorylation of Bcl10 after stimulation with PMA plus ionomycin. A comparison of wild-type and threonine 91 mutated Bcl10 was carried out by expressing them in the Jurkat T-cell derivative with down-regulated endogenous human Bcl10. The experiment led to the revelation of both non-phosphorylated and phosphorylated species of mouse Bcl10 in western blot of α-Bcl10 immunoprecipitates (II, Figure 6C). The over-expression of Bcl10 was responsible for the presence of phosphorylated Bcl10 in the transfected cells even before stimulation (II, Figure 6B and C). In cells transfected with the T91A mutant, the slowly migrating band of phosphorylated Bcl10 was strongly reduced, and it was lost in the T91D mutant (II, Figure 6C). This shows that CaMKII phosphorylates Bcl10 with the threonine 91 phosphorylation site seemingly being dominant. Over-expression of T91D mutant led to the accumulation of Bcl10 (II, Figure 6C and data not shown); giving the indication that the T91D mutant can be dominant negative for degradation of Bcl10.

**Bcl10 sites phosphorylated by CaMKII regulate phosphorylation of IKKα/β and JNK2**

Comparison of the phosphorylation status of IKK, JNK1/2 and ERK1/2 in Jurkat T-cells silenced for Bcl10 and reconstituted by either wild type or mutant mouse Bcl10 was carried out to identify if the significance of the T91
regulatory site of Bcl10 was solely in TCR signalling to NF-κB or if other signalling pathways downstream of the TCR were also affected.

Phosphorylation of IKKα/β could be induced with PMA plus ionomycin in the Jurkat T-cell derivative silenced for Bcl10, but to a reduced level compared to the parental Jurkat cells (II, Figure 6D). This is in line with earlier studies on IKK phosphorylation showing that it does not completely rely on Bcl10 and Carma1 and that it is mediated by TAK1 (Shambharkar et al., 2007). The induction of phosphorylation of IKKα/β was shown to be restored by expression of wild-type Bcl10 but the IKK phosphorylation was not restored by expression of the T91-mutated Bcl10 derivatives (II, Figure 6D and supplementary Figure 1A). Also after induction by stimulating the TCR with α-CD3 plus α-CD28 did expression of wild-type Bcl10 restore phosphorylation of IKKα/β but it was not restored when expressing the T91-mutated Bcl10 derivatives (II, Figure 6E). The deduction from these findings is clearly that T91A and T91D mutated Bcl10 are defective in signalling to phosphorylation of IKKα/β. The corresponding observations were made for JNK2 phosphorylation with the threonine 91-mutated Bcl10, whereas JNK1 and ERK1/2 phosphorylation were unaffected by silencing or mutation of Bcl10 (II, Figure 6D and E and supplementary Figure 1B and C). Earlier studies have reported Bcl10- and Carma1-dependent phosphorylation of JNK2 but not of JNK1 and ERK1/2 (Blonska et al., 2007; Shambharkar et al., 2007). In summary, the results of Figure 6D and E show that threonine 91 of Bcl10 is an important regulatory site for signalling to IKKα/β as well as for signalling through JNK2.

Defect CaMKII-induced phosphorylation of Bcl10 prevents K63-linked ubiquitination of Bcl10 and IKKγ

To determine the significance of the threonine 91 site of Bcl10 for the poly-ubiquitination of IKKγ and Bcl10, either wild-type or threonine 91-mutated Bcl10 was expressed together with hemagglutinin-tagged ubiquitin (HA-ubiquitin) in the Jurkat T-cell derivative with down-regulated endogenous Bcl10. Analysis was carried out selectively on K63-linked poly-ubiquitination of IKKγ using HA-ubiquitin with all lysines except K63 mutated to arginine. Western blot against HA-ubiquitin showed only weak K63-linked poly-ubiquitination of IKKγ in empty vector control transfected cells but a potent stimulation-induced poly-ubiquitination of IKKγ was found in cells transfected with wild-type Bcl10, as revealed by the appearance of high molecular weight species of ubiquitinated IKKγ (II, Figure 7A). In contrast, expression of either the T91A or T91D mutant of Bcl10 did not enable induction of K63-linked polyubiquitination of IKKγ (II, Figure 7A). This
shows that threonine 91 of Bcl10 is a vital regulatory site for the induction of K63-linked poly-ubiquitination of IKKγ. Immunoprecipitation of Bcl10 was used next to analyze K63-linked polyubiquitination of Bcl10. The Bcl10 was found to be heavily ubiquitinated by K63-linked poly-ubiquitination in stimulated cells (II, Figure 7B and supplementary Figure 2A). Of significance was the weak induction of K63-linked poly-ubiquitination in the T91A mutant of Bcl10 whereas the induction of K63-linked poly-ubiquitination was very poor or not present at all in the T91D mutant of Bcl10 (II, Figure 7B and supplementary Figure 2A). This shows that threonine 91 of Bcl10 takes part in the regulation of K63-linked poly-ubiquitination of Bcl10 as well as of IKKγ. An analysis of the effects of threonine 91 mutations on K48-linked poly-ubiquitination of Bcl10 was also carried out. HA-ubiquitin with all lysines except K48 mutated to arginine was used in these co-transfections. Western blot against HA-ubiquitin revealed the presence of K48-linked poly-ubiquitinated Bcl10 after immunoprecipitation with α-Bcl10 (II, Figure 7C and supplementary Figure 2B). The poly-ubiquitination of Bcl10 increased after stimulation with PMA plus ionomycin in cells expressing wild-type Bcl10, but remained weak for empty vector control-transfected cells (II, Figure 7C and supplementary Figure 2B). However, K48-linked poly-ubiquitination of Bcl10 was present already before stimulation for the T91A mutant of Bcl10 and did not increase any further upon the stimulation with PMA plus ionomycin (II, Figure 7C and supplementary Figure 2B). The K48-linked poly-ubiquitination was also present for the T91D mutant of Bcl10 before stimulation (II, Figure 7C and supplementary Figure 2B). These results show that CaMKII-induced Bcl10 phosphorylation at threonine 91 selectively is critical for K63-linked ubiquitination of Bcl10 and IKKγ and consequently for the activation of IKK and subsequently NF-κB.

In summary, CaMKII is recruited to the immunological synapse, a finding that agrees with Ishiguro et al. (Ishiguro et al., 2006). An activation-dependent recruitment of CaMKII to the interface between superantigen SEE coated antigen presenting cells (APCs) and Jurkat T-cells was demonstrated by them. Here inducible interaction of CaMKII with Bcl10 is demonstrated and also that CaMKII phosphorylates Bcl10 at serine 48 and threonine 91 and that these sites are valuable for the activation of NF-κB by Bcl10. Mutating serine 48 to alanine caused a reduction in the activating ability of Bcl10 on NF-κB-dependent transcription in Jurkat T-cells, whereas mutating threonine 91 to alanine caused a severe impairment in the function of Bcl10 in the activation. When over-expressed together with Carma1, both the S48A and the T91A mutant of Bcl10 were found to activate NF-κB to a similar extent as wild-type Bcl10. This implies that these mutations do not make the protein
non-functional per se but that they are not efficient at natural \textit{in vivo} concentrations of Carma1 and Bcl10. The mechanistic role of phosphorylation at serine 48 of Bcl10 in the activation of NF-κB after TCR activation was not clearly established. Analyzing the binding between purified Carma1 and wild-type or S48D mutated Bcl10 suggested that the modest role of phosphorylation at serine 48 is not a slight improvement in this interaction. There nevertheless is a possibility that phosphorylation of Bcl10 regulates the interaction between Bcl10 and Carma1 \textit{in vivo} at the immunological synapse. The activity of Bcl10 has been shown to be regulated by K63-linked poly-ubiquitination at lysines 31 and 63, which is important for the recruitment of IKKγ and subsequent NF-κB activation (Wu and Ashwell, 2008). Another possibility is therefore that phosphorylation of the nearby serine 48 could slightly improve this poly-ubiquitination, or its effect, consequently regulating the ability of Bcl10 to activate NF-κB. There is also a possibility that phosphorylation of serine 48 might have a slight effect on the interaction of the CARD domain of Bcl10 with a not yet characterized signalling mediator(s). Malt1 could be this mediator considering that the CARD domain of Bcl10 mediates a part of the interaction with Malt1, but it is known that the CaMKII phosphorylation site at serine 48 is far from the Malt1 binding part of the CARD domain (Langel et al., 2008). It is hard to experimentally discriminate between the possible alternative roles of this phosphorylation due to the modest level of the effect of mutating serine 48 of Bcl10. Mutations of the CaMKII site at threonine 91 gave much more dramatic results as compared to serine 48. There was not any significant NF-κB-dependent transcriptional activity induced by TCR/CD28 stimulation or by mimicking of TCR stimulation with PMA plus ionomycin that remained over the vector control when mutating threonine 91 of Bcl10 to alanine or to aspartate. The deleterious effect of mutation to alanine is as expected for an important phosphorylation site, whereas the negative effect of mutation also to aspartate is surprising. Neither of the threonine 91 mutations affected the CARD–CARD interaction with Carma1 detected \textit{in vitro}, which was in accordance with X-ray crystallography analyzes that indicate that the CARD–CARD interactions take place through interaction between the second and third α-helical face and between the first and fourth α-helical face of the proteins, whereas threonine 91 is in the sixth α-helix (Langel et al., 2008; Weber and Vincenz, 2001). Threonine 91 mutants have an intact interaction with Carma1, indicating that mutation of threonine 91 is not deleterious for the overall structure of the domain. Mutation of threonine 91 to alanine and to aspartate gave very sharply different effects. T91D mutation of Bcl10 as opposed to a T91A mutation was shown to be a dominant negative mutation because the co-expression of Carmal with the T91D mutant of Bcl10 but not
with the T91A mutant activated the NF-κB reporter even less than over-expression of Carma1 alone. Furthermore, the T91D mutation appeared also to be a dominant negative mutation for phosphorylation of other sites of Bcl10. The dominant negative effect of the T91D mutation of Bcl10 argues against an unspecific deleterious effect of mutation of threonine 91 on the structure of the protein. This study’s finding that mutation of threonine 91 to alanine partially blocks and mutation to aspartate abolishes the K63-linked poly-ubiquitination of Bcl10 establishes a reason for the defect NF-κB activation by the mutants, since this modification of Bcl10 is required for binding of IKKγ and subsequent phosphorylation of IκB and activation of NF-κB (Wu and Ashwell, 2008). The finding that K63-linked poly-ubiquitination of IKKγ is also inhibited in the T91A and T91D mutants is as expected from the block of the K63-linked polyubiquitination of Bcl10. This study’s finding that the expressed T91D mutant of Bcl10 was defective in signalling to NF-κB could be seen as a secondary effect in a sensitive signalling system that is subjected to negative feedback regulation. Lobry et al. (Lobry et al., 2007) have noted that phosphorylations of Bcl10 by IKK at amino acids 81 and 85, not far from threonine 91, recruited the ubiquitin ligase βTrCP, which caused a negative feedback regulation through K48-linked ubiquitin-mediated proteasomal degradation of Bcl10. An alternative explanation of the negative effect of the T91D mutation of Bcl10 would therefore be a dramatic increase in negative feedback regulation. But considering the findings of this study that mutation of threonine 91 had a negligible effect on the K48-linked poly-ubiquitination and did not decrease the expression level of the protein, the data do not support this alternative explanation. The conclusion that remains is that the dominant negative effect of the T91D mutation is due to the existence of a regulatory interaction that cannot be relieved when position 91 has a negative charge that is permanent. The nature of this regulatory interaction is not clear. A recent study by Carvalho et al. (Carvalho et al., 2010) reported that Bcl10, Malt1, and IKK inducibly associate in the cytosol with IκBα in a complex that is physically distinct from the early CBM complex at the plasma membrane. IKKβ-driven serine phosphorylation of BCL10 was proposed as a candidate for triggering expulsion of IκBα-activating structures into the cytosol due to its destabilizing effect on the CBM complex and its favoring of Bcl10 turnover. These authors didn’t speculate about a role for CaMKII, since Bcl10 was not known to be a CaMKII target. The present study’s findings indicate that phosphorylation of threonine 91 mediated by CaMKII could play an important role in triggering the expulsion of the IκBα activating complex, and a negative charge at threonine 91 that is not transient could trap an interaction in that process. This possibility has support from this study’s findings that
over-expression of CaMKII affects interactions within the CBM complex and that inactive CaMKII has distinct effects from active CaMKII. Further support for the need to remove phosphorylation(s) of Bcl10 comes from a recent study by Palkowitsch and co-workers (Palkowitsch et al., 2011). They showed that the inhibition of Ca\(^{2+}\)/CaM dependent phosphatase, CaN, leads to hyperphosphorylation of Bcl10 and affects the CBM complex. Their findings suggest the existence of a phosphorylation(s) of Bcl10 that is/are negative for the CBM complex and TCR-induced NF-κB activity. There is a need for further studies to be carried out in the area of the detailed mechanism of the expulsion of the activating complex into the cytosol.

The very strong negative effect of the T91A mutation of Bcl10 on NF-κB activation might seem surprising taking into consideration the more modest effect on the ubiquitination of Bcl10 (II, Figure 7B). However, threonine 91 is within the identified CaM interaction sequence of Bcl10, and the binding of CaM to Bcl10 is inhibitory for the interaction between Bcl10 and Carma1 (Paper I). The T91D mutant of Bcl10 reduced CaM binding \textit{in vitro} by approximately 45% (data not shown). This finding indicates that phosphorylation of threonine 91 of Bcl10 is negative for CaM binding. An important feature of CaM binding sites is a dominance of amino acids with positive charge over those with negative charge (Ishida and Vogel, 2006; Yamniuk and Vogel, 2004). A phosphorylation adds two negative charges, whereas the mutation to aspartate adds only one charge. Therefore, it is likely that phosphorylation of threonine 91 would be even more negative for CaM binding than the aspartate mutation. Thus, when CaMKII is recruited and activated, it might release CaM inhibition of the interaction between Bcl10 and Carma1 by phosphorylation at threonine 91. In the T91A mutant of Bcl10, however, negative charges cannot be added by phosphorylation and the inhibition by CaM may not be efficiently relieved, which would contribute to the negative effect of this mutant on NF-κB activation. This therefore leads to the assumption that phosphorylation of Bcl10 at threonine 91 by CaMKII could in part be a mechanism to remove CaM inhibition of the interaction between Bcl10 and Carma1 (II, Figure 8).

In conclusion, CaMKII recruited to the immunological synapse can regulate activation of NF-κB through phosphorylations in different ways (II, Figure 8):

1. Phosphorylation at S109 of Carma1 enhances the interaction between Bcl10 and Carma1 (Ishiguro et al., 2006).
2. Phosphorylation at serine 48 of Bcl10 promotes NF-κB activation by a not yet determined mechanism.

3. Phosphorylation at threonine 91 of Bcl10 promotes important K63-linked poly-ubiquitination of Bcl10 and subsequent activation.

4. Phosphorylation at threonine 91 of Bcl10 can release its inhibition by CaM, permitting its interaction with Carma1.

*CaMKII regulates the phosphorylation of IKK by TAK1*

The phosphorylation of IKKα/β and the ubiquitination of IKKγ after TCR stimulation are through two distinct signalling pathways. While the latter is based on CARMA1 and mediated by the downstream BCL10–MALT1–TRAF6 complex, PKC regulates TAK1, the IKKα/β kinase, through a CARMA1-independent pathway (Shambharkar et al., 2007). Although several studies have shown that TAK1 phosphorylates IKKα/β, the mechanism(s) of activation of TAK1 by PKC is not known. CaMKII has been shown to be involved in the regulation of TAK1 in a few other systems (Culver et al., 2010; Ishitani et al., 2003; Liu et al., 2008; Wang et al., 2009). I investigated therefore whether CaMKII has a functional role in regulation of IKKα/β phosphorylation by TAK1 in T-cells. To address whether CaMKII is involved in the phosphorylation of IKKα/β after PKC activation, Jurkat T cells induced by the PKC activator PMA plus the Ca$^{2+}$ ionophore ionomycin were used. Western analysis with anti-phosho-IKKα/β antibodies showed that the stimulation with PMA plus ionomycin induced phosphorylation of IKKα/β in Jurkat T cells (Figure 8A), as expected from previous studies. Importantly, this phosphorylation of IKKα/β was blocked in cells treated with either CaM inhibitor (W7) or CaMKII inhibitor (KN93). Analysis of IκBα degradation showed that it was also blocked in the cells treated with the inhibitors (Figure 8A). These results indicate that CaMKII is significant for the phosphorylation of IKKα/β after stimulation of PKC together with Ca$^{2+}$ stimulation.
Figure 8. CaMKII is important for IKKα/β phosphorylation in response to PMA plus ionomycin. Analysis of phosphorylation of IKKα/β was performed in Jurkat T cells stimulated with 50 ng/ml PMA plus 1 µg/ml ionomycin (P+I) for the indicated times with or without the presence of 10 µg/ml of the CaM inhibitor W7 or 20 µM of the CaMKII inhibitor KN93. The cell extracts were analyzed for phospho-IKKα/β, IKKα/β, and IκBα by western blot with α-phospho-IKKα/β,(16A6; Cell Signaling Technology), α-IKKα/β, (H-470; Santa Cruz) and α-IκBα (C-15; Santa Cruz), respectively.

Previous studies indicate the presence of an activating CaMKII phosphorylation site(s) in TAK1(Culver et al., 2010; Ishitani et al., 2003; Liu et al., 2008; Wang et al., 2009), and Liu and co-workers and Culver and co-workers showed that TAK1 can be phosphorylated by CaMKII. However, no CaMKII phosphorylation site has been identified in TAK1. By analyzing the mouse and human TAK1 sequence, I found conserved potentially good target sites for CaMKII (Hudmon and Schulman, 2002; White et al., 1998) at serine 82 (S82), serine 350 (S350), serine 553 (S553), and threonine 578 (T578) shown in Figure 9A. To find out the functional significance of these sites, they were mutated to alanine, which cannot be phosphorylated, or to aspartate, mimicking a phosphorylation.
Figure 9. *IκBα* degradation is blocked by TAK1 mutation. A) Sequence alignment of parts of human and mouse TAK1 with conserved potentially good target sites for CaMKII. Arrows indicate the four putative CaMKII target sites. Boxes represent a basic amino acid three amino acids N-terminal to the site, which is important for recognition by CaMKII. Serine 553 doesn’t have a basic amino acid three amino acids N-terminal to the site but other favorable amino acids around the serine makes it nevertheless a putative CaMKII target sites. B) Analysis of degradation of *IκBα* performed in HEK293 cells. The HEK293 cells were transiently transfected (Fugene HD; Promega) with 2 µg of pcDNA1 based expression vector for TAB1 alone, TAK1 wild-type (WT) alone, or TAB1 plus TAK1 WT or mutant derivative with alanine (A) at S82, S350, S553, or T578 as indicated. Twenty-four hours after transfection, cells were harvested. The cell extracts were analyzed for degradation of *IκBα* and for TAK1 expression and Tubulin control by western blot with α-*IκBα* (C-15; Santa Cruz), α-TAK1 (M-579; Santa Cruz), and α-tubulin (B-5-1-2; Sigma), respectively.
HEK293 cells have little TAK1 and exogenous TAK1 has been shown to be activated by co-expression of TAB1 in these cells (Mao et al., 2011; Sato et al., 2005). To determine the significance of the putative TAK1 sites, either TAB1 alone, TAK1 wild-type alone, or TAK1 wild-type or alanine mutants together with TAB1 were expressed by transient transfection in the HEK293 cells. Degradation of IκBα was seen in the cells expressing TAK1 wild-type together with TAB1 but not TAK1 or TAB1 alone (Figure 9B), which is in line with the earlier studies that TAB1 activates expressed TAK1 in these cells. The S553A and T578A mutants showed degradation of IκBα as the wild-type when co-expressed with TAB1, whereas the S82A mutant showed no IκBα degradation (Figure 9B) and the IκBα degradation was defect to different degrees in experiments with the S350A mutant. Thus, at least one of the mutants in putative CaMKII sites of TAK1, S82A, is defect in signaling to IκBα, and another one, S350A, is presumably partially defect. However, the lack of effect of the two other mutants at this experimental condition does not rule out the significance of the other sites either independently or in combination under physiological conditions and upon activation of TAK1 by various other inducers.

In summary, CaMKII is important for the phosphorylation of IKK by TAK1 and results indicate that at least one putative CaMKII target site of TAK1 is important in signaling to IκBα. Additional studies are needed to further analyze the significance of the putative CaMKII target sites of TAK1 and their relevance in different signaling pathways.
**Concluding Remarks**

Ca$^{2+}$ and CaM have been shown to be directly and indirectly involved in the regulation of NF-κB signalling in lymphocytes, and such regulation has been shown to occur at multiple levels. CaM has been found to directly and Ca$^{2+}$-dependently interact with the NF-κB proteins RelA and c-Rel after their signal-induced release from IκB, and thereby differentially regulate their nuclear localisation (Antonsson et al., 2003). CaM was found here to also have a direct and Ca$^{2+}$-dependent interaction with Bcl10, an interaction that was negative for the ability of Bcl10 to bind Carma1 and activate NF-κB after TCR stimulation. In addition, CaM positively regulates NF-κB activation through activation of CaMKII. CaMKII was found here to be recruited to the immunological synapse after TCR engagement. There was an inducible interaction with Bcl10 and phosphorylation of Bcl10 at sites significant for the ability of Bcl10 to activate NF-κB. It was interesting to find that CaM could play contrasting roles in the TCR-induced activation of NF-κB. This was achieved by direct inhibition of the interaction between Carma1 and Bcl10 versus indirect enhancement of the Bcl10 function. Changes in the concentration of Ca$^{2+}$ have been shown to be very important as they play a role in the regulation of T cell activation, and the different patterns of Ca$^{2+}$ signals created by TCR activation are known to regulate NF-κB (Dolmetsch et al., 1997; Dolmetsch et al., 1998; Feske, 2007; Quintana et al., 2005; Randriamampita and Trautmann, 2004). High Ca$^{2+}$ transients, such as those induced by pathogens stimulating the TCR, activate NF-κB in contrast to more modest Ca$^{2+}$ signals, such as those resulting from stimulation of the TCR by self-antigens that are not sufficient for NF-κB activation. I find it therefore likely that the inhibitory effect of CaM on NF-κB activation may play a significant role during incomplete TCR stimulations when activation of NF-κB should not be allowed. The contrasting outcome would occur when a proper immunological synapse is formed, since high fluctuating Ca$^{2+}$ in concert with other induced signalling events allowed CaM to positively regulate NF-κB through activation of CaMKII. CaMKII possesses unique features that allow it to ‘remember’ recent strong Ca$^{2+}$ signals in the form of auto-phosphorylations, which allows the activation of CaMKII by recurrent strong Ca$^{2+}$. This leads to the compelling suggestion that activation of CaMKII could be a “switch” that is part of the determination of T cell fate by antigenic stimulation. From earlier studies showing the potential of CaMKIIα in learning and memory in the brain (Colbran and Brown, 2004; Frankland et al., 2001; Mack et al., 2005; Merrill et al., 2005), it makes much sense that CaMKII could play a role in T cell memory by potentiating the response to
restimulation. More support for this model is attained from studies of transgenic mice expressing a constitutively active CaMKIIγB that suggested that CaMKII plays a role in modulation of the T cell response to antigen and formation of T cell memory (Bui et al., 2000; Lin et al., 2005; McGargill et al., 2005). There is a need for further studies in these areas, since the experimental evidences are still far from being completely conclusive. Another suggestion from my studies, yet to be analysed, is that similar regulation of CaMKII could regulate also responses of B cells to antigen. There is also a possibility that CaMKII might have additional targets besides Carma1, Bcl10, and TAK1 at the immunological synapse that have not yet been identified. Since Carma1 acts as a signalling platform recruiting Bcl10-MALT1, IKKγ, Caspase-8, TRAF6, and TAK1 (McCully and Pomerantz, 2008), there is a possibility that CaMKII also regulates an additional protein(s) at this synapse. CaMKII phosphorylates a number of proteins and regulates them in the neuronal synapse (Ishiguro et al., 2007; Söderling et al., 2001), so it wouldn't be surprising if it has a similar role in the immunological synapse. CaM plays a role in TCR signalling to NF-κB also through activation of the Ca^{2+}/CaM dependent phosphatase, CaN. CaN appears to accelerate phosphorylation and degradation of IκBα by controlling the CBM complex (Frantz et al., 1994; Palkowitsch et al., 2011; Steffan et al., 1995). CaMKIV can enhance stimulation of IκBα degradation by hydrogen peroxide and increase the transcriptional activity of RelA by phosphorylation, and it has been implicated in TCR-induced NF-κB activation (Howe et al., 2002; Jang et al., 2001).

In summing up the studies here together with previous analyses, it has been shown that Ca^{2+}/CaM plays multiple roles in the activation of NF-κB, and CaM appears to act as a modulator of NF-κB activation by different stimuli, thus creating specificity by which NF-κB can appropriately regulate gene transcription and cellular responses to different types of immune stimulations.
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