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REVIEW

Transcriptional regulation via the NF-κB signaling module

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Stimulus-induced nuclear factor-κB (NF-κB) activity, the central mediator of inflammatory responses and immune function, comprises a family of dimeric transcription factors that regulate diverse gene expression programs consisting of hundreds of genes. A family of inhibitor of κB (IκB) proteins controls NF-κB DNA-binding activity and nuclear localization. IκB protein metabolism is intricately regulated through stimulus-induced degradation and feedback re-synthesis, which allows for dynamic control of NF-κB activity. This network of interactions has been termed the NF-κB signaling module. Here, we summarize the current understanding of the molecular structures and biochemical mechanisms that determine NF-κB dimer formation and the signal-processing characteristics of the signaling module. We identify NF-κB–κB site interaction specificities and dynamic control of NF-κB activity as mechanisms that generate specificity in transcriptional regulation. We discuss examples of gene regulation that illustrate how these mechanisms may interface with other transcription regulators and promoter-associated events, and how these mechanisms suggest regulatory principles for NF-κB-mediated gene activation.
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Introduction

Transcription factor nuclear factor-κB (NF-κB) plays important roles in myriad physiological and pathological scenarios. It is the nuclear effector of a signaling pathway that is responsive to a large number of extracellular stimuli in many cells. These stimuli may trigger inflammation, innate immune responses, adaptive immune responses, secondary lymphoid organ development and osteoclastogenesis. Studies with knockout mice have revealed NF-κB's critical involvement in all of these processes (Gerondakis *et al*., 2006). NF-κB was first identified as a biochemical activity capable of binding to a 10 base pairs (bp) sequence within the transcriptional enhancer of the immunoglobulin (Ig) κ light chain gene. Consistent with that finding, NF-κB primarily mediates its functional effects through the transcriptional regulation of NF-κB target genes and has not been shown to have any cytoplasmic function.

Previous reviews have compiled lists of more than 100 genes that contain one or more κB-sequence elements that confer NF-κB-responsive transcriptional regulation (Pahl, 1999; www.nf-kb.org). Since then, high throughput technologies have revealed that the vast majority of the inflammatory gene expression program is NF-κB-dependent (Hoffmann and Baltimore, 2006), and that DNA-bound NF-κB can be detected at many chromosomal locations (Martone *et al*., 2003; Schreiber *et al*, 2006). Given NF-κB's critical involvement in so many physiological responses, a major challenge of current research is to elucidate how NF-κB signaling in response to diverse stimuli can mediate distinct cellular responses in particular physiological contexts. Two broad themes have emerged in recent years as playing important roles in allowing for specificity in NF-κB signaling: molecular interaction specificity and dynamic control.

Molecular components of the NF-κB family

In vertebrates, NF-κB connotes not a single protein, but a family of more than a dozen transcription factors that are comprised of homo- and heterodimers of five proteins: p50, p52, c-Rel, RelA/p65 and RelB, encoded by the *nfkb1*, *nfkb2*, *rel*, *rela* and *relb* genes (see Gilmore, 2006). These proteins share an approximately 300 residue long homologous domain near their N termini (Baldwin, 1996; Ghosh *et al*., 1998). This domain, called the Rel Homology Domain (RHD), is responsible for DNA binding, dimerization, inhibitor binding and nuclear localization (Huxford *et al*., 1999). Upon determination of the first X-ray crystal structures of the NF-κB RHD from p50 bound to DNA, it became clear that the RHD is composed of two-folded domains linked by a short polypeptide (Ghosh *et al*., 1995; Muller *et al*., 1995). Since then, the structures of several NF-κB family members have been determined either in their DNA-bound RHD form or as the dimerization domain (DimD) alone (Cramer *et al*., 1997; Chen *et al*., 1998a, b; Huang *et al*., 2001). These structures and biochemical studies have provided insights into the

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mechanisms of subunit association and DNA recognition by the NF-κB transcription factors.

Of the 15 possible dimers of NF-κB subunits (Figure 1), at least 12 are able to bind DNA and potentially regulate transcription. Interestingly, although κB-site sequences are diverse, the different dimers have broad sequence recognition specificities that largely overlap. Nevertheless, NF-κB–DNA interactions play an important role in determining transcriptional specificity, but, as discussed below, this specificity is not necessarily conferred by affinity differences.

A primary mechanism by which NF-κB activity is controlled is through regulation of its ability to bind DNA. Inhibitor of κB (IκB) proteins, a subfamily of the large Ankyrin Repeat Domain (ARD) containing superfamily, bind NF-κB dimers in a manner that prevents DNA binding and nuclear accumulation. Indeed, all cells examined so far contain stores of latent, largely cytoplasmic NF-κB activity, which is bound to IκB proteins. Cellular stimulation results in specific phosphorylation, ubiquitination and proteasome-mediated proteolysis of the NF-κB-bound IκB protein, which renders the NF-κB capable of binding DNA and localized to the nucleus.

Canonical and non-canonical NF-κB signaling via the NF-κB signaling module

Two major signaling pathways have been characterized that result in the activation of NF-κB activities via distinct kinases and IκB proteins (see Scheidereit, 2006). The canonical pathway is induced largely by IκB kinase (IKK)β, and utilizes the three canonical IκB proteins IκBα, IκBβ and IκBε. The non-canonical pathway is activated through IKKα, and utilizes the p52 precursor protein p100 (Senftleben *et al*., 2001), whose C-terminal ARD domain can function like an IκB protein to sequester NF-κB dimers in an inactive state (Derudder *et al*., 2003; Basak *et al*., 2006). As IκB proteins differ in their molecular interaction specificity towards the family of NF-κB dimers, specific NF-κB dimers may be activated via the degradation of specific IκB isoforms. For example, RelB-containing dimers are not well bound by canonical IκB proteins, but are bound by p100/IκBδ, which allows for NF-κB activation by stimuli that signal via the non-canonical pathway. There is surprisingly little information regarding the specificity of other NF-κB dimer–IκB interactions, given the potentially important role of these dynamic interactions in transcriptional specificity.

As regulators of NF-κB activity, IκB proteins markedly differ in the rates with which they are degraded in response to stimulus, and then subsequently resynthesized. All IκB proteins (IκBα, -β, -ε, p105/IκBγ and p100/IκBδ) have been reported to be NF-κB target genes, providing for potential negative feedback, yet with distinct temporal control. In recognition that these dynamic molecular interactions are critical for NF-κB signaling, the small network of IKK, IκB and NF-κB proteins has been termed the NF-κB signaling module (Figure 2) (Hoffmann *et al*., 2002; Basak *et al*., 2006). Indeed, NF-κB activation dynamics may be stimulus-specific (Werner *et al*., 2005) and can be a critical mechanism for stimulus-specific gene expression programs (Barken *et al*., 2006).

Transcriptional specificity is achieved via combinatorial control of signaling pathways: NF-κB activity does not function alone, but in conjunction with several other transcription factors and cofactors that function coordinately. As such promoters integrate

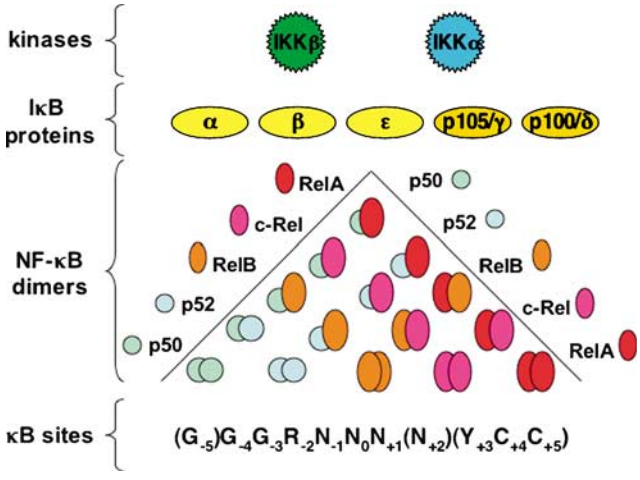

Figure 1 The molecular components of the NF-κB signaling module. Two IKKs control degradation and/or processing of IκB proteins, which bind and inhibit the DNA-binding activity of 15 possible NF-κB dimers, which bind to members of a large family of related κB sites that have a highly degenerate sequence consensus. In principle, every isoform of one molecule type can interact with every member of the adjacent family, but differential affinities make some interactions much more likely than others (see text).

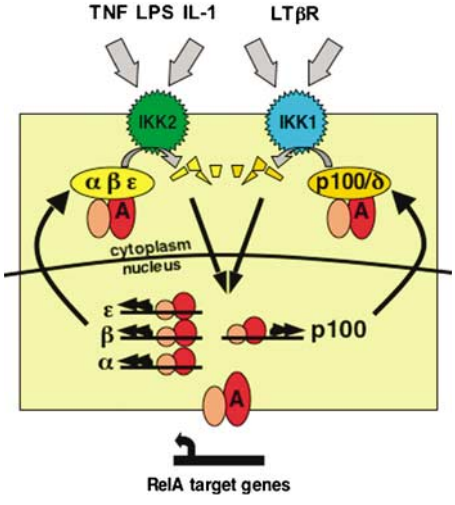

Figure 2 A representation of the NF-κB signaling module. The yellow box depicts the scope of the current version of a mathematical model capable of recapitulating p50:RelA activation in response to TNF, LPS, IL-1 and LT signaling (Basak *et al*., 2006). The model contains 99 differential equations representing interactions between p50:RelA, IκBα, IκBβ, IκBε, IκBδ/p100, IKKα and IKKβ, synthesis and degradation of IκB proteins and mRNAs and nuclear-cytoplasmic transport of these components and their complexes.

many molecular interactions (DNA–protein and protein–protein) involving multiple signaling effector proteins, which in turn show characteristic dynamic regulation. Understanding transcriptional regulation by NF-κB therefore involves the biophysical study of molecular interaction specificities and a systems biology approach to revealing combinatorial and dynamic control. In this light, we discuss the current knowledge of the NF-κB signaling module.

Formation of NF-κB dimers

The C-terminal Ig-like domain of approximately 100 amino acids within the RHD is wholly responsible for dimer formation. As a consequence, this is commonly referred to as the DimD. The overall domain fold and dimerization interfaces are highly conserved in all NF-κB proteins. Structural studies have revealed three primary mechanisms that regulate the assembly of monomers into active dimers.

The NF-κB dimer interface

In most NF-κB dimers, except for the RelB homodimer, each monomer contributes symmetrical β-strand elements that pack against each other to form a β-sheet dimer interface. Roughly, 12 side chains from each subunit mediate symmetrical (in the case of homodimers) or pseudosymmetrical (heterodimers) inter-subunit contacts (Huang *et al*., 1997; Huxford *et al*., 1999). The amino-acid residues at the dimer interface are highly conserved across the family of NF-κB proteins. The contribution made by each amino-acid position has been studied by alanine scanning mutagenesis and by *in vivo* selection for p50 (Sengchanthalangsy *et al*., 1999; Hart *et al*., 2001). Results from these experiments reveal that only a few of these interfacial residues (Y267, L269, D302 and V310; murine p50 numbering) contribute energy to the p50 homodimer formation. With the exception of Y267, which is substituted with a phenylalanine in RelA and c-Rel, these residues are identical in all NF-κB subunits. Involvement of the hydroxyl groups of tyrosine in cross-subunit hydrogen bonding may explain why the phenylalanine-containing RelA and c-Rel homodimers are weaker than the p50 homodimer. The identity of residues at position corresponding to 254 of p50 may contribute additional regulation to the stability of NF-κB dimers; D254 has a destabilizing effect. Alteration of this residue to an alanine enhances stability, whereas an asparagine at this position further destabilizes p50 homodimer. Another important dimer regulatory residue is alanine at position 307. Substitution of this residue with any other amino acid destabilizes the p50 homodimer.

Regulation of NF-κB dimerization

Positive and negative energetic contributions by amino-acid side chains at the dimer interface do not constitute the only mechanism that determines dimerization selectivity. Amino-acid sequences of the NF-κB DimD outside the dimer interface also seem to play a role, although these are not understood in atomic details. For example, although the subunit interfaces of RelA and c-Rel homodimers appear identical and all 12 subunit-contacting residues are in common, RelA and c-Rel display differences in their ability to heterodimerize with p50; the c-Rel homodimer appears to be more stable than the RelA homodimer. v-Rel, the oncogenic form of c-Rel, is even less prone to heterodimerization with p50 (Phelps and Ghosh, 2004). Although these observations are made qualitatively from *in vitro* co-refolding experiments, they are not inconsistent with *in vivo* results. From a vast body of work in many cell types, one can say with some confidence that the p50:RelA heterodimer is much more abundant than the RelA:RelA homodimer, with the abundance of the c-Rel:c-Rel homodimer and p50:c-Rel heterodimer being intermediate. However, relative expression levels of NF-κB proteins and therefore the relative abundance of different NF-κB dimers are dependent on cell type and cellular context and history.

Dimerization of the RelB protein is an extreme example of the case where non-interfacial residues play a significant role in determining NF-κB dimer formation (Ryseck *et al*., 1995). Every amino acid at the dimer interface of RelB is identical to that of p50 with the exception of residue N287 (equivalent to D254 in p50). However, RelB is unable to form a stable homodimer *in vivo* and forms a domain swapped, or intertwined homodimer *in vitro* (Figure 3a) (Huang *et al*., 2005b). Substitution of N287 to D or A does not alter the fate of the RelB homodimer *in vitro*, eliminating this residue as the sole determinant of RelB's unusual dimerization properties. Instead, we suggest that the unusually low number of surface hydrogen bonds present in the RelBD DimD (owing to substitution of surface polar by non-polar amino acids outside the dimer interface) weakens RelB's ability to form a classical NF-κB homodimers capable of binding DNA.

In contrast, we have recently determined by X-ray crystallography that p52:RelB and p50:RelB heterodimers exist as regular NF-κB dimers where two-folded monomers stack onto each other. Thus, RelB is capable of two alternate conformations and dimerization behaviors, one that allows for DNA binding and one that does not. What may be the biological significance of these two dimer forms? Although the intertwined dimer has low affinity and is unstable, such interactions may occur transiently within the dynamic molecular events of gene regulation. Intriguingly, RelB has been reported to have both positive and negative transcriptional activities (Saccani *et al*., 2003; Bonizzi *et al*., 2004; Jacque *et al*., 2005), but the molecular basis for alternate activities remains unknown.

IκB as a modulator of NF-κB dimerization

A third potential mechanism for the regulation of NF-κB dimer formation might occur at the level of IκB interaction. Although, as yet, there is no experimental evidence, one can imagine such a possibility if

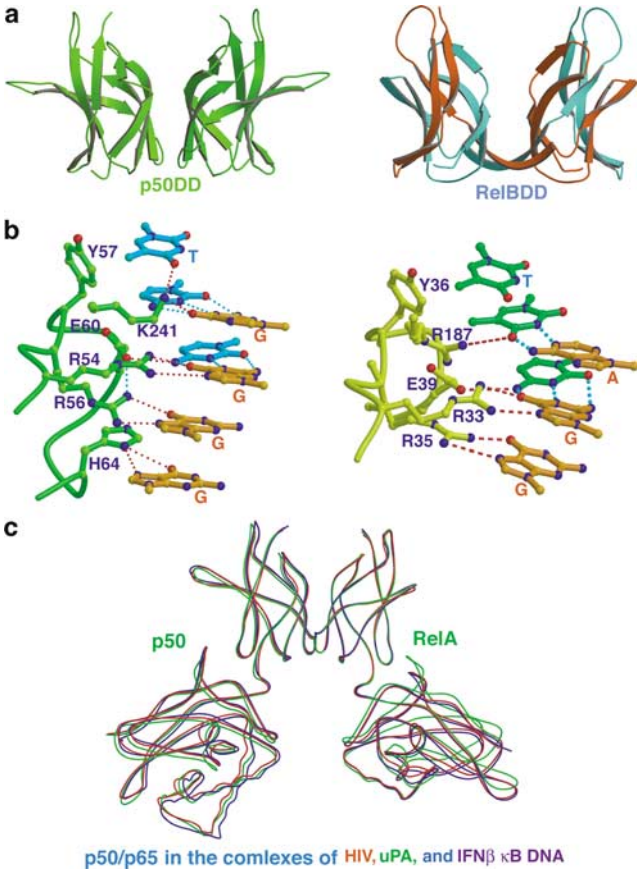

Figure 3 NF-κB subunit association and DNA contacts. (**a**) Ribbon presentations of the DimDs of NF-κB p50 (left) and RelB (right) homodimers. In the p50 dimer, two independently folded Ig domains are aligned side-by side whereas Ig domains are formed by co-folding of two peptides in the RelB homodimer. (**b**) Specific protein–DNA interactions between the p50 (left) and RelA (right) subunits are shown. p50 contacts a 5′-GGGAC half-site and RelA contacts a 5′-GGAA-3′ half-site. Two different colors for bases denote bases from two strands. (**c**) Conformational variations of the NTDs of p50 and RelA observed in the X-ray structures of three different p50/RelA:κB–DNA complexes (bottom). Three κB sequences are present in the promoters of human immunodeficiency virus (GGGACTTTCC), interferon-β (IFN-β-GGGAAAT TCC) and urokinase plasminogen activator (uPA-GGGAAACT AC) genes.

the comparative affinities between NF-κB monomers and between IκB and NF-κB dimers are considered. The affinities of physiological IκB–NF-κB complexes are much higher than that of the NF-κB dimers (pM versus nM) (Phelps *et al*., 2000a, b). In the absence of IκB proteins, a significant fraction of NF-κB proteins would be expected to exist as monomers. Thus, constitutive excess expression of IκB proteins may ensure NF-κB dimer formation.

If one considers that an IκB may have higher affinity for one NF-κB dimer over others, then that IκB can serve to redistribute prevalence of specific NF-κB dimers; alterations in relative IκB abundance may result in alterations of the available dimer pools. At present, surprisingly few data are available regarding the comparative affinity of IκB proteins for different NF-κB dimers. IκBα has a higher affinity for the p50:RelA heterodimer than the RelA homodimer (Malek *et al*., 2003), and RelB-containing dimers are not bound by the three canonical IκB proteins, but can be bound by the ARD of p100 (Solan *et al*., 2002; Derudder *et al*., 2003; Basak *et al*., 2006). Indeed, p100 deficiency reduces the amount of cellular RelB dimer (unpublished observation). Further biochemical analysis of the IκB–NF-κB interaction with recombinant proteins and of genetically perturbed signaling modules will clarify the role of IκBs in determining the pools of latent NF-κB dimers in unstimulated cells.

DNA recognition by NF-κB dimers

Extensive studies following diverse strategies over the past 20 years have helped to identify a large number of κB sequences as regulators of gene expression. Early structural and biochemical experiments shed some light on the rules that discern the selection of κB target sites by the various NF-κB dimers. Ongoing systematic analysis of interactions combined with quantitative biochemical and biophysical studies may improve our predictive understanding of the specificity rules (Udalova *et al*., 2002). Although gene knockout studies have shown that biochemical affinity data are often not sufficient to explain the functional specificity *in vivo* of NF-κB dimers in gene expression, κB DNA–NF-κB interactions are probably a critical prerequisite for NF-κB *in vivo* function. The following sections describe the current knowledge of κB site definition and its recognition by NF-κB dimers.

DNA target sites of NF-κB dimers

The DNA sequences that specifically bind NF-κB dimers are collectively known as κB sites. Most κB sites appear to be 10 bp in length with the consensus sequence 5′-GGGRN W YYCC-3′ (where R denotes a purine base, N denotes any base, W denotes an adenine or thymine and Y denotes a pyrimidine base) (Sen and Baltimore, 1986; Chen and Ghosh, 1999). However, this degenerate κB DNA consensus sequence may still be more restrictive than what may be revealed by systematic *in vitro* (Linnell *et al*., 2004) and *in vivo* (Martone *et al*., 2003; Schreiber *et al*, 2006) studies. (However, *in vivo* studies do not distinguish between direct DNA-binding events and protein–protein interaction-mediated promoter association by NF-κB). X-ray crystal structures of several κB DNA sequences are known both in free and NF-κB-bound states (Tisne *et al*., 1998, 1999; Berkowitz *et al*., 2002; Chen-Park *et al*., 2002; Huang *et al*., 2005a). The conformations of the κB DNA sites exhibit global structural likeness in their protein-bound forms. The DNA is slightly bent towards the major groove and the extent of bending is directly correlated to the length of the central tract of A•T base pairs. The AT-rich sequence is known to possess a higher propensity for bending, with longer stretches being more prone to bending. This region of the DNA also makes far fewer direct contacts with the

protein and the bending seems to facilitate NF-κB binding at the flanking recognition sequences. The minor groove is significantly narrowed in all these structures (Huang *et al*., 2005a).

DNA contacts by NF-κB

X-ray structures of several NF-κB dimers, including p50, p52, RelA and c-Rel homodimers, and the p50/RelA and p50/RelB heterodimers, are known. Together, these structures reveal that the p50 and p52 subunits bind to the 5 bp 5′-GGGRN-3′ half-site of the consensus sequence and the RelA, c-Rel and RelB subunit prefer the 4 bp 5′-YYCC-3′ half-site (Chen and Ghosh, 1999; Chen *et al*., 2000). Following this mode of binding, both the p50 and p52 homodimers appear to prefer an 11 bp κB site comprising of two 5 bp half-sites separated by a central A•T bp. Heterodimers containing one of these protein subunits prefer a 10 bp site. On the other hand, RelA and c-Rel homodimers bind 9 bp sites containing two 4 bp half-sites.

The N-terminal domain (NTD) of the RHD, which like the DimD folds into an Ig-like domain, makes both base-specific and non-specific contacts with the DNA. The DimD also makes DNA contacts, but these contacts are sequence non-specific. In addition, the linker peptide connecting the two domains also makes both specific and non-specific contacts with the DNA. A common theme shared by all NF-κB dimers is the use of loops, which connect secondary structure elements, to contact the DNA. The most significant contribution is made by loop L1, which is located near the start of the NTD (Figure 3b). This loop encompasses five base-contacting residues in murine p50 (R54, R56, Y57, E60 and H64) and four base-contacting residues in murine RelA (R33, R35, Y36 and E39) and c-Rel (R21, R23, Y24 and E27). Unlike conventional disordered loops, the loop L1 of NF-κBs is a highly structured region, which fits snugly in the DNA major groove contacting the distal end of both the DNA half-sites. The conserved tyrosine residue in loop L1, in addition to making van der Waals contacts with two central pyrimidines (preferably thymines), also interacts with the DNA phosphate backbone. The conserved histidine in loop L1 of both p50 and p52 directly contacts the first guanine in the κB site and this accounts for the requirement for an extra G•C bp at the −5 position in both p50 and p52 half-sites (Muller *et al*., 1995; Cramer *et al*., 1997; Chen *et al*., 1998a). The replacement of this histidine residue by an alanine in RelA and c-Rel is the primary determinant of 5 bp versus the 4 bp half-site selectivity for p50 and RelA (or c-Rel), respectively. Although the RelB DimD structure exhibits deviations from the canonical NF-κB structure, the recently solved structure of RelB/p50/DNA conforms to the other NF-κB/DNA structures.

Affinity differences between NF-κB dimers and κB site sequences

In spite of the apparent breadth of consensus sequence of κB sites, the κB sites within a gene show high evolutionary stability. This underlines a specificity, which may or may not arise from the one-to-one binding affinity between the NF-κB dimer and the κB DNA. Early studies used the electrophoretic mobility shift assay (EMSA) to select NF-κB-binding sequences from a pool random oligonucleotides to determine whether recombinant p50, RelA or c-Rel have distinct binding preferences (Kunsch *et al*., 1992). These studies found that p50 preferentially selected for 11 bp sites, whereas RelA and c-Rel selected for 10 bp sites, but the relative affinity of NF-κB dimers for different κB site sequences remained unclear.

More recent solution-based *in vitro* binding measurements revealed the following: p50:RelA binds to 10 or 11 bp consensus sequences with similar affinities (∼10 nM) (Phelps *et al*., 2000b). RelA and c-Rel homodimers bind the same sequences with at least 10-fold lower affinity. However, these homodimers bind more strongly to the 9 bp consensus sequences (∼50 nM). Homodimers of p50 and p52 or their heterodimers with RelB bind to all well-characterized κB sites belonging to both 10 and 9 bp consensus sites with similar affinities (∼50 nM) (Fusco and Ghosh, 2006, unpublished observation). Altogether, these studies suggest that (i) each NF-κB dimer has some preference for a specific set of κB sites, and (ii) some NF-κB dimers bind to most κB sites with reasonably high affinities.

In summary, solution-based DNA-binding data indicate that the NF-κB dimers bind κB sites with affinities ranging from 10–300 nM. In no cases are these affinities as high as seen for some other transcription factors, but even the lowest affinity complexes still exhibit roughly 10-fold higher affinity than that of the non-sequence-specific NF-κB/DNA complexes. Therefore, at a minimum *in vitro* binding data reveal the collection of κB site sequences to which a dimer is capable of binding. We discuss below whether relative binding affinities can provide some explanation for the transcriptional specificity of NF-κB *in vivo*.

Transcriptional specificity of NF-κB as determined by molecular interactions

Transcriptional control involves the coordinated function of many proteins, including DNA-bound transcription factors, co-activators and co-repressors in the context of nucleosomal chromatin. Given the large number of proteins located on DNA regulatory sequences, protein-protein interactions are likely as important as transcription factor–DNA interactions in determining transcriptional specificity.

Early studies employed transient co-transfection reporter assays to determine which particular NF-κB proteins alone or in combination are capable of activating a particular promoter. The results of such studies generally correlate with *in vitro* binding data by EMSA, but as others have observed this experimental approach may not allow for conclusions of transcriptional specificity of the endogenous gene. The transfected reporter gene is in molecular excess over

cooperating endogenous molecules, is not wrapped in nucleosomes or within its native chromatin environment. Therefore, recent studies have attempted to determine the transcriptional specificity rules of endogenous genes.

Using a panel of knockout cell lines deficient in one or two NF-κB protein genes, a genetic study did indeed reveal that different NF-κB-regulated genes have distinct requirements for NF-κB proteins (Hoffmann *et al*., 2003). In general, such genetic studies cannot determine which NF-κB dimer normally activates a given gene of interest, but when the available dimers in the mutant cell are identified by EMSA, the dimer requirement for a specific gene induction event can be determined.

NF-κB–DNA interaction affinity as the specificity determinant

Whereas RelA is a ubiquitous NF-κB protein required for many cellular or organismal functions as well as the expression of other NF-κB and IκB genes, c-Rel is generally confined to activated cells of the lymphoid system and is more amenable to genetic analysis. To characterize the molecular basis of the narrowly defined immunological phenotypes associated with c-Rel deficiency, several genes have been identified as specifically requiring c-Rel for activation. One such gene is that encoding interleukin (IL)-2 whose expression is induced by the so-called CD28 response element, which turns out to be a variant κB site that binds c-Rel homodimers and p50:c-Rel heterodimers with higher affinities than other NF-κB dimers (Verweij *et al*., 1991; Huang *et al*., 2001). This correlates with the fact that c-*rel*$^{-/-}$ T cells are deficient in IL-2 expression (and other CD28-responsive genes) and T-cell proliferation (Kontgen *et al*., 1995).

Similarly, interleukin-12 p40 expression in lipopolysaccharide (LPS)-treated macrophages requires c-Rel, and even additional ectopic expression of RelA is not able to rescue this c-*rel*$^{-/-}$ phenotype, although introduced c-Rel expression does (Sanjabi *et al*., 2000). Chimeras of RelA and c-Rel allowed mapping of the specificity region to a loop in the DNA-binding domain of the RHD. Subsequent EMSA with the functionally relevant κB-site revealed DNA-binding affinity differences between RelA and c-Rel and the chimeric proteins that correlate with their *in vivo* functionality on the IL-12 promoter (Sanjabi *et al*., 2005). Together, these studies suggest that c-Rel dimers can bind RelA dimer binding sites, but they also have broader sequence binding ability to include sequences that are not normally bound by RelA dimers. Hence, activation of c-Rel-containing dimers can result in the activation of specific genes that are not responsive to the classical p50:RelA NF-κB dimer.

In many cases, however, affinity differences in NF-κB–κB site interactions identified *in vitro* have not been predictive of gene expression specificity. For example, on the interferon-β promoter, both p50:RelA and RelA:RelA dimers can activate transcription even though these two dimers bind the β-interferon κB site with significantly different affinities (Phelps *et al*., 2000b). Considering the large assembly of activators and co-activators, a single activator bound to a single response element is rarely sufficient to induce transcription. Conversely, affinity differences at one site may be masked by protein–protein interactions. In the extreme, such protein–protein interactions may even obviate the need for direct DNA contacts altogether, as seen in the interaction between IRF-3 and RelA dimers on synthetic promoter constructs (Wietek *et al*., 2003; Leung *et al*., 2004).

Promoter-directed NF-κB–protein interactions as the specificity determinant

Early studies already concluded that not all κB-site consensus sequences that show high affinity for NF-κB *in vitro* confer NF-κB-responsiveness *in vivo*. Although the Ig κB site (5′-GGGACTTTCC-3′) and the IL-2Rα κB site are both high-affinity NF-κB-binding sites, they are not functionally equivalent, as determined by swap experiments (Cross *et al*., 1989). These observations suggested that the NF-κB dimer specificity of promoters may be context- and stimulus-dependent, making it unlikely that NF-κB–DNA interactions alone determine specificity of NF-κB-responsive promoters.

Using a systematic panel of single and double NF-κB gene knockouts, the NF-κB dimer requirement for a dozen well-characterized NF-κB target genes was determined (Hoffmann *et al*., 2003). Although transient transfection studies had been used to identify the relevant κB sites required for transactivation, the transiently transfected reporters did not recapitulate the specificity seen on the endogenous genes. Furthermore, within this limited set of promoters, the genetically determined NF-κB dimer requirement did not correlate with the sequence of the κB-binding site(s). Some promoters (e.g., RANTES, IP-10, macrophage colony-stimulating factor) with different sequences showed the same NF-κB dimer requirement, while other promoters (e.g., MIP-2, MCP-1) with identical κB-site sequences showed different NF-κB dimer requirements. These observations emphasized the importance of the promoter context in determining the specificity of a single κB site. In a subsequent study (Leung *et al*., 2004) the specific dimer requirement was further found to be stimulus-dependent. Although the RelA homodimer was found to be incapable of activating the IP-10 gene in response to tumor necrosis factor (TNF) stimulation, LPS stimulation did result in NF-κB-dependent transcription when only the RelA homodimer was available. As TNF and LPS induce distinct signaling events, these results suggest that different sets of synergizing transcription factors or co-activators can determine the functional specificity of the κB-site sequences.

Interestingly, chromatin immunoprecipitation (ChIP) analysis revealed that RelA was bound to the IP-10 promoter even when the RelA homodimer did not allow for gene activation (Leung *et al*., 2004), indicating that the interaction with presumed co-activators did not result in cooperativity at the level of DNA binding, but possibly at the level of downstream core promoter activation events. Similar conclusions were drawn for

the well-studied β-interferon enhanceosome (Merika *et al*., 1998). Although the ability of an NF-κB dimer to bind DNA is a requirement for gene activation, it is not sufficient for activation. Protein–protein interactions initiated or supervised by enhancer or promoter sequences play critical roles in NF-κB transcriptional specificity. Indeed, the importance of this level of transcriptional control has been revealed by the discovery that the glucocorticoid receptor is able to exert its inhibitory effect on a subset of inflammatory genes by interfering with protein–protein interactions of RelA dimers and IRF-3 (Ogawa *et al*., 2005; see De Bosscher *et al*., 2006).

Other cases in which promoter activity appears to require the interaction of specific co-activators have been described. Interestingly, the structural IκB homologs Bcl-3 and MAIL have been shown to have such co-activator activity for a subset of genes (Fujita *et al*., 1993; Leung *et al*., 2004; Yamamoto *et al*., 2004). In addition, post-translational modifications of NF-κB proteins are thought to play a role in regulated recruitment of co-activators or co-repressors (Campbell *et al*., 2004, see Perkins, 2006).

A role for κB site-mediated allostery in transcriptional specificity

Structural and biochemical studies have shown that NF-κB dimers are highly flexible and that each dimer can undergo conformational alterations to effectively interact with myriad κB sites. This inherent flexibility also allows for different NF-κB dimers to bind to the same κB site with comparable affinities. The bi-modular (NTD and DimD) architecture of the RHD provides a unique structural framework for NF-κB dimers. These two RHD subdomains are free to move with respect to each other allowing each dimer to assume different conformations upon binding to various related, but distinct, κB sites. For example, the structure of p50:RelA heterodimer bound to the Ig/human immunodeficiency virus-κB DNA site is different from that bound to the urokinase plasminogen activator-κB site (Figure 3c) (Chen-Park *et al*., 2002). The binding affinities for these two sites, however, are comparable. Sequence-dependent structural changes in DNA lead to alteration in the mode of NF-κB binding and conformation. Thus, a specific NF-κB/DNA complex can influence the cooperativity of interactions with nearby bound proteins through interactions with co-activators leading to the formation of a distinct enhanceosome. κB-site DNA can therefore, in principle, function as an allosteric regulator of NF-κB's interaction surfaces, an effect that is transmitted to other components in a transcription complex which may regulate the transcriptional activity of the complex.

By identifying two tandem κB sites as a specificity determining motif, Leung *et al*. (2004) were able to study NF-κB transcriptional specificity without the complex effects of the contextual promoter sequences. Indeed, the IP-10 tandem κB-site motif could be transplanted to a heterologous promoter to confer not only NF-κB responsiveness, but also the specific NF-κB dimer requirement in a retroviral transgene. As expected, inactive RelA homodimers were still found to be associated with the promoter, but altering the sequence of the κB sites could turn the bound RelA homodimer into a transcriptionally competent transcription factor–DNA complex. This dramatic alteration in functionality could be achieved with a single mutation of one of the central nucleotides within the κB-site motif. These findings await biophysical characterization of the hypothesized κB site-mediated allostery of the NF-κB dimer and its ability to interact with the obligate co-activator. Nevertheless, they powerfully illustrate why each endogenous κB-site sequence found in promoters, although being a member of a very broad family of potential κB site sequences with similar affinities for a specific NF-κB dimer, appears to be strictly conserved in evolution.

Transcriptional specificity as determined by NF-κB dynamics

As a central regulator of the transcriptional control of inflammatory genes, NF-κB function is not a steady-state or equilibrium phenomenon, but occurs in the context of highly dynamic signaling events. Indeed, the transient nature of gene expression is the basis for the resolution of inflammation and so the molecular attenuation mechanisms that limit inflammatory signaling have received attention (Han and Ulevitch, 2005). Within this context, it is notable (but not unexpected) that NF-κB response genes show distinct temporal expression profiles (Saccani *et al*., 2001; Hoffmann *et al*., 2003). Furthermore, NF-κB activities appear in the nucleus and on target genes with characteristic temporal profiles that may be stimulus-specific. Thus, the NF-κB dimer requirement revealed by genetic studies as described above may mask intricate specificity mechanisms that occur with the dynamic regulatory environment of gene control. Here, we discuss current research efforts to understand the dynamic control of NF-κB activity as a mechanism to achieve specificity in transcriptional control of target genes.

Dynamic control of NF-κB activities

NF-κB activation is controlled by the stimulus-induced degradation and re-synthesis of IκB proteins, and the processing and re-synthesis of the NF-κB precursor proteins p100 and p105. In response to inflammatory stimuli such as TNF, which functions through activation of IKKβ, these processes are sufficiently well understood and a mathematical model was constructed that recapitulates NF-κB activation dynamics as observed experimentally *in vitro* (Hoffmann *et al*., 2002). These initial studies utilized IκB-deficient cell lines and observed that continual application of TNF resulted in extended oscillations of NF-κB activity in IκBε$^{-/-}$β$^{-/-}$ cells owing to the powerful negative feedback of NF-κB-controlled IκBα expression. Similarly, overexpression of

RelA and RelA-responsive IκBα by transient transfection resulted in oscillatory activity in single cells as revealed by video microscopy in single cells (Nelson *et al*., 2004), although the resulting increase in the green fluorescence protein-fusion effector molecules can alter the dynamic behavior (Barken *et al*., 2005). However, in wild-type cells, NF-κB activity is subject to other negative feedback mechanisms. One, mediated by IκBε, appears with a 45 min delay which renders it in anti-phase to that mediated by IκBα (Kearns *et al*., 2006). The result is that in normal cells, induced NF-κB activity can be remarkably quickly and efficiently attenuated following termination of the signal, yet oscillatory ringing during chronic stimulation is counteracted to result in steadied and precisely controlled NF-κB activity.

NF-κB activation within the IκB-NF-κB signaling module is controlled by the activity of IKK, which can be thought of as the module's input. Probing the signaling module with a set of different IKK inputs revealed that the control of late NF-κB activity is remarkably sensitive to small differences in the levels of IKK activity, whereas early IKK activity (which is not yet subject to high feedback mechanisms) produces strong NF-κB activity independent of its level (Werner *et al*., 2005). Subsequent experimental studies have supported these predictions. Although a TNF dose–response study showed that even 0.1 ng/ml, about 100-fold lower than commonly used concentration of this cytokine, can produce strong NF-κB activity (Cheong *et al*., 2006), persistent or pulse stimulation with TNF produce remarkably different gene expression programs (Hoffmann *et al*., 2002).

Canonical IKK activities are generated by signaling pathways emanating from a variety of receptors, particularly those of the Toll-like receptor (TLR) and tumor necrosis factor receptor (TNF-R) superfamilies. Typical representatives TNF-R1 and TLR4, which mediate TNF and LPS signaling, respectively, were found to produce remarkably different IKK induction profiles (Werner *et al*., 2005), which in the case of TNF are independent of the stimulus concentration (Cheong *et al*., 2006). A current research focus is the elucidation of the mechanisms that produce stimulus-specific IKK activities. These reside in the specific signaling pathways upstream of IKK that are initiated by the specific ligand–receptor interactions, but they also may be subject to feedback regulation. Initial studies have identified A20 as a critical negative feedback regulator of TNF-induced IKK (Lee *et al*., 2000; Werner *et al*., 2005), whereas the LPS-induced IKK activity involves positive feedback regulation via an autocrine TNF loop (Covert *et al*., 2005; Werner *et al*., 2005). Importantly, both of these feedback mechanisms of IKK activity were shown to be critical for the NF-κB activation profile characteristic of the stimulus, and for stimulus-specific gene expression (Werner *et al*., 2005).

It is noteworthy that the activation profiles of NF-κB dimers via the canonical and non-canonical pathways are remarkably different. Whereas IKKβ-mediated NF-κB activation via the canonical IκBs occurs within minutes and is highly dynamic, IKKα-mediated NF-κB activation occurs within hours and is generally persistent. This may correlate with the distinct physiological functions in inflammation and immune signaling versus development and homeostasis of secondary lymphoid structures or control of bone homeostasis. Although the focus of past studies has been the activation of the non-canonical pathway-specific RelB dimer (Dejardin *et al*., 2002; Bonizzi *et al*., 2004), it is of interest to determine whether temporal control of NF-κB may play a role in distinguishing the developmental from the inflammatory gene expression programs.

Stimulus-specific gene expression via NF-κB dynamic control

If dynamic control of NF-κB activities has physiological significance, IκB proteins as regulators of the dynamic NF-κB activity may function as transcriptional specificity factors. First indications that this may be the case were found by comparing the gene expression response to transient and persistent NF-κB as elicited by pulse or chronic TNF stimulation (Hoffmann *et al*., 2002). Interestingly, cells deficient in IκBα were not only defective in limiting the NF-κB activity in response to pulse stimulation, they also expressed the gene for the chemokine RANTES, a hallmark for persistent NF-κB activity. Given the importance of stimulus-induced degradation and synthesis of IκB proteins in determining the NF-κB activation profile, it is likely that detailed analysis of the rate constants operating within the NF-κB signaling module will reveal molecular mechanisms with subtle effects on the stimulus-specific activation of NF-κB that may result in particular defects in stimulus-specific gene activation.

Recent studies directly examined the hypothesis that stimulus-specific dynamic control of NF-κB plays a role in determining the stimulus specificity of gene expression (Barken *et al*., 2006). Computational simulations with diverse IKK inputs were used to identify the molecular mechanisms within the module that confer the ability to distinguish between different inputs. These studies identified the negative feedback of IκBα and IκBε to be critical, and experimental studies confirmed that $I\kappa B\alpha^{-/-}\varepsilon^{-/-}$ cells are not capable of distinguishing between different IKK inputs. Strikingly, in these cells stimulus-specific gene expression programs dramatically collapsed into one, losing the distinction between LPS-, TNF-, or IL-1-specific genes (Barken *et al*., 2006). This finding demonstrates the ability of promoters to interpret stimulus-specific NF-κB dynamics to produce stimulus-specific gene expression.

Dynamic regulation of nuclear NF-κB activity can only play a regulatory role on gene control if the binding events on promoters reflect the nuclear-cytoplasmic dynamics. Contrary to commonly held assumptions, recent work has suggested that NF-κB interactions with its cognate DNA-binding sites are in fact very transient, implying a fast dissociation rate constant (Bosisio *et al*., 2006). Whereas protein complexes associated with gene regulatory sequences (enhanceosomes) have often been thought to be stable entities, these studies suggest that

high on and off rates allow for dynamically regulated protein complexes on enhancer/promoter regions. Other studies have revealed that the RelA protein itself is inducibly degraded via proteasome-mediated proteolysis (Saccani *et al*., 2004; Lawrence *et al*., 2005), enhancing the dynamic capacity of NF-κB promoter interactions. In addition, it has been proposed (but never precisely demonstrated *in vitro* or *in vivo*) that IκB proteins have a 'stripping' function, allowing them – as part of their negative feedback regulation – to actively remove (i.e., enhance the dissociation rate of) NF-κB dimers from the promoter DNA.

Whereas these studies were focused on the classical p50:RelA dimer, the NF-κB system is capable of producing a family of dimers. Given differential affinities of IκB proteins, differential synthesis, degradation and translocation rates, the dynamics of different NF-κB dimers are likely to differ. Cellular stimulation may be thought of to result in a dozen distinct (although partially overlapping) waves of different dimers, a subset of which may contact or probe the regulatory region of a particular gene, resulting in transcriptionally active or inactive complexes. Indeed, probing a handful of κB site-containing promoters by ChIP in a time course study detected the presence of RelA and RelB proteins at different times after LPS stimulation of myeloid cells (Saccani *et al*., 2003). On some genes, RelB was associated with sustained activation on other genes with attenuation following RelA-mediated activation.

Understanding NF-κB transcriptional control in context

NF-κB does not function on promoters in isolation. In spite of its obligate and central role on many promoters, NF-κB is only one component of a large and diverse set of molecular events that control gene activity. These events must be coordinated to produce specific regulation.

In *Drosophila*, the regulation of a large number of innate immune response genes can indeed be accounted for by the combinatorial regulation of NF-κB and the GATA factor serpent (Senger *et al*., 2004). By measuring DNA-binding affinities of these factors for diverse binding sites, it may be possible to construct a predictive model for the regulatory code. The classical view of cooperative transcription factor regulation is that protein–protein interactions between multiple sequence-specific transcription factors reciprocally support their recruitment (Ptashne and Gann, 1997). However, in mammalian cells, recruitment of NF-κB is often temporally dissociated from recruitment of cooperating transcription factors. For example, the assembly of the β-interferon enhanceosome occurs in a cooperative manner *in vitro* but in a progressive, stepwise manner *in vivo* (Munshi *et al*., 2001). Similarly, on the MIP-2 gene promoter, NF-κB recruitment parallels its nuclear localization dynamics, whereas activator protein-1 recruitment shows a progressive increase over time and remains bound between two pulses of NF-κB recruitment (Bosisio *et al*., 2006).

Sequential functions of NF-κB and cooperating transcription factors

An attractive model is that cooperating transcription factors promote NF-κB recruitment by prior preparation of the endogenous chromatin template. Support for this idea came from the observation that in murine macrophages, activation of all NF-κB-dependent genes induced with slow kinetics required chromatin remodeling, that is, double knock down of Brg1 and Brm1; the ATP-ase subunits of the SWI/SNF chromatin remodeling complexes resulted in a drop of the transcription rate of all slowly activated genes tested (including secondary genes and primary genes with slow activation kinetics), whereas immediate early genes were unaffected (Ramirez-Carrozzi *et al*., 2006). At some of the genes requiring remodeling for activation, RelA was indeed shown to be recruited at delayed times that coincided with the time of gene activation (Saccani *et al*., 2001). Moreover, in the specific case of the IL12p40 promoter, remodeling of a nucleosome covering several binding sites (including a κB site) was shown to be independent of c-Rel, which is required for transcriptional activation (Weinmann *et al*., 2001). Taken together, these results point to a linear pathway in which coordinated signaling events may lead to the recruitment of SWI/SNF complexes to organize a chromatin site for NF-κB binding, which in turn results in transcriptional activation. In this model, two different but coordinated signaling inputs must act in a defined temporal sequence on a promoter; stimulus specificity in gene expression could be achieved by triggering the same signaling pathways in an alternate sequence. An additional interesting aspect of this model is that it provides an example of how nucleosomes can act as mediators of transcription factor cooperativity.

Coincident functions of NF-κB and cooperating transcription factors

In an alternate model for gene control, coordinated signaling events may result in temporally coincident transcription factors or co-activators on target promoters. This model may best describe the case of the β-interferon enhancer, in which gene expression is dependent on the simultaneous presence of all required transcription factors on the enhancer (Maniatis *et al*., 1998). A less complex but apparently widespread phenomenon is the synergistic function of IRF-3 and RelA dimers. IRF-3 activation through phosphorylation by upstream kinases (Fitzgerald *et al*., 2003; Sharma *et al*., 2003) occurs in response to the LPS response-transducing TLR4, but not in response to many other NF-κB-inducing signaling pathways (Doyle *et al*., 2002). Not only is IRF-3 required for the induction of a subset of NF-κB-dependent LPS response genes, but the direct interaction between RelA and IRF-3 is required: hormone-bound glucocorticoid receptor was shown to disrupt the interaction, thereby mediating trans-repression of a subset of NF-κB target genes (Ogawa *et al*., 2005). Interestingly, IRF-3 is able to synergize with RelA homo- and heterodimers when bound to a wide variety of κB sites and itself does not need to contact DNA (Leung *et al*., 2004). About one-third of

glucocorticoid-inhibited NF-κB response genes also require IRF-3 for induction, suggesting the existence of other mechanisms by which the glucocorticoid receptor functions to dampen inflammatory responses.

Conclusions and perspectives

NF-κB denotes a family of transcriptional regulators that exhibit stimulus-specific dynamic activation profiles and bind to a large number of degenerate target sites to regulate the expression of hundreds of genes precisely and dynamically in response to changing environmental conditions. The regulatory regions of NF-κB-dependent genes have evolved binding sites and chromatin organization to achieve appropriate transcriptional control that is tissue and stimulus-specific.

Understanding transcriptional regulation by the NF-κB signaling module will require integration of the above-described different mechanisms that can generate specificity: dynamic control each member of the family of NF-κB dimers, their transient interactions with κB sites of different affinities, and alternate conformations of the resulting NF-κB–κB DNA complex allowing for alternate co-activator interactions. Current and future work may not only involve studies of single model promoters, but may benefit from systematic approaches that will identify groups of co-regulated genes and reveal basic regulatory principles. Multiple levels of analysis and investigation will be required. To understand the dynamic regulation of nuclear NF-κB activities and their interaction potentials, a comprehensive analysis of the biochemical and biophysical properties of IκB and NF-κB proteins is needed. Computational modeling of the IKK-IκB-NF-κB signaling module will be an increasingly important tool as the number of components and complexity of dynamic behavior increases. However, applying these models to *in vivo* regulation will be a challenge. Second, genome-wide measurements of mRNA levels and location analysis on NF-κB target genes will better define the range of NF-κB function and provide clues for the sequence motifs that are involved in determining transcriptional specificity. Defined genetic perturbation (e.g., knockouts) and a panel of well-defined stimulation conditions will prove useful. Third, high throughput measurements of coordinated signaling pathways, secondary and tertiary signaling events will be required. It is increasingly apparent that gene expression programs are specified through complex interactions of signaling networks involving feedback and autocrine cascades (Gaudet *et al*., 2005; Janes *et al*., 2006). Although NF-κB appears to be a central regulator in many physiological processes, accessory signaling pathways may prove critical in determining cell-, context- and stimulus-specific gene regulation.

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