UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
New Series No. 864 ISSN 0346-6612 ISBN 91-7305-549-2
From the Department of Molecular Biology,
Umeå University,
Umeå, Sweden.

REGULATION OF NF-κB BY CALMODULIN

by
Åsa Antonsson

Department of Molecular Biology
Umeå University, Umeå
2003

TABLE OF CONTENTS

PAPERS IN THIS THESIS

This thesis is based upon the following publications, which will be referred to in the text by their roman numerals (I-III).

I. **Antonsson, Å., Hughes, K., Edin, S. and Grundström, T. (2003)**
Regulation of c-Rel nuclear localization by binding of Ca^{2+}/calmodulin.
Mol Cell Biol. **23**:1418-27.

II. **Hughes, K.*, Antonsson, Å.* and Grundström, T. (1998)**
Calmodulin dependence of NFκB activation.
FEBS Lett. **441**:132-6.

III. **Hughes, K., Edin, S., Antonsson, Å. and Grundström, T. (2001)**
Calmodulin-dependent kinase II mediates T cell receptor/CD3- and phorbol ester-induced activation of IκB kinase.
J Biol Chem. **276**:36008-13.

* The first two authors contributed equally to this work.

ABBREVIATIONS

bHLH	basic-Helix-Loop-Helix
Ca^{2+}	Calcium ion
CaM	Calmodulin
CaMK	Calmodulin-dependent kinase
CBP	CREB-Binding Protein
CREB	cAMP-Response Element Binding Protein
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic Reticulum
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
Ig	Immunoglobulin
IKK	IκB kinase complex
IL-2	Interleukin-2
$InsP_3$	Inositol 1,4,5-trisphosphate
$InsP_3R$	Inositol 1,4,5-trisphosphate receptor
NES	Nuclear Export Signal
NLS	Nuclear Localisation Signal
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor κB
NPC	Nuclear Pore Complex
PKA	cAMP dependent protein kinase
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
Pol II	RNA polymerase II
RHD	Rel Homology Domain
SR	Sarcoplasmic reticulum
TCR	T-cell Receptor
TNFα	Tumour Necrosis Factor α

ABSTRACT

Cells experience numerous external signals which they must respond to. Such signals arriving at the cell surface are transduced via various signal transduction pathways and often ultimately result in regulation of transcription. NF-κB is a family of transcription factors involved in the regulation of genes important for processes such as immune and inflammatory responses, cell growth, development and cell survival. NF-κB proteins are normally kept inactive in the cytoplasm due to masking of their nuclear localisation signal (NLS) by inhibitory IκB proteins. A large number of stimuli lead to the activation of IκB-kinase (IKK). Active IKK phosphorylates IκB and thereby labels it for ubiquitination and, subsequently, degradation by the proteasome. Liberated NF-κB enters the nucleus, where it takes part in the regulation of its target genes.
Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein which is considered to be the predominant intracellular Ca^{2+} sensor. CaM plays a major role in the Ca^{2+}-dependent regulation of a wide variety of cellular processes, including transcription. CaM regulates transcription both indirectly through CaM-dependent kinases and phosphatases and directly through interaction with transcription factors.

CaM was found to bind directly and in a Ca^{2+}-dependent fashion to the two NF-κB family members c-Rel and RelA. The CaM-NF-κB interactions were strongly enhanced by NF-κB activating stimuli and this enhancement was blocked by the addition of IκB, suggesting that c-Rel and RelA can bind CaM after their signal-induced release from IκB. Compared to wild-type c-Rel, CaM binding-deficient mutants were shown to exhibit an increased nuclear accumulation and transcriptional activity on Ca^{2+}-regulated cytokine promoters. The results suggest that CaM can inhibit transport of c-Rel, but not of RelA, to the nucleus and thereby differentially regulate the activation of NF-κB proteins following cell stimulation. CaM was also found to affect NF-κB activity indirectly through the action of a CaM-dependent kinase (CaMK). Studies of the events leading to IκBα phosphorylation revealed that CaM and CaMKII inhibitors blocked phorbol ester induced activation of IKK. Furthermore, CaM and CaMKII inhibitors also blocked T cell receptor/CD3 induced IκBα degradation, and expression of an inhibitor-resistant derivative of the γ isoform of CaMKII caused the inhibitors lose their effect on phorbol ester induced IκBα degradation. Finally, expression of a constitutively active CaMKII resulted in the activation of NF-κB. These results identify CaMKII as a mediator of IKK activation, specifically in response to T cell receptor/CD3 and phorbol ester stimulation.

In conclusion, this thesis describes the identification of CaM as a dual regulator of NF-κB proteins, acting both directly and indirectly to affect the activity of this family of transcription factors.

INTRODUCTION

EUKARYOTIC TRANSCRIPTION

The genome contains thousands of genes and an enormously diverse regulatory system is necessary to obtain induction or repression of the expression of the right gene at the right time. The expression of a gene can be regulated at many different levels, e.g. initiation and elongation of transcription, RNA processing, RNA transport, RNA stability, initiation and elongation of translation, post-translational modification, protein transport and protein activity. The first step, initiation of transcription, represents a major control point for the regulation of gene expression.

Activation of transcription

The regulation of RNA polymerase II (Pol II) transcription of protein-encoding genes in eukaryotes is a very complex process (reviewed in (25, 96)). Pol II promoters are composed of the core promoter and promoter elements immediately upstream of the core promoter. In addition to these promoter regions, there are regulatory enhancer sequences which can be located either upstream or downstream of the gene, or even within introns. The core promoter surrounds the transcription start site and contains sequence elements, e.g. TATA box and initiator sequences, that are recognised by subunits of the general transcriptional machinery and thus direct Pol II to begin transcribing at the correct start site. The core promoter, however, is generally inactive until a gene-specific combination of transcriptional activators binds to other promoter elements and/or to the enhancer(s), and thereby enables recruitment of the general transcription machinery to the core promoter. The activators are sequence-specific transcription factors with distinct domains for DNA binding and transcriptional activation. The DNA-binding domain targets the activator to a specific DNA sequence and the transactivation domain interacts with proteins, e.g. the mediator complex and co-activators, that serve as bridges between the transcriptional activator and the general transcription machinery. The mediator complex and the co-activators, for example CBP and p300, have binding sites for multiple

transcriptional activators and can therefore participate in transcriptional activation at many different promoters.

In eukaryotes, the genome is arranged into a compact structure called chromatin. Condensed chromatin maintains genes in an inactive state by restricting access to RNA polymerase and its accessory factors. Thus, before any functional interactions between activators and the general transcription machinery can occur, the gene and its control regions must be made accessible for transcription. The process that accomplishes this is chromatin remodelling, which involves ATP-dependent remodelling and modification of histones. Many co-activators have enzymatic activities that can add various modifications to histone tails. For example, the above-mentioned co-activators CBP and p300 possess histone acetyl transferase (HAT) activity and, since they interact with transcriptional activators, the HAT activity is directed to the regulatory region to which the activator is bound.

Will gene X be transcribed in a certain cell type at a certain time? This depends on which transcription factor binding sites are present in the control regions of gene X, and on whether an appropriate set of transcription factors which is capable of binding to those DNA sequences is expressed and active in the cell type in question. Thus, regulation of the activity of transcription factors plays an important role in transcriptional regulation and is achieved at distinct levels in response to signals. For example, NF-κB transcription factors are, as will be discussed in section *Regulation of NF-kB activity,* to a large extent regulated at the level of sub-cellular localisation with an inactive cytoplasmic state and nuclear localisation following cellular stimulation. Phosphorylation has been shown to affect transcription factors at many levels, such as nuclear transport, DNA binding activity and transactivating function (86, 132). Many families of transcription factors, e.g. leucine zipper proteins, nuclear hormone receptors, basic-helix-loop-helix (bHLH) proteins and the NF-κB family, exist as dimers. The formation of various heterodimers within a family greatly increases the number of distinct functional transcription factors (95).

Repression of gene expression

When the expression of a gene product is no longer required, it is important to have means by which it can be turned off. Repression of eukaryotic transcription can be achieved through the action of repressor proteins. There are several mechanisms by which these DNA-binding proteins can negatively affect transcription. They can (i) compete with an activator protein for the same DNA sequence, (ii) bind to the activation domain of an activator protein and thereby prevent the activator protein from carrying out its activation function(s), (iii) interfere with the assembly of the general transcription machinery, (iv) recruit a chromatin remodelling complex that restores the repressed pre-transcriptional state of the promoter, or (v) attract a histone deacetylase (HDAC) to the promoter. Repression of transcription can also be achieved by negative regulation of the activity of transcriptional activators. When a signal to repress transcription reaches the cell, it might make a transcriptional activator lose its interaction with DNA, translocate to the cytoplasm or be modified in a way that is deleterious for transcription.

CALCIUM AND CALMODULIN

The calcium ion (Ca^{2+}) is the most versatile signal transduction element in cells. Highly regulated changes in the concentration of cytosolic Ca^{2+} control biological processes as diverse as muscle contraction, secretion, ion and nucleotide metabolism, and cell growth (162). Since Ca^{2+} is lethal to the cell in high concentrations and during prolonged exposure, the cells are forced to regulate the intracellular Ca^{2+} concentration efficiently.

Ca^{2+} homeostasis and regulation

Normal intracellular Ca^{2+} levels fluctuate at concentrations around 100 nM, which is approximately 20,000-fold lower than the 2 mM concentration found extracellularly (32). Generally speaking, the cell maintains this difference in concentration using two strategies: firstly, using Ca^{2+} pumps that remove cytosolic Ca^{2+} into specialised organelles and to the extracellular medium; and secondly, through proteins that are able to bind to and buffer the Ca^{2+}. The export of Ca^{2+} from the cytoplasm to the outside of the cell is taken care of by the plasma membrane Ca^{2+}-activated ATPase (PMCA) and, in some cells, also by a Na^{+}/Ca^{2+} exchange pump (reviewed in (24)). The endoplasmic reticulum (ER) and its muscle cell counterpart, the sarcoplasmic reticulum (SR), are the main Ca^{2+} storage compartments of eukaryotic cells. Ca^{2+} is pumped into the ER/SR by the sarco-endoplasmic reticulum Ca^{2+}-activated ATPase (SERCA). The Ca^{2+} concentration in the lumen of the ER is typically about 100-500 μM (2, 112), which is about 1000 to 5000-fold higher than in the surrounding cytoplasm. The perinuclear space is also an important Ca^{2+} storage compartment. The intracellular Ca^{2+} stores contain Ca^{2+}-binding buffering proteins (i.e. calsequestrin, calreticulin, etc.) that sequester Ca^{2+} and maintain a low free Ca^{2+} concentration in the lumen of the Ca^{2+} store. Mitochondria, acting as local Ca^{2+} buffers, are additional cellular tools for the regulation of Ca^{2+} homeostasis.

Ca^{2+} as a second messenger

The low cytoplasmic Ca^{2+} concentration of resting cells makes Ca^{2+} very well suited for signalling purposes, since the influx of even small amounts of Ca^{2+}

into the cytoplasm will result in a relatively large increase in the intracellular Ca^{2+} concentration.

Various extracellular signals can promote the movement of Ca^{2+} into the intracellular milieu, either from outside the cell via plasma membrane Ca^{2+} channels, or from intracellular stores via specific receptors in the ER/SR or nuclear membranes (Figure 1). The mechanisms by which this occurs differ between excitable and non-excitable cells. In non-excitable cells such as blood cells, hepatocytes and endothelial cells, two stages of Ca^{2+} entry into the cytoplasm have been distinguished. The first stage is through $InsP_3$-mediated release of Ca^{2+} from intracellular stores. Activation of receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GCRs) at the cell surface results in release of $InsP_3$. GCRs stimulate phospholipase Cβ (PLCβ) and RTKs activate phospholipase Cγ (PLCγ), both resulting in the conversion of phosphatidylinositol-(4,5)-bisphosphate into inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ acts as an intracellular second messenger by binding to the $InsP_3$ receptor ($InsP_3R$) in the ER membrane and, thus, triggers the release of Ca^{2+} from the ER. $InsP_3$-mediated signal transduction can increase the intracellular Ca^{2+} concentration from approximately 100 nM to approximately 1 μM (14, 32). In addition to $InsP_3R$, the ER membrane contains the ryanodine receptor (RyR). $InsP_3R$ and RyR are both structurally and functionally related, but while $InsP_3R$ is activated by $InsP_3$, RyR is activated by either Ca^{2+}, cADPr or depolarisation of the plasma membrane. The release of Ca^{2+} from the ER is closely followed by the second stage of Ca^{2+} entry into the cytoplasm, which involves a sustained influx of Ca^{2+} through Ca^{2+} channels in the plasma membrane. The signal which leads to opening of the plasma membrane Ca^{2+} channels appears to be the actual decrease in Ca^{2+} concentration in the ER lumen (123), but the mechanism behind this so-called "capacitative calcium entry" or "store-operated calcium entry" is not entirely clear (reviewed in (156) and references therein). In excitable cells such as neuronal and muscle cells, there is an additional system to import Ca^{2+} to the cytoplasm and this is via voltage-gated Ca^{2+} channels. Upon depolarisation of the plasma membrane, the voltage-gated Ca^{2+} channels change conformation, allowing Ca^{2+} to enter the cell.

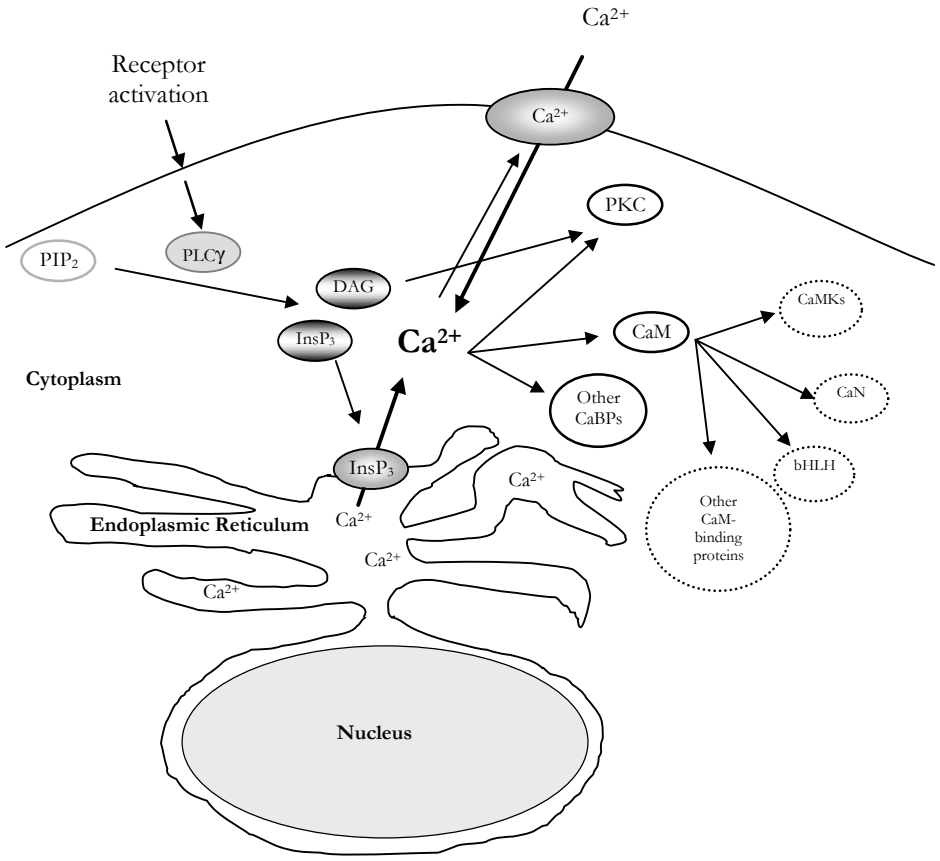

Figure 1. Regulation of intracellular calcium in response to receptor activation. CaBPs = Calcium-binding proteins.

Processes as diverse as muscle contraction, fertilisation, cell proliferation, vesicular fusion, apoptosis and transcription are all, in part, regulated by changes in the concentration of intracellular Ca^{2+}. One might ask what gives specificity to a Ca^{2+} signal. How does the cell discriminate between, for instance, an "apoptotic" and a "proliferative" Ca^{2+} signal? The cellular response to a Ca^{2+} signal depends on how the Ca^{2+} ions entered the cell, the cellular localisation of the Ca^{2+} increase and the modulation of the Ca^{2+} signal itself. The particular membrane channel or intracellular receptor responsible for the release of Ca^{2+} has a great influence on the eventual effects of the Ca^{2+}

signal. The mode of cellular entry also influences the site of action of the Ca^{2+} signal. To a great extent, Ca^{2+} signalling can be localised to distinct parts of the cell (1).

Ca^{2+} signals can take on many different forms. They can be single transient peaks of $[Ca^{2+}_i]$, they can be sustained plateaux of elevated $[Ca^{2+}_i]$, or they can – because of the feedback regulation of the pathways responsible for Ca^{2+} mobilisation – be organised in the form of oscillations. As opposed to a static increase in $[Ca^{2+}_i]$, Ca^{2+} oscillations are capable of transducing a more complex message. Ca^{2+} oscillations have been reported to reduce the effective Ca^{2+} threshold for activating transcription factors, thereby increasing signal detection at low levels of stimulation (50, 99). In addition, Dolmetsch et al. have shown that part of the specificity in transcription factor activation is encoded by the frequency of the Ca^{2+} oscillations (50). Furthermore, Hu et al. have shown that by tuning the frequency of Ca^{2+} oscillations in HAEC cells, the activity of NF-κB is altered as a consequence (68). The frequency, however, is not the only important modulation of Ca^{2+} oscillations. It is evident that the amplitude, duration and location of the Ca^{2+} increase are also parameters that will affect the outcome of the signal. For instance, the two stages of Ca^{2+} entry into the cytoplasm described above are differentially coupled to signalling pathways in B cells. The transient spike of Ca^{2+} that results from depletion of the Ca^{2+} stores is sufficient to activate certain signalling pathways and transcription factors such as NF-κB and JNK (49), but is insufficient for the activation of NFAT. Instead, activation of NFAT, which is another Ca^{2+}-activated transcription factor, requires a sustained increase in Ca^{2+} concentration through capacitative calcium entry (49).

Ca^{2+}-binding proteins

Ca^{2+}-binding proteins in eukaryotes can be subdivided into two broad categories, Ca^{2+} buffers and Ca^{2+} sensors. Ca^{2+} buffer proteins such as calreticulin and calsequestrin bind Ca^{2+} and modulate the $[Ca^{2+}_i]$, for instance, by transporting or storing Ca^{2+}. These proteins typically bind Ca^{2+} with low affinity (K_d in the high μM to mM range) and high capacity (20-100 mol Ca^{2+}/mole of protein) and their main task is to decrease the free Ca^{2+} concentration in the lumen of the ER (calreticulin) and SR (calsequestrin).

Besides this passive function, many Ca^{2+} storage proteins also participate in the control of Ca^{2+} homeostasis and are involved in Ca^{2+}-dependent cellular processes.

Ca^{2+} sensors are proteins that bind Ca^{2+} and decode the information of the Ca^{2+} signal. Ca^{2+}-regulated mechanisms start with the detection of increased intracellular concentrations of Ca^{2+} by specific Ca^{2+} sensors, which, in turn and more or less directly, transduce the Ca^{2+} signal. Some of these proteins, such as DRE antagonist modulator (DREAM) and some of the protein kinase C (PKC) family members, are directly regulated in a Ca^{2+}-dependent manner – and are themselves effectors of the Ca^{2+} signal. Other Ca^{2+} sensors, however, are intermediaries in the Ca^{2+} signalling pathway. They translate and transduce the Ca^{2+} signal, resulting in cellular changes through biochemical changes in other proteins. Calmodulin, troponinC and the S100 protein family are examples of this second group of Ca^{2+} sensors.

Calmodulin

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein which may be considered as the primary intracellular Ca^{2+} sensor. CaM plays a major role in the Ca^{2+}-dependent regulation of a wide variety of cellular processes such as secretion, cell motility and contraction, ion homeostasis, energy and nucleotide metabolism, cell cycle progression and transcription (162). Several studies have shown that CaM is essential for viability. Deletion or disruption of the CaM gene results in growth arrest in *Schizosaccharomyces pombe*, and a recessive lethal phenotype in *Saccharomyces cerevisiae* and *Aspergillus nidulans* (44, 124, 154). In line with the essential function of CaM, its amino acid sequence is highly conserved throughout evolution and across species. In fact, all known vertebrate calmodulins are identical, and it is only when diverse organisms such as vertebrates and protozoans are compared that the proportion of non-identical amino acids exceeds 10% (92). CaM belongs to the EF-hand family of Ca^{2+}-binding proteins and has four EF-hand Ca^{2+}-binding motifs. The affinity of these Ca^{2+}-binding sites is such that at resting cell Ca^{2+} concentrations, CaM will predominantly be in a non-Ca^{2+} bound form, whereas upon stimulation all four EF-hands will be occupied. As a result of the binding of Ca^{2+}, CaM undergoes a conformational change that (for most target proteins) enables it to bind and activate the protein. This is the conventional behaviour, but there are

additional modes of target regulation by CaM. CaM binding proteins can be divided into at least five different categories, based on their modes of regulation in the presence or absence of Ca^{2+} (31). These are summarised in Table 1.

Table 1. Different classes of CaM-binding proteins.

Class:	**Examples:**
A. Proteins that bind essentially irreversible to CaM, both in presence and absence of increased $[Ca^{2+}_i]$	phosphorylase kinase
B. Proteins that bind to CaM in the absence of Ca^{2+} and dissociate in the presence of Ca^{2+}.	neuromodulin, neurogranin (81)
C. Proteins that bind CaM with low affinity and are inactive at low $[Ca^{2+}]$. At high $[Ca^{2+}]$ these proteins form high affinity complexes with CaM and are activated.	smooth-muscle myosin-light-chain-kinase (MLCK), calcineurin (89, 103)
D. Proteins that bind to CaM in the presence of Ca^{2+} but are inhibited by this interaction.	some G-protein-receptor kinases (73), $InsP_3R$ type 1 (65)
E. Proteins that bind CaM in the presence of Ca^{2+} and are activated by this interaction.	CaM-dependent kinases I, II and IV

Calmodulin action through calmodulin-dependent kinases and phosphatase

CaM exerts many of its functions through the activation of CaM-dependent kinases and phosphatase (162). The group of CaM-dependent kinases (CaMKs) (Table 2) can be divided into two general categories: dedicated and multifunctional CaM kinases (142). The dedicated CaM-dependent kinases, such as myosin light chain kinase (MLCK) and elongation factor 2 kinase (eEF-2K), are, as their names suggest, dedicated to phosphorylate only one substrate, the regulatory light chain (RLC) of myosin and elongation factor 2 (eEF-2), respectively. CaMKK has three known substrates and CaMKII and IV are multifunctional – meaning that they have a broad spectrum of cellular substrates. All CaMKs have some structural features in common, the catalytic (kinase) domain and a regulatory domain. The regulatory domain regulates the kinase activity and consists of two somewhat overlapping domains: an autoinhibitory sequence resembling a CaMK substrate and a CaM binding site. In the absence of Ca^{2+}/CaM, the autoinhibitory sequence interacts with the catalytic domain and acts as a pseudosubstrate, thereby blocking kinase activity. Binding of Ca^{2+}/CaM to the CaM binding site induces structural changes that release the inhibition. Regulation of CaMKs also involves phosphorylation events. To date, only one CaM-dependent phosphatase, calcineurin (CaN), has been identified.

Table 2. Properties of major mammalian Ca^{2+}/calmodulin-dependent protein kinases (142)**.**

CaM kinase	**MW**	**Holoenzyme**	**Substrates**	**Regulation by phosphorylation**
CaMKI	42 kD	monomer	unknown	phosphorylation by CaMKK leads to increased activity
CaMKII	50-60 kD	multimer	multiple	autophosphorylation leads to constitutive activity and enhanced binding of Ca^{2+}/CaM
eEF-2K (CaMKIII)	100 kD	monomer	eEF-2	autophosphorylation and phosporylation by PKA both lead to constitutive activity
CaMKIV	65-67 kD	monomer	multiple	phosphorylation by CaMKK leads to increased total and constitutive activity, autophosphorylation leads to activation
CaMKK	55-65 kD	monomer	CaMKI and IV, PKB	phosphorylation by PKA is inhibitory
MLCK	67-210 kD	monomer	RLC	phosphorylation by PKA, PKC, CaMKII and PAK increases K_{CaM} or V_{max}

CaMKII

The CaMKII family

CaMKII is the most intensely studied member of the multifunctional CaMKs. The CaMKII family (70, 71) is encoded by four closely related, yet distinct genes termed α, β, γ and δ. The γ and δ isoforms have a broad tissue distribution, whereas the α and β isoforms are most abundant in the brain, constituting up to 2% of total protein in the hippocampus. The general structure of CaMKII is shown in figure 2a. All of the isoforms of CaMKII have a similar domain organisation with an N-terminal catalytic domain, a central regulatory region with partly overlapping autoinhibitory and CaM-binding domains, two variable regions and a C-terminal subunit association domain. The four isoforms share approximately 89-93% sequence similarity in their catalytic and regulatory domains (159), and the primary difference between the CaMKII isoforms results from insertions or deletions in the variable regions. The CaMKII family is further expanded by alternative splicing. Each of the CaMKII genes gives rise to multiple isoforms. Today, there are in total 30 known isoforms of CaMKII: 3 α, 6 β, 8 γ and 13 δ isoforms (references in (70)). CaMKIIs form multi-subunit holoenzymes (see Figure 2b), composed of identical or mixed isoforms. Each holoenzyme has been suggested to be composed of twelve subunits, and electron microscopic images of the CaMKII holoenzyme have revealed its hub-and-spoke-like assembly in which the association domains are gathered in the centre and the catalytic domains are arranged in a peripheral ring (83, 93, 114). Hoelz et al. have recently solved the crystal structure of the association domain of CaMKIIα and their results support the hub-like assembly of the holoenzyme, but they found that each holoenzyme is composed of fourteen subunits, arranged as two seven-membered rings stacked head to head (66).

Regulation of CaMKII

Regulation of the activity of CaMKII occurs at several levels, including Ca^{2+}/CaM-binding, autophosphorylation and multimerisation (reviewed in (71)). In the absence of Ca^{2+}-loaded CaM, autoinhibition restricts the enzymatic activity of CaMKII to basal levels, which are 100-1000 fold lower than the maximal Ca^{2+}/CaM-stimulated level. Ca^{2+}/CaM binding disrupts the interaction between the catalytic and the autoinhibitory domains, leading to kinase activation. This is followed by a rapid autophosphorylation of Thr 286.

The autophosphorylation takes place between adjacent Ca^{2+}/CaM-bound subunits in the oligomeric complex. Following autophosphorylation, the dissociation rate for Ca^{2+}/CaM upon removal of Ca^{2+} is decreased by several orders of magnitude, a phenomenon which is called CaM trapping and which allows the kinase to remain active even when the Ca^{2+}-concentration has returned to basal levels. A second consequence of the autophosphorylation is that even after full dissociation of Ca^{2+}/CaM, the kinase retains partial activity (called autonomy) owing to the fact that the autophosphorylation inhibits the interaction between the autoinhibitory and the catalytic domains. Subunit composition of the holoenzymes works as another level of regulation of CaMKII, and has been shown to affect the sensitivity of CaMKII to activation by Ca^{2+}/CaM (19).

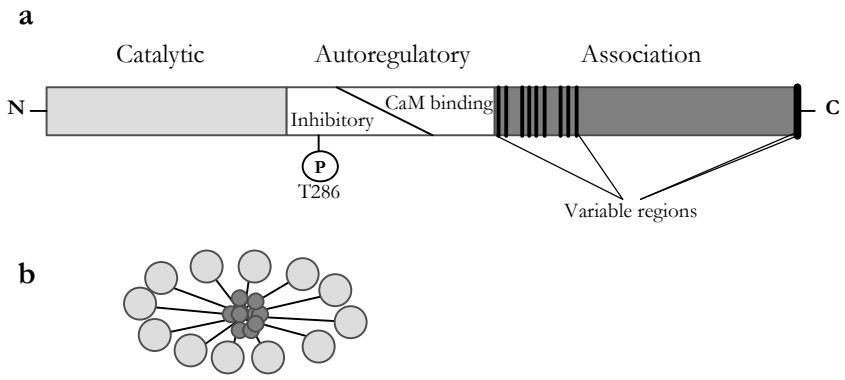

Figure 2. CaMKII. a) Domain organisation of the CaMKIIs. **b)** The CaMKII holoenzyme.

The calmodulin-dependent kinase cascade: CaMKK, CaMKI and CaMKIV

Some of the CaMKs are, like many other protein kinases, involved in a kinase cascade. The CaM-kinase cascade appears to consist of the three related CaMKs: CaMKI, CaMKIV and their upstream activator CaMKK. CaMKI has a broad tissue distribution in mammals and is cytosolic (122), whereas the two splice variants of CaMKIV are strongly expressed in neural tissue, T cells and testis (141). CaMKIV is predominately localised in the nucleus but may also be detected in the cytoplasm (79). In the absence of Ca^{2+}/CaM, CaMKI and CaMKIV are inactive due to intramolecular steric inhibition of the active sites by a C-terminal autoinhibitory domain. The binding of Ca^{2+}/CaM releases this autoinhibition, and the activity of the kinases can be further increased 10-50-fold following phosphorylation of a single threonine residue in the activation loop. The kinase responsible for this activating phosphorylation, CaMKK, is also subject to Ca^{2+}/CaM-mediated relief of autoinhibition (160). CaMKIV has been suggested to play a role in (i) long-term potentiation (16), (ii) the Ca^{2+}-dependent switch of Epstein-Barr virus from latency to viral replication (26), and (iii) thymocyte development and activation of mature T cells (3). The physiological function of CaMKI is, however, not yet known.

Calcineurin

As far as is known, the serine/threonine protein phosphatase calcineurin, also called protein phosphatase 2B, is the only phosphatase controlled by Ca^{2+}/CaM. Calcineurin is a heterodimer of a 58-64 kD catalytic and CaM-binding subunit, calcineurin A, tightly bound to a 19 kD Ca^{2+}-binding regulatory subunit, calcineurin B (91). Like CaM, calcineurin B is a member of the EF-hand family of Ca^{2+}-binding proteins and has four Ca^{2+}-binding sites, one high affinity site ($k_d < 10^{-7}$ M) and three with affinities in the micromolar range (82). The Ca^{2+} dependence of the phosphatase activity of calcineurin is controlled by both calcineurin B and CaM. At Ca^{2+} concentrations less than 10^{-7} M, calcineurin B, with its high affinity site occupied, is bound to calcineurin A, but the enzyme is inactive. At higher Ca^{2+} concentrations (micromolar), all Ca^{2+}-binding sites of calcineurin B are occupied and this results in a small degree of activation. To achieve full activation of calcineurin, however, Ca^{2+}-dependent binding of CaM to calcineurin A is required. As with most CaM-regulated enzymes, the mechanism of activation of calcineurin is thought to be

one whereby CaM, by binding to the CaM-binding domain, displaces an autoinhibitory domain (38, 91).
Calcineurin has important roles in many Ca^{2+}-dependent cellular processes, ranging from the pheromone response pathway in yeast (40) to regulation of expression of interleukin-2 and other cytokines in T cells (37). In fact, inhibition of calcineurin by the two immunosuppressive agents cyclosporin A and FK506 blocks T cell activation and is a very important and widely used tool for prevention of organ rejection after transplants.

Calmodulin as a regulator of transcription
CaM has been shown to regulate transcription both indirectly via CaM dependent kinases and phosphatase, and directly via interaction with transcription factors.

- **Indirect action of calmodulin**

The Ca^{2+}/CaM-dependent phosphatase calcineurin is involved in the regulation of a number of transcription factors (reviewed in (37)) but by far the most well-studied example is the nuclear factor of activated T-cells (NFAT) family of proteins. The sub-cellular localisation of NFAT is regulated by phosphorylation. Phosphorylation of serines within the SP repeats and the serine-rich region of NFAT proteins hides the nuclear localisation sequences needed for nuclear import. T cell receptor occupancy results in both an increase in the intracellular Ca^{2+} concentration and activation of a kinase cascade. The increased Ca^{2+} activates calcineurin, leading to dephosphorylation and subsequent nuclear translocation of NFAT. In the nucleus, GSK3 has been suggested to be the main kinase that re-phosphorylates NFAT, thereby bringing about nuclear export.
Calcineurin plays a positive role also in the regulation of NF-κB/Rel transcription factors. In this case, calcineurin seems to act at two different levels. Firstly, calcineurin apparently indirectly promotes the phosphorylation that leads to degradation of the inhibitory IκB proteins and, secondly, calcineurin affects the expression of c-Rel in a positive way. Calcineurin is also involved in the regulation of other transcription factors. It interacts with the AML1 transcription factor and enhances transcriptional activation of the GM-CSF promoter by AML1 (H. Liu, M. Holm, X. Xie and T. Grundström,

unpublished results). Furthermore, calcineurin has been reported to be required for AP-1 mediated transcription from some promoters and, finally, calcineurin has been suggested to mediate Ca^{2+}-dependent control of the activity of MEF2 (37).

The multifunctional Ca^{2+}/CaM-dependent kinases CaMKI, CaMKII and CaMKIV have all been attributed functions in the regulation of transcription. One common feature of these kinases is that they can phosphorylate the activating Ser 133 residue of the transcription factor cAMP responsive element binding protein (CREB) *in vitro* (39, 41, 138, 149). Ser 133 phosphorylation enables the interaction between CREB and the transcriptional co-activator CREB-binding protein (CBP) and is absolutely required for transcriptional activation by CREB. Over-expression of CaMKI or CaMKIV stimulates CREB-dependent transcription (107, 149, 150), but since CaMKI is a cytoplasmic protein, CaMKIV, and not CaMKI, is likely to be a physiological CREB kinase. Despite its ability to phosphorylate Ser 133, over-expression of CaMKII does not activate CREB-dependent transcription (107, 149, 150). This is believed to be due to phosphorylation of an additional CREB residue, Ser 142, by CaMKII (149). This phosphorylation is inhibitory to CREB and dominates over the activating phosphorylation of Ser 133, suggesting that CaMKII may regulate CREB negatively by phosphorylation of Ser 142. In addition to CREB, there is also evidence for CaMKIV-mediated phosphorylation of the transcription factors SRF and ATF-1 (reviewed in (3)). Interestingly, the co-activator CBP appears to require phosphorylation by CaMKIV for its activity (27). CBP is a co-activator used by many transcription factors, suggesting that phosphorylation of CBP by CaMKIV may represent a mechanism for Ca^{2+}-dependent regulation of the expression of numerous genes.

- **Direct action of calmodulin**

In addition to affecting transcription in an indirect way, via CaM-dependent phosphatase and kinases, CaM also regulates transcription by direct interaction with some transcription factors. Transcription factors of the basic helix-loop-helix (bHLH) family are key regulators during many processes including myogenesis, neurogenesis and hematopoiesis (105). Ca^{2+}-loaded CaM interacts with certain bHLH proteins, resulting in a block in DNA binding and, subsequently, transcriptional repression (36). Smad proteins are intracellular

mediators of transforming growth factor β (TGF-β) and activin signalling. Zimmerman et al. have shown that a number of both *Xenopus* and human Smad proteins can interact with CaM (175), and Scherer & Graff have found that the activities of Smad1 and Smad2 are regulated by interaction with CaM (131). In this case, CaM was shown to have opposite effects on the two Smad family members, namely increasing Smad1 activity while inhibiting Smad2 function. CaM-binding dependent regulation has been suggested also for the glucocorticoid receptor (118) and the testis determining factor SRY (62).

NUCLEAR TRANSPORT

Eukaryotic cells are divided into distinct compartments such as the cytoplasm, nucleus and mitochondria. This makes intercompartmental transport of molecules necessary and specialised systems have evolved which mediate this. In this context, nuclear transport is unusual since it goes in both directions, whereas in other organelles, transport is mostly a one-way process. Nuclear proteins are made in the cytoplasm and must be imported to the nucleus. RNA is transcribed in the nucleus, so to get any protein synthesis at all, export of RNA from the nucleus to the cytoplasm is required. Many proteins shuttle between the cytoplasm and the nucleus more or less continuously. Altogether, there is a massive flux of molecules between the cytoplasm and nucleus. One estimate suggests that more than 1 million macromolecules per minute pass the nuclear pore complexes (NPCs) in a growing mammalian cell (59). NPCs are the structures that connect the nucleus with the cytoplasm (reviewed in (129)). These large protein complexes (estimated size in vertebrates is 125 MDa) have a highly conserved structure with cytoplasmic fibrils, a central channel and a nuclear basket. The proteins that make up the NPC are called nucleoporins.

a

	NLS
SV40 large T antigen	PKKKRKV
Nucleoplasmin	KRPAATKKAGQAKKKK
RelA	KRKR
c-Rel	KKQK

b

	NES
PKI	LALKLAGLDIN
HIV-1 Rev	LQLPPLERLTLD
IκBα	IQQQLGQLTLENL

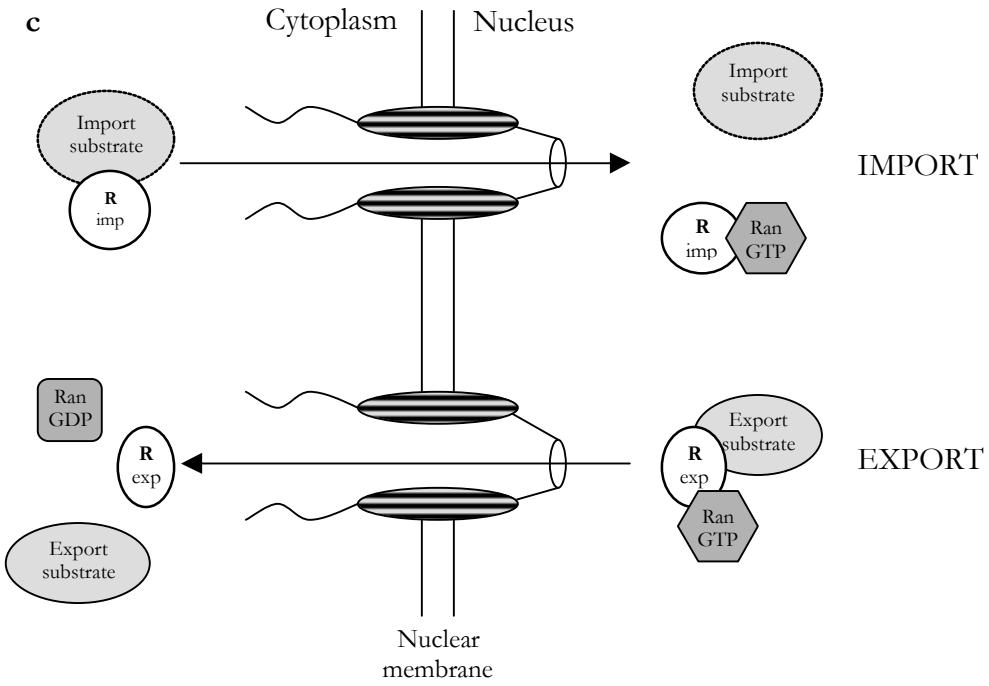

Figure 3. Nuclear transport. Examples of **a)** nuclear localisation signals (NLS) (12, 171) and **b)** nuclear export signals (NES) (8, 171). **c)** A model of nuclear import and export, see text for details. R imp = import receptor, R exp = export receptor.

Nuclear import

The transport of proteins across the nuclear envelope during nuclear import is a highly regulated process (reviewed in (106, 115, 144)). Proteins which undergo regulated nuclear import harbour specific nuclear localisation signals (NLSs) that act as markers for the import machinery. Since the topic of this thesis is regulation of NF-κB proteins, and NF-κB proteins have so-called "classical" NLSs, this introduction to nuclear import will focus on nuclear import mediated by this type of NLS. The classical NLS is either a short stretch of basic amino acids (monopartite NLS), or a longer sequence containing two clusters of basic amino acids separated by a flexible spacer (bipartite NLS) (for examples, see Figure 3a). Classical NLSs are recognised and bound by importin-α, a member of the importin family of proteins (also termed karyopherins). The importins are dedicated transport receptors which are able to move NLS-containing proteins from the cytoplasm to the nucleus through the nuclear pores. Importin-α cannot mediate nuclear import by itself, but acts as an adaptor by linking the cargo protein to importin-β. The cargo protein/importin-α/importin-β complex is then believed to dock at the cytoplasmic fibrils of the NPC via interactions between importin-β and certain nucleoporins. The subsequent translocation of the cargo protein/importin-α/importin-β complex through the NPC is not understood, but may be through facilitated diffusion controlled by association and dissociation between importin-β and nucleoporins located in the interior of the NPC. Finally, the cargo protein/importin-α/importin-β complex ends up in the so-called nuclear basket, which is an extension of the NPC into the nucleus. The small GTPase Ran is required for nuclear transport (Figure 3c). The concentration of Ran bound to GTP is high in the nucleus and low in the cytoplasm, and the reverse is true for GDP-bound Ran. This is a result of the nuclear localisation of the Ran GTP-GDP exchange factor (RanGEF) and the cytoplasmic localisation of the RanGTPase activating protein (RanGAP). It is this differential cellular distribution of its GTP- and GDP-bound forms that promotes the directionality of nuclear transport. The direct binding of RanGTP to importin-β induces the release of the imported protein into the nuclear compartment. The importin-β/RanGTP complex is subsequently exported to the cytoplasm where GTP hydrolysis turns RanGTP into RanGDP. RanGDP dissociates from importin-β, leaving importin-β free to start a new round of import, while RanGDP is delivered back to the nucleus by NTF2, a RanGDP-specific import receptor.

Nuclear export

Protein transport through the NPCs is bidirectional. As discussed above, protein import to the nucleus is a regulated process and this holds true also for the "mirror process", protein export (reviewed in (106, 115, 144)). The import and export processes are, in fact, quite similar (Figure 3c). Nuclear export also depends on specific localisation signals, in this case nuclear export signals (NESs) (for examples, see Figure 3b), and nuclear export is also dependent on the RanGTPase. Furthermore, the nuclear export receptors CAS and CRM1 are functionally related to importin-β. Export receptors also bind RanGTP but, in contrast to the effect on importin-β, RanGTP binding *induces* the interaction between the export receptor and the cargo protein. So the fact that the concentration of RanGTP is high in the nucleus makes it possible to form the export complexes. These cargo protein/export receptor complexes pass through the NPC into the cytoplasm where they dissociate due to GTP hydrolysis. Like import receptors, the export receptors are also recycled and used for new rounds of transport.

Role of nuclear transport in regulation of transcription factors

Regulation of sub-cellular localisation plays an important role in determining the activity of a number of transcription factors, for example the STAT (90), NFAT and NF-κB protein families are regulated in this way (see sections: *Calmodulin as a regulator of transcription* and *Regulation of NF-kB activity,* respectively). Activating signals result in the translocation of these transcription factors from the cytoplasm into the nucleus where they can bind to their target DNA sequences and regulate transcription. This type of regulation allows a very rapid induction of transcription, since it does not require new synthesis of the transcription factors.

THE NF-kB TRANSCRIPTION FACTORS

History

The first publication on nuclear factor-κB (NF-κB) was from Sen and Baltimore and came in 1986 (134). Using electrophoretic mobility shift assay (EMSA), the authors detected a protein that bound to a decameric sequence present in the intronic enhancer element of the immunoglobulin κ light chain (Igκ) gene, called the κB sequence. Because this protein was constitutively present in the nuclei of certain B cells, it was first believed to be a nuclear protein with a B cell-restricted expression pattern. Later it became clear that NF-κB is present in other cell types in an inactive cytoplasmic form which, upon cellular stimulation, could be induced to translocate into the nucleus and bind to and regulate many different enhancers and promoters. The subsequent cloning of the genes encoding the NF-κB proteins p50 and p65 (RelA) revealed a striking homology with members of the Rel family, namely the viral oncogene *v-rel,* the proto-oncogene *c-rel* and the *Drosophila* morphogen *dorsal* (18, 20, 23, 55, 60, 88, 110, 119, 127, 145, 146, 167). Since these reports, more members from different species have been added (58) and NF-κB, Rel, Rel/NF-κB and NF-κB/Rel are different ways of naming this family of transcription factors. In this thesis, the name "NF-κB" is used to refer to all family members.

The discovery of NF-κB was the starting point of what has to be recognised as a very broad, intense and fast-growing field of research. In 1996, 10 years after the initial finding, a Medline search for NF-κB resulted in approximately 2 000 publications. In an NF-κB-meeting review that year, Baeuerle and Baltimore concluded that "…this transcription factor system is still a hot discovery zone and far from having reached a state of clean-up experimentation" (10). This remark has proven to be true. Today, after seven more years, the NF-κB field has produced more than 14 000 publications and the end is still not in view.

Family members

NF-κB is a family of dimeric transcription factors that all share a highly conserved Rel homology domain (RHD). The approximately 300 amino acid RHD is located in the amino terminal parts of the NF-κB proteins and is responsible for dimerisation, DNA binding, nuclear import and interactions with a family of inhibitory κB proteins, the IκBs (see Figure 4). Five NF-κB proteins have been identified in mammalian cells: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel, RelA (also called p65) and RelB (57). All mammalian NF-κB proteins can form both homodimers and heterodimers, except for RelB which can only form heterodimers (109). In *Drosophila*, three family members have been identified, Dorsal, Dif (75) and Relish (51).

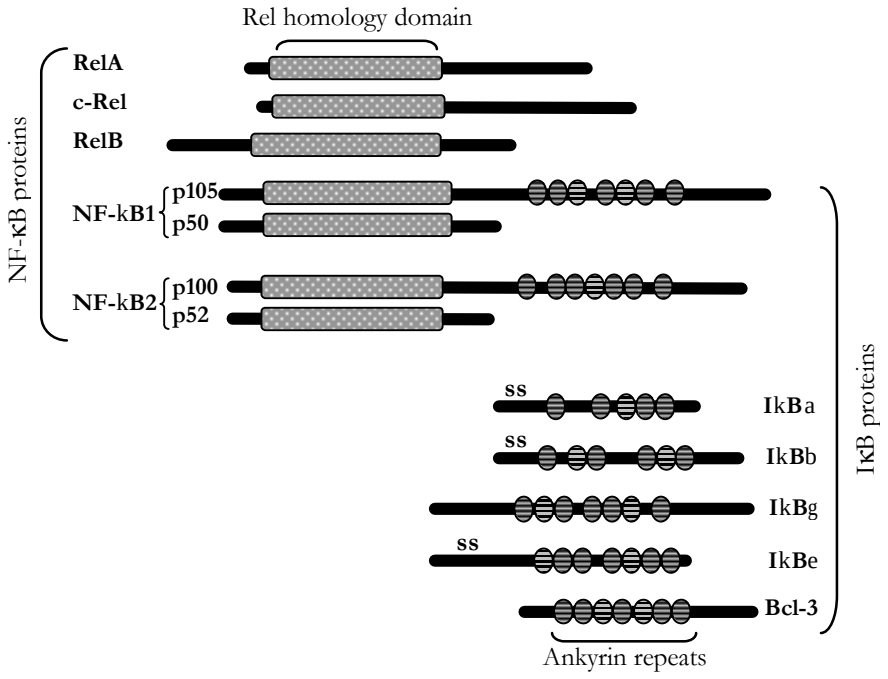

Figure 4. The mammalian NF-kB and IkB families.

The NF-κB proteins are divided into two groups. c-Rel , RelA, RelB, Dorsal and Dif belong to the first group, and have carboxy-terminal transactivation domains and are therefore able to activate transcription by themselves. The second group of NF-κB proteins consists of NF-κB1 (p105/p50), NF-κB2 (p100/p52) and Relish (139). These proteins are synthesised as large precursor proteins that, in addition to the amino-terminal RHD, contain an autoinhibitory IκB-like ankyrin repeat domain (see Figure 4). Activation of p105 and p100 involves their proteasome-dependent processing into the shorter active variants (p50 and p52, respectively), either constitutively (p105) or in a regulated fashion (p100) (139). It has also been suggested that co-translational dimerisation of the RHD of p50 with p105 generates p50/p105 heterodimers and that this mechanism is important for effective p50 production (101). p50 and p52 lack transcriptional activation domains but can still participate in transcriptional activation when in a heterodimeric complex with RelA, c-Rel or RelB. Relish is activated by signal-induced proteasome-independent endoproteolysis which generates two stable products, the RHD that translocates to the nucleus where it activates transcription, and the IκB-like domain which remains cytoplasmic (147, 148).

NF-κB/Rel proteins bind to DNA sequences that are collectively referred to as κB sites. The consensus sequence of the κB sites is GGGRNNYYCC, where R is a purine, Y is a pyrimidine and N is any base (111). As mentioned above, all mammalian NF-κB proteins can form heterodimers with each other and all but RelB can form homodimers. Moreover, each NF-κB dimer has its own binding preferences towards variants of the κB site. These features of the NF-κB proteins contribute to the regulatory diversity that enables this fairly small group of proteins to regulate a vast number of target genes in the right cells and under the right conditions.

Regulation of NF-kB activity

In contrast to many other transcription factors, NF-κB is always present in cells, awaiting a signal that leads to its activation. This makes a rapid activation process possible, but also calls for a tight control of its regulation in order to avoid unwanted "outbreaks" of activated NF-κB. Dysregulation of NF-κB activity contributes to many different diseases (see the section *NF-kB and disease*), which emphasises the importance of keeping NF-κB under strict control. Figure 5 is a model showing the various steps of the NF-κB activation pathway, which will be described in some detail below.

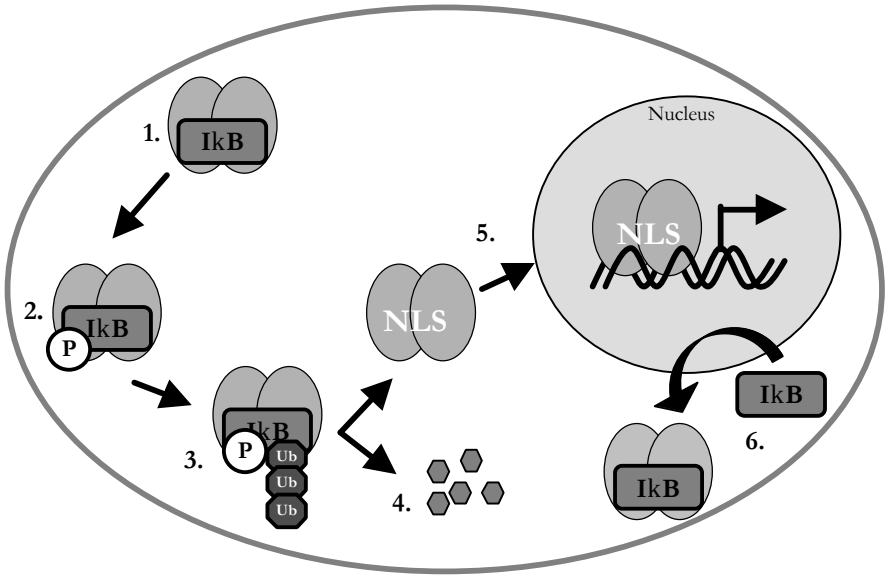

Figure 5. A schematic view of NF-kB activation. In most resting cells, NF-κB is sequestered in the cytoplasm through its interaction with IκB **(1).** Signal induced phosphorylation of IκB **(2)** leads to its ubiquitination (Ub) **(3)** and proteasome-dependent degradation **(4).** After IκB has been removed, the NLS of NF-κB is exposed and NF-κB can localise to the nucleus and regulate its target genes **(5).** The IκB gene is one such target gene. Newly synthesised IκB can enter the nucleus, remove NF-κB from the DNA and export NF-κB to the cytoplasm **(6).**

- **The basal state of NF-kB activity: the role of the IkBs**

In all unstimulated cells, except mature B-cells, NF-κB transcription factors are kept inactive by members of a second protein family, the inhibitory κB proteins (IκBs). By binding to the RHD of NF-κB, IκB masks the nuclear localisation sequence (NLS), causing cytoplasmic retention and thus inactivation of NF-κB. The IκB family consists of IκBα, IκBβ, IκBε, IκBγ, Bcl-3 and the *Drosophila* protein cactus (see Figure 4). A common feature of all IκB family members is that they contain protein interaction motifs called ankyrin repeats. The number of ankyrin repeats differs between IκB family members but the function is the same, namely to mediate interaction with the RHD of NF-κB. As mentioned above, the NF-κB proteins p100 and p105 also contain ankyrin repeats. In this way, p100 and p105 can actually repress themselves and any other NF-κB protein that is their heterodimeric partner.
IκBα was the first family member to be cloned (43, 63) and is also the best-characterised IκB protein. Its structure consists of three basic elements: (i) an N-terminal signal response domain containing two serines that become phosphorylated in response to signals, (ii) a central ankyrin repeat domain, and (iii) a C-terminal PEST (Pro, Glu, Ser, and Thr-rich) domain which is involved in regulation of the basal turnover of the protein. When IκBβ and IκBε were later cloned (100, 140, 158, 166), it was found that their structures were very similar to that of IκBα - apart from the fact that IκBε lacks a PEST domain. Despite these structural similarities, IκBα, IκBβ and IκBε are somewhat different functionally. IκBα regulates rapid but transient induction of NF-κB, whereas IκBβ regulates persistent activation in response to a different set of activators (57). Regulation by IκBε is less well understood, but it has been suggested to be a specific inhibitor of c-Rel and RelA (166). IκBγ is actually the C-terminal half of NF-κB1 (p105) resulting from alternate promoter usage (57). The function of IκBγ in regulation of NF-κB has not yet been clarified. Bcl-3 is very different from the other IκBs in that it is nuclear and that, in complex with NF-κB2 (p52), it can function as a transactivator – in contrast to the canonical inhibitory role of the IκBs (57).

- **Phosphorylation of IkB: regulation of the IkB kinase**

Signals which activate NF-κB result in phosphorylation of the two N-terminal Serine residues in the signal response domain of IκB. The kinase responsible for this initiating step of NF-κB activation is the IκB kinase (IKK) complex (reviewed in (85)). IKK is composed of two catalytic subunits, IKKα and

IKKβ (also named IKK1 and IKK2), and a regulatory subunit, IKKγ (also named NEMO or IKKAP1). IKKα and IKKβ are highly homologous proteins (50% sequence identity) and they are both capable of phosphorylating IκB *in vitro*. Despite their similarities *in vitro*, IKKα and IKKβ have distinct *in vivo* functions in the regulation of NF-κB activity. IKKβ is essential for the inducible phosphorylation of IκB, whereas IKKα is required for phosphorylation-induced processing of NF-κB2 (p100) (135, 168). Furthermore, IKKα plays an essential role in skeletal morphogenesis and epidermal differentiation through NF-κB activation- and phosphorylation-independent pathways (69, 153)). The exact mechanisms by which IKKα and IKKβ are activated in response to external signals are not clear, but it appears that the two kinases are differentially regulated. The IKKα-mediated processing of NF-κB2 (p100) is activated by two specific members of the TNF family, lymphotoxin B (LTβ) and BAFF, and by CD40 ligation (33, 35, 45). The IKKβ-dependent, canonical NF-κB activation pathway is activated by many other stimuli, including TNFα and most members of the TNF family, IL-1, innate immune stimuli such as lipopolysaccharide (LPS) and double stranded RNA, and B- and T-cell receptor (BCR and TCR) ligation (reviewed in (56, 128); for a comprehensive list of NF-κB inducers, see (58)). The regulatory subunit IKKγ is thought to link the IKK complex to upstream signalling molecules that regulate its activity. It seems, however, that IKKγ is only essential for the canonical NF-κB activation pathway that uses IKKβ (56). Activation of IKK involves the phosphorylation of regulatory serines located within the activation loops of IKKα and IKKβ, and often the recruitment of IKK to the activated receptor complex. In the case of IKKα-mediated phosphorylation of NF-κB2 (p100), the IKK-activating kinase is believed to be the NF-κB inducing kinase (NIK) (56). As regards IKKβ, it is not clear whether the activating phosphorylation is performed by an IKK-kinase or by transautophosphorylation within the IKK complex, but since knock-outs of putative IKK-kinases have failed to affect IKK activation, transautophosphorylation is the more likely alternative. The induction of transautophosphorylation is thought to be a result of increased proximity between the subunits of the IKK complex (56).

A large number of more or less receptor-specific proteins are involved in transmitting signals from cell surface receptors to the activation of the IKKs. Figure 6 shows some of the more well-established signalling pathways that start with receptor ligation and result in NF-κB activation. TCR-mediated

activation of NF-κB (128, 157) is of special interest in this thesis and is therefore described in greater detail. Binding of the TCR to antigen together with CD28 co-stimulation initiates the activation of TCR-associated tyrosine kinases such as Lck, Fyn and ZAP-70. These kinases phosphorylate adaptor proteins (LAT, SLP-76 and Grb-2) and signalling molecules (Vav and PLCγ) that induce the formation of an immunological synapse. The PKC isoform PKCθ is then recruited into the immunological synapse, resulting in an enrichment of PKCθ, IKK and the ligated TCR in lipid rafts (membrane microdomains that concentrate signalling mediators). The signal is then transmitted further from PKCθ to the IKK complex by way of a three-membered complex containing Bcl-10, Carma1 and MALT1. It is not known whether these proteins are substrates or interaction partners of PKCθ. Neither is the precise mechanism by which Bcl-10/Carma1/MALT1 transduces the signal to the IKK complex known.

Tyrosine phosphorylation of IκBα in response to reoxygenation of hypoxic cells or hydrogen peroxide treatment has also been shown to result in NF-κB activation. Interestingly, tyrosine phosphorylation does not lead to degradation of IκBα (74, 152), but rather induces its dissociation from NF-κB.

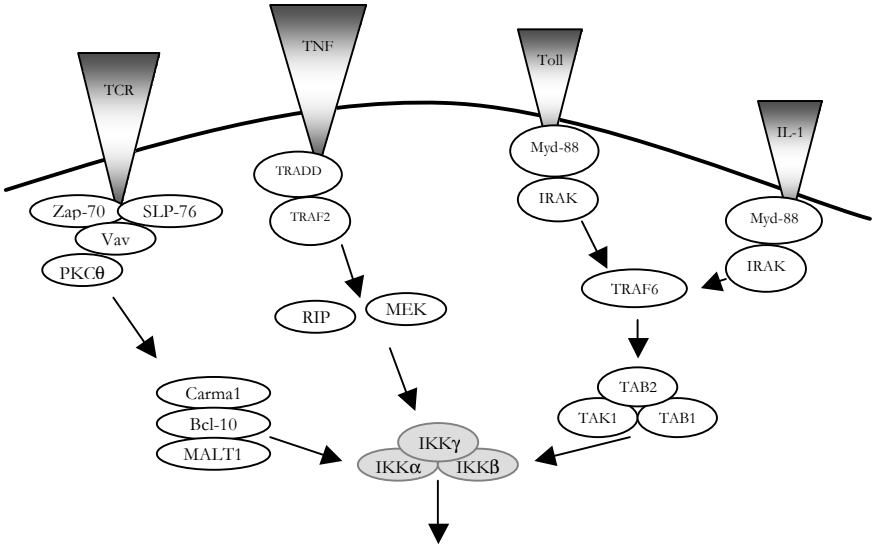

Figure 6. Examples of signalling pathways leading to NF-kB activation (7, 47, 98).

- **Ubiquitination and degradation of IkB**

Phosphorylation does not in itself lead to IκB degradation, but it creates a recognition motif for the ubiquitination system. Phosphorylated IκB is recognised by the F-box WD repeat protein β-TrCP which, in turn, is the receptor for the ubiquitin ligase complex $SCF^{\beta TrCP}$. $SCF^{\beta TrCP}$ collaborates with a ubiquitin conjugating enzyme in building chains of ubiquitin on two N-terminal lysine residues in IκB (K21 and K22 in IκBα). The ubiquitin chains are then recognised by the 26S proteasome which degrades IκB (13).

- **Nuclear translocation of NF-kB**

After IκB has been degraded, the NLS of NF-κB is exposed, thus allowing nuclear import. The NF-κB proteins all contain classical NLSs and are imported to the nucleus by the help of importin-α/β.

- **Regulation of NF-kB activity by modification of the NF-kB proteins**

Research on the regulation of NF-κB has been focused primarily on the events leading to the phosphorylation and degradation of IκB. It is clear, however, that the removal of IκB is only one of many steps in the process of NF-κB activation. To ensure full transactivating activity, modifications such as phosphorylations and acetylations of the NF-κB proteins themselves and the surrounding chromatin environment are required (reviewed in (29, 56, 132)). NF-κB1 (p105), NF-κB 2 (p100), c-Rel and RelA are all constitutively phosphorylated in an unstimulated cell (97, 116, 117) and this basal phosphorylation is increased further by a broad range of stimuli. NF-κB1 (p105) is phosphorylated in response to PMA, PHA, H_2O_2 and TNFα (64, 97, 116); RelA is phoshorylated in response to H_2O_2, TNFα, PMA, IL-1 and LPS (5, 15, 116, 130, 133, 163, 164, 173); and in T-cells, c-Rel is phosphorylated in response to PMA/CD28 and TNFα (22, 104). The kinases that are responsible for these phosphorylation events, the exact phosphorylation sites and the mechanisms by which these phosphorylations enhance NF-κB transcriptional activity are in most cases unknown. Regulation of RelA by protein kinase A (PKA) has, however, been extensively studied by Zhong and co-workers (173, 174) and their results suggest that signal-induced PKA phosphorylation at Ser 276 in RelA makes RelA transcriptionally active by recruiting CBP/p300. Further studies by the same group have demonstrated that phosphorylation of RelA determines whether it associates with either CBP

to form a transcriptionally active complex, or with HDAC-1 to form a transcriptionally inactive complex (172). The phosphorylation sites have also been mapped in the case of TNFα-induced phosphorylation of RelA and c-Rel, and mutation of these greatly impairs TNFα-induced NF-κB dependent transcription (104, 163).

RelA and p50 have both been shown to be targets for protein modification by acetylation, and the acetyltransferases responsible are the co-activators p300 and CBP (28, 30, 46, 53). Acetylation of RelA and p50 increases their DNA binding and impairs the interaction between IκBα and RelA. Furthermore, acetylation of lysine 310 in the transactivation domain of RelA is required for full transcriptional activity of RelA. Taken together, these studies show that acteylation is positive for NF-κB mediated transcriptional activation.

Recently, one of the IκB kinases, IKKα, has been shown to have an unexpected nuclear function. Anest et al. and Yamamoto et al. reported that TNFα induces nuclear import of IKKα. This nuclear pool of IKKα associates with certain κB site-containing promoters/enhancers and phosphorylates histone H3, leading to enhanced transcription of these NF-κB-responsive genes (4, 170).

- **Termination of the NF-kB response**

Since one of the NF-κB target genes is IκBα, NF-κB activation results in new production of its own inhibitor. IκBα contains both an NLS and an NES, allowing an effective negative feedback regulation of NF-κB activity. Newly synthesised IκBα enters the nucleus, where it binds to and blocks the DNA binding of NF-κB. IκBα then mediates the nuclear export of the inactivated NF-κB dimer. IκBα is an inhibitor of NF-κB which is relevant for the majority of activation pathways. There are, however, also pathway-specific NF-κB inhibitors such as A20 and CYLD which both interfere with TNFα induced NF-κB activation (17, 21, 94, 161). A20 is regulated in a way similar to IκB, in that it is induced by TNFα in an NF-κB dependent manner and is detectable after 15 minutes of TNFα stimulation. The mechanism by which A20 blocks NF-κB activation is at present unknown. The tumour suppressor CYLD is a deubiquitinating enzyme that can prevent signalling from the TNF receptor, probably by removing ubiquitins from TRAF2.

- **Not the whole truth…..**

Regulation of NF-κB can be much more complex than what has been revealed in the above section. Structural and functional studies have indicated that in some NF-κB-IκBα complexes, the NLS of one of the two NF-κB subunits is still exposed (72, 76, 102), allowing the complex to shuttle between the cytoplasm and the nucleus. In contrast, IκBβ and IκBε are able to bind to and mask both NLSs in the NF-κB dimer and thus inhibit nucleocytoplasmic shuttling (102, 155). The function of nucleocytoplasmic shuttling of IκBα-NF-κB complexes is not obvious. Since IκB blocks the DNA binding function of NF-κB, the shuttling complex is inactivated just like the cystoplasmically retained IκBβ-bound or IκBε-bound NF-κB. It has been suggested that proteasome-dependent degradation of IκBα can occur also in the nucleus (80, 126). If this is the case, the function of shuttling of IκBα-NF-κB complexes could be to provide a target-proximal activation of NF-κB. An alternative viewpoint comes from studies showing that the protein which directs ubiquitination to IκBα, β-TrCP, is predominately a nuclear protein (42). It may be that nucleocytoplasmic shuttling of IκBα-NF-κB is required in order to recruit β-TrCP and enable ubiquitination of IκBα.

Target genes and differences between family members

To date, close to 200 NF-κB target genes have been described (see Table 3 for examples, and (58) for an extensive list) and many more have κB sites in their promoters, but have not yet been clearly shown to be controlled by NF-κB. Many NF-κB target genes are involved in the regulation of immune and inflammatory responses. NF-κB function is critical for for the rapid induction of expression of acute-phase antimicrobial defence genes in response to invading pathogens. NF-κB also up-regulates the expression of many cytokines that are essential for the immune response. Studies of genetic disruptions of NF-κB genes in mice show that NF-κB also plays important roles in the development of adaptive immunity. Lymphocytes from mice lacking individual NF-κB proteins have defects in proliferation, activation and cytokine and antibody production (54). NF-κB2 also appears to be involved in B cell maturation and the formation of secondary lymphoid organs (54, 135).

NF-κB promotes cell proliferation, mainly by activating the expression of D-cyclins, which lead to G1 entry, and by up-regulation of growth factors such as IL-2, GM-CSF and CD40-ligand. NF-κB also regulates the expression of many

anti-apoptotic genes, including cIAPs, c-FLIP, A1/BFL1 and Bcl-X_L, and its central role in protecting cells from apoptosis has been demonstrated by studies of mice lacking either RelA, IKKβ or IKKγ (reviewed in (87)). These animals die during embryogenesis from severe TNF-dependent liver apoptosis. In contrast, some reports have suggested a pro-apoptotic role for NF-κB. This is based on the involvement of NF-κB in expression of the pro-apoptotic proteins death receptor 6 (DR6), DR4, DR5 and Fas. The relevance of this for apoptosis is unclear, however, since NF-κB simultaneously induces the expression of anti-apoptotic factors that neutralise the activity of the pro-apoptotic proteins (87).

In addition to having distinct functions, such as RelA in protection against apoptosis, the NF-κB family members are also functionally redundant when it comes to certain aspects of transcriptional regulation. This is evident from the more severe phenotypes seen in mice that lack more than one NF-κB protein (54). For instance, double knock-out of NF-κB1 and NF-κB2 results in mice that die postnatally and also lack osteoblasts, whereas the single knock-outs survive to adulthood and show no sign of a bone remodelling phenotype.

Many viruses, including human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 (HTLV-1), take full advantage of the services that powerful NF-κB transcription factors provide. They utilise NF-κB for the expression of their own genes, and also to stimulate growth and survival of the cells they have invaded.

Table 3. Examples of NF-kB target genes (84)**.**

genes involved in negative feedback control of NF-kB	IκBα, A20
immunoregulatory genes	chemokines, cytokines, antimicrobial peptides, adhesion molecules, iNOS, COX2
anti apoptotic genes	cIAPs, A1/BFL1, BCL-X_L, c-FLIP
genes promoting cell proliferation	cyclin D1, c-MYC

NF-kB and disease

Defects in the NF-κB system have been reported to contribute to the pathogenesis of a variety of human diseases (reviewed in (6, 11, 84, 151, 169)).

Due to homology with the potent viral oncogene v-Rel, the NF-κB transcription factors have always been considered to be proto-oncogenes. Indeed, many links between the NF-κB family and human cancer have been identified, including chromosomal amplifications, overexpression and rearrangement of NF-κB genes (125). Aberrant NF-κB activity as a result of constitutive activation of upstream signalling kinases or inactivating mutations of IκBs has also been described in several human cancer cell types (125, 151). Many oncogenes activate NF-κB one way or another and several oncogenic viruses, such as human T-cell leukemia virus type I (HTLV-1) and Epstein-Barr virus (EBV), activate NF-κB as part of the transformation process. There are three main mechanisms by which NF-κB proteins can contribute to tumorigenesis: (i) by promoting cell proliferation, (ii) by inhibiting apoptosis, and (iii) by promoting metastasis and angiogenesis through the function of target genes encoding cell-surface proteases, cell-adhesion molecules and chemokines (84).

Activation of NF-κB has been implicated in the pathogenesis of inflammatory diseases. Patients with asthma, rheumatoid arthritis, atherosclerosis and inflammatory bowel disease have increased levels of NF-κB in the affected tissues, and proteins encoded by certain NF-κB target genes are likely to be of importance for the inflammatory responses associated with these diseases (169). The generation of reactive oxygen species (ROS) and ROS-mediated NF-κB activation has been implicated in pancreatic beta cell death, the hallmark of insulin-dependent diabetes mellitus (IDDM) (169). A number of genetic disorders are believed to be caused by defects within or upstream of the NF-κB activation pathway, including ectodermal dysplasia (ED), familial expansile osteolysis (FEO), primary lymphoedema (PL) and incontinentia pigmenti (IP) (6).

Finally, altered NF-κB regulation may also be involved in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (108).

Involvement of calmodulin in the regulation of NF-kB

This thesis concentrates on the role of calmodulin (CaM) in the regulation of NF-κB. CaM has previously been attributed a positive indirect role in the regulation of NF-κB. The Ca^{2+}/CaM-dependent phosphatase calcineurin (CaN) has been implicated in antigen receptor-mediated NF-κB activation for a long time (37). More recently, and in parallel with the studies in this thesis, the Ca^{2+}/CaM-dependent kinase IV (CaMKIV) has been attributed roles in NF-κB activation. Over-expression of CaMKIV has been shown to augment H_2O_2-induced IκB phosphorylation (67). In a different study, CaMKIV was found to interact with and phosphorylate RelA, leading to increased NF-κB dependent transactivation (9, 78).

AIMS OF THIS THESIS

- To analyse the Ca^{2+} /CaM dependence of different NF-κB-inducing stimuli.
- To study properties and function of the interactions between calmodulin and the NF-κB proteins RelA and c-Rel.

RESULTS AND DISCUSSION

The following section is a summary and discussion of the three articles in this thesis. The figures are referred to by the Roman numeral of the article in which they are found, followed by their figure number in the article.

NF-kB TRANSCRIPTION FACTORS ARE REGULATED BY DIRECT INTERACTION WITH CALMODULIN (CaM) (PAPER I).

CaM interacts with RelA and c-Rel.

In a previous study from the group (36), members of the basic helix-loop-helix family of transcription factors were found to interact directly with CaM. This led us to investigate whether other transcription factors could also interact with CaM. The approach was to pass a calf thymus nuclear extract over a series of sepharose columns that purify proteins with DNA-binding properties. The resulting DNA-binding proteins were then tested for their ability to bind to CaM-sepharose. Proteins binding to the CaM-sepharose in the presence of Ca^{2+} were eluted and then screened for DNA-binding activities by electrophoretic mobility shift assay (EMSA) using consensus DNA sequences for different transcription factors. The eluate contained a protein(s) that bound specifically to a DNA sequence from the Ig κ light chain enhancer (**I**, Figure 1A). The Ig κ light chain enhancer is known to be a target for NF-κB, so we tested the ability of NF-κB proteins synthesised *in vitro* to bind to CaM-sepharose and found that RelA and c-Rel, but not NF-κB1/p50, could bind and that the binding was Ca^{2+}-dependent (**I**, Figure 1B).

The next step was to investigate whether RelA and c-Rel from cellular extracts could bind to CaM, whether NF-κB inducing stimuli could affect such an interaction and, finally, whether these interactions occur in the cell. We found that the interactions between CaM-sepharose and RelA and c-Rel are predominately cytoplasmic, and that NF-κB-inducing stimuli, e.g. phorbol ester/Ca^{2+}ionophore or TNFα, induce the interaction (**I**, Figure 2). Addition of recombinant IκBα to the binding reaction abolished the induced interaction (**I**, Figure 2C). Taken together, these results suggest that these NF-κB proteins

can bind to either CaM or IκB and that binding to CaM is dependent on the induced degradation of IκB. Immunoprecipitation analysis showed that these interactions occur in cells. Figure 3D in paper I shows that over-expressed RelA and c-Rel can be co-immunoprecipitated with endogenous CaM in DG75 B cells. In these cells, IκB degradation is a continuous process, so stimulation is not needed for the interaction between CaM and NF-κB.
After having established that CaM and certain NF-κB proteins do interact, we wanted to localise the CaM-binding sites of RelA and c-Rel and make mutants in order to assess the function of the interactions. The CaM-binding site of RelA was identified by deletion mapping (**I**, Figure 3A) and a similar region was found in c-Rel. Knowing the nature of many other CaM targets (121), we could predict that hydrophobic and basic amino acids were likely to be critical for the CaM interaction. We made point mutants in which such amino acids were substituted for by the uncharged polar amino acid asparagine and these mutants were indeed all, more or less, affected in their ability to bind to CaM (**I**, Figure 3B-D).

Ca^{2+} regulation of IL-2 and GM-CSF transcription is partly mediated by the CaM-binding site of c-Rel.

To analyse the phenotype of the CaM-binding deficient mutants of RelA and c-Rel, we decided to use a system in which both Ca^{2+} and NF-κB play well-defined roles. The transcriptional regulation of the two cytokines interleukin-2 (IL-2) and granulocyte macrophage-colony stimulating factor (GM-CSF) are such systems (77, 136, 137). In the transcriptional control regions of these genes, there are both NF-κB sites and sites for other Ca^{2+}-responding transcription factors, e.g. NFAT. Reporter plasmids with the transcriptional control regions of IL-2 and GM-CSF driving the expression of luciferase were co-transfected into Jurkat T cells together with wild-type or CaM-binding deficient c-Rel or RelA. The ability of these NF-κB proteins to regulate the expression of the reporter genes was assayed in the presence of a Ca^{2+} signal. We found that c-Rel increases Ca^{2+}-induced transcription of the IL-2 and GM-CSF reporter genes and that c-Rel mutants that are unable to bind to CaM are more potent activators of this transcription than the wild-type protein (**I**, Figure 5A and B). The interaction between CaM and c-Rel is apparently one way by which Ca^{2+} can regulate the expression of the cytokines IL-2 and GM-

CSF, suggesting that the CaM-binding site of c-Rel is of functional importance *in vivo*.

CaM regulates nuclear localisation of c-Rel.

The above analysis of the CaM-binding deficient c-Rel mutants supports a model in which CaM binding has a negative effect on transcriptional activation by c-Rel. In an attempt to elucidate the mechanism underlying this, we chose to analyse the sub-cellular distribution of wild-type c-Rel and its mutated derivatives in Jurkat cells, both in the absence and presence of NF-κB activating stimuli. In a resting cell, the vast majority of c-Rel is retained in the cytoplasm and, thus, is not available for activation of transcription in the nucleus. We found that mutation of the CaM binding site had no effect on the sub-cellular localisation of c-Rel in unstimulated cells or in cells stimulated with phorbol ester alone (**I**, Figure 6A and B). In cells stimulated with either phorbol ester in combination with the Ca^{2+}-ionophore ionomycin or with TNFα, however, mutation of the CaM binding site of c-Rel resulted in a markedly increased nuclear accumulation. The high nuclear levels of the c-Rel mutants explains their increased ability to activate transcription as seen in **I,** Figure 5.

We have yet to identify the mechanism by which CaM regulates nuclear accumulation of c-Rel. There are many possible mechanisms, but we favour a model in which CaM negatively regulates nuclear import of c-Rel. As import of proteins to the nucleus is a complex process, there are several levels at which CaM could act. (i) There is evidence that the c-terminal half of c-Rel contains sequences that, by an unknown mechanism, prevent c-Rel from entering the nucleus (23). Perhaps CaM is part of this regulatory mechanism and when the CaM-binding site of c-Rel is mutated, this negative regulation is overcome. (ii) The conformation of an NLS bound to its nuclear import receptor importin-α is an extended chain (34). However, CaM targets are usually α-helical structures (121), so it is conceivable that by binding to c-Rel, CaM imposes an α-helical structure on the nearby NLS which is incompatible with importin-α interaction and nuclear import. (iii) The CaM-binding site that we have identified in c-Rel is directly adjacent to the NLS. It is therefore possible that CaM could have a negative effect on nuclear import of c-Rel by competing for binding to amino acids that the nuclear import receptor also binds to.

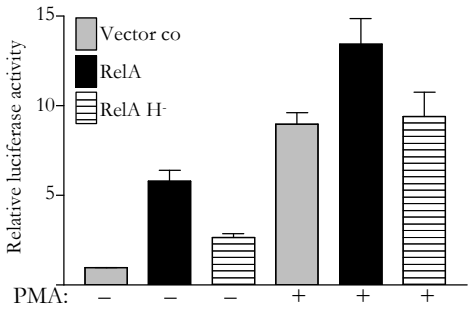

Figure 7. Disruption of the CaM binding site of RelA impairs the ability of RelA to activate transcription. Jurkat T cells were transfected as described in paper **II**, using a luciferase reporter plasmid controlled by two κB sites, and vectors expressing RelA or its CaM-binding deficient derivative RelA H^-.

Transcriptional activation by RelA may be regulated by CaM.

In contrast to c-Rel, mutation of the CaM-binding site in RelA did not result in a more potent activation of transcription of the IL-2 and GM-CSF reporter genes (**I**, Figure 5A and B). Accordingly, the CaM-binding deficient RelA mutant accumulated in the nucleus to the same extent as the wild-type protein (**I**, Figure 6D and E). The RelA mutant was studied further by assessing its ability to activate transcription of a luciferase reporter gene controlled by two NF-κB-binding sites. In this system, the CaM-binding deficient RelA was reduced in its ability to activate transcription to only 30% of the wild-type activity (Figure 7), suggesting a positive role for CaM in the regulation of RelA transcriptional activity. As mentioned in the section: *Involvement of calmodulin in the regulation of NF-**k**B*, CaM has previously been implicated in the regulation of RelA transcriptional activity. Jang and Bae and their co-workers found that the CaM-dependent kinase CaMKIV is capable of phosphorylating RelA on serine 535, leading to increased transcriptional activity (9, 78). CaMKIV is present in the nucleus and requires CaM for its activation (142). A model that would explain the phenotype of RelA H^-, is that RelA, by help of its CaM binding site, brings CaM into the nucleus where it can activate CaMKIV, which in turn phosphorylates RelA on an amino acid residue critical for transcriptional activity.

The differential effect of CaM on c-Rel and RelA.

As far as we can tell from the experiments that we have performed, the modes by which CaM interacts with c-Rel and RelA are similar. Still, the outcome is very different, with CaM acting negatively on the activity of c-Rel while – at least in some contexts – acting positively on RelA. The main homology between NF-κB proteins lies within the rel homology domain (RHD). Sequences C-terminal to the RHD are not conserved within the family. It is possible that these C-terminal sequences determine the effect of the CaM interaction. It has been shown that RelA is transported to the nucleus much faster than c-Rel following stimulation ((48, 113) and data not shown). It may be that this difference between RelA and c-Rel is a result of the differential effect of CaM-binding.

Do other NF-kB proteins bind to CaM?

In this study we found that NF-κB1/p50, unlike RelA and c-Rel, is unable to bind to CaM-sepharose, but one question that remains unanswered is whether the two remaining NF-κB proteins, RelB and NF-κB2, are able to bind to CaM. Neither RelB nor NF-κB2/p52 were available for testing, but we do not believe that it is likely that they bind since they both have α-helix destabilising residues in their domains which correspond to the CaM-binding site of RelA and c-Rel (**I**, Figure 7), and α-helical structures have been shown to be important features of CaM targets.

CaM-DEPENDENT KINASE II MEDIATES T CELL RECEPTOR/CD3-INDUCED AND PHORBOL ESTER-INDUCED ACTIVATION OF THE IkB-KINASE (PAPERS II AND III).

CaM is involved in the activation of NF-kB.

During the course of investigation of the interaction between NF-κB proteins and CaM, we found that CaM plays more than one role in the intricate system of NF-κB regulation. While studying activation of endogenous NF-κB by the phorbol ester PMA in reporter gene assays, we found that treatment of cells with W7, a drug that inhibits CaM, led to inhibition of NF-κB activation (**II**, Figure 1). Several CaM-inhibitors were assessed and they were all found to be capable of inhibiting PMA-induced NF-κB activity (**II**, Figure 2 A and B).

CaM is required for phorbol ester- and T cell receptor/CD3-induced phosphorylation and degradation of IkBa.

In a simplified view, inhibition of NF-κB activation could be achieved by blocks at any of three general levels: (i) at the level of IκB phosphorylation and degradation, (ii) at the level of nuclear import of the NF-κB complex, or (iii) at the level of regulation of the transcriptional activation properties of the NF-κB protein. Examination of the phosphorylation status and/or degradation of IκBα, in the presence or absence of NF-κB inducers and CaM inhibitor, revealed that CaM is required for PMA- and T cell receptor/CD3-induced phosphorylation and degradation of IκBα (II, Figure 4 and III, Figures 1A and 5A).

CaMKII is required for activation of IKK.

CaM exerts its regulatory roles not only by direct binding to target proteins, but also indirectly through the action of CaM-dependent enzymes. Since we found that CaM was somehow involved in the phosphorylation of IκB, it was tempting to hypothesise that in this case CaM was acting through a CaM-dependent kinase. Analysis of the status of IκBα after stimulation in the presence or absence of a CaMKII inhibitor, KN93, revealed that this was indeed the case (**III**, Figures 1B and 5A). In agreement with these findings, the activity of the IκB kinase complex (IKK) was found to be dependent on

CaMKII, as the CaMKII inhibitor could block phorbol ester-induced IKK activation (**III**, Figure 6). Evidence of the specificity of the CaM inhibitor W7 and the CaMKII inhibitor KN93 is given in **III,** Figure 2. In this experiment, it was found that over-expression of the CaM-independent and inhibitor-resistant constitutively active form of CaMKIIγ, CaMKIIγ T286D, but not over-expression of wild-type CaMKIIγ, overrides the ability of W7 and KN93 to inhibit PMA-induced IκBα degradation. Further evidence supporting placement of CaMKII in the pathway leading from phorbol ester to NF-κB activation came from ectopic expression of CaMKIIγ derivatives in Jurkat cells. During activation of NF-κB, IκBα is initially degraded but, as IκBα is an NF-κB target gene, it is later up-regulated and transported to the nucleus to terminate the NF-κB activity. These features make the amount and localisation of IκBα suitable as markers of NF-κB activity. From immunohistochemical analysis of the amount and sub-cellular localisation of IκBα, it was evident that over-expression of either wild-type or constitutively active CaMKIIγ mimicked the effect of phorbol ester, albeit at different levels (**III**, Figure 7). Our results strongly suggest that CaMKIIγ is required for IKK and, subsequently, NF-κB activation. It is possible, however, that the physiological kinase is a different CaMKII isoform or perhaps even a closely related kinase, the function of which can be substituted for by over-expression of CaMKIIγ and the activity of which is inhibited by CaM and CaMKII inhibitors.

The CaMKII requirement of NF-kB activation is restricted to certain signalling pathways.

There is a multitude of activators of NF-κB and even though some of them share signalling pathways to NF-κB activation, there is also a multitude of different pathways. In paper III, we showed that NF-κB activation by both T cell receptor/CD3 and phorbol ester requires the action of CaMKII. We have also found that CaM inhibitor can block NF-κB activation by hydrogen peroxide (Figure 8). To determine whether other NF-κB activation pathways also require the action of CaMKII, the effects of the CaMKII inhibitor on NF-κB stimulation by TNFα and the phosphatase inhibitor calyculin A were examined. The CaMKII inhibitor had no effect on IκB degradation induced by either TNFα or calyculin A (**III**, Figure 3), suggesting that the CaMKII dependence is specific to certain signalling pathways. Although CaMKII is activated by Ca^{2+}-loaded CaM, we have found that the action of CaMKII in

PMA-induced NF-κB activation is independent of increased intracellular Ca^{2+}. It may be that, in this case, CaM functions independently of Ca^{2+} - as has been shown for some other CaM-regulated processes (reviewed in (81)) – or that in this case CaM/CaMKII has a higher affinity for Ca^{2+} than that of typical CaMKII-regulated processes. The latter alternative has actually been demonstrated for another CaMK, myosin light chain kinase, where the affinity of CaM for Ca^{2+} is increased upon binding to the kinase and further increased when the CaMK binds its substrate (61, 120). Increased Ca^{2+} affinity or Ca^{2+} independence could perhaps be facilitated through specific association with another protein or proteins in the signalling pathway.

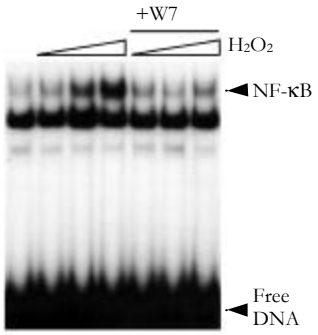

Figure 8. H_2O_2-induced NF-kB activation is dependent on calmodulin. Jurkat T cells were stimulated with increasing concentrations of H_2O_2 (0.1, 0.2 and 0.3 mM) for 60 minutes in the absence or presence of the CaM inhibitor W7 (25 μM). The NF-κB DNA-binding activity in nuclear extracts was analysed by EMSA performed as described in paper **II**.

What is the function of CaMKII in the NF-kB activation pathway?

At present, we do not know by which mechanism CaMKII acts in TCR/CD3 or PMA-induced NF-κB activation. Many players have been identified in this pathway (Figure 6 and (128)), but their respective roles are unclear in many cases. Our data suggest that CaMKII is required for activation of IKK (**III**, Fig. 6), and from the use of PKC-inhibitor we also know that CaMKII acts downstream of PKC (**III** Fig. 4B and 5B). This means that we should search for the function of CaMKII in the steps between PKC and IKK. One possible role for CaMKII is to activate IKK directly. This theory is supported by the finding that PKC can phosphorylate CaMKII at T286 *in vitro* (165). Phosphorylation of T286 is required for CaMKII activation, and thus one attractive model is that PKC directly activates CaMKII, which in turn activates IKK. As discussed in the section *Phosphorylation of IkB: regulation of the IkB kinase,* Bcl-10, Carma1 and MALT1 also act between PKC and IKK in this pathway, and perhaps CaMKII co-operates with one or more of these proteins in mediating NF-κB activation.

CONCLUSIONS

- The NF-κB proteins c-Rel and RelA interact with calmodulin.
    - The interaction is negative for c-Rel nuclear localisation but has no effect on the localisation of RelA.
    - The interaction between CaM and c-Rel is part of the Ca^{2+}-mediated transcriptional regulation of the cytokines IL-2 and GM-CSF.
    - The interaction may influence transcriptional activity of RelA.

- CaMKII is required for TCR/CD3- and phorbol ester-induced NF-κB activation, acting at a step between PKC and IKK.

FINAL REMARKS

The results obtained in this thesis and work of others point towards CaM having multiple roles in the regulation of NF-κB transcription factors (model in Figure 9).

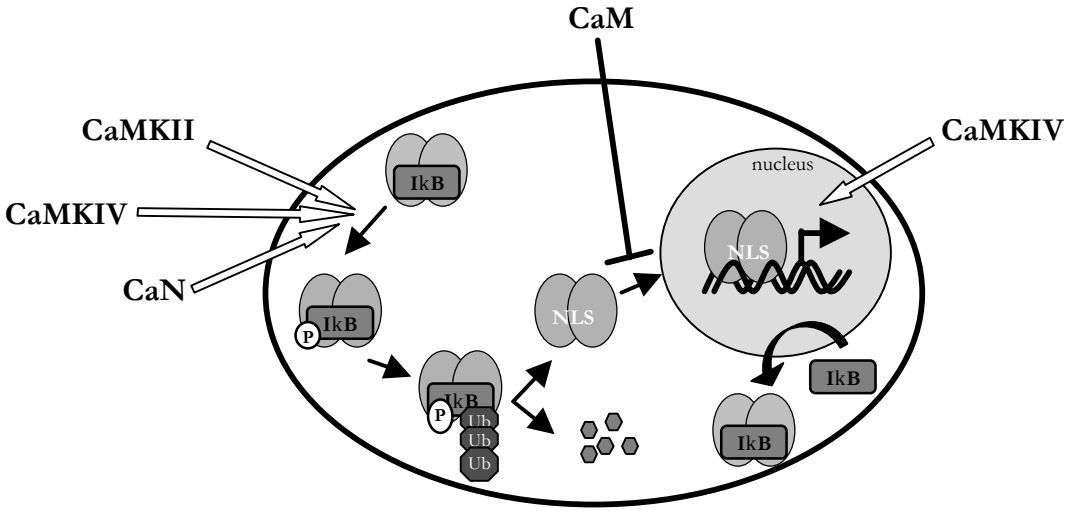

Figure 9. A schematic view of NF-kB activation indicating the steps at which CaM or CaM-dependent enzymes are involved.

The first step in which CaM acts is in the process of phosphorylation of the inhibitory IκB protein IκBα after certain stimuli. At this level, CaM acts both via the CaM-dependent kinase CaMKII (paper **III**) and via the CaM-dependent phosphatase calcineurin (CaN). The role of CaN in NF-κB activation is not really understood, but it appears to accelerate IκB phosphorylation (52, 143). Regulation of c-Rel nuclear accumulation by direct CaM binding is the second level at which CaM has a function in the regulation of NF-κB (paper **I**). NF-κB are powerful transcription factors that are able to quickly activate the transcription of target genes in response to stimuli. Even though there are several IκBs available, it seems they are not sufficient to obtain full control of NF-κB activity. As mentioned in the section *Termination*

of the NF-kB response, there are also pathway-specific inhibitors of NF-κB, and with our finding of CaM inhibition of c-Rel nuclear localisation, we might perhaps add subunit-specific inhibitors to the growing list of NF-κB regulators. In parallel with our studies, other groups have found roles for the Ca^{2+}/CaM-dependent kinase IV (CaMKIV) in NF-κB activation. Firstly, CaMKIV was found to interact with and phosphorylate RelA, leading to increased NF-κB-dependent transactivation (78). Secondly, over-expression of CaMKIV was shown to augment H_2O_2-induced IκB phosphorylation (67).

The many effects of Ca^{2+}/CaM in this system may appear somewhat confusing and, in some cases, contradictory. For instance, what does the cell gain from responding to Ca^{2+} signals by the two seemingly contradictory events of (i) degradation of the NF-κB inhibitor IκBα, and (ii) blockage of nuclear accumulation of the NF-κB protein c-Rel? It may be that the Ca^{2+} signals that result in these events are themselves distinct in terms of amplitude, frequency and/or location, and that these characteristics decide whether the outcome of the signal should be activation or inactivation of NF-κB-mediated transcription. It is also plausible that both processes respond to the same Ca^{2+} signal but with temporal differences, or that cell-type differences decide what the effect will be. It is clear that more work is required to fully understand NF-κB regulation by Ca^{2+} and CaM.

ACKNOWLEDGEMENTS

I was for a while considering to settle for a big fat **THANKS** on this page, but since this section of a thesis attracts more attention than any of the other sections, I will try to do this the proper way....

I believe that most things in life happen by chance, and that–even though things may look bad–everything turns out OK, eventually. It so happens that both these mottos of mine are correct descriptions of my life as a PhD student. It was really by chance that I ended up in Thomas' group when I was looking for a place to do my project work (I would not be honest if I claimed that all I ever wanted to do at that time was to study how transcription factors are influenced by calmodulin...). But, even though it wasn't planned, it seems (I don't know yet if I'll pass the dissertation..) it turned out OK and I have enjoyed this time in group TG!!

I would like to thank the following persons for having contributed in different ways to my work, to the atmosphere at the department and in making outside life enjoyable:

My supervisor **Thomas Grundström** for finding explanations to unexplainable results, for fantastic ideas, for being the human calculator, for giving absolute freedom in the lab and for making fantastic ice cream Easter-eggs.

The NF-kB girls. During my years at the department, I have had the fortune of sharing the NF-κB projects with two wonderful persons, Kate and Sofia!
I want to thank **Kate** for introducing me into the NF-κB field, for great teamwork and companionship, and for many nice times outside work (on holiday, at parties etc).
Thanks goes to **Sofia** for great teamwork and for being a good friend, a perfect travel-companion, a reliable baby-sitter and much, much more.

Juha for all instant lab discussions, for all nice sessions of skitsnack, for knowing not to ask the wrong questions and for having been a PhD student for just as long as I have!
Christine for being such a nice and kind person, the lab would not be the same without you!
Stefan for all the lively colours, for nice drinks and for dancing like no one else...
Xiao-Qi for your good mood that lightened up the lab and for all nice gifts you brought us from China. Good luck with the twins!
Jackie, Magnus, Rich, Natalia, Amir, Hebin, Xiaowen and **Jannek** for making our lab an interesting place.

Jenni, for knowing what is important, for being a great friend and for sharing and encouraging my food and drink obsession.

Mike & Eric for important friendship, Tuesdays at Lottas' and for introducing me to crispy rice and true irish "cooking". I miss you both!!

Pelle, for bouncing around with a smile, for solving the cover-problem and for keeping my blood-sugar on acceptable levels by constantly providing me with mazariner.

Lisa for friendship and for sharing the knowledge of what is NOT important. Strangely enough, we both spent a lot of time and sweat on doing this extremely non-important thing...

Jenny & Christer for being great neighbours with excellent taste. I'm also grateful for having a daughter who knew how a lighter works at the suitable age of ~6 months...

The Thursday lunch club: **Sara, Petra, Jeanette, Christina & Sofia** (again). Thursdays are now the highlight of the week at work, I have never had such nice lunches before! (The company isn't bad either....)

Johnny, Ethel, Ewa-Christine and the dish/media department for making everything work smoothly around here.

People who have left the department, in particular **beer-corner enthusiasts** and members of the fantastic brännboll team: **" Dr. Lippmanns pigga möss",** there will never be a better-looking team than us dressed as Spice Girls!

Karin (Plupp), for being wise enough to ask me the way to hörsal C, and for (ever since that day) being a very good friend.

Bodil, my oldest friend for being such a good friend!

Music is an important part of my life, and being a member of **Umeå Studentkör, Bettans Brudar** and **Kvart i Bettan** has been a true pleasure and an important factor in keeping sane. Thanks!

Henrik "Hans, Hasse, Hinken" Josefsson, min olycksbroder. Tack för att du alltid kommer att vara nästan exakt lika gammal som jag. Det är en tröst!

Ett stort tack till mina föräldrar **Inger och Torbjörn** som alltid har trott på mig och stöttat mig på alla sätt. Denna bok skulle nog ha blivit avsevärt försenad om det inte varit för all hjälp jag fått. TACK!!!
Syster yster **Camilla,** den slinke & slärte **Anders** och mosters ögonstenar **Ebba och Wiktor** har på olika sätt bidragit till att jag inte blivit allt för konstig under dessa år.

Och slutligen, tack till min lilla familj **Fredrik** och **Sigrid!** Ni är fina ni, det är knappt man tror att det är sant! Nu kan vi åka på semester!!!

REFERENCES

1. **Allbritton, N. L., and T. Meyer** 1993. Localized calcium spikes and propagating calcium waves *Cell Calcium*. **14:**691-7.
2. **Alvarez, J., M. Montero, and J. Garcia-Sancho** 1999. Subcellular Ca(2+) Dynamics *News Physiol Sci*. **14:**161-168.
3. **Anderson, K. A., and C. D. Kane** 1998. Ca2+/calmodulin-dependent protein kinase IV and calcium signaling *Biometals*. **11:**331-43.
4. **Anest, V., J. L. Hanson, P. C. Cogswell, K. A. Steinbrecher, B. D. Strahl, and A. S. Baldwin** 2003. A nucleosomal function for IkappaB kinase-alfa in NF-kappaB-dependent gene expression *Nature*. **423:**659-663.
5. **Anrather, J., V. Csizmadia, M. P. Soares, and H. Winkler** 1999. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells *J Biol Chem*. **274:**13594-603.
6. **Aradhya, S., and D. L. Nelson** 2001. NF-kappaB signaling and human disease *Curr Opin Genet Dev*. **11:**300-6.
7. **Arendt, C. W., B. Albrecht, T. J. Soos, and D. R. Littman** 2002. Protein kinase C-theta;: signaling from the center of the T-cell synapse *Curr Opin Immunol*. **14:**323-30.
8. **Arenzana Seisdedos, F., P. Turpin, M. Rodriguez, D. Thomas, R. T. Hay, J. L. Virelizier, and C. Dargemont** 1997. Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm *J. Cell Sci.* **110:**369-378.
9. **Bae, J. S., M. K. Jang, S. Hong, W. G. An, Y. H. Choi, H. D. Kim, and J. Cheong** 2003. Phosphorylation of NF-kappa B by calmodulin-dependent kinase IV activates anti-apoptotic gene expression *Biochem Biophys Res Commun*. **305:**1094-8.
10. **Baeuerle, P. A., and D. Baltimore** 1996. NF-kappa B: ten years after *Cell*. **87:**13-20.
11. **Baldwin, A. S., Jr.** 2001. Series introduction: the transcription factor NF-kappaB and human disease *J Clin Invest*. **107:**3-6.
12. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin Jr** 1992. I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention [published erratum appears in Genes Dev 1992 Dec;6(12B):2664-5] *Genes Dev*. **6:**1899-1913.

13. **Ben-Neriah, Y.** 2002. Regulatory functions of ubiquitination in the immune system *Nat Immunol.* **3:**20-26.
14. **Berridge, M. J.** 1993. Inositol trisphosphate and calcium signalling *Nature.* **361:**315-25.
15. **Bird, T. A., K. Schooley, S. K. Dower, H. Hagen, and G. D. Virca** 1997. Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit *J Biol Chem.* **272:**32606-12.
16. **Bito, H., K. Deisseroth, and R. W. Tsien** 1996. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression *Cell.* **87:**1203-14.
17. **Boone, D. L., E. G. Lee, S. Libby, P. J. Gibson, M. Chien, F. Chan, M. Madonia, P. R. Burkett, and A. Ma** 2002. Recent advances in understanding NF-kappaB regulation *Inflammatory Bowel Diseases.* **8:**201-212.
18. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist** 1990. Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs *Nature.* **348:**76-80.
19. **Brocke, L., L. W. Chiang, P. D. Wagner, and H. Schulman** 1999. Functional implications of the subunit composition of neuronal CaM kinase II *J Biol Chem.* **274:**22713-22.
20. **Brownell, E., N. Mittereder, and N. R. Rice** 1989. A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. *Oncogene.* **4:**935-42.
21. **Brummelkamp, T. R., S. M. B. Nijman, A. M. G. Dirac, and R. Bernards** 2003. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB *Nature.* **424:**797-801.
22. **Bryan, R. G., Y. Li, J. H. Lai, M. Van, N. R. Rice, R. R. Rich, and T. H. Tan** 1994. Effect of CD28 signal transduction on c-Rel in human peripheral blood T cells *Mol Cell Biol.* **14:**7933-42.
23. **Capobianco, A. J., D. L. Simmons, and T. D. Gilmore** 1990. Cloning and expression of a chicken c-rel cDNA: unlike p59v-rel, p68c-rel is a cytoplasmic protein in chicken embryo fibroblasts *Oncogene.* **5:**257-265.
24. **Carafoli, E., L. Santella, D. Branca, and M. Brini** 2001. Generation, control, and processing of cellular calcium signals *Crit Rev Biochem Mol Biol.* **36:**107-260.
25. **Carey, M., and S. T. Smale** 1999. Transcriptional regulation in eukaryotes. Concepts, strategies and techniques. Cold Spring Harbor Laboratory Press, New York.

26. **Chatila, T., N. Ho, P. Liu, S. Liu, G. Mosialos, E. Kieff, and S. H. Speck** 1997. The Epstein-Barr virus-induced Ca2+/calmodulin-dependent kinase type IV/Gr promotes a Ca(2+)-dependent switch from latency to viral replication *J Virol.* **71:**6560-7.
27. **Chawla, S., G. E. Hardingham, D. R. Quinn, and H. Bading** 1998. CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV *Science.* **281:**1505-9.
28. **Chen, L.-f., W. Fischle, E. Verdin, and W. C. Greene** 2001. Duration of Nuclear NF-kappa B Action Regulated by Reversible Acetylation *Science.* **293:**1653-1657.
29. **Chen, L.-F., and W. C. Greene** 2003. Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation *Journal of Molecular Medicine.* **Epub ahead of print**.
30. **Chen, L.-f., Y. Mu, and W. C. Greene** 2002. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-{kappa}B *EMBO J.* **21:**6539-6548.
31. **Chin, D., and A. R. Means** 2000. Calmodulin: a prototypical calcium sensor *Trends Cell Biol.* **10:**322-8.
32. **Clapham, D. E.** 1995. Calcium signaling *Cell.* **80:**259-68.
33. **Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist** 2002. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells *Nat Immunol.* **3:**958-65.
34. **Conti, E., M. Uy, L. Leighton, G. Blobel, and J. Kuriyan** 1998. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha *Cell.* **94:**193-204.
35. **Coope, H. J., P. G. P. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. B. Klaus, L. H. Johnston, and S. C. Ley** 2002. CD40 regulates the processing of NF-{kappa}B2 p100 to p52 *EMBO J.* **21:**5375-5385.
36. **Corneliussen, B., M. Holm, Y. Waltersson, J. Onions, B. Hallberg, A. Thornell, and T. Grundström** 1994. Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains *Nature.* **368:**760-764.
37. **Crabtree, G. R.** 2001. Calcium, calcineurin, and the control of transcription *J Biol Chem.* **276:**2313-6.
38. **Crivici, A., and M. Ikura** 1995. Molecular and structural basis of target recognition by calmodulin *Annu Rev Biophys Biomol Struct.* **24:**85-116.
39. **Cruzalegui, F. H., and A. R. Means** 1993. Biochemical characterization of the multifunctional Ca2+/calmodulin- dependent protein kinase type IV expressed in insect cells *J Biol Chem.* **268:**26171-8.

40. **Cyert, M. S., and J. Thorner** 1992. Regulatory subunit (CNB1 gene product) of yeast Ca2+/calmodulin- dependent phosphoprotein phosphatases is required for adaptation to pheromone *Mol Cell Biol.* **12:**3460-9.
41. **Dash, P. K., K. A. Karl, M. A. Colicos, R. Prywes, and E. R. Kandel** 1991. cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase *Proc Natl Acad Sci U S A.* **88:**5061-5.
42. **Davis, M., A. Hatzubai, J. S. Andersen, E. Ben-Shushan, G. Z. Fisher, A. Yaron, A. Bauskin, F. Mercurio, M. Mann, and Y. Ben-Neriah** 2002. Pseudosubstrate regulation of the SCFbeta -TrCP ubiquitin ligase by hnRNP-U *Genes Dev.* **16:**439-451.
43. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H. C. Liou, D. Baltimore, and H. R. Bose Jr** 1991. Rel-associated pp40: an inhibitor of the rel family of transcription factors *Science.* **253:**1268-1271.
44. **Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner** 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein *Cell.* **47:**423-31.
45. **Dejardin, E., N. M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z. W. Li, M. Karin, C. F. Ware, and D. R. Green** 2002. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways *Immunity.* **17:**525-35.
46. **Deng, W.-G., Y. Zhu, and K. K. Wu** 2003. Up-regulation of p300 Binding and p50 Acetylation in Tumor Necrosis Factor-alpha -induced Cyclooxygenase-2 Promoter Activation *J. Biol. Chem.* **278:**4770-4777.
47. **Dixit, V., and T. W. Mak** 2002. NF-kappaB signaling. Many roads lead to madrid *Cell.* **111:**615-9.
48. **Doerre, S., P. Sista, S. C. Sun, D. W. Ballard, and W. C. Greene** 1993. The c-rel protooncogene product represses NF-kappa B p65-mediated transcriptional activation of the long terminal repeat of type 1 human immunodeficiency virus *Proc. Natl. Acad. Sci. U. S. A.* **90:**1023-1027.
49. **Dolmetsch, R. E., R. S. Lewis, C. C. Goodnow, and J. I. Healy** 1997. Differential activation of transcription factors induced by Ca2+ response amplitude and duration *Nature.* **386:**855-8.
50. **Dolmetsch, R. E., K. Xu, and R. S. Lewis** 1998. Calcium oscillations increase the efficiency and specificity of gene expression *Nature.* **392:**933-6.
51. **Dushay, M. S., B. Asling, and D. Hultmark** 1996. Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of *Drosophila. Proc. Natl. Acad. Sci. U S A.* **93:**10343-10347.

52. **Frantz, B., E. C. Nordby, G. Bren, N. Steffan, C. V. Paya, R. L. Kincaid, M. J. Tocci, S. J. O'Keefe, and E. A. O'Neill** 1994. Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B *Embo J.* **13:**861-70.
53. **Furia, B., L. Deng, K. Wu, S. Baylor, K. Kehn, H. Li, R. Donnelly, T. Coleman, and F. Kashanchi** 2002. Enhancement of Nuclear Factor-kappa B Acetylation by Coactivator p300 and HIV-1 Tat Proteins *J. Biol. Chem.* **277:**4973-4980.
54. **Gerondakis, S., M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont** 1999. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts *Oncogene.* **18:**6888-95.
55. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore** 1990. Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal *Cell.* **62:**1019-29.
56. **Ghosh, S., and M. Karin** 2002. Missing pieces in the NF-kappaB puzzle *Cell.* **109:**S81-96.
57. **Ghosh, S., M. J. May, and E. B. Kopp** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses *Annu Rev Immunol.* **16:**225-60.
58. **Gilmore, T. D.**, Rel/NF-kappaB transcription factors (http://people.bu.edu/gilmore/nf-kb/index.html).
59. **Gorlich, D., and I. W. Mattaj** 1996. Nucleocytoplasmic transport *Science.* **271:**1513-8.
60. **Grumont, R. J., and S. Gerondakis** 1989. Structure of a mammalian c-rel protein deduced from the nucleotide sequence of murine cDNA clones. *Oncogene Res.* **4:**1-8.
61. **Haiech, J., M. C. Kilhoffer, T. J. Lukas, T. A. Craig, D. M. Roberts, and D. M. Watterson** 1991. Restoration of the calcium binding activity of mutant calmodulins toward normal by the presence of a calmodulin binding structure *J. Biol. Chem.* **266:**3427-3431.
62. **Harley, V. R., R. Lovell Badge, P. N. Goodfellow, and P. J. Hextall** 1996. The HMG box of SRY is a calmodulin binding domain *FEBS Lett.* **391:**24-28.
63. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson Johannes, K. Mondal, P. Ralph, and A. S. Baldwin Jr** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity *Cell.* **65:**1281-1289.

64. **Heissmeyer, V., D. Krappmann, F. G. Wulczyn, and C. Scheidereit** 1999. NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes *Embo J.* **18:**4766-78.
65. **Hirota, J., T. Michikawa, T. Natsume, T. Furuichi, and K. Mikoshiba** 1999. Calmodulin inhibits inositol 1,4,5-trisphosphate-induced calcium release through the purified and reconstituted inositol 1,4,5- trisphosphate receptor type 1 *FEBS Lett.* **456:**322-6.
66. **Hoelz, A., A. C. Nairn, and J. Kuriyan** 2003. Crystal Structure of a Tetradecameric Assembly of the Association Domain of Ca(2+)/Calmodulin-Dependent Kinase II *Mol Cell.* **11:**1241-51.
67. **Howe, C. J., M. M. LaHair, J. A. Maxwell, J. T. Lee, P. J. Robinson, O. Rodriguez-Mora, J. A. McCubrey, and R. A. Franklin** 2002. Participation of the calcium/calmodulin-dependent kinases in hydrogen peroxide-induced Ikappa B phosphorylation in human T lymphocytes *J Biol Chem.* **277:**30469-76.
68. **Hu, Q., S. Deshpande, K. Irani, and R. C. Ziegelstein** 1999. [Ca(2+)](i) oscillation frequency regulates agonist-stimulated NF- kappaB transcriptional activity *J Biol Chem.* **274:**33995-8.
69. **Hu, Y., V. Baud, M. Delhase, P. Zhang, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin** 1999. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase *Science.* **284:**316-20.
70. **Hudmon, A., and H. Schulman** 2002. Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function *Annu Rev Biochem.* **71:**473-510.
71. **Hudmon, A., and H. Schulman** 2002. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II *Biochem J.* **364:**593-611.
72. **Huxford, T., D. B. Huang, S. Malek, and G. Ghosh** 1998. The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation [see comments] *Cell.* **95:**759-770.
73. **Iacovelli, L., M. Sallese, S. Mariggio, and A. de Blasi** 1999. Regulation of G-protein-coupled receptor kinase subtypes by calcium sensor proteins *Faseb J.* **13:**1-8.
74. **Imbert, V., R. A. Rupec, A. Livolsi, H. L. Pahl, E. B. Traenckner, C. Mueller-Dieckmann, D. Farahifar, B. Rossi, P. Auberger, P. A. Baeuerle, and J. F. Peyron** 1996. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha *Cell.* **86:**787-98.

75. **Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei, and M. Levine** 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. *Cell.* **75:**753-63.
76. **Jacobs, M. D., and S. C. Harrison** 1998. Structure of an IkappaBalpha/NF-kappaB complex *Cell.* **95:**749-758.
77. **Jain, J., C. Loh, and A. Rao** 1995. Transcriptional regulation of the IL-2 gene *Curr Opin Immunol.* **7:**333-42.
78. **Jang, M. K., Y. H. Goo, Y. C. Sohn, Y. S. Kim, S. K. Lee, H. Kang, J. Cheong, and J. W. Lee** 2001. Ca2+/calmodulin-dependent protein kinase IV stimulates nuclear factor-kappa B transactivation via phosphorylation of the p65 subunit *J Biol Chem.* **276:**20005-10.
79. **Jensen, K. F., C. A. Ohmstede, R. S. Fisher, and N. Sahyoun** 1991. Nuclear and axonal localization of Ca2+/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex *Proc Natl Acad Sci U S A.* **88:**2850-3.
80. **Johnson, C., D. Van Antwerp, and T. J. Hope** 1999. An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha *Embo J.* **18:**6682-6693.
81. **Jurado, L. A., P. S. Chockalingam, and H. W. Jarrett** 1999. Apocalmodulin *Physiol Rev.* **79:**661-82.
82. **Kakalis, L. T., M. Kennedy, R. Sikkink, F. Rusnak, and I. M. Armitage** 1995. Characterization of the calcium-binding sites of calcineurin B *FEBS Lett.* **362:**55-8.
83. **Kanaseki, T., Y. Ikeuchi, H. Sugiura, and T. Yamauchi** 1991. Structural features of Ca2+/calmodulin-dependent protein kinase II revealed by electron microscopy *J Cell Biol.* **115:**1049-60.
84. **Karin, M., Y. Cao, F. R. Greten, and Z. W. Li** 2002. NF-kappaB in cancer: from innocent bystander to major culprit *Nat Rev Cancer.* **2:**301-10.
85. **Karin, M., and M. Delhase** 2000. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling *Semin. Immunol.* **12:**85-98.
86. **Karin, M., and T. Hunter** 1995. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus *Curr Biol.* **5:**747-57.
87. **Karin, M., and A. Lin** 2002. NF-kappaB at the crossroads of life and death *Nat Immunol.* **3:**221-7.
88. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel** 1990. The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product *Cell.* **62:**1007-18.

89. **Kincaid, R. L., and M. Vaughan** 1986. Direct comparison of Ca2+ requirements for calmodulin interaction with and activation of protein phosphatase *Proc Natl Acad Sci U S A.* **83:**1193-7.
90. **Kisseleva, T., S. Bhattacharya, J. Braunstein, and C. W. Schindler** 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges *Gene.* **285:**1-24.
91. **Klee, C. B., G. F. Draetta, and M. J. Hubbard** 1988. Calcineurin *Adv Enzymol Relat Areas Mol Biol.* **61:**149-200.
92. **Klee, C. B., and T. C. Vanaman** 1982. Calmodulin *Adv Protein Chem.* **35:**213-321.
93. **Kolodziej, S. J., A. Hudmon, M. N. Waxham, and J. K. Stoops** 2000. Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIalpha and truncated CaM kinase IIalpha reveal a unique organization for its structural core and functional domains *J Biol Chem.* **275:**14354-9.
94. **Kovalenko, A., C. Chable-Bessia, G. Cantarella, A. Israël, D. Wallach, and G. Courtois** 2003. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination *Nature.* **424:**801-805.
95. **Lamb, P., and S. L. McKnight** 1991. Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerization *Trends Biochem Sci.* **16:**417-22.
96. **Levine, M., and R. Tjian** 2003. Transcription regulation and animal diversity *Nature.* **424:**147-51.
97. **Li, C. C., M. Korner, D. K. Ferris, E. Chen, R. M. Dai, and D. L. Longo** 1994. NF-kappa B/Rel family members are physically associated phosphoproteins *Biochem J.* **303:**499-506.
98. **Li, Q., and I. M. Verma** 2002. NF-kappaB regulation in the immune system *Nat Rev Immunol.* **2:**725-34.
99. **Li, W., J. Llopis, M. Whitney, G. Zlokarnik, and R. Y. Tsien** 1998. Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression *Nature.* **392:**936-41.
100. **Li, Z., and G. J. Nabel** 1997. A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription *Mol. Cell. Biol.* **17:**6184-6190.
101. **Lin, L., G. N. DeMartino, and W. C. Greene** 2000. Cotranslational dimerization of the Rel homology domain of NF-kappaB1 generates p50-p105 heterodimers and is required for effective p50 production *Embo J.* **19:**4712-22.
102. **Malek, S., Y. Chen, T. Huxford, and G. Ghosh** 2001. Ikappa Bbeta , but Not Ikappa Balpha , Functions as a Classical Cytoplasmic Inhibitor of

NF-kappa B Dimers by Masking Both NF-kappa B Nuclear Localization Sequences in Resting Cells *J. Biol. Chem.* **276:**45225-45235.
103. **Mamar-Bachi, A., and J. A. Cox** 1987. Quantitative analysis of the free energy coupling in the system calmodulin, calcium, smooth muscle myosin light chain kinase *Cell Calcium.* **8:**473-82.
104. **Martin, A. G., and M. Fresno** 2000. Tumor necrosis factor-alpha activation of NF-kappa B requires the phosphorylation of Ser-471 in the transactivation domain of c-Rel *J Biol Chem.* **275:**24383-91.
105. **Massari, M. E., and C. Murre** 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms *Mol Cell Biol.* **20:**429-40.
106. **Mattaj, I. W., and L. Englmeier** 1998. Nucleocytoplasmic transport: the soluble phase *Annu Rev Biochem.* **67:**265-306.
107. **Matthews, R. P., C. R. Guthrie, L. M. Wailes, X. Zhao, A. R. Means, and G. S. McKnight** 1994. Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression *Mol Cell Biol.* **14:**6107-16.
108. **Mattson, M. P., and S. Camandola** 2001. NF-kappaB in neuronal plasticity and neurodegenerative disorders *J Clin Invest.* **107:**247-54.
109. **May, M. J., and S. Ghosh** 1997. Rel/NF-[kappa]B and I[kappa]B proteins: an overview *Seminars in Cancer Biology.* **8:**63-73.
110. **Meyer, R., E. N. Hatada, H. P. Hohmann, M. Haiker, C. Bartsch, U. Rothlisberger, H. W. Lahm, E. J. Schlaeger, A. P. van Loon, and C. Scheidereit** 1991. Cloning of the DNA-binding subunit of human nuclear factor kappa B: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha *Proc Natl Acad Sci U S A.* **88:**966-70.
111. **Miyamoto, S., and I. M. Verma** 1995. Rel/NF-kappa B/I kappa B story. *Adv. Cancer Res.* **66**.
112. **Mogami, H., A. V. Tepikin, and O. H. Petersen** 1998. Termination of cytosolic Ca2+ signals: Ca2+ reuptake into intracellular stores is regulated by the free Ca2+ concentration in the store lumen *Embo J.* **17:**435-42.
113. **Molitor, J. A., W. H. Walker, S. Doerre, D. W. Ballard, and W. C. Greene** 1990. NF-kappa B: a family of inducible and differentially expressed enhancer-binding proteins in human T cells *Proc. Natl. Acad. Sci. U. S. A.* **87:**10028-10032.
114. **Morris, E. P., and K. Torok** 2001. Oligomeric structure of alpha-calmodulin-dependent protein kinase II *J Mol Biol.* **308:**1-8.
115. **Nakielny, S., and G. Dreyfuss** 1999. Transport of proteins and RNAs in and out of the nucleus *Cell.* **99:**677-90.

116. **Naumann, M., and C. Scheidereit** 1994. Activation of NF-kappa B in vivo is regulated by multiple phosphorylations *Embo J.* **13:**4597-607.
117. **Neumann, M., K. Tsapos, J. A. Scheppler, J. Ross, and B. R. Franza, Jr.** 1992. Identification of complex formation between two intracellular tyrosine kinase substrates: human c-Rel and the p105 precursor of p50 NF-kappa B *Oncogene.* **7:**2095-104.
118. **Ning, Y. M., and E. R. Sanchez** 1995. Evidence for a functional interaction between calmodulin and the glucocorticoid receptor *Biochem. Biophys. Res. Commun.* **208:**48-54.
119. **Nolan, G. P., S. Ghosh, H. C. Liou, P. Tempst, and D. Baltimore** 1991. DNA binding and I kappa B inhibition of the cloned p65 subunit of NF- kappa B, a rel-related polypeptide *Cell.* **64:**961-9.
120. **Olwin, B. B., A. M. Edelman, E. G. Krebs, and D. R. Storm** 1984. Quantitation of energy coupling between Ca2+, calmodulin, skeletal muscle myosin light chain kinase, and kinase substrates *J. Biol. Chem.* **259:**10949-10955.
121. **O'Neil, K. T., and W. F. DeGrado** 1990. How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices *Trends Biochem. Sci.* **15:**59-64.
122. **Picciotto, M. R., M. Zoli, G. Bertuzzi, and A. C. Nairn** 1995. Immunochemical localization of calcium/calmodulin-dependent protein kinase I *Synapse.* **20:**75-84.
123. **Putney, J. W., Jr.** 1986. A model for receptor-regulated calcium entry *Cell Calcium.* **7:**1-12.
124. **Rasmussen, C. D., R. L. Means, K. P. Lu, G. S. May, and A. R. Means** 1990. Characterization and expression of the unique calmodulin gene of Aspergillus nidulans *J Biol Chem.* **265:**13767-75.
125. **Rayet, B., and C. Gelinas** 1999. Aberrant rel/nfkb genes and activity in human cancer *Oncogene.* **18:**6938-47.
126. **Renard, P., Y. Percherancier, M. Kroll, D. Thomas, J.-L. Virelizier, F. Arenzana-Seisdedos, and F. Bachelerie** 2000. Inducible NF-kappa B Activation Is Permitted by Simultaneous Degradation of Nuclear Ikappa Balpha *J. Biol. Chem.* **275:**15193-15199.
127. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen** 1991. Isolation of a rel-related human cDNA that potentially encodes the 65- kD subunit of NF-kappa B *Science.* **251:**1490-3.
128. **Ruland, J., and T. W. Mak** 2003. Transducing signals from antigen receptors to nuclear factor-kappaB *Immunol Rev.* **193:**93-100.
129. **Ryan, K. J., and S. R. Wente** 2000. The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm *Curr Opin Cell Biol.* **12:**361-71.

130. **Sakurai, H., H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi** 1999. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain *J Biol Chem.* **274:**30353-6.
131. **Scherer, A., and J. M. Graff** 2000. Calmodulin differentially modulates Smad1 and Smad2 signaling *J Biol Chem.* **275:**41430-8.
132. **Schmitz, M. L., S. Bacher, and M. Kracht** 2001. I[kappa]B-independent control of NF-[kappa]B activity by modulatory phosphorylations *Trends in Biochemical Sciences.* **26:**186-190.
133. **Schmitz, M. L., M. A. dos Santos Silva, and P. A. Baeuerle** 1995. Transactivation domain 2 (TA2) of p65 NF-kappa B. Similarity to TA1 and phorbol ester-stimulated activity and phosphorylation in intact cells *J Biol Chem.* **270:**15576-84.
134. **Sen, R., and D. Baltimore** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences *Cell.* **46:**705-16.
135. **Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S.-C. Sun, and M. Karin** 2001. Activation by IKKalpha of a Second, Evolutionary Conserved, NF-kappa B Signaling Pathway *Science.* **293:**1495-1499.
136. **Serfling, E., A. Avots, and M. Neumann** 1995. The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation *Biochim Biophys Acta.* **1263:**181-200.
137. **Shannon, M. F., L. S. Coles, M. A. Vadas, and P. N. Cockerill** 1997. Signals for activation of the GM-CSF promoter and enhancer in T cells *Crit Rev Immunol.* **17:**301-23.
138. **Sheng, M., M. A. Thompson, and M. E. Greenberg** 1991. CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases *Science.* **252:**1427-30.
139. **Silverman, N., and T. Maniatis** 2001. NF-{kappa}B signaling pathways in mammalian and insect innate immunity *Genes Dev.* **15:**2321-2342.
140. **Simeonidis, S., S. Liang, G. Chen, and D. Thanos** 1997. Cloning and functional characterization of mouse IkappaBepsilon *Proc. Natl. Acad. Sci. U. S. A.* **94:**14372-14377.
141. **Soderling, T. R.** 1996. Structure and regulation of calcium/calmodulin-dependent protein kinases II and IV *Biochim Biophys Acta.* **1297:**131-8.
142. **Soderling, T. R., and J. T. Stull** 2001. Structure and regulation of calcium/calmodulin-dependent protein kinases *Chem Rev.* **101:**2341-52.
143. **Steffan, N. M., G. D. Bren, B. Frantz, M. J. Tocci, E. A. O'Neill, and C. V. Paya** 1995. Regulation of IkB alpha phosphorylation by PKC-and Ca(2+)-dependent signal transduction pathways *J. Immunol.* **155:**4685-4691.

144. **Steggerda, S. M., and B. M. Paschal** 2002. Regulation of nuclear import and export by the GTPase Ran *Int Rev Cytol.* **217:**41-91.
145. **Stephens, R. M., N. R. Rice, R. R. Hiebsch, H. R. Bose, Jr., and R. V. Gilden** 1983. Nucleotide sequence of v-rel: the oncogene of reticuloendotheliosis virus *Proc Natl Acad Sci U S A.* **80:**6229-33.
146. **Steward, R.** 1987. Dorsal, an embryonic polarity gene in Drosophila, is homologous to the vertebrate proto-oncogene, c-rel *Science.* **238:**692-4.
147. **Stöven, S., I. Ando, L. Kadalayil, Y. Engström, and D. Hultmark** 2000. Activation of the Drosophila NF-{kappa}B factor Relish by rapid endoproteolytic cleavage *EMBO Reports.* **1:**347-352.
148. **Stöven, S., N. Silverman, A. Junell, M. Hedengren-Olcott, D. Erturk, Y. Engström, T. Maniatis, and D. Hultmark** 2003. Caspase-mediated processing of the Drosophila NF-kappa B factor Relish *PNAS.* **100:**5991-5996.
149. **Sun, P., H. Enslen, P. S. Myung, and R. A. Maurer** 1994. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity *Genes Dev.* **8:**2527-39.
150. **Sun, P., L. Lou, and R. A. Maurer** 1996. Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca2+/calmodulin-dependent protein kinases type I, II, and IV *J Biol Chem.* **271:**3066-73.
151. **Sun, S. C., and G. Xiao** 2003. Deregulation of NF-kappaB and its upstream kinases in cancer *Cancer Metastasis Rev.* **22:**405-22.
152. **Takada, Y., A. Mukhopadhyay, G. C. Kundu, G. H. Mahabeleshwar, S. Singh, and B. B. Aggarwal** 2003. Hydrogen Peroxide Activates NF-{kappa}B through Tyrosine Phosphorylation of I{kappa}B{alpha} and Serine Phosphorylation of p65: EVIDENCE FOR THE INVOLVEMENT OF I{kappa}B{alpha} KINASE AND Syk PROTEIN-TYROSINE KINASE *J. Biol. Chem.* **278:**24233-24241.
153. **Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira** 1999. Limb and skin abnormalities in mice lacking IKKalpha *Science.* **284:**313-6.
154. **Takeda, T., and M. Yamamoto** 1987. Analysis and in vivo disruption of the gene coding for calmodulin in Schizosaccharomyces pombe *Proc Natl Acad Sci U S A.* **84:**3580-4.
155. **Tam, W. F., and R. Sen** 2001. Ikappa B Family Members Function by Different Mechanisms *J. Biol. Chem.* **276:**7701-7704.
156. **Taylor, C. W.** 2002. Controlling calcium entry *Cell.* **111:**767-9.

157. **Thome, M., and J. Tschopp** 2003. TCR-induced NF-[kappa]B activation: a crucial role for Carma1, Bcl10 and MALT1 *Trends in Immunology*. **24:**419-424.
158. **Thompson, J. E., R. J. Phillips, H. Erdjument Bromage, P. Tempst, and S. Ghosh** 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B *Cell*. **80:**573-582.
159. **Tobimatsu, T., and H. Fujisawa** 1989. Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs *J Biol Chem*. **264:**17907-12.
160. **Tokumitsu, H., M. Muramatsu, M. Ikura, and R. Kobayashi** 2000. Regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase *J Biol Chem*. **275:**20090-5.
161. **Trompouki, E., E. Hatzivassiliou, T. Tsichritzis, H. Farmer, A. Ashworth, and G. Mosialos** 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members *Nature*. **424:**793-796.
162. **Van Eldik, L., and D. M. Watterson** (eds.) 1998 Calmodulin and signal transduction. Academic Press, New York.
163. **Wang, D., and A. S. Baldwin, Jr.** 1998. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529 *J Biol Chem*. **273:**29411-6.
164. **Wang, D., S. D. Westerheide, J. L. Hanson, and A. S. Baldwin, Jr.** 2000. Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II *J Biol Chem*. **275:**32592-7.
165. **Waxham, M. N., and J. Aronowski** 1993. Ca2+/calmodulin-dependent protein kinase II is phosphorylated by protein kinase C in vitro *Biochemistry*. **32:**2923-30.
166. **Whiteside, S. T., J. C. Epinat, N. R. Rice, and A. Israel** 1997. I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity *Embo J*. **16:**1413-1426.
167. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin** 1984. Nucleic acid sequences of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel *J Virol*. **52:**172-82.
168. **Xiao, G., E. W. Harhaj, and S. C. Sun** 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100 *Mol Cell*. **7:**401-9.
169. **Yamamoto, Y., and R. B. Gaynor** 2001. Role of the NF-kappaB pathway in the pathogenesis of human disease states *Curr Mol Med*. **1:**287-96.

170. **Yamamoto, Y., U. N. Verma, S. Prajapati, Y.-T. Kwak, and R. Gaynor** 2003. Histone H3 phosphorylation by IKK-alfa is critical for cytokine-induced gene expression *Nature*. **423:**655-659.
171. **Yoneda, Y., M. Hieda, E. Nagoshi, and Y. Miyamoto** 1999. Nucleocytoplasmic protein transport and recycling of Ran *Cell Struct Funct*. **24:**425-33.
172. **Zhong, H., M. J. May, E. Jimi, and S. Ghosh** 2002. The Phosphorylation Status of Nuclear NF-kappaB Determines Its Association with CBP/p300 or HDAC-1 *Molecular Cell*. **9:**625-636.
173. **Zhong, H., H. SuYang, H. Erdjument Bromage, P. Tempst, and S. Ghosh** 1997. The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism *Cell*. **89:**413-424.
174. **Zhong, H., R. E. Voll, and S. Ghosh** 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300 *Mol Cell*. **1:**661-671.
175. **Zimmerman, C. M., M. S. Kariapper, and L. S. Mathews** 1998. Smad proteins physically interact with calmodulin *J Biol Chem*. **273:**677-80.