Chapter 4

REGULATION OF NF-κB TRANSCRIPTIONAL ACTIVITY

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Abstract: Nuclear factor κB (NF-κB) is regarded as a key regulator of inflammation; hence, several inflammatory diseases result from deregulation of NF-κB signaling. There is, however, also increasing evidence for a preponderant role of NF-κB in tumor development and progression. Constitutive activation of NF-κB activity by signaling defects, mutations or chromosomal rearrangements can be found in a wide variety of cancers. Additionally, a causal link between inflammation and cancer has been noted, which makes NF-κB an interesting target for development of both anti-inflammatory and anti-cancer therapeutics. Here, we review current knowledge of NF-κB signal transduction, focusing on the regulation of its transcriptional activity by post-translational modification of the NF-κB subunits.

Keywords: NF-κB; signal transduction; acetylation; phosphorylation; inflammation; cancer

1. INTRODUCING NF-κB

NF-κB comprises a family of inducible transcription factors that serve as important regulators of immune and inflammatory responses. In addition, the relevance of NF-κB to the pathogenesis and treatment of cancer is becoming more evident. The homo- or heterodimeric NF-κB complexes are composed of members of the Rel family of proteins, which all contain an N-terminal Rel homology domain (RHD). According to their modular structure, Rel family members can be divided in 2 groups. The first one contains p65 (RelA), cRel and RelB. These proteins represent the transactivation function of the complexes, since they all posses one or more transactivation domain. The second subgroup, lacking transactivation domains, is produced cotranslationally or upon processing of precursor proteins (p100 to p52, p105 to p50). Activation of NF-κB is regulated by a variety of IκB proteins (IκBα, IκBβ, IκBγ, IκBε, BCL-3). They share C-terminal ankyrin repeats, which are also found in the p100 and p105 precursor proteins. In most cells, the majority of NF-κB complexes are retained in the cytoplasm by IκB molecules, which mask nuclear localization sequences. Translocation of NF-κB to the nucleus and engagement of NF-κB enhancer sequences generally results from signal-induced phosphorylation of IκB by the IκB kinase (IKK) complex (reviewed in Israel, 2000). Following subsequent ubiquitination, the inhibitor gets degraded by the 26S proteasome. Among the IκB molecules, IκBα is the only one which displays nucleocytoplasmic shuttling. NF-κB complexes bound to IκBα can freely translocate to the nucleus, where an efficient export machinery assures that only very low amounts of NF-κB can be found in the nucleus of unstimulated cells. Additionally, IκBα can actively relieve DNA-bound NF-κB complexes and send them back to the cytoplasm, hereby playing an important role in the termination of the signal. Full transcriptional activation of NF-κB in the nucleus is ensured by additional post-translational modification (phosphorylation/acetylation) of the transcription factor (Chen and Greene, 2004; Schmitz *et al.*, 2001; Vermeulen *et al.*, 2002). Genes activated by NF-κB include cytokines, chemokines, adhesion molecules and receptors involved in immune recognition. Deregulation of expression of these molecules is associated with a large variety of inflammatory and autoimmune diseases like rheumatoid arthritis, asthma and inflammatory bowel disease. Various scientists have suggested a causal link between inflammation and cancer (Farrow *et al.*, 2004; Greten *et al.*, 2004). However, the exact mechanism by which

inflammation may promote tumorigenesis has not been entirely revealed, yet.

Constitutive NF-κB activity has been found in several breast cancers, tumor cell lines and lymphoid malignancies (Biswas *et al.*, 2004; Karin *et al.*, 2002). The first evidence that linked NF-κB to hematopoietic cancers came from studies with the avian REV-T retrovirus oncoprotein v-Rel (a viral mutant of c-Rel) that causes B-cell lymphomas in young birds. Other viruses that have evolved to affect NF-κB regulation and lead to viral transformation are human T-cell leukemia virus type 1 and Epstein-Barr virus (reviewed in Hiscott *et al.*, 2001). In addition, genetic alterations like amplification, deletion or chromosomal rearrangements of regions encoding NF-κB or IκB family members are found in several lymphomas (reviewed in Karin *et al.*, 2002). Furthermore, NF-κB is directly involved in protecting cells from undergoing apoptosis in response to DNA damage or cytokine treatment through the induction of anti-apoptotic genes. Unfortunately, many chemotherapeutics or radiation used in the treatment of cancer also induces NF-κB. This undesired property leads to anti-apoptotic signals which actually counteract the activity of the anti-cancer therapies.

Taken together, all these findings suggest that NF-κB inhibitors could be developed into a new class of anti-cancer therapeutics. It is therefore of extreme importance to fully dissect the mechanisms of activation and post-translational regulation of NF-κB to be able to design specific drugs.

2. REGULATING NF-κB TRANSCRIPTIONAL ACTIVITY

Although the primary mechanism of regulation of NF-κB activity is the liberation of the transcription factor from its inhibitor IκB leading to nuclear translocation, the transcriptional activity of NF-κB in the nucleus (that is the ability to recruit the transcriptional apparatus and stimulate target gene expression) is ensured by additional post-translational modification (i.e. phosphorylation, acetylation etc.) of the transcription factor itself and its surrounding chromatin environment.

To date, it has not been determined if altered post-translational regulation of either NF-κB family member lies at the basis of any of the diseases which involves deregulation of NF-κB.

2.1 Regulation by phosphorylation

2.1.1 p65 (RelA)

Phosphorylation of p65, the transactivating subunit of the most common NF-κB complex p50/p65, has been widely studied. In response to different stimuli, five phosporylated Ser residues have been identified (Figure 1).

Ser276 situated in the RHD can be phosphorylated both by PKAc and MSK1 (mitogen- and stress-induced protein kinase-1). PKAc, included in some fractions of the NF-κB/IκB complex, is held inactive by IκB. Upon LPS treatment, PKAc becomes activated after degradation of IκB and is able to phosphorylate Ser276 (Zhong *et al.*, 1997). MSK1, a nuclear kinase that is itself activated by both ERK and p38 kinases, phosphorylates p65 at the same site in response to TNF treatment (Vermeulen *et al.*, 2003). Conformational changes in p65 triggered by Ser276 phosphorylation enable binding to CBP/p300 cofactors. These proteins stimulate gene expression via their intrinsic histone acetyl transferase (HAT) activity (see 2.2) (Zhong *et al.*, 1998).

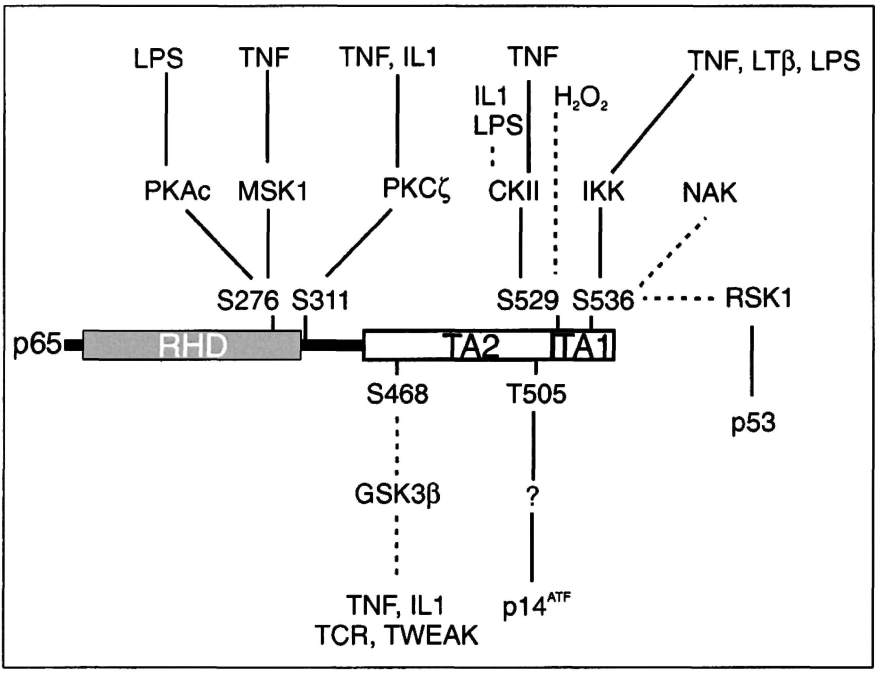

Figure 1. p65 phosphorylation

In endothelial cells where constitutive Ser276 phosphorylation was found, overexpression studies point to a possible role for PKCζ in the phosphorylation of the p65 RHD (Anrather *et al.*, 1999). Later, the role of PKCζ in p65 phosphorylation was confirmed using $PKC\zeta^{-/-}$ embryonal fibroblasts. The lack of functional PKCζ clearly inhibited cytokine-induced phosphorylation and transcriptional activity of p65 (Leitges *et al.*, 2001). Recently, Ser311 was identified as the PKCζ target. Its phosphorylation is essential for p65/CBP interaction and recruitment of CBP and RNA polymerase II to the NF-κB-dependent IL-6 promoter (Duran *et al.*, 2003).

Recently, Ser468 was also detected to be phosphorylated in response to TNF, IL-1 or phorbolester plus ionomycin treatment and T-cell receptor co-stimulation (Buss *et al.*, 2004a; Mattioli *et al.*, 2004b). In contrast to p65 phosphorylation at other sites, Ser468 phosphorylation appears to regulate NF-κB dependent gene expression in a negative way (Buss *et al.*, 2004a). Ser468 is one of the four GSK3β concensus phosphorylation sites that were detected in the C-terminal portion of p65, which are phosphorylated in vitro by GSK3β (Schwabe and Brenner, 2002). Furthermore, NF-κB-dependent transcription is impared in $GSK3\beta^{-/-}$ cells. Depletion of GSK3β did not alter IκB phosphorylation nor p65 nuclear translocation, but clearly decreased NF-κB/DNA-binding activity (Hoeflich *et al.*, 2000). Recently, we found that GSK3β also plays an important role in signaling from TWEAK (a TNF-family member) to NF-κB (De Ketelaere *et al.*, 2004). However, in response to TWEAK, GSK3β positively regulates NF-κB-dependent gene expression.

In response to TNF treatment, p65 can be phosphorylated in HeLa cells at Ser529 by a casein kinase II (CKII) fraction that is itself part of the NF-κB/IκB complex (Wang and Baldwin, 1998; Wang *et al.*, 2000). IL-1 can also mediate p65 phosphorylation through CKII (Bird *et al.*, 1997). In addition, phosphorylation of Ser529 was observed when KBM-5 cells were treated with H_2O_2 (Takada *et al.*, 2003). By comparing EMT-6 clones with different ability to induce the NOSII gene upon IL-1β or LPS treatment, it was found that absence of CKII-mediated p65 phosphorylation was responsible for a decreased NF-κB-mediated activation of the NOSII gene in some of these cells (Chantome *et al.*, 2004).

Phosphorylation of Ser536 is mediated by the IKK complex (Sakurai *et al.*, 1999). Dependent on the cell line and treatment, Ser 536 phosphorylation is either transient or sustained. In HeLa cells, phosphorylation is maximal at 5 minutes of TNF treatment. It was suggested that p65 is dephosphorylated once it enters the nucleus (Sakurai *et al.*, 2003). Cytoplasmic Ser536 phosphorylation also takes place after T-cell costimulation within an intact NF-κB/IκBα complex. Prior phosphorylation of IκBα is required. Reconstitution experiments in $p65^{-/-}$ MEF cells showed that Ser536 phosphorylation negatively regulates the kinetics of nuclear import during

the shuttling of NF-κB/IκBα complexes. Concomitantly, cells expressing the Ser536 mutant form of p65 showed significantly higher amounts of IκBα in the nucleus, suggesting a contribution of Ser536 for cytoplasmic retention of IκBα (Mattioli *et al.*, 2004a). Recently, it was suggested that Ser536 phosphorylation enhances interaction with the TATA-binding protein-associated factor II31 (TAFII31), a component of TFIID of the basal transcription machinery. In the absence of phosphorylation, p65 favours binding of the corepressor amino-terminal enhancer of split (AES) (Buss *et al.*, 2004b). Other stimuli that induce Ser536 phosphorylation through the IKK complex are LPS (Yang *et al.*, 2003) and lymphotoxin β (LTβ) (Jiang *et al.*, 2003). In vitro, NAK (NF-κB-activating kinase), an IKK-related protein kinase was also found to phosphorylate Ser536 (Fujita *et al.*, 2003). Recently, Ser536 has been identified as a site that is phosphorylated by RSK1 as well. This happens in response to DNA damage through signaling by the tumor suppressor p53. p53-mediated activation of NF-κB does not occur via the classical activation pathway. Neither inducible degradation of IκB, nor the activation of the IKK complex is involved in this process. The lower affinity of RSK1-phosphorylated p65 for IκBα decreases IκBα-mediated nuclear export of shuttling forms of NF-κB, thereby promoting NF-κB/DNA-binding (Bohuslav *et al.*, 2004). It has also been suggested that intact Ser529 and Ser536 residues are necessary for an Akt-mediated increase in p65 transcriptional activity, although no kinase assays were performed to prove this (Madrid *et al.*, 2001). The contribrution of the Akt/PI3K pathway to either NF-κB translocation or p65 transcriptional activation is still controversial and has been discussed in detail in Vermeulen *et al.*, 2002. With respect to regulation of p65 transcriptional activity, Sizemore and colleagues observed specific inhibition of IL-1-induced p65 phosphorylation by PI3K inhibitors in HepG2 cells (Sizemore *et al.*, 1999). Later, they and others showed that this effect depends on IKK (Madrid *et al.*, 2000; Sizemore *et al.*, 2002) and p38 kinase activity (Madrid *et al.*, 2001).

Alternatively, phosphorylation can inhibit p65 transcriptional activity. Upon activation of the tumor suppressor gene ARF, Thr505 phosphorylation triggers association with the HDAC1 corepressor protein. Simultaneously, expression of TNF-induced anti-apoptotic genes, like Bcl-xL, are repressed (Rocha *et al.*, 2003).

Overexpression of CaMKIV also enables phosphorylation in the C-terminal domain (Jang *et al.*, 2001), whereas phosphorylation of the transactivation domain 2 (TA2) is visible upon PMA (phorbol myristate acetate) treatment (Schmitz *et al.*, 1995); however, the exact phosphorylation sites have not been identified.

Finally, it should be noted that the entire p65 phosphorylation status is not only determined by kinase activities, but results from the interplay of both

kinases and phosphatases. Indeed, protein phosphatase 2A (PP2A) is physically associated with p65 in unstimulated melanocytes and is thus able to dephosphorylate p65 after IL-1 stimulation (Yang *et al.*, 2001). PP4 also interacts with p65 in cervical carcinoma cells. Overexpression of PP4 has been shown to activate NF-κB by p65 dephosphorylation. Phospho-Thr435 has been suggested as a target for PP4 (Yeh *et al.*, 2004).

2.1.2 Other NF-κB family members

Investigations into the effect of phosphorylation on the transactivation capacity of cRel or RelB have been rare. cRel-TD kinase, a 66 kDa Ser/Thr kinase has been identified to bind cRel both *in vitro* and *in vivo*. Ser451 in the C-terminal part of cRel was suggested to be the target of this kinase which has not been further characterized up until now. Mutation of Ser451 or deletion of the surrounding consensus sequence inhibited transcriptional activity of c-Rel (Fognani *et al.*, 2000). In the case of RelB, phosphorylation has not been found to alter its transcriptional activity. Instead, phosphorylation is involved in RelB degradation (Marienfeld *et al.*, 2001) and Ser368 has been shown to be crucial in dimerisation and p100 stabilization (Maier *et al.*, 2003).

2.2 Regulation by acetylation

Phosphorylation of p65 Ser276 and Ser311 is important for the recruitment of CBP/p300 cofactors. CBP and p300 are histone acetyl transferases (HATs). The levels of histone acetylation have been correlated with the transcription status of many genes. Transcriptionally active euchromatin is often bound to hyperacetylated histones (Jenuwein and Allis, 2001). Long-standing models have suggested that histone phosphorylation and/or acetylation disrupts electrostatical interaction between neighbouring histones or between the basic histone tails and the negatively charged DNA (Wolffe and Hayes, 1999). As a consequence, the accessibility of the underlying genome for nuclear factors is increased. Alternatively, reversible modification of histone tails might encode a histone 'language' that is specifically recognized by other proteins or protein modules to elicit appropriate downstream responses (Cheung *et al.*, 2000; Jenuwein and Allis, 2001; Strahl and Allis, 2000). Recently, NF-κB family members have been shown to be directly targeted by acetylation (reviewed in Quivy and Van Lint, 2004). It has been shown that p65 also interacts with distinct HDAC (histone deacetylase) isoforms to negatively regulate gene expression

(Ashburner *et al.*, 2001; Chen *et al.*, 2001; Ito *et al.*, 2000). HDACs counteract HAT activity. It has been suggested that shifting the balance between acetylation and deacetylation contributes to the regulation of NF-κB-dependent gene expression. Indeed, in a defined subset of genes, p50 homodimers, associated with HDAC-1, are bound to DNA in unstimulated cells. Following stimulation, p50/p65 heterodimers enter the nucleus and displace DNA-bound p50/p50/HDAC-1 complexes. Phosphorylation of nuclear NF-κB determines whether it is associated with CBP/p300 (HATs) or HDAC-1, ensuring that only signal-induced NF-κB entering the nucleus can activate transcription (Zhong *et al.*, 2002).

Recently, it was observed that HATs can also target non-histone proteins. To date, five acetylation sites have been detected in p65. Chen *et al.* identified lysines 218, 221 and 310 as target for acetylation by p300 (Chen *et al.*, 2002). Mutation of K218 did not result in a significant difference in activation of the NF-κB-dependent E-selectin promoter compared to wt p65. In contrast, activation by K221R or K310R mutant p65 was clearly impaired. Acetylation of K221 enhances the binding affinity of the NF-κB complex for the κB consensus sequence. It was suggested that Ac-K221, alone or in combination with Ac-K218, plays a key role in impairing the assembly of p65 with IκBα, thereby preventing nuclear export (Chen *et al.*, 2002). Alternatively, cotreatment of cells with deacetylase inhibitors was suggested to prolong induced NF-κB/DNA-binding activity by delaying the replenishment of the cytoplasmic pool of IκBα by newly synthesized protein (Adam *et al.*, 2003). As DNA-binding was not altered when K310 was mutated, acetylation of K310 seems to enhance p65 transactivation potential (Chen *et al.*, 2002). SIRT1, a nicotinamde adenosine dinucleotide-dependent histone deacetylase, regulates the transcriptional activity of NF-κB through deacetylation of p65 K310 (Yeung *et al.*, 2004).

In vitro acetylation assays using p300 or p/CAF identified K122 and K123 as acetyl-acceptor sites (Kiernan *et al.*, 2003). Acetylation of these sites, however, lowers the affinity of NF-κB for the κB-concensus sequence, which facilitates the removal of the transcription factor from enhancer elements by newly synthesized IκBα. In this case, p65 acetylation inhibits the overall transcriptional activity of the NF-κB complex. Deacetylation of these lysine residues can be accomplished by HDAC3.

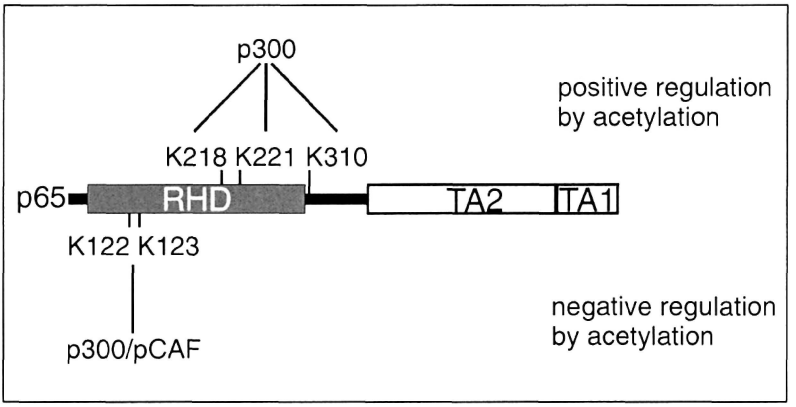

Figure 2. p65 acetylation

Similar to p65, acetylation of p50 increases its DNA-binding properties, which coincides with an increased rate of transcription (Deng *et al.*, 2003; Furia *et al.*, 2002). *In vitro* assays revealed 3 acetylation sites: K431, K440 and K441 (Furia *et al.*, 2002).

2.3 Pinning and SUMOylation

Pin1 is a peptidyl-prolyl isomerase that binds and isomerizes specific phosphorylated serine or threonine residues that precede proline (pSer/Thr-Pro) in certain proteins. This process induces conformational changes that can effect transcriptional regulation, cell cycle progression, RNA processing, cell proliferation and differentiation (Lu, 2003 and 2004). Recently, Pin1 has been shown to interfere with NF-κB signaling. Upon cytokine treatment, Pin1 binds to the pThr254-Pro motif in p65 and inhibits p65 binding to IκBα, which leads to increased nuclear accumulation and enhanced NF-κB activity. Additionally, association of Pin1 with p65 was shown to stabilize wild type p65 but not a Thr254-mutant. In Pin1-deficient cells, steady-state levels of p65 were found to be much lower when compared to wild type cells. This resulted in reduced NF-κB signaling in response to cytokine treatment (Ryo *et al.*, 2003). Thr254 phosphorylation, as suggested by these authors, has not been observed earlier, nor has the kinase been identified.

Small ubiquitin-related modifier (SUMO) proteins function by covalent binding to other proteins (Johnson, 2004). Next to a large variety of cellular processes, SUMO is also involved in the regulation of NF-κB activity. SUMO-1 conjugation to IκBα is able to inhibit proteasome-mediated degradation of IκBα since it targets the same lysine residue (K21) that is

normally ubiquitinated in response to cytokine treatment. Consistently, SUMO-1 overexpression inhibits NF-κB-dependent transcription (Desterro *et al.*, 1998). SUMOylation of IKKγ has also been observed in response to DNA damage, where it is an essential step in the activation of the IKK complex (Hay, 2004; Huang *et al.*, 2003).

2.4 p65, a potential target for future therapies?

Small molecule NF-κB inhibitors are being developed by many pharmaceutical companies to be tested as anticancer drugs either alone or in combination with classical chemotherapeutics and/or radiation to counteract activated NF-κB-dependent anti-apoptotic properties. Most of these target IKKβ (reviewed in Haefner, 2002; Karin *et al.*, 2004). Several natural compounds have been proposed to have a role in the treatment or prevention of cancer. Among these are resveratrol, curcumin and green tea extracts. All of these agents have been described to inhibit IKK/NF-κB activation (Surh, 2003). Although NF-κB appears as a logical target for anti-cancer therapeutics, it is not desirable to block NF-κB signaling for prolonged periods of time since the transcription factor also plays an important role in the maintenance of host defense responses. Therefore, intervening selectively with p65 transcriptional activity without completely blocking NF-κB activation, might be a superior strategy.

Despite numerous studies, researchers have not yet reached a consensus as to the extent by which the various p65 residues are phosphorylated/acetylated within a particular cell line, nor as to the contribution and dynamics of each modification to the transcriptional control of the different classes of NF-κB-regulated genes. Indeed, although many genes contain NF-κB-responsive elements, their expression pattern may vary from both a kinetic and quantitative point of view. In addition, post-translational regulation of p65 transcriptional activity appears to be time-dependent and cell type-specific. Further studies directing attention to these intriguing aspects of gene regulation are needed. One might speculate that a specific phosphorylation event is needed for the full expression of certain subclasses of NF-κB-regulated genes but not for that of others. Therefore, it would be interesting to make a list of genes which need specific p65 kinases or phosphorylation at specific sites for full transcriptional activation. This information could be very useful in the search for more specific NF-κB inhibitors as therapeutic agents. Indeed, when a certain kinase is involved in the transcriptional regulation of anti-apoptotic genes but is not essential for the expression of pro-inflammatory genes, inhibition of such kinases might

lead to safer drugs for cancer treatment. Alternatively, specific inhibitors of pro-inflammatory genes could be desirable in inflammatory disease therapy.

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