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Regulation of the transcriptional activity of the nuclear factor-κB p65 subunit

Linda Vermeulen, Gert De Wilde, Sofie Notebaert, Wim Vanden Berghe, Guy Haegeman*

Department of Molecular Biology, University of Gent-VIB, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

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Abstract

Nuclear factor-κB (NF-κB) is well known for its role in inflammation, immune response, control of cell division and apoptosis. The function of NF-κB is primarily regulated by IκB family members, which ensure cytoplasmic localisation of the transcription factor in the resting state. Upon stimulus-induced IκB degradation, the NF-κB complexes move to the nucleus and activate NF-κB-dependent transcription. Over the years, a second regulatory mechanism, independent of IκB, has become generally accepted. Changes in NF-κB transcriptional activity have been assigned to phosphorylation of the p65 subunit by a large variety of kinases in response to different stimuli. Here, we give an overview of the kinases and signalling pathways mediating this process and comment on the players involved in tumour necrosis factor-induced regulation of NF-κB transcriptional activity. Additionally, we describe how other posttranslational modifications, such as acetylation and methylation of transcription factors or of the chromatin environment, may also affect NF-κB transcriptional activity.

Keywords: NF-κB; Gene regulation; Phosphorylation; MSK; Histone modification; Transcriptional activity

1. Introduction

NF-κB transcription factors are key regulators of immune and inflammatory responses [1,2], which appear as homo- or heterodimers composed of members of the NF-κB/Rel family (Fig. 1). They bind selectively to the κB consensus sequence GGGRNNYYCC (R = purine, Y = pyrimidine, N = any base), which is found in the promoter of a large variety of genes [3]. Proteins belonging to the NF-κB/Rel family all share an N-terminal Rel homology domain and can be divided into two classes based on their C-terminal sequences [2,4]. In vertebrates, one group includes RelA (p65), RelB and cRel. These proteins contain one or more transactivation domains in their C-terminal end. Members of the second class (p50 and p52), which do not exert transactivation functions, are produced cotranslationally or by limited proteolysis from larger precursor proteins (i.e. p105 and p100). The C-terminal domain of these precursors consists of multiple copies of ankyrin repeats. NF-κB activity is tightly regulated by members of the IκB family, which also contain ankyrin repeats. In unstimulated cells, NF-κB is generally found in the cytoplasm. This subcellular localisation results from efficient masking of the nuclear localisation signal of NF-κB by IκBβ and IκBε [5,6]. Complexes bound by IκBα continuously shuttle between the cytoplasm and the nucleus. A highly efficient nuclear export of these complexes ensures very low levels of nuclear NF-κB in the uninduced state [7,8]. Activation of the IκB kinase complex, consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit IKKγ (reviewed in [9,10]), leads to phosphorylation of the IκB molecules at two conserved N-terminal Ser residues. Subsequent ubiquitination and degradation of the inhibitor liberates the NF-κB complex, which is now able to migrate to the nucleus and to activate NF-κB-regulated gene expression.

* Corresponding author. Tel.: +32-9-264-51-66; fax: +32-9-264-53-04.
E-mail address: guy.haegeman@dmb.rug.ac.be (G. Haegeman).

Abbreviations: CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; HDAC, histone deacetylase; IKK, IκB kinase; IL, interleukin; MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PKAc, catalytic domain of protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RHD, Rel homology domain; RSK, ribosomal S6-kinase; TNF, tumour necrosis factor.

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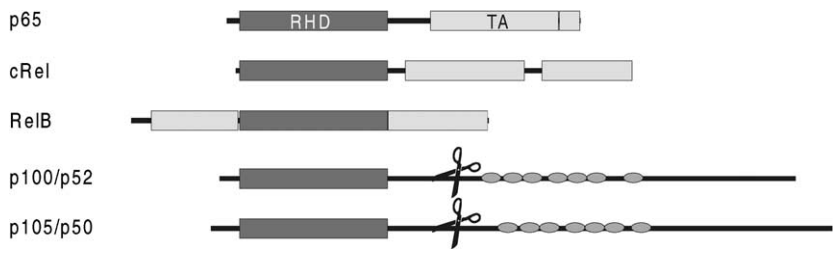

Fig. 1. The NF-κB family. Dark grey area show the Rel homology domain (RHD), which is homologous in all transcription factor members of the NF-κB/Rel family. Light grey regions are more variable and represent transactivation domains (TA). The largest family members contain so-called ankyrin repeats, which are processed to generate smaller, non-transactivating family members.

2. Enhanced NF-κB transcriptional activity by p65 phosphorylation

Although the primary level of regulation of NF-κB activity lies in the cytoplasm by liberating the factor from its inhibitory subunits and nuclear translocation, the transactivation potential of NF-κB in the nucleus, i.e. the ability to recruit the transcriptional apparatus and stimulate target gene expression, is ensured by additional modification of the transcription factor itself and its surrounding chromatin environment. In this respect, inducible p65 phosphorylation has been found both in the C-terminal transactivation domains and in the RHD [11] (Fig. 2). In response to TNF treatment, p65 can be phosphorylated in HeLa cells at Ser529 by a casein kinase II (CKII) fraction being part of the NF-κB/IκB complex [12,13]. IL1 can also mediate p65 phosphorylation through CKII [14]. Apart from this, Ser536 was shown to be phosphorylated by the IKK complex [15]. Phosphorylation of the transactivation domain 2 (TA2) is visible after PMA treatment [16], whereas overexpression of Ca^{2+}/calmodulin kinase IV also enables phosphorylation in the C-terminal domain [17]; however, the exact phosphorylation sites have not yet been determined. Ser276, situated in the RHD, can be phosphorylated by PKAc as well as by MSK1. PKAc, included in some fractions of the NF-κB/IκB complex, is kept inactive by IκB. When IκB is degraded, PKAc becomes activated and phosphorylates Ser276 [18]. 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.[1] The N-terminal domain of p65 was shown to be phosphorylated by PKCζ [19]; correspondingly, IL1- or TNF-induced p65 phosphorylation is inhibited in PKCζ$^{-/-}$ embryonal fibroblasts [20]. It should be noted that the entire p65 phosphorylation status is not only determined by kinase activities, but also 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 IL1 stimulation [21].

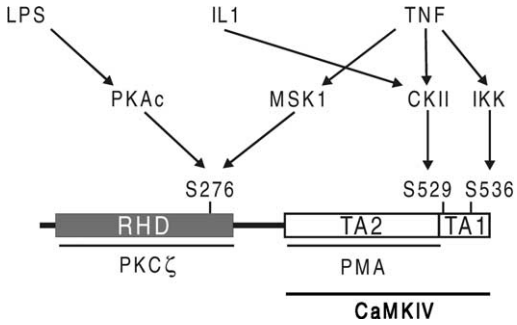

Fig. 2. Phosphorylation scheme of p65. The main inflammatory stimuli, as well as the proposed kinases and target sites involved, are indicated.

Additionally, the transactivation function of p65 might also be regulated by reversible acetylation. Overexpression of HDAC1 and HDAC2 reduces TNF-mediated p65 transcriptional activity, associated with impaired NF-κB-dependent gene expression [22]. Stimulus-induced acetylation of p65, probably mediated by CBP/p300, has been shown to control the duration of the NF-κB transcriptional response [23]. Deacetylation of p65 through specific interaction with HDAC3 promotes effective binding to newly synthesised IκBα, which subsequently leads to IκBα-dependent nuclear export. Alternatively, cotreatment of cells with deacetylase inhibitors prolonged induced NF-κB DNA-binding activity by delaying the replenishment of the cytoplasmic pool of IκBα with newly synthesised protein.[2]

3. The controversial role of PI3K signalling in the transcriptional activation of NF-κB

The possible role of PI3K signalling in cytoplasmic NF-κB activation or nuclear p65 transactivation is still a matter of debate. Using specific PI3K inhibitors, dominant-negative PI3K or kinase-dead Akt (a downstream kinase of PI3K), TNF-induced DNA binding was impaired in 293, HeLa and ME180 cells [24]. Moreover, Akt was able to

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phosphorylate IKKα and to enhance IκB degradation [24,25]. Others could show the involvement of PI3K signalling in NF-κB nuclear translocation after lipopolysaccharide and PMA treatment, but not in response to TNF, ceramide, H_2O_2 or ocadaic acid [26]. These experiments point to a possible role for PI3K signalling pathways in activating the IKK complex. Alternatively, the involvement of PI3K pathways in p65 transactivation has been proposed: specific PI3K inhibitors block IL1-induced p65 phosphorylation and transcriptional activity in HepG2 cells without affecting IκB degradation, nuclear translocation or NF-κB DNA-binding capacity [27]. An enhanced p65 transactivation potential, induced by oncogenic Ras, is also mediated by the PI3K/Akt pathway in NIH3T3 cells [28]. Moreover, overexpression studies with activated Akt revealed that IKKβ and p38 are both necessary for enhanced p65 transactivation, whereas mutation of either Ser529 or Ser536 abolishes this effect [29]. The involvement of both IKKα and IKKβ in C-terminal p65 phosphorylation was recently confirmed using $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ MEF cells [30]. In contrast, a number of research groups, including ours, found no evidence for the involvement of PI3K signalling in NF-κB-mediated gene expression in endothelial cells, smooth muscle cells and fibroblasts [31–33]. More particularly, by using the inhibitors wortmannin and LY294002, we investigated the possible involvement of the PI3K pathway in TNF-induced NF-κB regulation in the mouse fibrosarcoma cell line L929sA. LY294002 and wortmannin had no effect on the translocation or DNA-binding capacity of the p50/p65 NF-κB complex (Fig. 3A). Furthermore, the effect of the PI3K pathway on the p65 transcriptional activity was tested using the Gal4-'one hybrid' system. TNF clearly enhanced p65 transcriptional activity by 3-fold. Pretreatment with LY294002 and wortmannin did not significantly alter this effect, whereas inhibitors of the MAPK pathways or H89 (a PKA and MSK inhibitor) clearly do (Fig. 3B). Taken together, we find that, in the fibroblasts tested, inhibitors of the PI3K pathway do not change TNF-induced NF-κB DNA-binding activity nor interfere with p65 transactivation in the TNF-signalling pathway.

In conclusion, although PI3K pathways might be involved in NF-κB activation or regulation of p65 transcriptional activity in particular cell systems or depending on the stimuli used, it is certainly not a general phenomenon.

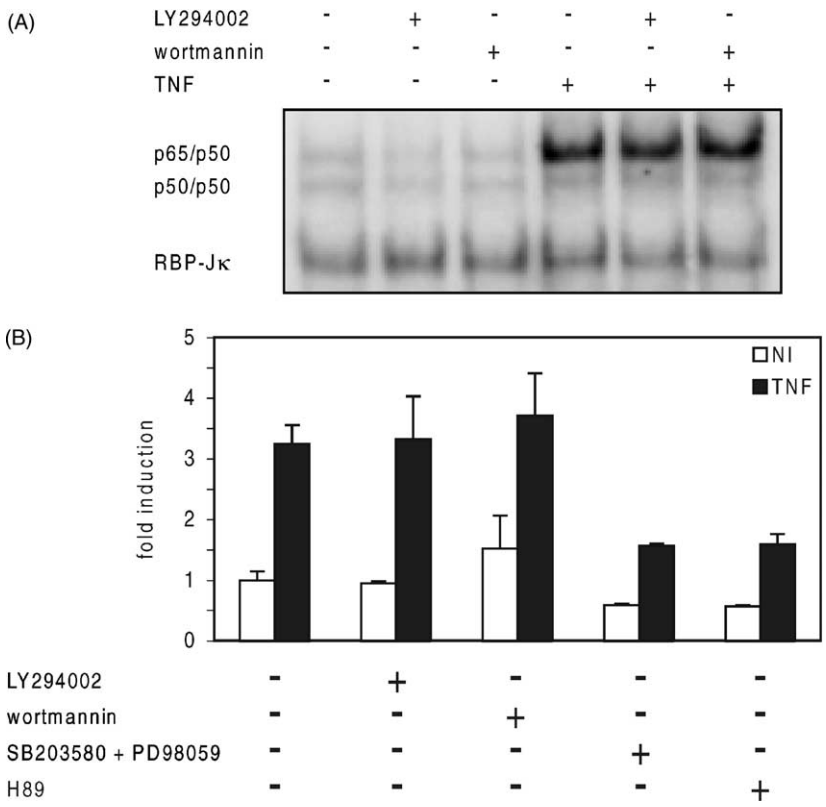

Fig. 3. The PI3K pathway is not involved in NF-κB-dependent gene regulation. (A) L929sA cells were left untreated, or were treated with 2000 IU/mL TNF for 30 min, either or not following a 2-hr pretreatment with 20 μM LY294002 or 100 nM wortmannin. Total cell lysates were incubated with a ^{32}P-labelled IL6-κB site-containing probe. Loading of equal protein amounts was verified by comparison with the binding activity of the repressor molecule RBP-Jκ [85]. (B) L929sA cells, stably expressing Gal4-p65 and transfected with a $p(gal4)_2$huIL6-luc+ reporter construct, were left untreated, or were induced with 2000 IU/mL TNF for 6 hr, either or not following a 2-hr pretreatment with 20 μM LY294002, 100 nM wortmannin, 10 μM SB203580 + 10 μM PD98059 or 10 μM H89.

4. Identification of MSKs as nuclear p65 kinases

Mutation analysis of p65 Ser276 showed the extreme importance of this residue for engagement of the cofactor CBP and its paralogue p300 (see footnote 1) [34]. Although PKAc is able to phosphorylate Ser276 in the cytoplasm, this cannot explain the inhibitory effect of SB203580 and PD98059 (inhibitors of the p38 and ERK signalling pathways, respectively) on TNF-induced p65 transcriptional activity. MSK1 is a direct target for both p38 and ERK MAP kinases and was first identified as a very potent CREB kinase [35]. Recently, MSK1 activity was shown to be inhibited by H89, mainly known as a potent PKA inhibitor [36]. Alignment of the CREB sequence containing the MSK1 phosphorylation site (Ser133) and the sequence surrounding Ser276 of p65 revealed an unexpectedly high degree of similarity (Fig. 4). The fact that phosphorylation of CREB and/or p65 is essential for recruitment of CBP/p300 led us to investigate the role of MSK1 in p65 phosphorylation. Indeed, MSK1 was shown to be involved in Ser276 phosphorylation and subsequent gene activation in response to TNF treatment (see footnote 1).

MSK2, also termed RSK-B, is approximately 75% identical to MSK1 and seems to share some functional properties. Both kinases are involved in CREB phosphorylation and activation [35,37]. Additionally, TNF is able to activate MSK2 activity, which can also be counteracted by H89 treatment [38]. In view of these data, one might also suggest a role for MSK2 in p65 phosphorylation. However, MSK1 activation is a highly transient process, reaching a peak at 10–15 min after induction and returning to basal level after 30 min (see footnote 1) [35]. MSK2 activation occurs as fast, but is sustained for a much longer period of time, as 3 hr post-induction, MSK2 activity still reaches 50% of its maximal activity [38]. The most straightforward way to further investigate the physiological role of both MSKs in p65 phosphorylation and concomitant regulation of NF-κB transcriptional activity will include studies in MSK1$^{-/-}$, MSK2$^{-/-}$ and MSK1/2 double knockout cells.

5. MSK: a trendsetter of induced gene expression?

As mentioned above, CREB was the first MSK1 substrate to be identified [35]. CREB phosphorylation at Ser133 is a prerequisite for attracting the cofactor CBP [39–42]. In turn, CBP was proposed to activate genes by associating with the RNA polymerase complex [43,44], and by exerting an intrinsic histone acetyl transferase activity that acetylates promoter-bound histones [45–47]. Other proteins that might be activated by MSK1 phosphorylation include the transcription factor STAT3 [48] and the kinase Akt [49] in response to UV A and UV B treatment, respectively, as well as the NF-κB p65 subunit in response to TNF (see footnote 1). Besides, MSK1 is involved in the rapid induction of immediate-early genes in response to mitogen and stress stimuli by phosphorylation of histone H3 at Ser10 [36]. It has been proposed that Ser10 phosphorylation renders H3 more susceptible to acetylation at Lys14 [50–52], although later *in vivo* experiments showed that these processes are independently regulated [53]. The levels of histone acetylation have been correlated with the transcription status of many genes. Transcriptionally inactive regions in the genome (such as heterochromatin and the 'barr body,' which is the inactive X chromosome in female mammalian cells) are generally associated with hypoacetylated histones. On the contrary, transcriptionally active euchromatin is often bound to hyperacetylated histones [54]. 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 [55]. 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' specifically recognised by other proteins or protein modules to elicit appropriate downstream responses [54,56,57].

The multitude of MSK1 functions, thus, poses researchers for a real puzzle: (i) is the chromatin environment, determining gene expression, set by recruiting MSK1 prior to transcription factor activation or (ii) are specific chromatin modifications introduced by transcription factor complexes which subsequently adopt MSK1, or else (iii) are both these processes independently regulated?

6. NF-κB: switchboard for differential gene activation?

Several growth factor and stress signals (forskolin, PMA, EGF, UV, arsenite, NGF) have been shown to promote phosphorylation of CREB at Ser133, with com-

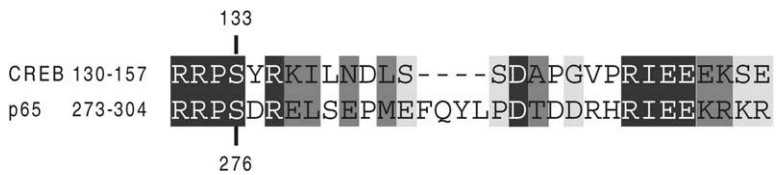

Fig. 4. Comparison of the amino acid sequences surrounding Ser276 of p65 and Ser133 of CREB.

parable stoichiometry and kinetics. Coincident with this wide profile of inducibility, CREB is a substrate for various cellular kinases including pp90rsk, PKA, PKC, Akt, MSK1, MSK2, MAPKAPK-2, and Ca^{2+}/calmodulin kinases II and IV. The question can be raised if, p65 Ser276, considering its high homology with the CREB Ser133 motif, will display a similar kinase promiscuity (PKA, MSK and others?) and how p65 can distinguish between various stimuli to perform different gene responses. The ability of two stimuli to activate distinct genetic programmes through a common component has now been noted in eukaryotes and yeast [58]. Recent data suggest that gene expression variations in response to different signals—despite an identical site-specific transcription factor phosphorylation—may reflect different potencies of recruitment of the transcriptional apparatus. It has to be noted that, besides promoter architecture and transcription factor phosphorylation, involvement of other surrounding modifications, such as acetylation, ubiquitination, methylation or SUMOylation may affect cofactor-complex formation due to conformational changes, intramolecular allosteric switches or attraction of additional adaptor proteins inhibiting or promoting cofactor binding [58–63]. Furthermore, regulation of some modifications may be linked; acetylation of NF-κB has recently been reported to be connected with its phosphorylation, as deacetylase inhibitors prolong its phosphorylation status [22]. Similar results were observed with acetylation-dependent phosphorylation of CREB [64]. Another level of specificity may result from segregation of transcription factor complexes in specialised nuclear compartments (transcription territories) [65–68]. Kinase-specific NF-κB phosphorylation effects may only affect a designated compartment without influencing the same factors in other compartments associated with different kinases [69,70]. More particularly, NF-κB/IκB modification by SUMO has been proposed to play a role in protein targeting [71]. Various experimental approaches (such as transient transfection, microinjection), that overload cells with transcription components (transcription factors, cofactors, kinases), neglect the dynamic stoichiometry of transcription complexes and may not reflect an appropriate regulation with respect to nuclear architecture [69,70].

In the last few years, more and more evidence has accumulated that combinations of transcription factor modifications may also reveal a particular transcription factor code, by analogy with combinations of histone tail modifications resulting in a particular histone language [58,59]. The recent observation of tandem complexes of acetylases and kinases, such as CBP–RSK or CBP–MSK, which modify transcription factors and histone tails, is intriguing; their specific role in the transcriptional regulation of CREB or p65, respectively, needs further investigation [72].

Finally, the activation kinetics (either fast/slow or transient/sustained) of regulatory kinases may result in signalling specificity [35,38,73–76]. In this respect, different kinases may be acting at various stages of the transcription process (nuclear transport, DNA-binding capacity, transcription initiation vs. elongation) [72,77–79]. Part of the gene expression specificity may also be codetermined by the surrounding chromatin modifications; NF-κB-dependent promoter activity has now been associated with increased H3/H4 acetylation and H3 phosphorylation levels [80–82]. Differences in NF-κB response kinetics (early vs. late genes) have been associated with differences in H4 acetylation patterns at particular promoters (MnSOD, MIP1 vs. IL6, IL8) [83], whereas histone H3 phosphorylation has been linked to expression of inflammatory-type NF-κB-dependent genes [84]. The various relationships in space and time between multiple enzymatic cofactor complexes vs. nucleosome and transcription factor modifications remain to be further investigated in order to better understand the basis for gene-specific regulation and to design selective targets for therapeutic intervention.

7. Summary

NF-κB transcriptional activity is regulated at multiple levels. First, NF-κB activity is regulated by cytoplasmic degradation of the IκB inhibitor and nuclear translocation. Second, the nuclear p65 transactivation potential can be further influenced by posttranslational modifications, such as phosphorylation and/or acetylation. The p65 phosphorylation is a process highly regulated by both cell- and stimulus-dependent activating kinases. Ser276 phosphorylation seems to be highly important considering its crucial role in interaction with and engagement of the cofactor CBP/p300. We identified MSK1 as an acting kinase in the TNF-signalling pathway, where it is responsible for p65 phosphorylation at Ser276, as well as for H3 phosphorylation of Ser10 in IL6 promoter-associated chromatin (see footnote 1). To our knowledge, this is the first report that identifies one particular kinase involved in transcription factor phosphorylation and histone modification at the level of a single promoter in order to establish gene activation. The question of which element takes the initial step to recruit and to assemble the activated transcription complex still remains unanswered.

Acknowledgments

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