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NF-κB signalling: Embracing complexity to achieve translation

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NF-κB is a dimeric transcription factor that has emerged as an important regulator of liver homeostasis and is mechanistically implicated in a variety of liver pathologies including hepatitis, steatosis, fibrosis, and hepatocellular carcinoma. The question remains as to whether NF-κB can really be exploited for the development of therapeutics for these pathologies in the diseased human liver. This review casts a critical eye on the experimental evidence gathered to date and in particular questions the rationale for the current focus on components of the upstream IKK complex as therapeutic targets. We make the argument that translation of NF-κB biology to new therapies is more likely to emerge from a re-focus of basic research back to the NF-κB/Rel subunit functions and the complexities of their post-translational modifications and context-dependent co-regulator interactions.

Introduction

The past decade has seen an explosion of new experimental data that suggest important functions for the NF-κB signalling system in liver physiology and disease. Hepatic NF-κB is implicated in at least three normal homeostatic processes: (1) clearance of microbial pathogens, (2) protection of hepatocytes from TNFα-induced cell death and (3) compensatory proliferation of hepatocytes in response to loss of hepatic mass through liver injury. These "healthy" functions of NF-κB contrast with a large and growing body of work suggesting a variety of "unhealthy" activities that span a wide spectrum of pathologies found in chronic liver disease [1]. These include steatosis, insulin resistance, hepatitis, biliary disease, fibrosis, and hepatocellular carcinoma [2–10]. Not surprisingly there is considerable interest for the prospect of targeting NF-κB in liver disease, especially as there are already a vast number of pharmacological and biological inhibitors available (Table 1) [11–25]. However, there are serious caveats to the published experimental work on NF-κB and liver disease that urge caution and possibly even a return to the drawing board. Firstly, studies that describe NF-κB activities and functions in human liver disease are rare and have been hampered by lack of appropriate investigative technologies. Second, the most well cited and popular experimental work is with mouse models of liver disease utilising knockout technology to disarm the IKK complex which is certainly a crucial activator of NF-κB but which has a variety of non-NF-κB targets that can also impact on liver pathology. Furthermore, these mouse models largely rely on the Cre/lox targeting technology which has implications for interpretation and physiological relevance of data. Third, the "healthy" functions of NF-κB are at considerable risk if we were to use currently available inhibitors. While there is literature claiming that mice lacking key components of the hepatic NF-κB system thrive perfectly well [6,9,10,26], one must be alert to the fact that these mice live in a highly controlled environment with strict dietary regimens and protection from environmental pathogens and toxins that would be near impossible to achieve for patients. This review aims to critically reassess where we currently are with respect to translation of NF-κB to therapies for prevention or treatment of liver disease and cancer.

A brief overview of the NF-κB signalling system

Numerous recent reviews provide highly detailed descriptions of the NF-κB system and the signalling pathways that lead to its activation in response to environmental cues [27,28]. As such we will only provide a brief description here but will highlight the complexities that may eventually point the way towards opportunities for therapeutic targeting. NF-κB functions as a dimeric DNA binding complex generated from interactions between the protein products of 5 structurally related members of the Rel gene family, namely relA (or p65), c-rel, relB, nfkb1 (which encodes for the p50 subunit and its precursor p105) and nfkb2 (encoding for p52 and its p100 precursor). Most of what we have learned about NF-κB in the liver has come from studies focused on the so-called canonical activation pathway which results in stimulation of inflammatory gene transcription by the RelA:p50 heterodimer. This dimer cycles between the cytoplasm and the nucleus complexed with an inhibitory protein IκBα which reduces the efficiency of nuclear transport and prevents DNA binding of RelA:p50. Upon interaction with their cell surface receptors, extracellular stimuli such as bacterial LPS and TNFα

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Abbreviations: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IKK, IkappaB kinase; CBP, CREB binding protein; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted.

Table 1. List of inhibitors targeting NF-κB in correlation with liver diseases.

Names	Pharmacological origin	Mode of action
Sulfasalazine	Derivative of mesalazine (5-aminosalicylic acid)	IKKα and IKKβ inhibitor [11]
Gliotoxin	*Aspergillus fumigatus*	Prevent IκBα degradation [12]
Curcumin	Polyphenol from *Curcuma longa*	Induce oxidative stress and inhibits NF-κB activation [13]
(−)-Epigallocatechin-3-gallate	Antioxidant present in *Camellia sinensis*	Abrogates p300-induced p65 acetylation [14]
Resveratrol	Phytoalexin from *Polygonum cuspidatum*	Prevent nuclear translocation of NF-κB [15]
Silymarin	Flavonoid isolated from *Silybum marianum*	Inhibits NF-κB activation by inhibiting upstream kinases [16]
Captopril	Angiotensin-converting enzyme (ACE) inhibitor	Inhibits NF-κB activation [17]
Pyrrolidine dithiocarbamate	Synthetic	Inhibits IκB release [18]
Over-expression of IκBα	Synthetic I-κBα superrepressor	Mutation at 32nd and 36th amino acid,prevent phosphorylated degradation of IκB [19]
Thalidomide	Synthetic derivative of Glutamic acid	Inhibit degradation of IκB [20]
Proteasome inhibitor (a) Bortezomib (b) NPI-0052	Synthetic molecule	Inhibits IκB degradation [21]
Cell-permeable peptides	Synthetic peptides	Block the association of IKKγ/NEMO with IKKβ and thereby inhibit NF-κB activation [22]
(a) Glucocorticoids (e.g. dexamethasone; prednisolone) (b) Selective estrogen receptor modulator	Corticosteroids	Glucocorticoid when binds to its receptor it can interact with NF-κB transcription factors, forming transexpression complexes or induces IκBa expression and retain NF-κB in the cytoplasm [23]
Decoy oligonucleotides	Synthetic double stranded oligonucleotides	Contains the consensus sequence and thus inhibits NF-κB binding to its promoter region [24]
Small interfering ribonucleic acid	Double stranded RNA molecule	Inhibit NF-κB protein synthesis [25]

activate RelA:p50 via a complex series of signalling events that are channelled through the IKK complex. The IKK complex is comprised of three major components known as IKK1 (or IKKα), IKK2 (or IKKβ) and NEMO (or IKKγ). The general consensus is that the catalytic component IKK2 and the scaffold component NEMO transduce signals to the canonical pathway. IKK2 catalyses phosphorylation of N-terminal serine residues of IκBα which leads to polyubiquitination and degradation by the 26S proteasome to generate "free" RelA:p50. Loss of IKK2 or NEMO results in diminished activation of RelA:p50 and reduced expression of pro-inflammatory cytokines, this explains the intense focus on these molecules in experimental mouse studies [2,10]. However, because NF-κB is a regulator of so many other important physiological processes (immunity; cell differentiation, growth and life-span; metabolism etc.), it is critical that additional regulatory checkpoints help control its activity. For example, for RelA:p50 to be fully transcriptionally active the RelA subunit undergoes a series of post-translational modifications including phosphorylation and acetylation [29]. Details of the kinases, phosphatases, acetyltransferases and deacteylases that control these modifications of RelA are emerging but are not well understood. At many genes RelA:p50 competes for its DNA target motif (5′-GGGPuNNNPyPyCC-3′) with the p50:p50 homodimer which lacks transcriptional activity and when in association with the histone deacteylase HDAC1 can actively repress transcription [30]. How p50 and RelA interactions are regulated is poorly defined, as are the molecular events that control competition for DNA binding between RelA:p50 and p50:p50. Discovering the answers to these questions may enable selective experimental control over the type of NF-κB that is recruited to inflammatory, fibrogenic and tumour-regulating genes.

The IKK complex and liver disease

Initial efforts to investigate the canonical NF-κB pathway in the mouse liver were problematic since non-conditional knockout of RelA, IKK2 or NEMO resulted in massive and fatal TNF-mediated death of hepatocytes during foetal development [31–34]. This led to the use of Cre/lox technology to target knockout of the canonical pathway to specific liver cell types. Unfortunately the data that has emerged from different laboratories that have used this technology to target IKK/NEMO has generated unclear and often contradictory conclusions [35]. This confusion may be explained by different environmental conditions in the investigator laboratories and/or may also directly relate to the Cre/lox technology itself. As described in more detail elsewhere, Cre is associated with cellular toxicity including induction of DNA damage and growth arrest which unfortunately were often not appropriately controlled for in many high profile studies. If we ignore these caveats, then the current popular opinion is that targeted knockout of IKK2 in hepatocytes removes a cytoprotective property of RelA:p50 which prevents excessive cell death in response to toxic damage of the liver. When the toxin is also a carcinogen such as diethylnitrosamine the high degree of cell death occurring in the absence of IKK2 leads to compensatory proliferation of hepatocytes including transformed cells [10]. As a result, mice lacking hepatocyte IKK2 develop liver cancer more rapidly than wild type mice. By contrast, if IKK2 knockout is targeted to the myeloid cell lineage (including macrophages) then the expression of hepatomitogens by these cells is blunted and the outcome is attenuated development of liver cancer [10]. Targeted knockout of NEMO to parenchymal liver cells using an Alfp-Cre system was reported to result in spontaneous steatohepatitis and fibrosis

followed by development of a severe liver cancer phenotype [2]. Why targeting of NEMO should generate this phenotype when the same group showed that similar targeting of IKK2 did not is unclear although the authors suggested that NEMO knockout generates more complete loss of NF-κB activity. However, this suggestion is at odds with a later report which concluded that although NEMO-deficient hepatocytes are more sensitive to TNF-induced death, no mice actually died from *in vivo* challenge with TNF [5].

Perhaps more worrying, a different group of investigators targeted RelA knockout to hepatocytes and showed that although all animals died upon challenge with TNF, they did not report any spontaneous development of liver pathology [6]. It is therefore difficult to be sure that defective NF-κB is the only explanation for the liver pathology and cancer observed in mice lacking NEMO expression in hepatocytes. Indeed, we should in general exercise caution with the assumption that liver disease phenotypes obtained with IKK/NEMO-deleted mice can be directly attributed to functions of NF-κB. Why should this be so? Firstly, the IKK1, IKK2 and NEMO have multiple targets outside of the NF-κB system (e.g. p53, TSC1 and FOXO3a) which if defective in the knockout mice would be expected to at least contribute to the observed phenotypes [36]. In addition, these mice will still have the capability of generating NF-κB through alternate pathway, particularly in cancer cells where such signalling pathways may evolve during transformation if they are advantageous to cell growth. As an example, the signal transducer and activator of transcription 3 (STAT3), a major conduit for IL-6 signalling has recently been reported to stimulate RelA-dependent transcription in human and rodent tumours via an IKK/NEMO-independent route involving stimulation of acetylation of the NF-κB subunit which reduces its affinity for IκBα [37].

Many of the major pharmaceutical companies have now invested heavily in the development of increasingly specific and efficacious IKK inhibitors including design of IKK1- and IKK2-selective drugs. Pre-clinical studies have demonstrated the ability of such drugs to be of potential benefit in the treatment of steatosis, hepatitis and fibrosis in rodents [8,38]. However, the potential for side-effects of long-term IKK inhibitor therapy has not been addressed in these models or in experimental models of liver cancer. In this context, the discovery of an increasing number of targets for the IKKs, both inside and outside the NF-κB system, raises the issue of safety. A particular concern is the report that IKK2 is required for normal mitosis and bipolar spindle formation, and the discovery of increased evidence for micro-nuclei in IKK-deficient cells which is a signature for chromosomal instability and potential for transformation [39]. If IKK inhibitors have a similar impact on mitosis and chromosomal stability then there would be significant danger for their long-term therapeutic application in liver disease.

Turning the complexity of the NF-κB system to our advantage

Concurrent with our increased understanding of the IKK complex is the realisation of the vast complexity of NF-κB signalling and the additional regulatory checkpoints that operate at the level of the Rel subunits, including context-dependent events that may be exploited to suppress specific types of NF-κB activities within specific cell types in a diseased tissue.

(i) Subunit interactions and specific functions. We currently have no understanding of how Rel subunits select their dimeric partners and yet this is important particularly for control of inflammatory gene expression where the switch from RelA:p50 to p50:p50 dimers is important for resolution of inflammation. Mice lacking the *nfkb1* gene which encodes p50 develop normally but develop severe hepatitis and fibrosis upon chronic liver injury, a phenotype associated with over-expression of TNFα [40]. Experimental approaches to tipping the balance of NF-κB dimers in favour of p50:p50 may encourage resolution of hepatitis in chronic disease, but this requires some understanding of the post-translational events that regulate subunit selection during dimer formation which is currently lacking. There is also a need to carefully explore the activities of the other Rel subunits in the healthy and diseased liver, in particular c-Rel, RelB and p52 which to date have been ignored but have been implicated in the pathology of a variety of human cancers including hepatocellular carcinoma [41–43]. As we have recently demonstrated with *c-rel* knockout mice which display attenuated inflammation, fibrosis, and hepatocyte proliferation, the individual Rel factors have non-redundant physiological functions in the liver that may only be revealed when these otherwise healthy animals are challenged [44]. Ruddell et al. recently reported pro-fibrogenic properties for the lymphotoxin β receptor (LTβR) expressed on fibrogenic hepatic stellate cells [45], and sustained activation of this receptor has recently been implicated in the development of viral-induced hepatitis and cancer [46]. The LTβR is known to activate non-canonical RelB:p52, as well as canonical NF-κB dimers implicating RelB and p52 in fibrogenesis and oncogenesis of the liver. However, direct investigations on the hepatic functions of p52 and RelB are currently lacking. These factors provide a form of NF-κB that is distinct from the product of the canonical pathway and may offer solutions to the conundrum of how to target the NF-κB system without removing its hepato-protective properties which are principally regulated by RelA-containing dimers.

(ii) Modulation of subunit activities by post-translational modification and recruitment of co-regulators. Each Rel subunit is subject to a variety of post-translational modifications including phosphorylation, acetylation, ubiquitination, sumoylation and nitrosylation [29]. These modifications vary according to the cell type and stimulus and can have profound influence over NF-κB activity. Phosphorylation of RelA has received the most attention. At least 8 phosphorylation sites have been identified on RelA and of these the evolutionary conserved Ser-276 and Ser-536 residues have emerged as important functional biomarkers of canonical NF-κB activity. Some data on the regulation and functions of RelA phosphorylation in liver physiology and disease are starting to emerge but this remains an under-explored area of research. Ser-276 lies within the Rel homology domain and is phosphorylated by the mitogen- and stress-activated protein kinase (MSK1) and protein kinase A (PKA) and is required for recruitment of the transcriptional co-activator CREB binding protein (CBP)/p300 and displacement of histone deacetylase 1 (HDAC1) which functions as a transcriptional repressor [46–48]. Ser-276 phosphorylation is observed in response to many stimuli including LPS and TNF and is a good surrogate marker for active NF-κB in human tissues and cancers that we should exploit for improved analysis of NF-κB activation status in diseased human liver [49,50]. Knock-in mice in which Ser-276 is switched to an Alanine residue are embryonic lethal and display several severe development defects

probably resulting from loss of important long-range epigenetic influences of DNA-bound NF-κB:HDAC1 complexes [51]. Ser-276 may therefore not be an ideal therapeutic target. Ser-536 is present in the transactivation 1 domain of RelA and is phosphorylated by a variety of kinases including IKK1, IKK2, IKKi (also known as IKKε) and p90 ribosomal S6 kinase (RSK) [29]. Ser-536 appears to regulate multiple functions of RelA including its cytoplasmic/nuclear transport, strength of transcriptional activity and its rate of degradation [52–54]. Intriguingly depending on which kinase stimulates Ser-536 phosphorylation there may be a distinct functional outcome. For example, IKK2-stimulated phosphorylation of Ser-536 enhances NF-κB activity [55], whereas at least in macrophages, IKK1-mediated phosphorylation of Ser-536 promotes degradation of RelA and termination of NF-κB-dependent transcription [54]. Ser-536 phosphorylation may be an important biomarker for active NF-κB and fibrogenic signalling in myofibroblasts derived from activation of hepatic stellate cells. Immunohistochemical staining for phospho-Ser-536 in diseased liver reveals close association with myofibroblasts and fibrotic tissue which correlates with reports that these cells express high levels of constitutive NF-κB required for prevention of apoptosis [56]. An autocrine pathway in which Angiotensin II stimulates IKK2-dependant Ser-536 phosphorylation drives this activity and can be targeted with angiotensin-converting enzyme (ACE) inhibitors, Angiotensin 1 receptor blockers or IKK inhibitors. Moreover, high levels of constitutive phospho-Ser-536 in fibrotic tissue may provide a predictive marker for fibrosis that is amenable to effective treatment with inhibitors of the rennin-angiotensin pathway [56]. Phosphorylation of Ser-536 can also be stimulated by ligation of CD40 receptors on hepatic stellate cells suggesting that this modification may also be responsive to interaction of fibrogenic liver cells with immune effector cells [57]. Ser-536 phosphorylation by IKKi/IKKε has been implicated in generating high constitutive NF-κB activity in a variety of cancer cells [58], this has not yet been investigated in hepatocellular carcinoma but further supports development of Ser-536 as a therapeutic target. Cell-permeable peptides carrying sequences spanning either Ser-276 or Ser-536 have been used to inhibit NF-κB *in vitro* [59]. The Ser-536 inhibitory peptide is able to stimulate apoptosis of hepatic stellate cells and has anti-fibrotic activity *in vivo* [56]. Such peptides may be used as the basis for design of drugs that specifically block Ser-276- or Ser-536-dependent NF-κB activities and provide more selective inhibitors than those targeting the IKKs. There is equal potential to be gained by investigating functions of other Rel subunit modifications in all liver pathologies. These types of studies will be possible as antibody reagents improve and knock-in mice are generated.

As already alluded to above, the recruitment of co-activators such as CBP/p300 or co-repressors like HDAC1 is important in dictating the nature of the transcriptional response to NF-κB DNA binding. An interesting case in point is the p50:p50 homodimer. Due to its absence of a transactivation domain this form of NF-κB has no intrinsic ability to influence gene transcription; however, its ability to recruit a variety of co-regulators enables it to operate either as a transcriptional activator or repressor in a gene-context-dependent manner [8,30,60–62]. At the IL-6 and TNFα gene promoters, p50:p50 dimers recruit HDAC1 and this repressive complex prevents inappropriate expression of the powerful pro-inflammatory cytokines and may be important in repressing these genes during the resolution phase of inflammatory responses [8,30]. The IκB-like protein Bcl3 forms inhibitory complexes with p50:p50 in LPS-stimulated macrophages to ensure down-regulation of TNFα and IL-1β in the resolving phase of the LPS response. As a consequence of loss of this mechanism, Bcl3-deficient mice lack tolerance to LPS challenge [63]. By contrast p50:p50 dimers are transcriptionally active at the anti-inflammatory IL-10 gene due to recruitment of histone acetyltransferase CBP [61]. The basis for gene-context dependant recruitment of co-regulators to Rel subunits is not fully understood although Leung et al. have demonstrated that the sequence of the κB DNA binding motif within a gene promoter can determine selective co-regulator recruitment presumably by altering the conformation of the NF-κB proteins to enable specific recognition by co-regulators [64]. Synthetic so-called "decoy" κB oligonucleotides that compete for NF-κB binding with genomic κB sites have been successfully employed *in vivo* to inhibit hepatic NF-κB [24,65]. This technology may be further developed to sequester specific types of NF-κB:co-regulator complexes that promote pathological states. As there is remarkable sequence conservation in κB sites between mouse and human genes [64], this idea can be first tested in mouse models with high confidence of translation to humans.

(iii) Oscillatory NF-κB activities. The discovery that cytoplasmic to nuclear shuttling of NF-κB occurs even in resting cells and that interaction with IκBα modulates this movement has moved us away from the static concept that the inhibitor simply "traps" NF-κB in the cytoplasm. This more dynamic view of NF-κB signalling has recently been further refined towards a new model of oscillatory NF-κB signalling studied by real-time fluorescence imaging of NF-κB in single cells [66]. These oscillations are asynchronous between cells which is why they have not previously been detected using population-level biochemical methods such as western blot and gel shift assays. The oscillations arise from negative feedback loops generated by NF-κB-dependent transcription of its inhibitor IκBα and a functionally related protein IκBε (Fig. 1). A recent study by the group of Mike White at Liverpool University has shown that repeated short pulses of TNFα stimulation result in synchronisation of oscillatory NF-κB activity between cells in the population and furthermore, the frequency of cytokine pulsing has a remarkable influence on target gene expression [67]. If pulses of TNFα stimulation are spaced apart by greater than 100 min then the Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES) chemokine is not expressed; however, at pulse frequencies of greater than 100 min RANTES expression increases as a function of shortening of the intervals between stimulatory pulses. What the data tell us is that NF-κB signalling is cyclical and requires greater than 100 min to reset itself to resting state once activated. However, under conditions of persistent high frequency firing of the pathway, such as may be seen in a chronic injured liver, disruption of cyclical NF-κB generates synchronised waves of transcriptional activity which targets genes such as RANTES to drive inflammation. If this concept is confirmed *in vivo* then it has important implications for therapeutic targeting of NF-κB. We may need to develop a new generation of biomarkers that predict "healthy" asynchronised oscillatory NF-κB activity versus "unhealthy" synchronised oscillatory NF-κB. Distinguishing between these variables may enable us to effectively target NF-κB to promote resolution of inflammation and wound-healing in the context of ongoing injury.

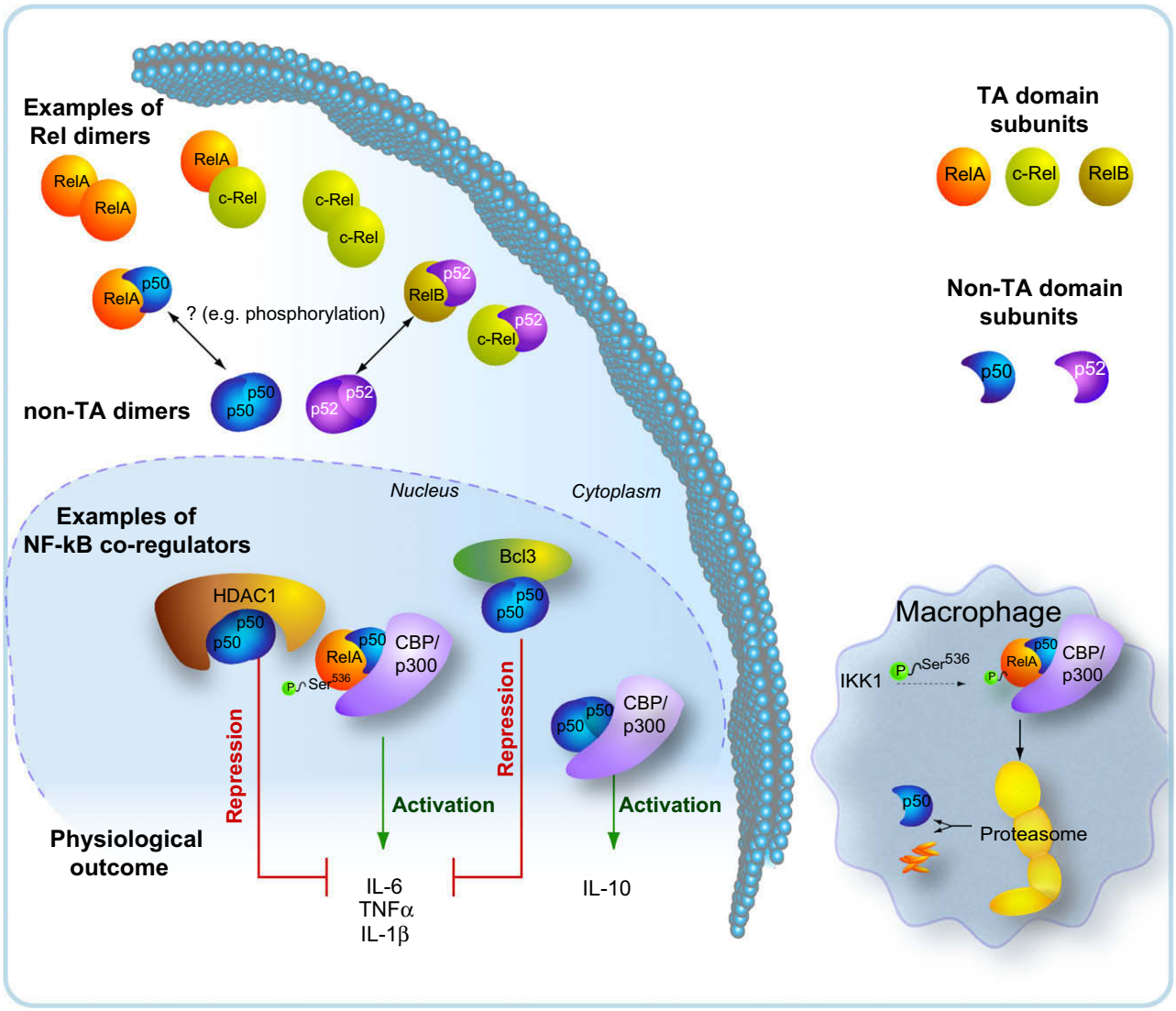

Fig. 1. Five NF-κB/Rel subunits are able to form a variety of functionally distinct heterodimeric or homodimeric NF-κB complexes that have activities subject to further modification by context-dependent post-translational modifications and co-regulator interactions. The RelA, RelB and c-Rel subunits have intrinsic transcriptional activities provided by a transactivation (TA) domain that is absent in the p50 and p52 proteins. How choice of dimerisation of individual subunit partners is controlled is not understood. The context-dependent (cellular- and gene-specific) selection of phosphorylation (e.g. P-Ser-536) and co-regulator recruitment that dictates physiological outcome of NF-κB signalling is also poorly defined.

Summary

Since its discovery as a regulator of immunoglobulin light chain gene expression in B lymphocytes 24 years ago, NF-κB has become one of the most intensively studied transcription factors and one that has held much promise for translation to improved treatment of chronic disease. However, this promise has not yet delivered, in part due to lack of appreciation of the complexity of the NF-κB signalling system and also an over-simplistic assumption that the IKKs tell us all we need to know about NF-κB. By embracing the complexity of NF-κB signalling and unearthing the subtle tissue, cell, gene or even disease context-dependent variables in subunit composition, subunit phosphorylation, co-regulator recruitment and timing of signalling, we will position ourselves better to exploit NF-κB in the clinic.

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