Specification of DNA Binding Activity of NF-κB Proteins

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Nuclear factor-κB (NF-κB) is a pleiotropic mediator of inducible and specific gene regulation involving diverse biological activities including immune response, inflammation, cell proliferation, and death. The fine-tuning of the NF-κB DNA binding activity is essential for its fundamental function as a transcription factor. An increasing body of literature illustrates that this process can be elegantly and specifically controlled at multiple levels by different protein subsets. In particular, the recent identification of a non-Rel subunit of NF-κB itself provides a new way to understand the selective high-affinity DNA binding specificity of NF-κB conferred by a synergistic interaction within the whole complex. Here, we review the mechanism of the specification of DNA binding activity of NF-κB complexes, one of the most important aspects of NF-κB transcriptional control.

Nuclear factor-κB (NF-κB), a collective term for a family of transcription factors, was originally detected as a transcription-enhancing, DNA-binding complex governing the immunoglobulin (Ig) light chain gene intronic enhancer (Sen and Baltimore 1986; Lenardo et al. 1987). NF-κB is evolutionarily and structurally conserved and has representative members in a wide range of species. In essentially all unstimulated nucleated cells, NF-κB complexes are retained in latent cytoplasmic form through binding to a member of the inhibitor of NF-κB (IκB) proteins (Lenardo and Baltimore 1989; Lenardo et al. 1989; Hayden and Ghosh 2004; Hayden and Ghosh 2008). NF-κB induction typically occurs following the activation of the IκB kinase (IKK) signalosome, resulting in the phosphorylation and subsequent dispatch of the inhibitory IκBs to the proteasome for protein degradation (Hacker and Karin 2006). This cytoplasmic "switch" liberates NF-κB complexes for subsequent nuclear translocation and target gene transcription (Scheidereit 2006). It provides a pre-established genetic switch that is independent of new protein synthesis and triggered by a biochemical change in the cell. This adaptability and versatility no doubt underlies its broad use. A diverse spectrum of modulating stimuli can activate this pleiotropic transcription factor; furthermore, the fundamental use of NF-κB has been highlighted with an ever-increasing array of genetic targets, responsible for diverse biological activities including immune response, inflammation,

cell proliferation, and death (Grilli et al. 1993) (also see http://www.nf-kb.org).

The best known subunits of mammalian NF-κB consist of five proteins in the Rel family: RelA (p65), RelB, c-Rel, p50, and p52, which are capable forming homo- and heterodimeric complexes in almost any combination (Hayden and Ghosh 2004). Each of these subunits harbors a prototypical amino-terminal sequence of roughly 300 amino acids, termed the Rel homology domain (RHD), that mediates dimerization, DNA-binding, nuclear localization, and cytoplasmic retention by IκBs (Rothwarf and Karin 1999; Chen and Greene 2004). In contrast, the transcription activation domain (TAD) necessary for the target gene expression is present only in the carboxyl terminus of p65, c-Rel, and RelB subunits. NF-κB complexes have long been thought to function dimerically; but functional and biochemical information belied this simple conceptualization. The native complex of NF-κB from nuclear extracts is more than 200 kDa, significantly higher than that reconstituted from purified p50 and p65 proteins (115 kDa) (Urban et al. 1991). Moreover, native NF-κB complexes have a >100-fold higher affinity for Ig κB motif DNA than reconstituted p65−p50 heterodimers (Phelps et al. 2000). A new study shows that another essential subunit of NF-κB complex, ribosomal protein S3 (RPS3), cooperates with Rel dimers to achieve full binding and transcriptional activity (Wan et al. 2007). As an integral component, RPS3 plays a critical role in determining the DNA binding affinity and specificity of NF-κB, which will be discussed in more detail in the following discussion (Wan et al. 2007). Therefore, the molecular machine known as NF-κB consists of both Rel and non-Rel subunits that actually comprise multiple protein complexes with different gene activation specificities, masquerading as a single NF-κB complex in the nucleus.

NF-κB exerts its fundamental role as transcription factor by binding to variations of the consensus DNA sequence of 5′-GGGRNYYYCC-3′ (in which R is a purine, Y is a pyrimidine, and N is any nucleotide) known as κB sites (Chen et al. 1998). How NF-κB selectively recognizes a small subset of relevant κB sites from the large excess of potential binding sites (about 1.4×10^4 estimated in human genome) is a critical step for stimulus-specific gene transcription. Increasing evidence suggests that specific chromatin modifications and configurations are required for NF-κB proteins to access the chromosomally embedded cognate κB motifs (Natoli et al. 2005; Natoli 2006). The presence of κB sites, however, appears to be a minimal requirement for NF-κB regulation but not sufficient for gene induction (Wan et al. 2007). We will attempt to decipher the elegant but recondite control of DNA binding activity of NF-κB proteins at multiple levels, which is one of the most important, yet complex, aspects of NF-κB function. This process, more abstruse than initially considered, involves IκBs, Rel subunits, Rel-associating proteins, and non-Rel subunits in both the cytoplasm and the nucleus, as well as complicated associations in the nuclear chromatin. This synopsis will highlight our current knowledge of the DNA binding activity of NF-κB complex, focusing on its liberation from cytoplasmic sequestration complexes to recruitment to cognate κB regulatory sites in the genome.

IκB FAMILY PROTEINS

The evidence that induction of NF-κB activity does not require new protein synthesis, and that the detergent deoxycholate liberated active κB-site DNA binding activity in cytosolic extracts from unstimulated cells, revealed a key property of this gene regulatory system (Baeuerle and Baltimore 1988). This established a new regulatory paradigm involving the specific and reversible DNA binding of NF-κB proteins governed by NF-κB inhibitor protein(s), known as IκB. The IκB protein family currently consists of eight members: IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, and the Rel protein precursors p105 and p100, all of which possess a characteristic structural feature of ankyrin repeats. The centerpiece of both classical and alternative NF-κB pathways is the IκBs, functioning at the primary level to regulate the DNA binding activity of NF-κB proteins.

Specific modification and subsequent degradation of IκBs is critical in regulating NF-κB DNA binding activity, particularly in the classic or canonical pathway (Table 1). In unstimulated cells, NF-κB complexes are sequestered with various binding preferences by the IκBs in the cytoplasm because IκBs physically mask the nuclear localization signal (NLS) of NF-κB. On stimulation, whether intra- or extracellular, multiple intracellular signaling pathways converge on a tripartite IκB kinase (IKK) complex consisting of two functionally nonredundant kinases IKKα and IKKβ, as well as a regulatory subunit IKKγ (Fig. 1A). These mediate the phosphorylation of IκBs at specific amino acid residues (for instance, Ser-32 and Ser-36 in IκBα) predominantly through the action of the IKKβ subunit in the activated IKK complex. These phosphorylation events are a prerequisite for their successive

Table 1. Post-translational modification of IκB family proteins

IκB family proteins	Target residues	Enzymes	References
Phosphorylation			
IκBα	S32 and S36	IKKβ	(Hayden and Ghosh 2004)
IκBα	S283, S289, T291, S293, and T299	CKII	(Lin et al. 1996; McElhinny et al. 1996; Schwarz et al. 1996)
IκBα	Y42	p56-lck, Syk, and c-Src	(Koong et al. 1994)
	Y42	Unknown	(Schoonbroodt et al. 2000)
	Y42 and Y305	Unknown	(Waris et al. 2003)
IκBα	Unknown	PI3K/Akt	(Sizemore et al. 1999)
IκBβ	S19 and S23	IKKβ	(Hayden and Ghosh 2004)
IκBβ	S313 and S315	CKII	(Chu et al. 1996)
IκBε	S18 and S22	IKKβ	(Hayden and Ghosh 2004)
Bcl-3	S394 and S398	GSK3β	(Viatour et al. 2004)
p100	S99, S108, S115, S123, S866, S870, and S872	IKKα	(Senftleben et al. 2001; Xiao et al. 2001; Xiao et al. 2004)
p105	S927 and S932	IKKβ	(Lang et al. 2003)
p105	S903 and S907	GSK3β	(Demarchi et al. 2003)
p105	S337	PKAc	(Hou et al. 2003)
Ubiquitination			
IκBα	K21 and K22	βTrCP	(Hayden and Ghosh 2004)
IκBβ	K6	βTrCP	(Hayden and Ghosh 2004)
IκBε	K6	βTrCP	(Hayden and Ghosh 2004)
p100	K856	βTrCP	(Amir et al. 2004)
p105	Multiple Ks	βTrCP	(Cohen et al. 2004)
Sumoylation			
IκBα	K21	Unknown	(Desterro et al. 1998)
p100	K90, K298, K689, and K863	Ubc9	(Vatsyayan et al. 2008)
Acetylation			
p100	Unknown	P300	(Hu and Colburn 2005; Deng et al. 2006)
p105	K431, K440, and K441	P300	(Furia et al. 2002; Deng and Wu 2003)
S-nitrosylation			
p105	C62		(Matthews et al. 1996)

IKKβ, IκB kinase β; CKII, Casein kinase II; p56-lck, lymphocyte-specific protein tyrosine kinase; Syk, spleen tyrosine kinase; c-Src, normal cellular Src kinase; PI3K, phosphoinositide 3-kinases; GSK3β, glycogen synthase kinase 3 β; IKKα, IκB kinase α; PKAc, catalytic subunit of protein kinase A; βTrCP, β-transducin repeat-containing protein; Ubc9, E2 small ubiquitin-like modifier (SUMO)-conjugating enzyme Ubc9; p300, E1A binding protein p300.

F. Wan and M.J. Lenardo
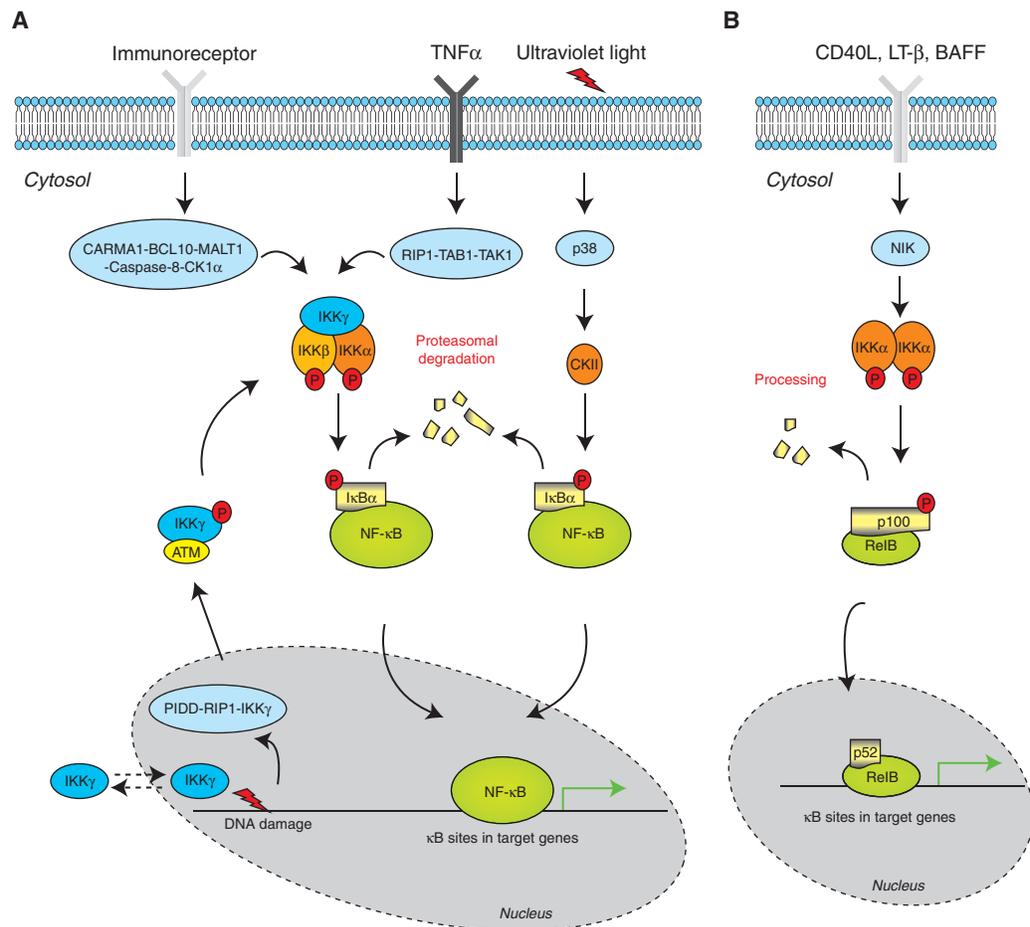
Figure 1. A schematic representation of how NF-κB DNA binding is regulated by IκB degradation and processing following activating stimuli. (*A*) IκB degradation mechanism that depends on site-specific phosphorylation of IκBs mediated by activated IKKβ in the IKK complex as well as CKII. Unique intracellular signaling complexes (which contains more components as shown, e.g., CARMA1-BCL10-MALT1-Caspase-8-CK1α complex downstream of T cell receptor [TCR] ligation [Bidere et al. 2009]) phosphorylate and activate the IKK complex depending on the initial stimulus (e.g., TNF, immunoreceptors, and DNA damage). IKKβ phosphorylates serine residues located in the amino-terminus of IκBs following TNF and TCR stimulation. In contrast, NF-κB induction by ultraviolet light and select chemotherapeutic agents requires the MAP kinase p38 and CKII. CKII phosphorylates serine residues located in the carboxyl terminus of IκBs. Phosphorylation of IκBs leads to successive ubiquitinylation and proteasomal degradation. The removal of IκBs liberates NF-κB complexes for nuclear translocation and binding to cognate regions in target genes. (*B*) The alternative pathway, which is induced by a few stimuli including CD40L, LT-β, and BAFF. NIK and IKKα are critical kinases for the phosphorylation of p100 at regulatory serine residues within its carboxyl terminus. This causes proteasomal degradation of the carboxyl terminus of the p100 molecule, generating a DNA binding NF-κB complex containing RelB and processed p52.

ubiquitinylation by β-transducin repeat-containing protein (βTrCP) and degradation in the 26S proteasome. The removal of IκBs liberates NF-κB for nuclear translocation and binding to cognate sites in target genes.

Additionally, various kinases other than the IKK complex may also mediate site-specific modification of IκBs, contributing to the diversity of signals regulating the latent DNA binding activity of NF-κB. In ultraviolet (UV) light-induced NF-κB activation, the phosphorylation-dependent IκBα degradation process does not rely on the IKK complex but the alternative serine/threonine kinase, casein kinase II (CKII) (Fig. 1A). Several studies have shown that CKII phosphorylates IκBα at a cluster of serine and threonine residues in the carboxyl terminus through a p38-MAPK kinase-dependent pathway, which is critical for degradation of IκBα and induced NF-κB DNA binding activity in cells exposed to UV light (Lin et al. 1996; McElhinny et al. 1996; Schwarz et al. 1996). Moreover, tyrosine kinases p56-lck, Syk, and c-Src and the serine/threonine kinase GSK3β have also been reported to phosphorylate IκBs and control the DNA binding activity of NF-κB (Table 1) (Koong et al. 1994; Viatour et al. 2004). Along with phosphorylation, modifications such as sumoylation, acetylation, and s-nitrosylation of the IκBs at specific residues can also trigger the degradation process (Table 1). However, these modifications lead to the final common pathway of ubiquitination of IκBs at specific lysine residues as the essential requirement for IκB degradation and release of NF-κB for nuclear translocation (Table 1).

p100 and p105, the precursor proteins for NF-κB subunits p52 and p50 respectively, contain multiple ankyrin repeats (similar to those of the IκB family) in their carboxy-terminal domains. In some circumstances, these keep NF-κB complexes cytoplasmically anchored. The regulation of NF-κB DNA binding activity by p105 mirrors IκBs of the classical pathway by undergoing inducible, complete degradation, particularly when it is bound to NF-κB complexes (Heissmeyer et al. 2001). By contrast, partial processing, but not full degradation, of p100 represents another mechanism by which IκB regulates the DNA binding activity of NF-κB proteins. This was discovered recently in an alternative pathway of NF-κB regulation involving RelB and p52 (Fig. 1B) (Hayden and Ghosh 2004). A few select stimuli including CD40 ligand (CD40L), lymphotoxin β (LT-β), and B-cell activating factor (BAFF) phosphorylate p100 on carboxy-terminal serine residues, triggering successive polyubiquitination and proteasomal degradation of the carboxyl terminus of the p100 molecule. The resulting complex, containing RelB and processed p52, functions as the main NF-κB species with selected DNA binding activity in the alternative pathway. Of interest, the kinase signalosome mediating p100 phosphorylation contains the NF-κB-inducing kinase (NIK) and IKKα, and functions completely independently of IKKβ and IKKγ. Moreover, recent evidence suggests that IKKα phosphorylates p100 at several serine residues within the RHD of p52, and these modifications can augment p52 DNA binding, in addition to inducing the efficient processing of p105 (Xiao et al. 2004).

Besides their hallmark sequestration function in cytoplasm, IκBs can also regulate the DNA binding properties of NF-κB proteins in the nucleus. IκBζ localizes to the nucleus following its inducible expression (Yamamoto et al. 2004) and Bcl-3 is also found in the nucleus associated with p50- and p52-containing NF-κB complexes (Cogswell et al. 2000). IκBs such as IκBα, IκBβ, and IκBε are transcriptional targets of NF-κB itself, creating a negative feedback loop. This facilitates restoration of latent NF-κB complexes in the cytoplasm and maintains the cell's responsiveness to subsequent stimuli. With variant kinetics of degradation and resynthesis, IκBs compete NF-κB complexes off their chromosomal locations and export them back into cytoplasm. By affecting the cytoplasmic–nuclear shuttling of NF-κB proteins, these titrate the occupancy of gene regulatory elements in nuclear DNA. Strikingly, IκBβ may also function to directly regulate NF-κB DNA binding activity on relevant promoters or enhancers, as evident by

its stable nuclear association with NF-κB complexes that are already bound to κB sites in chromatin (Thompson et al. 1995; Suyang et al. 1996). Moreover, both IκBζ and Bcl-3 primarily interact with specific NF-κB complexes and subvert their DNA binding activity. IκBζ was reported to associate preferentially with p50 homodimers and also negatively regulate p65-containing NF-κB complexes (Kang et al. 1992; Motoyama et al. 2005; Hayden and Ghosh 2008), which suggests it may possess the capability to selectively inhibit or activate specific NF-κB species. However, the role of Bcl-3 in regulating DNA binding seems controversial. It has been proposed to remove the repressive p50 homodimers from κB DNA, allowing transcriptionally active NF-κB species access to those cognate elements (Lenardo and Siebenlist 1994; Hayden and Ghosh 2008). However, recent evidence indicates that Bcl-3 might also stabilize p50 homodimers and inhibit NF-κB activation by preventing transcriptionally active NF-κB complexes from binding to κB DNA (Carmody et al. 2007). Regardless of the inhibited or enhanced NF-κB transactivation consequences, IκBs regulate the DNA binding activity of NF-κB proteins by controlling the high-affinity interactions of NF-κB species to cognate DNA regulatory sites.

POST-TRANSLATIONAL MODIFICATIONS OF REL PROTEINS

Like IκB proteins, Rel subunits are subject to numerous post-translational modifications, which represents another mechanism of regulating NF-κB activation that has been extensively studied recently. These modifications, within conserved RHD, TAD or linker sequences, convey various physiological functions of NF-κB (Viatour et al. 2005; Perkins 2006; Neumann and Naumann 2007; O'Shea and Perkins 2008). This includes control of NF-κB DNA binding activity through modifications, particularly phosphorylation and acetylation, of Rel subunits or adjusting the association of NF-κB complexes to either IκBs or κB DNA (Table 2). This could be caused by steric alternations of the Rel proteins

themselves or to controlling their associations with other proteins in transcriptional regulatory complexes.

The phosphorylation of p65 under basal and activated conditions is by far the best-characterized modification among Rel proteins, and p65 is the transcriptionally active component of the NF-κB species that is most abundant and has the broadest function. So far, nine phosphorylation sites have been identified in p65 located in both the RHD and TAD, with several sites remarkably critical for DNA binding capability. Ser-536 phosphorylation by various kinases leads to a substantially weaker interaction of p65 with newly synthesized IκBα, leading to a dramatic decrease of nuclear p65 export, which enhances the duration of κB DNA access (Table 2). Furthermore, Ser-536-phosphorylated p65 does not interact with cytosolic IκBα at all, which could accelerate p65 nuclear localization and attachment to DNA (Sasaki et al. 2005). Phosphorylation of p65 at Ser-276 and Ser-536 together with successive acetylation at Lys-310 alters its affinity to IκB proteins, resulting in different kinetics of p65 cytoplasmic-nuclear shuttling and subsequent DNA binding activity (Chen et al. 2005). Phosphorylation of Thr-254 by the nuclear peptidylprolyl isomerase Pin1, followed by isomerization of specific amino acid residues, also strongly increases p65 nuclear translocation and DNA binding activity. Pin1-induced conformational changes increase the structural stability of p65 with dramatic decrease in IκBα affinity (Ryo et al. 2003). Recently, phosphorylation of Ser-276 by the mitogen- and stress-activated protein kinase-1 (MSK1) was shown to be essential for p65 binding to the κB intronic enhancer site of the mast cell growth factor *SCF* (stem cell factor) gene in inflammation (Reber et al. 2009). Phosphorylation of p65 can also repress the transcriptional activity of other Rel subunits. For instance, Ser-536-phosphorylated p65 was reported to specifically interact with RelB in the nucleus, forming a complex that cannot bind to κB DNA (Jacque et al. 2005).

Post-translational modification of c-Rel and its effect on DNA binding activity is similar

Table 2. Post-translational modifications of Rel subunits that regulate NF-κB DNA binding activity

Rel family proteins	Target residues	Enzymes	Functional effect on DNA binding activity	References
Phosphorylation				
p65	T254	Unknown	Enhancement	(Ryo et al. 2003)
	S529	CKII	Unknown	(Bird et al. 1997; Wang et al. 2000; O'Mahony et al. 2004)
	Unknown	PI3K/Akt	Enhancement	(Sizemore et al. 1999)
	S276	MSK1	Enhancement	(Reber et al. 2009)
	S536	IKKα	Enhancement	(Jiang et al. 2003; O'Mahony et al. 2004)
		IKKβ	Enhancement	(Sakurai et al. 1999)
		IKKε	Enhancement	(Buss et al. 2004; Adli and Baldwin 2006; Mattioli et al. 2006)
		TBK1	Enhancement	(Fujita et al. 2003; Buss et al. 2004)
		RSK1	Enhancement	(Bohuslav et al. 2004)
c-Rel	Unknown	TBK1	Enhancement	(Harris et al. 2006)
	Unknown	IKKε	Enhancement	(Sanchez-Valdepenas et al. 2006)
p50	S337	PKAc	Enhancement	(Guan et al. 2005)
Acetylation				
p65	K218, K221, and K310	p300	Enhancement	(Chen et al. 1998)
	K122 and K123	PCAF and p300	Reduce	(Kiernan et al. 2003)
p50	K431, K440, and K441	p300	Enhancement	(Furia et al. 2002)

CKII, Casein kinase II; MSK1, Mitogen- and stress-activated protein kinase-1; PI3K, Phosphoinositide 3-kinases; IKKα, IκB kinase α; IKKβ, IκB kinase β; IKKε, IκB kinase epsilon; TBK1, TANK-binding kinase 1; RSK1, Ribosomal protein S6 kinase; 90 kDa, polypeptide 1; PKAc, catalytic subunit of protein kinase A; p300, E1A binding protein p300; PCAF, p300/CREB binding protein-associated factor.

to p65, but with fewer characterized kinase(s) or target residue(s). c-Rel can be specifically phosphorylated by the serine/threonine kinases NIK, TBK1, and IKKε on its carboxyl terminus, which dissociates the IκBα-c-Rel complex and enhances binding to cognate DNA (Harris et al. 2006; Sanchez-Valdepenas et al. 2006). Furthermore, tyrosine phosphorylation of c-Rel within its carboxyl terminus also increased its ability to bind κB sites in human neutrophils stimulated with granulocyte-colony-stimulating factor (Druker et al. 1994). More importantly, dysregulated Ser-525 phosphorylation located within c-Rel TAD, caused by a serine to proline point mutation discovered in two patients with follicular and mediastinal B-cell lymphoma, might slightly enhance c-Rel DNA binding activity, at least

for κB motifs (Starczynowski et al. 2007). These data show that the carboxy-terminal modifications, especially in the TAD, are vital in regulating c-Rel DNA binding activity.

The RelB monomer is easily degraded and preferentially binds to p52 and p50, making it an unusual NF-κB subunit (Hayden and Ghosh 2004). Although the kinases have not been identified yet, phosphorylation of RelB at multiple residues including Thr-84, Ser-254, Ser-368, and Ser-552, have been reported as essential for its dimerization and degradation (Viatour et al. 2005; Neumann and Naumann 2007). It certainly deserves further investigation whether these or other unknown modifications of RelB regulate DNA binding, given the crucial role of RelB in the alternative pathway of NF-κB activation. Although inducible

phosphorylation of p50, similar to its precursor p105, has been noticed in various cell types (Neumann and Naumann 2007), site-specific modifications of transactivation repressive p50 and p52 have been rarely reported. One recent study showed the catalytic subunit of PKA (PKAc) specifically phosphorylated mature p50 at Ser-337 located within RHD and augmented DNA binding activity, which may be important for maintaining stable negative regulation of NF-κB gene expression in unstimulated cells (Guan et al. 2005).

Acetylation is another key modification of Rel subunits that regulates their association with IκBs and direct binding to cognate DNA (Table 2). Five residues, Lys-122, Lys-123, Lys-218, Lys-221, and Lys-310, can be specifically acetylated in p65 in response to TNFα stimulation. Acetylation at Lys-218 and Lys-221 markedly diminishes the binding of p65 to IκBα thereby increasing its DNA binding (Chen et al. 2002). In particular, Lys-221 of p65 directly interacts with Met-279 of IκBα in the crystal structure of p65-p50-IκBα complex (Huxford et al. 1998); therefore, acetylation of Lys-221 may result in a conformational change in p65 and lessen its interaction with IκBα (Chen et al. 2002). In contrast, acetylation of p65 at Lys-122 and Lys-123 accelerates its dissociation from DNA and successive nuclear export by IκBα, attenuating NF-κB transactivation (Kiernan et al. 2003). In addition, acetylation of Rel subunits may modulate their binding with DNA directly. Acetylation of p65 at Lys-221 apparently causes a conformation change favoring κB DNA binding. This is supported by the crystal structure of p65-p50-κB DNA complex illustrating that Lys-221 directly contacts the DNA backbone (Chen et al. 1998). Various mutational analyses strengthen the concept that this acetylation influences DNA-binding properties. Mutation of Lys-221 produces a sharp decline in the DNA binding affinity of p65 homodimers and a substantial decrease in that of p65-p50 heterodimers (Chen et al. 2002). Strikingly, p50 can be acetylated in vitro by p300/CBP at Lys-431, Lys-440, and Lys-441, and this modification augments its DNA binding properties, as evidenced by

pull-down assays using κB oligonucleotides (Furia et al. 2002). Collectively, site-specific acetylation of Rel proteins, at least p65 and p50, augments their intrinsic DNA binding activity, as shown for other transcription factors (Boyes et al. 1998).

REL SUBUNIT-ASSOCIATING PROTEINS

For full NF-κB transactivation to occur at a given target DNA locus, a successive enhanceosome complex needs to be assembled by multiple coactivators, corepressors, other transcription factors, and basal transcription machinery proteins through protein–protein interactions with Rel subunits (Hayden and Ghosh 2004; Hayden and Ghosh 2008). For instance, over 100 proteins can modulate NF-κB association with chromatin or the assembly of an NF-κB enhanceosome via their interaction with full length, RHD, TAD, or the central region of p65 (O'Shea and Perkins 2008). Until a short time ago, Rel subunits were widely regarded as the only executers to recognize and bind κB DNA. However, several Rel-associating proteins, albeit with no κB DNA binding capability themselves, have recently been illustrated to be essential in regulating NF-κB binding to cognate DNA (Table 3). These Rel-associating proteins feature restricted tissue distribution, specific expression profiles, or unique pathophysiological circumstances and inhibit, as currently reported, the DNA binding activity of NF-κB proteins. This establishes Rel-associating proteins as a group of tissue- or cell context-specific negative regulators of NF-κB beyond the IκB family, adding another level of complexity to the exquisite spatiotemporal control of DNA binding activity of NF-κB proteins.

Some Rel-associating proteins attenuate NF-κB DNA binding in cotransfection experiments. RelA-associated inhibitor (RAI), isolated in a yeast two-hybrid screen utilizing the central region of p65 as bait, interacts with p65 in vitro and in vivo, and specifically inhibits p65 binding to the cognate DNA when cotransfected in human embryonic kidney 293 cells (Yang et al. 1999). A similar function was ascribed to the aryl hydrocarbon receptor

Table 3. Rel-associating proteins that regulate NF-κB DNA binding activity

NF-κB subunits	Associating/ interacting proteins	Domain/portion of Rel proteins sufficient for binding	Evidence for integration into DNA binding complexes	Functional effect on DNA binding activity	References
p65					
	RAI	RHD, aa 176–405	No	Inhibition	(Yang et al. 1999)
	AhR	Not mapped	No	Inhibition	(Tian et al. 1999; Kim et al. 2000; Ruby et al. 2002)
	β-Catenin	Not mapped	No	Inhibition	(Deng et al. 2002)
	Cdk9	Not mapped	No	Inhibition	(Amini et al. 2002)
	PIAS1	TAD, aa 299–551	No	Inhibition	(Liu et al. 2005)
	VP1686	Not mapped	No	Inhibition	(Bhattacharjee et al. 2006)
	Myocardin	RHD, aa 1–276	No	Inhibition	(Tang et al. 2008)
	RPS3[a]	RHD, aa 21–186	Yes	Enhancement	(Wan et al. 2007)
p50					
	β3-Endonexin	Not mapped	No	Inhibition	(Besta et al. 2002)
	Cdk9	Not mapped	No	Inhibition	(Amini et al. 2002)
	β-Catenin	Not mapped	No	Inhibition	(Deng et al. 2002)

RAI, RelA associated inhibitor; AhR, Aryl hydrocarbon receptor; Cdk9, Cyclin-dependent kinase 9; PIAS1, Protein inhibitor of activated STAT1; VP1686, *Vibrio parahaemolyticus* type III secretion protein; RPS3, Ribosomal protein S3; β3-Endonexin, Integrin β 3 binding protein.

[a]RPS3, an integral subunit of certain NF-κB DNA binding complex that also physically interacts with p65, is listed here to compare with Rel-associating proteins in their DNA-binding regulatory functions.

(AhR), a ligand-activated transcription factor. When cotransfected in 293 cells, AhR specifically inhibited the DNA binding activity of p65, but not the p50–p50 complex (Tian et al. 1999; Ruby et al. 2002), suggesting that synergic interaction between AhR and NF-κB, mainly the p65 subunit, is critical for suppression of immune responses and xenobiotic metabolism. Some pathogen-encoded proteins also possess the ability to regulate host NF-κB binding to cognate DNA, hampering immune responses directed against the microbes that express them. For instance, *Vibrio parahaemolyticus* is a causative agent of human gastrointestinal diseases and significantly suppresses the induction of the DNA binding activity of NF-κB via the physical interaction between its effector protein VP1686 and p65. Such attenuation of NF-κB DNA binding activity by VP1686 is sufficient to sensitize infected-macrophages for death, because of the diminished expression of many antiapoptosis-related NF-κB target genes (Bhattacharjee et al. 2006).

The regulatory effects of Rel-associating proteins have been studied not only in the cellular context of NF-κB signaling, but also in in vitro biochemical investigations with purified proteins. One of the first studies described a surprising cross-regulation of NF-κB by β-catenin (Deng et al. 2002). Both p65 and p50 could complex with β-catenin independently of IκBα, but seemed to require additional cellular factors. β-catenin markedly attenuated the DNA binding of both p50–p65 and p50–p50 complexes as shown by electrophoretic mobility shift assays (EMSA), causing reduced NF-κB target gene expression. Of note, β-catenin is not directly integrated into the NF-κB-DNA complex in spite of its strong association with NF-κB subunits, suggesting it may interact with NF-κB proteins to disrupt their DNA binding ability (Deng et al. 2002).

Inhibition of NF-κB DNA binding activity by β-catenin could be important in oncogenesis, as hinted at by an inverse correlation between the β-catenin expression levels and levels of the NF-κB target gene *Fas* in colon and breast tumor tissues (Deng et al. 2002). Furthermore, the inhibitory effects on NF-κB DNA binding by Cyclin-dependent kinase 9 (Cdk9), the PIAS1 (protein inhibitor of activated STAT [signal transducers and activator of transcription] 1) protein, and myocardin were also shown using EMSA, in which these proteins significantly reduced the DNA binding activity of NF-κB (Amini et al. 2002; Liu et al. 2005; Tang et al. 2008). Cdk9 markedly suppressed the association of the p50–p50, p65–p65, and p50–p65 complexes to κB DNA, and the ability of NF-κB to modulate HIV-1 gene transcription was controlled by this inhibitory function (Amini et al. 2002). Both PIAS1 and myocardin specifically inhibited the DNA binding activity of p65-containing complexes, serving as negative regulators of NF-κB in certain conditions. PIAS1 was originally identified in the Jak/STAT signaling pathway, whereas myocardin is expressed specifically in cardiac and smooth muscle cells (Liu et al. 2005; Tang et al. 2008). Of interest, their effects on NF-κB DNA binding appear essential for tuning cytokine-induced NF-κB target gene expression and cardiomyocyte proliferation and differentiation, respectively (Liu et al. 2005; Tang et al. 2008).

As described above, β-catenin and Cdk9 both complex with p50 and dramatically inhibit its DNA binding activity (Amini et al. 2002; Deng et al. 2002). β3-endonexin is another p50-interacting molecule that inhibits p50–p65 complex binding to κB DNA. Moreover, binding of β3-endonexin to p50 was inhibited in the presence of wild-type but not mutated κB oligonucleotides, suggesting a steric competition between β3-endonexin and κB DNA for the p50–p65 complex. Despite the association with the transcription repressive p50 subunit, β3-endonexin negatively regulates expression of the urokinase-type plasminogen activator receptor that is essential for endothelial migration (Besta et al. 2002).

INTEGRAL NON-REL SUBUNITS IN NF-κB COMPLEXES

As for the unresolved and important question of how regulatory specificity of NF-κB is achieved, it has long been regarded that the variability of κB sequences may govern the usage of certain Rel dimers at specific promoters. Principally, each κB site variant could preferentially recruit one type of Rel dimer over other species (Natoli et al. 2005; Natoli 2006). Selective NF-κB gene expression, however, cannot be completely explained by a simple correlation between the sequence of κB sites in target genes and the requirement for a specific Rel dimer (Hoffmann et al. 2003). However, κB sites may still impart a specific configuration for NF-κB binding, because a single nucleotide change in an NF-κB binding site affected the formation of productive interactions between Rel dimer and coactivators (Leung et al. 2004). Therefore, other protein components beyond Rel subunits could form integral parts of the NF-κB binding complex, thus controlling its recognition and action on target genes. Support for this hypothesis comes from observations made in seminal previous studies. First, the contradiction in the size of native NF-κB from nuclear extracts ($>$ 200 kDa) and that reconstituted from purified p50 and p65 proteins (115 kDa) implies the presence of other proteins in the native complex (Urban et al. 1991). Second, reconstituted p65–p50 heterodimers from purified proteins have a $>$100-fold lower affinity for DNA than native NF-κB complexes, at least for binding to the Ig κB motif (Phelps et al. 2000). Third, distinct variants of the κB motifs display different responses to various NF-κB inducers in different cell types implying selective regulation. Finally, a large number of NF-κB binding motifs beyond either canonical or variant κB site sequences were revealed throughout the human genome by mapping with chromatin immunoprecipitation-coupled microarray or sequencing (Martone et al. 2003; Schreiber et al. 2006; Lim et al. 2007). Collectively, these findings strongly suggested that other non-Rel proteins could not only regulate NF-κB DNA binding

activity, but also participate in DNA binding as an essential component.

This hypothesis was confirmed by a recent study showing that RPS3, an integral non-Rel subunit in certain NF-κB DNA binding complexes, is essential for the recruitment of NF-κB p65 to selected κB sites (Wan et al. 2007). RPS3 prominently features a heterogeneous nuclear protein K (hnRNP K) homology (KH) domain, a structural motif that binds single-stranded RNA and DNA with some sequence specificity (Siomi et al. 1993). Indeed, the KH domain within RPS3 is essential for association with p65 (Wan et al. 2007). This study underscored the inherent complexity of NF-κB binding to κB sites by demonstrating that DNA binding capability is not conferred strictly by Rel subunits, as has been long assumed. Rather, the integral non-Rel subunit RPS3 represents a newly recognized subunit that potently contributes to DNA binding

activity. These observations suggest a new regulatory paradigm in which DNA binding activity could be regulated within NF-κB complexes through synergistic interactions between Rel and non-Rel subunits (Fig. 2).

RPS3 was found to physically interact with p65 in a proteomic screen, and shown to be critical in NF-κB transactivation. Under conditions of reduced RPS3, the DNA binding activity of NF-κB complexes was significantly attenuated. Knockdown of RPS3 resulted in failed recruitment of p65 to selected endogenous gene regulatory sites and abortive induction, despite normal p65 nuclear translocation. Therefore, RPS3 facilitates p65 binding to cognate DNA, which is essential for normal expression of specific NF-κB target genes involved in key physiological processes. This was dramatically shown for the induction of immunoglobulin κ light chain gene expression in B cells and cell proliferation and cytokine secretion in T cells that were
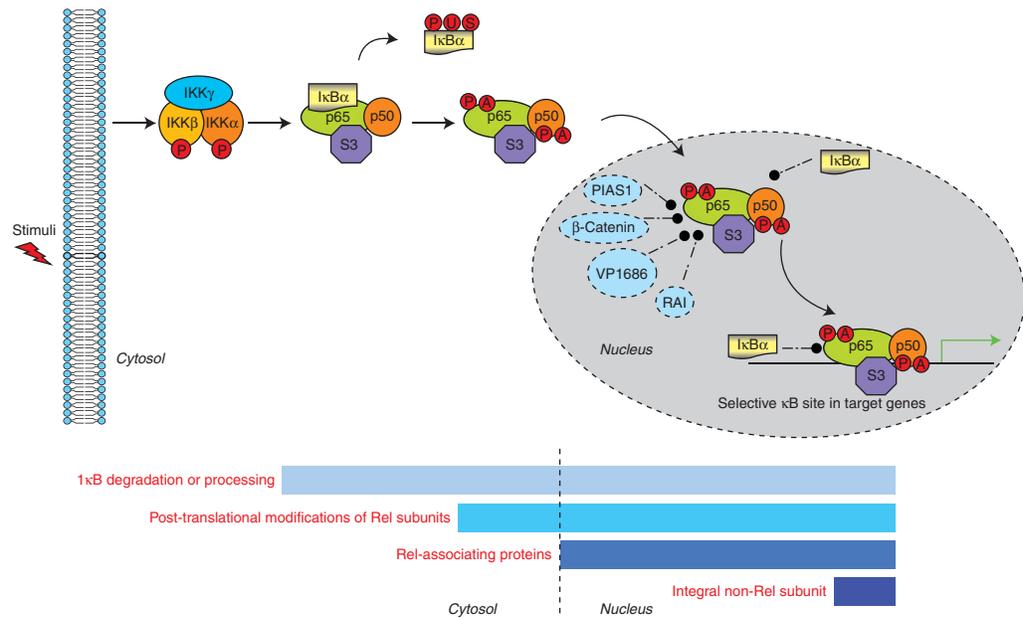
Figure 2. Complex regulation of NF-κB DNA binding activity. As illustrated in one of the most abundant NF-κB complexes, the DNA binding activity of NF-κB is controlled in both the cytoplasm and nucleus at multiple levels: involving degradation or processing of inhibitory IκBs; the post-translational modification of Rel subunits; Rel-associating proteins that modulate NF-κB DNA binding potential; and an integral non-Rel subunit RPS3 required for selective NF-κB target gene transcription based on enhanced DNA binding affinity. The bars represent the intracellular locations where the DNA binding activity of NF-κB proteins is regulated by indicated proteins.

markedly impaired by RPS3 knockdown (Wan et al. 2007). Strikingly, purified RPS3 protein exerted a dramatic synergistic effect on the DNA binding activity of both p65–p65 and p50–p65, but not p50–p50 complexes in EMSA (Wan et al. 2007). As a DNA-binding facilitator, RPS3 dramatically stabilizes NF-κB association with certain cognate sites. This could explain the extremely high affinity of semipurified NF-κB complexes for DNA, which is not manifested by complexes formed solely of purified p50 and p65 subunits. By contrast, none of the aforementioned Rel-associating proteins has been shown to integrate into NF-κB DNA binding complexes, and all of them inhibit the p65 DNA binding activity, although several were assessed in vitro using recombinant proteins in EMSA (Amini et al. 2002; Deng et al. 2002; Liu et al. 2005; Tang et al. 2008).

It is important to recognize that RPS3 is not an NF-κB-associated transcriptional coactivator, which by definition reorganizes chromatin templates and recruits the basal transcriptional machinery to the promoter region. RPS3 possesses little, if any, intrinsic transcriptional activating ability in a standard coactivation assay (Wan et al. 2007). Furthermore, the ability of RPS3-specific antibodies to dramatically supershift or diminish p65-containing DNA complexes in EMSAs strongly suggests that RPS3 is an integral part of NF-κB DNA binding complexes (Wan et al. 2007). By contrast, administration of a specific antibody against p300, one of well-characterized transcriptional coactivators that complex with NF-κB, did not alter NF-κB-DNA complexes, suggesting that p300 is not incorporated into the DNA binding complex (Deng et al. 2002). Further lines of evidence support the notion that RPS3 is an integral subunit of NF-κB: RPS3 physically associates with p65, p50, and IκBα in resting cells (guided through its interaction with p65); RPS3 can specifically translocate to the nucleus in response to T cell receptor (TCR) and TNFα stimulation; RPS3 is recruited to κB sites in a large number of NF-κB-driven genes in vivo upon stimulation; and RPS3 and p65 are significantly correlated in transcribing

a subset of TCR ligation-induced NF-κB genes (Wan et al. 2007). Whether the RPS3 subunit is essential in other stimuli-induced NF-κB signal pathways and whether it targets certain NF-κB complexes to specific κB sites in different cell types certainly deserves further investigation. Because RPS3 is only required for selected particular genomic κB sites to be activated under certain conditions and preferentially directs binding to κB sites with some sequence specificity, we call it a "specifier" subunit of NF-κB. This also lends credence to the idea that there are actually multiple molecular complexes containing RPS3-like "specifier" subunits with different gene activation specificities that all masquerade as single NF-κB complex in the nucleus. Indeed, another KH domain protein, Src-associated in mitosis, 68 kDa (Sam68), was found to be essential for some NF-κB gene transcription where RPS3 is not required (F. Wan and M. Lenardo, unpubl.). Sequence specificity preferred by various KH domains could confer different gene activation patterns via diverse NF-κB complexes. These findings may unveil a novel regulatory paradigm in which KH domain proteins serve as essential functional components in regulating the DNA binding activity of not only NF-κB, but also other transcription factors, because several other KH domain proteins have been shown to bind to DNA recognition motifs and to promote transcription (Tomonaga and Levens 1996; Ostrowski et al. 2003; Moumen et al. 2005).

CONCLUDING REMARKS

Since it was originally identified as a regulator of κ light chain expression in B cells over 20 years ago, NF-κB has served as a paradigm for signaling associated with inflammation, autoimmunity, and cancer. Recruitment of NF-κB proteins to regulatory DNA sites within the chromatin is fundamental and crucial for their target gene transcription. The complexity inherent in the DNA binding activity of NF-κB proteins is essential for achieving a fine-tuned regulatory specificity. An increasing body of literature illustrates that

the DNA binding activity of NF-κB proteins can be elegantly and specifically controlled at multiple levels by different protein subsets, including IκBs, Rel subunits, Rel-associating proteins, and integral non-Rel subunits (Fig. 2). In particular, the expanding list of Rel-associating proteins constitutes a unique category of tissue- or cell type-specific negative regulators of NF-κB beyond IκBs that control DNA binding activity under certain circumstances. Furthermore, the recent identification of a non-Rel subunit of NF-κB itself provides a new way to understand the selective high-affinity DNA binding specificity of NF-κB conferred by a synergistic interaction within the whole complex.

Despite extensive studies on the control of NF-κB DNA binding activity, numerous issues are still unresolved and warrant further investigation. These include the identity of regulatory kinases and site-specific modulating target residues in IκBs that mediate their degradation/processing; additional post-translational modifications of Rel subunits beyond p65 and their affect on DNA binding; and specific associating and regulatory proteins for RelB and c-Rel. Because the inclusion of RPS3 does not fully explain the size of native NF-κB or its high affinity to cognate κB sites, the identification of other unknown non-Rel subunits of NF-κB is well worth further investigation. Undoubtedly, a more complete understanding of the complex control of the DNA binding activity of NF-κB proteins will not only revise or add to our fundamental knowledge of gene regulation, but also elucidate novel target molecules for pharmacological interventions.

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