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Diversity and regulation in the NF-κB system

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The nuclear factor (NF)-κB family of transcription factors is a key participant in multiple biological processes, most notably in the immune and inflammatory response. Five proteins make up the NF-κB family, and these proteins can hetero- and homo-dimerize, giving rise to diversity. Recently, it has been shown that certain members can also interact directly with other transcription factors such as signal transducers of activated transcription, interferon regulatory factor family members and p53, providing further diversity. We propose that this promiscuity might help explain the many of roles of NF-κB in specialized cell function and fate. Furthermore, the state of a cell and its cellular background in addition to overall promoter structure and variations in the κB target sequence will all define the composition and activity of multimeric NF-κB complexes.

The breadth of the NF-κB family

The family of nuclear factor (NF)-κB transcriptional regulators is a truly remarkable one in that it is the target of multiple stimuli and participates in a wide range of important physiological and pathological processes, notably, in immunity, inflammation and development of diverse tissues. There are five proteins in the NF-κB family: NF-κB1 (also known as p105 or p50 depending on whether or not it has been processed), NF-κB2 (a.k.a. p100 or p52 when processed) and the Rel subfamily of RelA (a.k.a. p65), RelB and c-rel (Figure 1). All members have a conserved Rel-homology domain (RHD; see Glossary), which contains a DNA-binding motif, a dimerization region and a nuclear location signal (NLS) [1]. In addition, members of the Rel subfamily contain a transactivation domain (TAD), which is crucial for target gene activation (Figure 1). The five proteins have been shown to homo- and hetero-dimerize, with examples of different functions being ascribed to different dimers [2,3]. This provides a starting point to explain the diversity of functions within the family. But how do cells achieve stimuli-specific gene activation with a limited set of transcriptional activators?

Recent studies have begun to confirm what had been suspected – that there would be subtle differences in the NF-κB motif recognized by different dimers and that these motifs would occur in specific genes [4]. However, it has also become clear that the binding motif is not the sole determining factor for specific NF-κB complex recruitment. In addition, it emerged that particular NF-κB subunits can associate with other transcription factors, notably signal transducers of activated transcription (STATs), interferon regulatory factors (IRFs) and the tumour suppressor protein p53. Complex formation with some of these transcription factors is influenced by the κB target sequences. Moreover, there have been new insights into regulation through phosphorylation of certain dimers and into the recruitment of co-activators. Here, we argue that these recent insights provide a model for diversity in the capacity of one family of transcriptional regulators to control a wide range of biological processes. Rather than being a comprehensive review of protein complexes containing NF-κB proteins, which are extensively reviewed elsewhere (see, for example, Ref. [5]), we only describe more recently discovered complexes and their regulation.

Dimerization and regulation of NF-κB family members

NF-κB family members function as either homo- or hetero-dimers. In this section, we describe the characteristics of those dimers that are best understood, together with their regulation.

The two best-characterized NF-κB dimers are p50–p65 and p52–RelB [1]. In unstimulated cells, p50–p65 is bound to inhibitory IκB proteins. These inhibitors interact with the RHD and, at least partly, mask the NLS with ankyrin repeats. Of the eight known IκBs, the three best-characterized are IκBα, IκBβ and IκBε. The precursors to p50 and p52, termed p105 and p100, respectively, also have ankyrin repeats and have to be processed to gain the respective active p50 and p52 NF-κB proteins. Because p105 can be found in a complex with p65, this processing provides another alternative mechanism to the removal of IκB for

Glossary

Enhanceosome: a protein complex binding to a defined enhancer region to promote transcription of a target gene.
κB-binding site: DNA sequence targeted by individual NF-κB complexes.
Rel-homology domain (RHD): a domain composed of β-barrel subdomains found in families of eukaryotic transcription factors, including NF-κB and NF-AT.
Transactivation domain (TAD): the acidic and amphipathic domain consisting of β-sheets and α-helices that is essential for induction of target gene transcription.
Ankyrin repeats: common structural motifs in proteins consisting of two α-helices separated by loops that mediate protein–protein interactions.
Leucine zipper: a type of structural motif found in parallel coiled-coils that facilitates dimerization and DNA binding of transcription factors.

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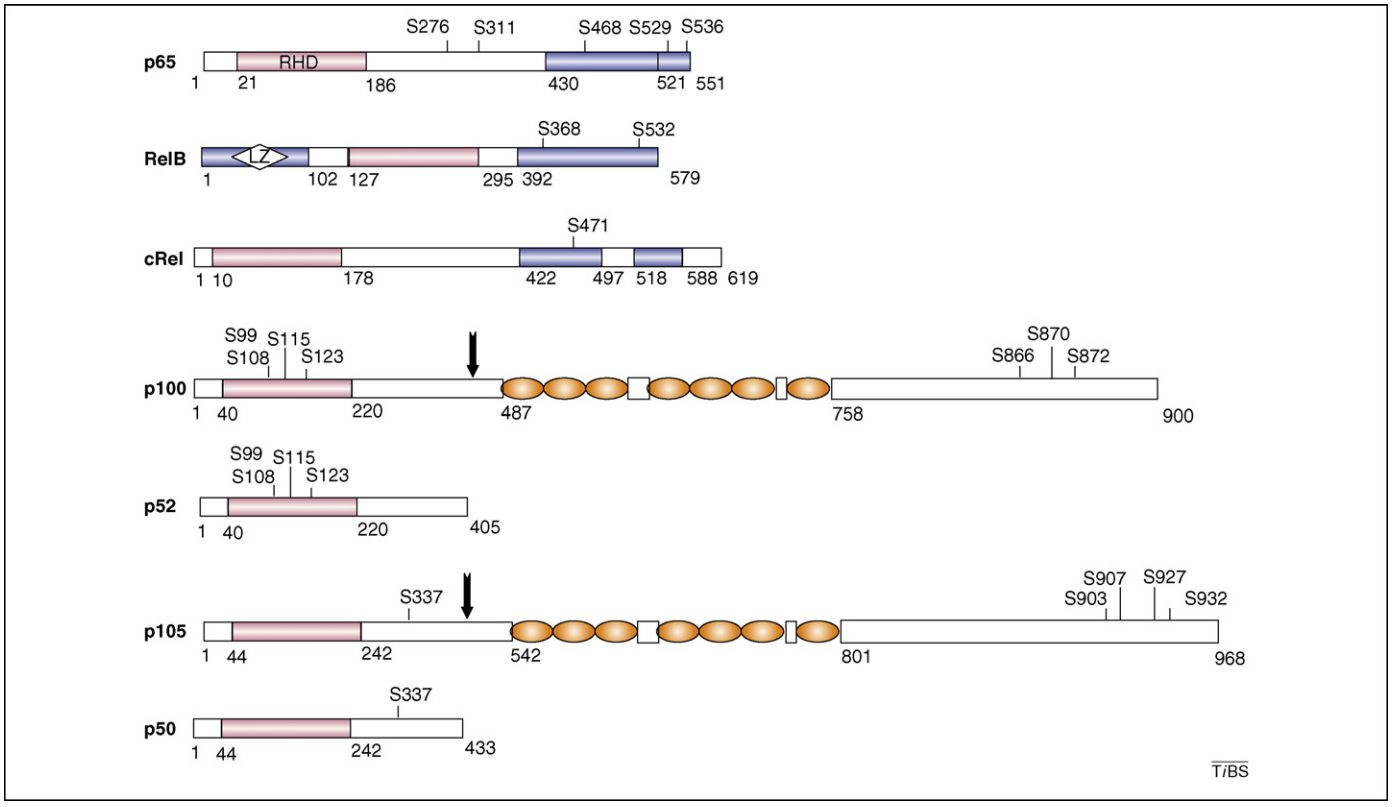

Figure 1. The NF-κB family of transcription factors. Schematic overview of the NF-κB family depicting the RHD (brown), TADs (blue) and inhibitory ankyrin repeats (orange ovals). The Rel subfamily (p65, RelB and c-Rel) is characterized by proline- and glutamine-rich or acidic amphipathic regions, TADs, which are essential for transactivation of target genes. A leucine zipper motif (LZ) in RelB is shown as white diamond. Size of proteins and locations of RHDs, TADs and ankyrin repeats are indicated with residue number, as are the phosphorylation sites (see also Table 1). Black arrows indicate processing positions of p100 and p105.

the generation of active p50–p65 dimers. Owing to these inhibitory features, the ankyrin-repeat-containing C termini of p105 and p100 have been termed IκBγ and IκBδ, respectively.

The p50–p65 dimers

Many different stimuli activate the p50–p65 complex, most notably, inflammatory cytokines, bacterial and viral products acting via Toll-like receptors (TLRs) and various cell stressors. Activation of this so-called classical or canonical pathway crucially involves IκB subunits becoming phosphorylated by IκB-kinases (IKKs), mainly IKKβ and IKKα, which form a multimeric complex with the structural protein NF-κB essential modulator (NEMO, also termed IKKγ). Phosphorylation of IκBs results in ubiquitylation and degradation of these inhibitors [6]. The released NF-κB dimers then undergo further transactivating phosphorylation, bind to their cognate target sites and recruit co-factors into their activation complexes. Transactivating phosphorylation of NF-κB is essential to induce transcription of target genes (Figure 2a and Box 1).

Two additional non-canonical kinases, IKKε or IKK-i and TANK-binding kinase 1 (TBK-1) or NF-κB- activating kinase (NAK), have been characterized in recent years. Both kinases have been mainly implicated in the interferon pathway through phosphorylation of interferon regulatory factors (IRFs) but can also phosphorylate p65 in its transactivation domain (TAD). A recent study describes c-Rel as another substrate for IKKε and TBK-1 [7]. In addition, IKKε can phosphorylate IκBα. This is, however, different to IKKβ. With IKKβ, IκBα becomes phosphorylated on both Ser32 and Ser36, whereas, with IKKε, only Ser36 is phosphorylated [8]. Interestingly, IKKε is not required for activation of the NF-κB following tumour necrosis factor-α (TNF-α) or interleukin-1 (IL-1) stimulation but is involved in T-cell-receptor-induced NF-κB activation [8].

The p52–RelB dimer

The p52–RelB dimer was originally shown to be activated by the TNF family member lymphotoxin-β (LT-β) (Figure 2b). Stimulation with LT-β also activates the canonical NF-κB pathway, resulting in transcription of p65–p50-inducible genes [9]. This initial rapid response was, however, shown to be followed by a delayed activation of the alternative or non-canonical pathway, which causes a shift from p65–p50 to RelB–p52 dimer activation (Box 1). This slower activation results in a sustained activation of RelB–p52-dependent genes. The alternative NF-κB pathway does not require NEMO and IKKβ but relies on IKKα and NF-κB-inducing kinase (NIK) for processing of p100 and activation of p52 [9,10]. It is activated by several members of the TNF-α family such as LT-β and CD40 ligand (CD40L). Similar to LT-β, CD40L can induce activation of both pathways [9,11]. The alternative pathway regulates B-cell maturation and is crucial for lymphoid organogenesis [12–14]. Interestingly, the alternative pathway is also involved in cell-cycle regulation. A recent study found that the RelB–p52 dimer controls progression from G1- to S-phase through regulation of S-phase kinase-associated protein gene expression [15].

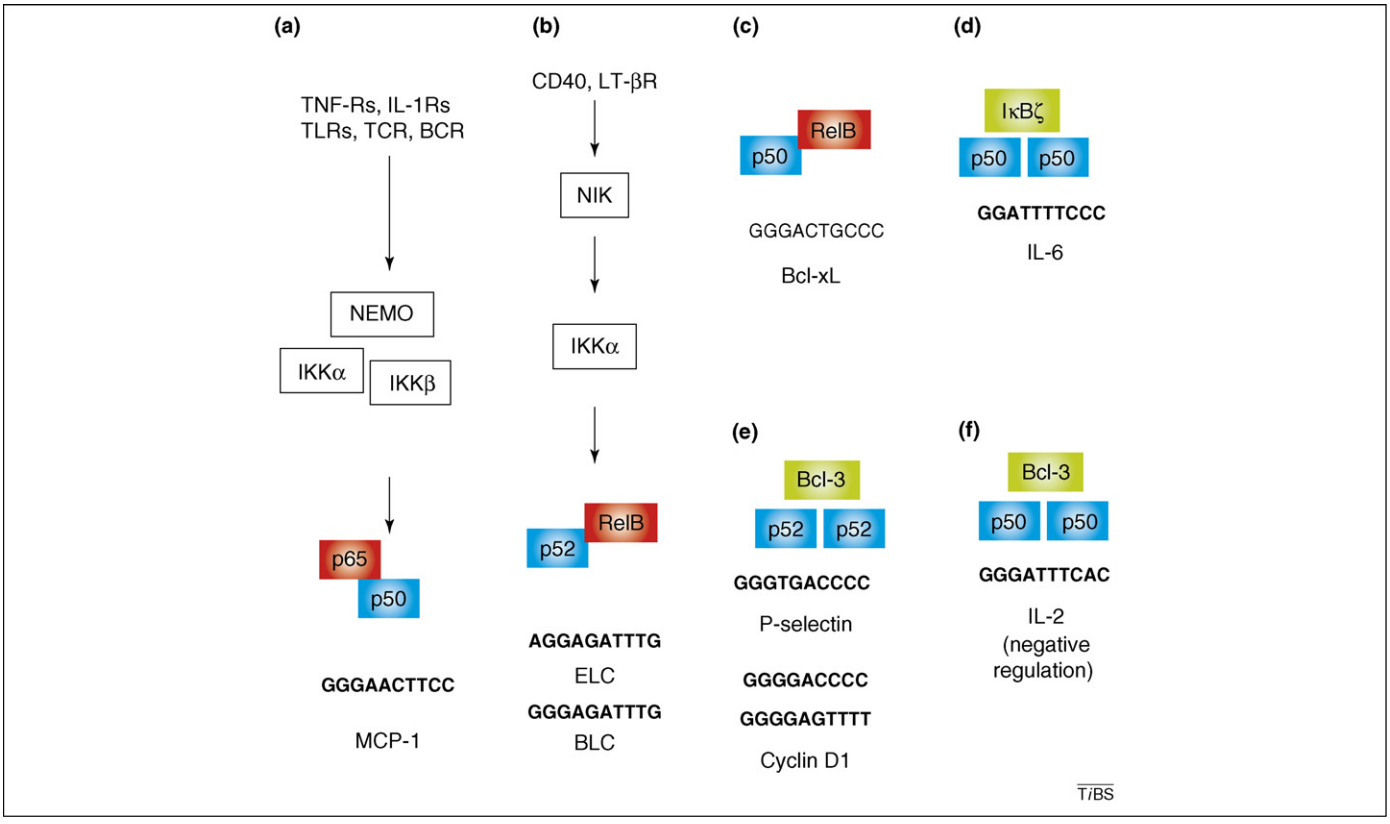

Figure 2. Dimers of the NF-κB family. Illustrated are the six NF-κB dimers discussed in this article. The κB-DNA motifs in the target gene promoters are shown. Members of the Rel subfamily are shown in red, and p50 and p52 are shown in blue. The unusual IκB proteins Bcl3and IκBζ are green. **(a)** The canonical activation pathway is activated by inflammatory stimuli such as TNF, IL-1 and TLR ligands, in addition to B-cell receptors (BCR) and T-cell receptors (TCR), all of which activate IKKβ and lead to activation of p50–p65. Many inflammatory genes are regulated by this complex. **(b)** The alternative pathway is activated by TNF-R family members such as CD40 and LT-βR in a signalling pathway involving NIK, which activates IKKα [9,65]. The activated NF-κB complex comprises RelB–p52, which regulates genes encoding proteins such as B-lymphocyte chemoattractant (BLC) and ELC, which are involved in lymph-node development and function [12–14]. **(c)** The p50–RelB dimer can induce transcription of the gene encoding Bcl-xL [35,36]. **(d)** In a complex with p50, IκBζ binds to a defined site and regulates genes such as that encoding IL-6. **(e)** p52 dimers in complex with Bcl3 will regulate genes such as that encoding cyclin D1 [31,32]. **(f)** Finally, p50 dimers in complex with Bcl3 negatively regulate IL-2 gene expression.

In vitro studies indicate that the p50–p65 and p52–RelB dimers activate different target sites, which could enable fine tuning of the cellular response to the stimulus [16]. IKKα might have an essential role in these dimer-shifting events. The kinase has recently been found to accelerate clearance of p65- and c-Rel-containing complexes from NF-κB-dependent promoters during resolution of the inflammatory response [17]. A shift in dimer activity has also been observed following extended lipopolysaccharide (LPS) and TNF-α stimulations [18]. Replacement of short-lived activated p65–p50 dimer with the IκB-insensitive RelB–p52 resulted in sustained activation of some target genes such as those encoding Epstein–Barr virus (EBV)-induced molecule 1 ligand chemokine (ELC) and macrophage-derived chemokine (MDC) in dendritic cells. Interestingly, expression of other genes such as that encoding IL-12p40 is downregulated by RelB–p52, indicating a promoter-specific function of this dimer. Dejardin [19] has proposed a 'hybrid pathway' that leads to activation of a p52–c-Rel or p52–p65 dimers. Activation of this pathway through TNF-R-related receptors triggers NIK- and IKKα-dependent processing of p100 to gain p52, which then dimerizes with p65 or c-Rel. This complex is IκB-bound and, therefore, has to be activated via the classical NF-κB activation pathway. Furthermore, we have recently found that p65 might be a transactivating partner for p52-dependent gene expression [20]. Dimer formation and binding to the κB-target sequence was observed upon sustained stimulation with TNF-α. Interestingly, the p52–p65 dimer associates with IKKε, which inducibly phosphorylates p65 in its TAD [20]. p52 also interferes with p65–p50-dependent gene expression: increased p52 levels in a human B-cell line have been observed to strongly repress p65-dependent TNF-α promoter transactivation, hinting at an inhibition of p65–p50 by p52 [21].

RelB and other NF-κB subunits

To add further complexity, RelB can also interact with p65 or p50. RelB can inactivate the canonical p65–p50 dimer [22]. This inhibitory effect is due to the formation of inactive RelB–p65 heterodimers, which do not bind DNA and are, hence, transcriptionally inactive. Here, RelB functions as a decoy partner for p65 and stochiometrically prevents active p65–p50 dimer formation. LPS-stimulated RelB-deficient fibroblasts show increased expression of several chemokines, which correlates with increased p65 DNA-binding in the absence of RelB, highlighting the physiological relevance of this inhibition [23]. Interestingly, RelB is inducibly degraded upon T-cell activation in a manner similar to IκB proteins, which could possibly control these RelB effects [24].

Sequestration of RelB in the RelB–p65 complex reciprocally prevents formation of the transcriptionally active RelB–p50 dimer (Figure 2c), causing repression of a subset of RelB-dependent genes. The formation of the

Box 1. Canonical and non-canonical NF-κB activation pathways

The canonical or classical NF-κB pathway leads to activation of the p65–p50 heterodimer (Figure I). Signalling events in this pathway occur rapidly upon receptor stimulation to induce transcription of immediate response genes. TNF-α, IL-1 and ligands to Toll-like receptors (TLRs), such as LPS and bacterial DNA, trigger this pathway through activation of IKKβ in the IKKα–IKKβ–NEMO complex. IKKβ phosphorylates IκBα, which forms an inactive complex with the p65–p50 heterodimer. This complex is predominantly found in the cytosol [66]. Although the complex can translocate into the nucleus, the inhibitory IκBα prevents its binding to the κB-target sequence and promotes nuclear export of the NF-κB dimer [67].

Once phosphorylated, IκBα is marked for ubiquitylation and proteasome-mediated proteolysis to release the p65–p50 heterodimer. Consecutive phosphorylation of the p65 transactivation domain renders the dimer fully active and recruitment of co-factors such as CBP or p300 to the activation complex at the kB-target site enable it to induce transcription. Regulated target genes of this pathway encode for crucial players in inflammation, such as IL-2.

The non-canonical or alternative pathway is induced by members of the TNF-α family such as LT-β, CD40L, BAFF, receptor activator of NF-κB ligand (RANKL), TNF-like weak inducer of apoptosis (TWEAK) or upon viral infection with EBV or human T-cell leukemia virus (HTLV). Activation is mediated by IKKα, which phosphorylates p100 to induce its processing to p52, releasing the p52–RelB heterodimer. Induced activation of this pathway is dependent on the upstream kinase NF-κB-inducing kinase (NIK), which links IKKα to its substrate. However, pathogenic activation of this pathway by HTLV occurs independently of NIK [65]. In contrast to the canonical pathway, the alternative RelB–p52 dimer mainly regulates lymphoid organogenesis and B-cell maturation. Constitutively elevated levels of p52 have been shown to be involved in B- and T-cell leukaemias.

Some stimuli such as CD40L and LT-βR can induce activation of both the classical and the alternative pathway, and crosstalk between these pathways is likely to occur.

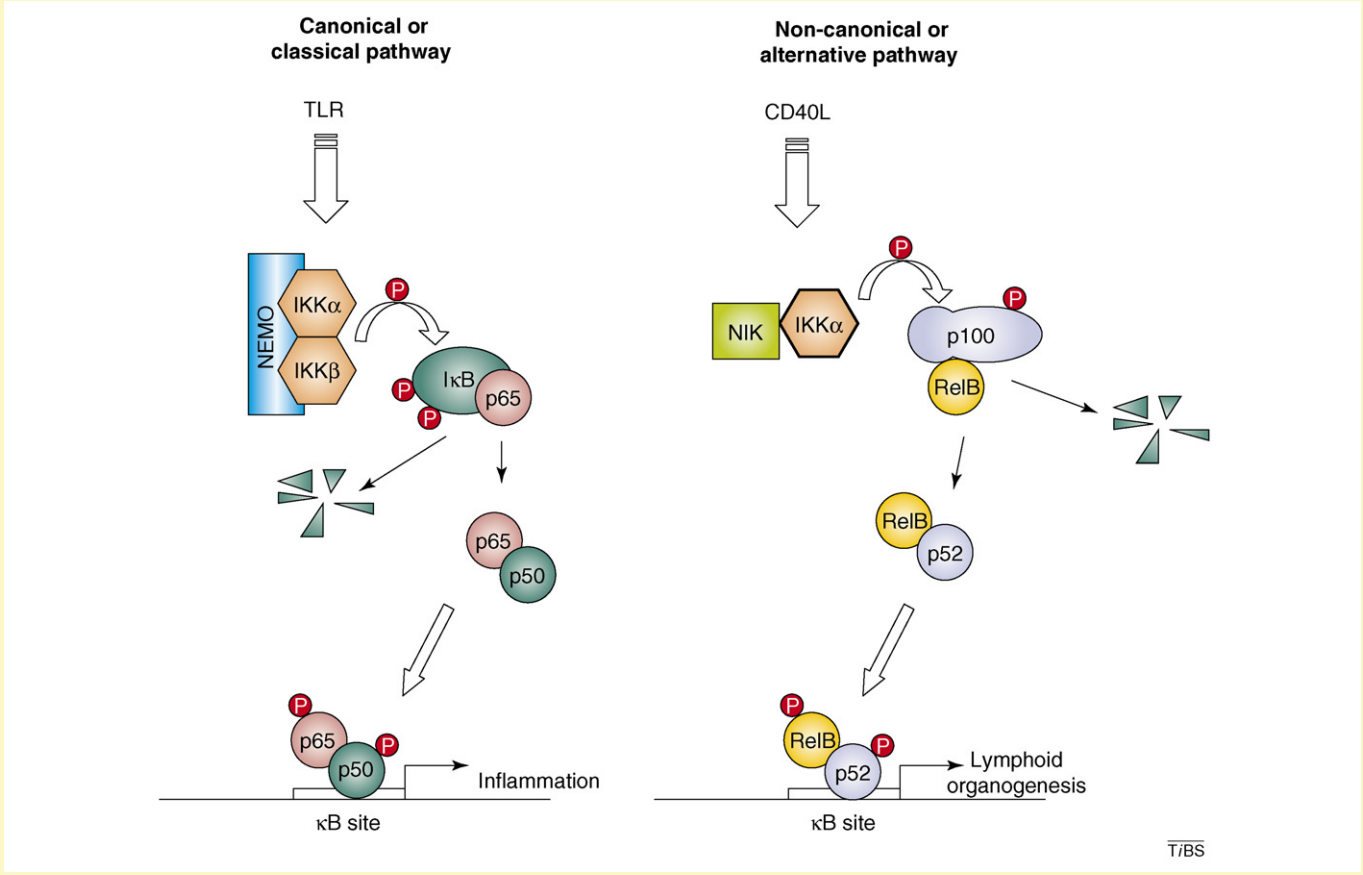

Figure I. Classical and alternative NF-κB activation pathways. The classical or canonical pathway is activated by TNF-α, IL-1 or TLRs, and results in activation of the p65–p50 heterodimer. Members of the TNF family stimulate activation of the RelB–p52 dimer via NIK and IKKα.

inhibitory heterodimer is crucially regulated through phosphorylation of Ser276 in the RHD of p65 [25].

Despite the fact that p50 and p52 specifically activate similar but non-identical κB target sequences, they also compensate for each other's loss [26]. p65–p52 and RelB–p50 dimers can replace p65–p50 and RelB–p52 dimers, respectively, in the absence of either p50 or p52 [14,27]. These findings indicate that κB target sites are not exclusive for one specific subunit but can recruit different activating NF-κB complexes.

Apart from the respective interactions of p50 and p52 with p65 and RelB, homodimeric complexes of p52–p52 and p50–p50 have also been observed. p50 and p52 lack a TAD domain and their homodimers can therefore function as transcriptional repressors [28].

c-Rel activates its target genes as a homodimer or as a heterodimer with either p50 or p65. Its activation, dimerization and function in B-cell development has been reviewed recently (see Ref. [29]).

IκBζ and Bcl-3

The final two dimers involving the NF-κB family to be examined in detail are somewhat different from the others in that they involve two IκBs, IκBζ and Bcl-3. These are

unusual IκB proteins because their elevated expression can either activate or suppress expression of a subset of NF-κB-dependent genes [30–32]. IκBζ production is considerably upregulated by ligands of the TLRs and by IL-1β [30]. Unlike other IκB proteins, IκBζ levels are controlled at the level of their mRNA stability and expression rather than by inducible protein degradation. The preferred NF-κB partner for IκBζ is p50, which lacks a TAD but can induce expression of inflammatory cytokines such as IL-6 and IL-12p40 in complex with IκBζ [30]. Phosphorylation of IκBζ has been observed *in vivo* and could contribute to transactivation of p50-dependent genes (Figure 2d).

IκBζ is also an inhibitor for p65-dependent gene expression through direct interaction with p65. Association with IκBζ prevents transactivation and DNA-binding of the transcription factor [33].

Bcl-3 is known as a proto-oncogene and a transactivating partner of p52 [31,32] (Figure 2e). Its oncogenic potential is controlled through phosphorylation by glycogen synthase kinase 3β (GSK3β), which leads to degradation of Bcl-3 through the proteasome pathway [34]. Interestingly, Bcl-3 can also interact with the p50 homodimer and either reinforce its repressive effect or cause dissociation of the repressive dimer from its binding site [35,36] (Figure 2f).

Taken together, these discoveries paint a complicated picture of the functioning of NF-κB dimers and reveal an elaborate subunit cross-regulation that we are only just beginning to understand.

Dimerization and regulation with other transcription factors and co-activators

The activation of NF-κB dimers alone is insufficient to induce gene expression, which requires recruitment by the active dimers of transcriptional co-activators such as CREB-binding protein (CBP) and p300. CBP and p300 are versatile co-activators that link a variety of different transcription factors in addition to NF-κB, including IRFs and STATs, to the transcriptional apparatus. The N- and C-terminal domains of these paralogue proteins interact with p65 in a region containing the TAD. This interaction is crucial for p65-driven gene expression with consequences for immune regulation [37]. An interferon-γ- and TNF-α- inducible trimeric complex of p65, CBP and STAT-1 controls transcription of the chemokine CXC ligand 9 (CXCL9) (Figure 3a). The binding sequences for the two transcription factors in the promoter region of this gene are essential for optimal gene expression as is direct interaction between the factors, which is promoted by CBP [38].

CBP is also likely to mediate the interaction of p65 with another transcription factor, IRF-3. Interaction between IRF-3 and p65 is essential for gene expression from the interferon-stimulated response element (ISRE) following TLR4 stimulation and for activation of the IP-10 promoter [4,39] (Figure 3b). Glucocorticoid receptors target and disrupt this interaction and, thus, block expression of a specific subset of inflammatory genes [40], although other genes are also subject to regulation by glucocorticoids that are independent of this mechanism.

A recent report demonstrates that the canonical p65–p50 dimer can also function as a repressor for gene expression. The heterodimer is basally bound to the promoters of a series of anti-viral and immune-modulatory genes and dampens their full activation via interactions with STAT1/2 and IRF-1 [41] (Figure 3c).

A final interaction recently reported is p52 with the tumour suppressor protein p53. p52 can, in fact, be recruited by p53 to multiple p53-regulated promoters independently of the DNA-binding activity of p52. An important functional consequence of this is inhibition of cyclin-dependent kinase inhibitor p21 expression. The p52–p53 complex can, however, upregulate other genes such as those encoding p53-upregulated modifier of apoptosis (PUMA) and death receptor 5 (DR5) [42].

In addition, NF-κB factors have been reported to interact with other transcription factors such as CREB, CCAATT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1) and Specificity protein 1 (SP-1). These findings provide plenty of evidence for intense regulatory cross-talk of different signalling pathways [43–45].

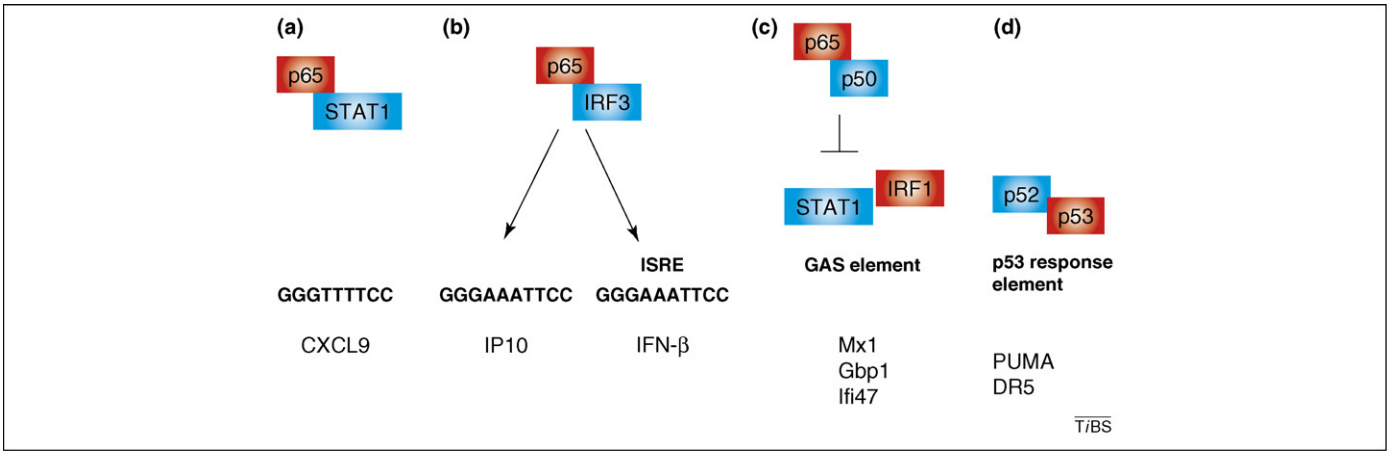

Figure 3. NF-κB family members and other transcription factors. NF-κB family members interact with and modulate other transcription factors. A p65–STAT1 complex regulates the expression of the gene encoding the chemokine CXCL9 [38]. A p65–IRF3 complex regulates several genes including that encoding another chemokine IP10 via a particular NF-κB motif that differs in one base pair from the canonical NF-κB motif that binds p50–p65 [4]. It can also bind to ISRE and regulate genes such as that encoding interferon-β [39]. This complex is inhibited by the glucocorticoid receptor [40]. The p65–p50 dimer can interact with and displace the STAT1–IRF1 dimer on the promoters of genes encoding such proteins as Mx1 [41]. Finally, a p52–p53 complex can regulate the expression of genes encoding proteins such as PUMA and DR5 [42]. For clarity, interactions with monomeric NF-κB subunits is shown, although it is likely that dimeric complexes are involved.

Regulation of NF-κB subunits by phosphorylation

Recently, new insights have emerged into the regulation of NF-κB dimers by phosphorylation, which provides additional understanding of specificity in the NF-κB system. Activity of the NF-κB subunits p50 and p52 is controlled through phosphorylation and processing of their ankyrin-motif-containing precursor proteins p105 and p100. p105 is phosphorylated on the C-terminal Ser927 and Ser932 by the IKK complex, which leads to its ubiquitylation and processing to gain NF-κB p50 [46]. In unstimulated cells, GSK3β counteracts this processing event by stabilizing the phosphorylation of p105, which prevents its degradation [47] (Figure 1; Table 1).

Processing of p100 to p52 occurs constitutively as a co-translational event and is also induced by stimulation with CD40L, LT-β and B-cell-activating factor (BAFF). In both cases, direct phosphorylation by IKKα is required to trigger degradation of the inhibitory C terminus. Inducible processing is controlled by NIK, which activates IKKα and, importantly, links IKKα to its substrate p100 [10].

Transactivating phosphorylation of the NF-κB factors p65, RelB and c-Rel is essential to activate transcription of NF-κB-dependent genes through the initiation complex. The constitutive basal phosphorylation of p65 in unstimulated cells is not sufficient to drive this complex. Upon stimulation of the canonical pathway, a variety of serine residues in both RHD and TAD are phosphorylated through different activated kinases. For example, Ser276 is targeted by protein kinase A (PKA) in response to LPS and mitogen-stress-activated protein kinase-1 (MSK-1) following TNF-α stimulation. Phosphorylation of this residue enhances recruitment of co-factors CBP and p300 [48,49]. Another residue in the RHD, Ser311, is phosphorylated by PKCζ and similarly increases the transactivation potential of p65 through enhanced co-factor binding [50]. The TAD of p65 harbours several additional serine residues that have been demonstrated to be relevant for gene transactivation, most importantly Ser536. Several different kinases have been identified to target this residue, including IKKβ, IKKα, IKKε and TBK-1 [51] (Figure 1; Table 1).

Recent findings demonstrate that IKKε phosphorylates Ser536 in p65 upon ligand binding to IL-1R [51]. In addition, IKKε phosphorylates Ser468 in the TAD upon T-cell-receptor stimulation [52]. We have recently found that IKKε also phosphorylates p65 in its TAD following sustained stimulation with TNF-α, leading to enhanced transactivation of p52-dependent gene expression [20]. Our results indicate that TNF-α stimulates the early activation of the canonical p65–p50 dimer, which is followed by formation of a late p65–p52 DNA-binding complex regulated by IKK- ε [20] (Figure 4).

Two regulatory serine residues have been identified in RelB. Phosphorylation of Ser368 induces dimerization of RelB with p100 [53], whereas phosphorylation of Ser552 promotes degradation of RelB through the proteasome pathway [22]. To date, the understanding of how RelB is regulated in the alternative activation pathway is limited and activating kinases for RelB have yet to be identified.

c-Rel activity is mainly regulated through phosphorylation of Ser471 in the TAD of this protein [54]. It remains to be clarified whether PKA-Cβ, which promotes c-Rel-dependent transactivation, targets this crucial residue [55]. In addition, NIK has recently been shown to phosphorylate c-Rel in its TAD and promote transactivation of c-Rel-dependent genes such as IL-2 [56]. The main function of c-Rel is regulation of B- and T-cell development, and overexpression of this transcriptional activator leads to lymphoid cell malignancies [29,57].

Recognition of κB-binding sites by protein complexes

Given the diversity in the composition of NF-κB dimers and interactions between NF-κB and other transcription factors, a key question concerns the nature of the DNA motifs that these complexes recognize. Information here remains limited but recent studies are providing new insights.

NF-κB dimers bind to a remarkably varied 9-bp consensus sequence, GGRNNYYCC. It is tempting to assume that this heterogeneity of κB-target sites confers specificity for gene regulation through different affinities to NF-κB dimer isoforms. Several attempts have been undertaken to decipher a specific code in the κB-sequence for dimer binding, but the universal key to this code remains obscure. A few systematic approaches have been undertaken to solve this problem. An *in vitro* study using purified recombinant RHD proteins showed that the dimers display differential preferences for DNA-binding sequences [58].

Table 1. Phosphorylation of NF-κB subunits

NF-κB subunits	Kinase[a]	Phospho-acceptors	Functional consequence	Refs
p65	PKA and MSK-1	Ser276	DNA binding	[49,68]
	PKCζ	Ser311	Transactivation	[50]
	IKKα,β,ε and TBK1	Ser536	Transactivation	[51]
	CKII	Ser529	Transactivation	[69]
	IKKε	Ser468	Transactivation	[52]
RelB	?	Ser538	Dimerization with p100	[53]
	?	Ser532	Processing	[24]
cRel	?	Ser471	Transactivation	[54]
p50	PKA	Ser337	DNA binding	[70]
p105	IKK complex	Ser927 and Ser932	Processing to p50	[46]
	GSK3β	Ser903 and Ser907	Stabilization	[47]
p100	IKKα	Ser99, Ser108, Ser115, Ser123, Ser866, Ser870 and S872	Processing to p52	[10]

[a]Question marks indicate that the kinase is unknown.

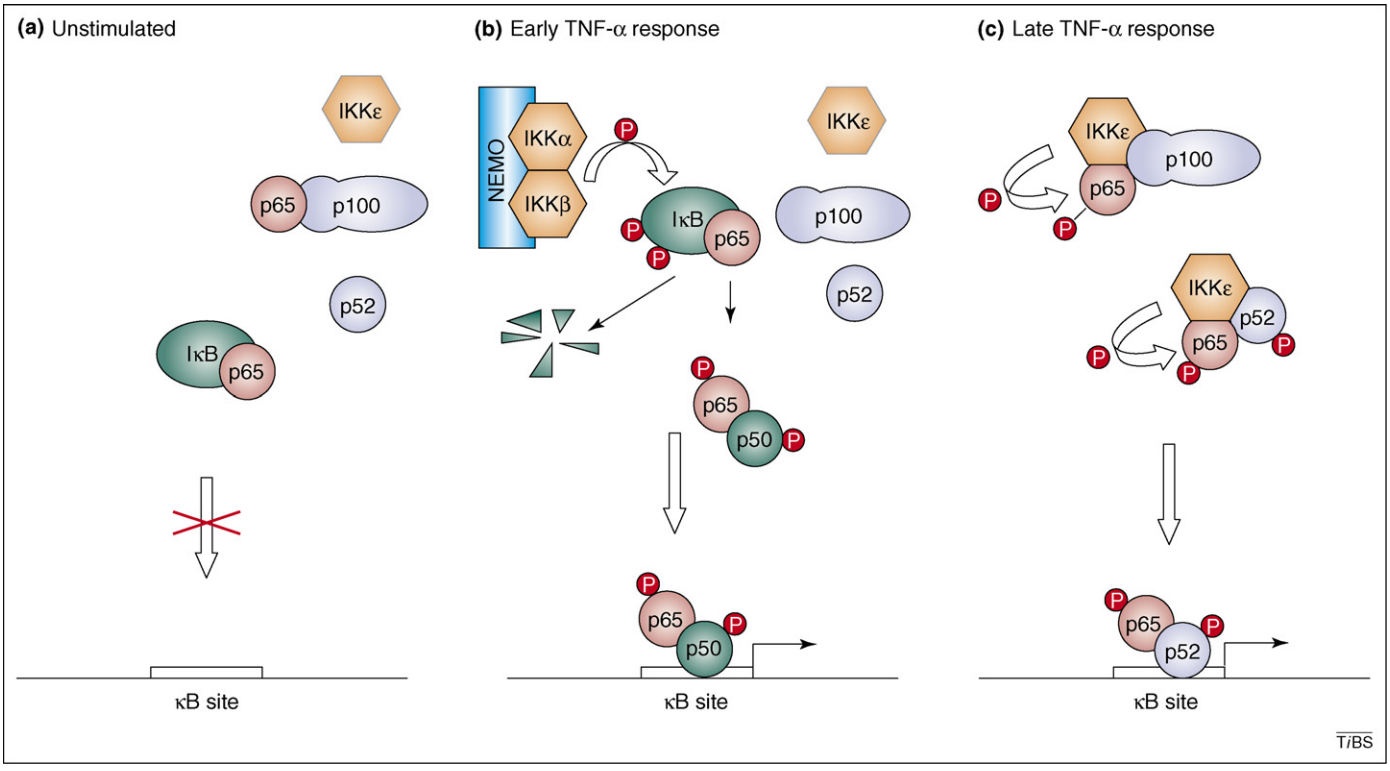

Figure 4. IKKα,β and IKKε in the early and late activation of NF-κB complexes by TNF. Activation of NF-κB following TNF-α stimulation probably occurs in two phases, as depicted in this model. In the unstimulated cell, p65 is bound in an inactive complex by IκB **(a)**. At early time points, p50–p65 is activated via the canonical pathway involving IKK-α–β kinase complex and proteasomal degradation of phosphorylated and ubiquitinated IκB, which results in the release of the p65–p50 dimer **(b)**. Extended stimulation with TNF-α, however, gives rise to a p52–p65-containing DNA-binding complex. IKK-ε interacts with this dimer and inducibly phosphorylates p65 in this complex [20] **(c)**. Although IKK-ε is not involved in processing of p100 to p52, in this pathway, the kinase might be involved in transactivation of this late p65–p52 complex. The specific genes targeted by this complex are not known and the biological relevance of these events has yet to be evaluated.

Another reporter-based assay demonstrated that p50 and p65 hetero- and homo-dimers have different κB-sequence-specific effects on gene expression [59]. DNA affinities of p65 and p52 have been shown to be affected by minor changes in the κB-target sequence [60]. Moreover, a comparison between the canonical p65–p50 heterodimer and the alternative RelB–p52 dimer has demonstrated that two complexes bind to different variations of the κB-consensus-binding site [61]. But minor changes in the κB-binding sequence can also alter co-factor binding to the NF-κB dimer, as recently shown with the IP-10 promoter [4]. In this case, the p65–IRF3 dimer binds 5′-GGGAAATTCC-3′, as occurs in the inflammatory protein 10 (IP-10) promoter, whereas p50–p65 binds to the canonical motif 5′-GGGAACTTCC-3′ in the MCP-1 promoter. Such subtle differences might also be evident for other dimers.

Structures of p65–p50 heterodimers obtained by X-ray crystallography have revealed that the dimer can bind to a variety of κB sequences, indicating a remarkable permissiveness of NF-κB–DNA interactions. However, changes in the target sequence can cause structural and functional changes of the binding NF-κB dimer [62]. In addition, a genetic analysis of NF-κB dimer specificity has revealed little correlation between the κB-target site and the RHD dimer [27]. In fact, the promoter context of the κB-target sequence and protein interactions in the enhanceosome seem to be at least equally defining for the nature of the dimer as the specific binding site. We mentioned earlier that extended exposure to certain stimuli can cause a shift in NF-κB subunits bound to their targeted promoter regions [18]. Whether this isomeric dimer shift occurs at the same κB- target site after disassembly of the initial enhanceosome or whether the delayed NF-κB dimer binds to adjacent κB-sites in the promoter region remains to be clarified.

NF-κB-dependent genes usually have more than one κB site in their promoter regions. This indicates that gene expression is either under the control of different dimer isoforms or that a stimulus- or time-dependent change in the enhanceosome composition alters the affinity of NF-κB to its target site.

The affinity of DNA–protein interaction is defined by the number of ionic interactions, which, in turn, depends on site sequence and the structure of the dimer. Interestingly, the structure of the NF-κB dimer is influenced by its κB-target sequence [62,63]. Structural changes provoked by transactivating phosphorylation and DNA binding have further implications for interactions with other components in the activation complex. It has also been demonstrated that κB-sequence-specific structural alterations of the NF-κB dimer correlate with functional differences in HIV transcription [64]. However, these DNA-binding-induced structural changes do not affect dimerization properties. The structure of the dimerization domains and dimer interface of the p65–p50 heterodimer does not alter substantially upon binding to the κB- target sequence [63].

Taken together, these findings indicate that the overall structure of the promoter region and the protein interactions within this region are more defining for NF-κB

specificity than the actual specific κB-binding sequence. The sequence does, however, affect the structural features of the binding dimer with consequences for interactions with other transcription factors and co-activators.

Concluding remarks and future perspectives

Coordinated and timely control of transcription-factor activities is crucial to cell survival and development. Both deletion and hyperactivity of NF-κB have severe consequences for systemic responses. Protein–protein interactions of the NF-κB subunits with other isoforms, co-activators and other transcriptional factors are the key to NF-κB specificity. These interactions are controlled via different signalling pathways through activation and phosphorylation of components of transactivation complexes. The interactions of these components in enhanceosomes provide specific control over target gene expression. We can, therefore, conclude that the overall nature of the enhanceosome is influenced by three main factors:

(i) The architecture or context of the promoter region with its specific binding sites for transcriptional regulators defines which components can actually bind in the regulatory region of the cognate gene. As a result, the same NF-κB dimer can have different functions in different promoters. Although minor changes in the κB-target sequence can determine the composition of the binding complex, it has also become clear that these sequences are usually permissive for different NF-κB dimers.

(ii) External stimuli activate specific signalling pathways to mobilize transcriptional regulators, which can then either bind to their target sequences or interact with other regulators to modify their activity. We propose that this cross-regulation is a major mechanism for NF-κB-dependent gene expression.

(iii) The cell type and overall state of the cell provide a specific background setting in which these signalling events take place. Not all regulatory pathways are active in different cell types, resulting in cell-type-specific alteration of expression patterns. The same is true for a particular cell that expresses a different set of proteins according to its state, that is, primed cells or mature cells differ from immature, untreated cells.

The NF-κB system continues to reveal important new insights into the regulation of gene expression in health and disease, which will hopefully enable the rational design of novel therapeutics to use in the treatment of diseases in which the NF-κB system is dysregulated.

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