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Signal Transduction by Tumor Necrosis Factor and Gene Regulation of the Inflammatory Cytokine Interleukin-6

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ABSTRACT. Interleukin (IL)-6 is a multifunctional cytokine that can be induced by a plethora of chemical or physiological compounds, including the inflammatory cytokines tumor necrosis factor (TNF) and IL-1. The molecule TNF has a trimeric configuration and thus binds to membrane-bound, cellular receptors to initiate cell death mechanisms and signaling pathways leading to gene induction. Previously, we showed that induced clustering of the intracellular domains of the p55 TNF receptor, or of their respective 'death domains' only, is sufficient to activate the nuclear factor κB (NF-κB) and several mitogen-activated protein kinase (MAPK) pathways. NF-κB is the exclusive transcription factor for induction of the *IL-6* gene in response to TNF and functions as the final trigger to activate a multiprotein complex, a so-called 'enhanceosome', at the level of the IL-6 promoter. Furthermore, the enhanceosome displays histone acetylation activity, which turned out to be essential for *IL-6* gene activation via NF-κB. However, activation of NF-κB alone is not sufficient for *IL-6* gene induction in response to TNF, as inhibition of the coactivated extracellular signal-regulated kinase and p38 MAPK pathways blocks TNF-mediated gene expression. Nevertheless, the transactivating NF-κB subunit p65 is not a direct target of MAPK phosphorylation. Thus, we postulated that other components of the enhanceosome complex are sensitive to MAPK cascades and found that MAPK activity is unequivocally linked to the histone acetylation capacity of the enhanceosome to stimulate gene expression in response to TNF. In contrast, glucocorticoid repression of TNF-driven *IL-6* gene expression does not depend on abrogation of histone acetyltransferase activity, but originates from interference of the liganded glucocorticoid receptor with the contacts between NF-κB p65 and the promoter configuration around the TATA box. BIOCHEM PHARMACOL **60**;8:1185–1195, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. interleukin-6; tumor necrosis factor; NF-κB; coactivator; acetyltransferase; corepressor; deacetylase; glucocorticoid

IL-6† is a multifunctional cytokine that plays a central role in host defence due to its wide range of immune and hematopoietic activities as well as its potent ability to induce the acute phase response [1–3]. It is normally tightly regulated and expressed at low levels, except during infection, trauma, or other stress. Under these circumstances, strongly enhanced IL-6 expression contributes to a cascade of events typical of inflammation, including leukocytosis, thrombocytosis, lymphocyte activation, acute phase protein synthesis, etc. Apart from its hematologic, immune, and hepatic effects, it also has many endocrine and metabolic actions. Specifically, it is a potent stimulator of the hypothalamic-pituitary-adrenal axis under tight negative control of glucocorticoids, but is positively controlled by catecholamines. It has also become apparent that, in the quiescent state, gene expression is kept in check by a complex network that involves the secondary sex steroids, including estrogen and testosterone. After menopause or andropause, loss of the normally inhibiting sex steroids results in elevated IL-6 levels, which accounts for certain of the phenotypic changes of advanced age, particularly those that resemble chronic inflammatory diseases. Accumulating evidence further supports an essential role of IL-6 in the development, differentiation, regeneration, and degeneration of neurons in the peripheral and central nervous system, where it can exert completely opposite actions on neurons, triggering either neuronal survival after injury or causing neuronal degeneration and cell death in disorders such as Alzheimer's disease [4–7]. The age-associated rise in IL-6 has been linked to lymphoproliferative disorders,

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† *Abbreviations:* IL, interleukin; CBP, CREB-binding protein; CRE, CREB-responsive element; CREB, cAMP-responsive element-binding protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; RBP-Jκ, recombination signal sequence-binding protein Jκ; RHD, rel homology domain; TNF, tumor necrosis factor; and PKA, protein kinase A.

multiple myeloma, neoplasia, rheumatoid arthritis, bowel disease, psoriasis, late-life diseases, frailty, dementia, chronic stress disease, and postmenopausal osteoporosis. Serum IL-6 levels are currently also regarded as a diagnostic marker for tumor progression and prognosis in various cancers (renal cell carcinoma, as well as breast, lung, ovarian, and gut cancer) [8, 9]. Hence, selective interference with IL-6 activation may offer therapeutic benefits [7, 10–15].

FROM TNF RECEPTOR TO IL-6 GENE INDUCTION

Regulation of the *IL-6* gene has been intensely investigated in recent years [16–21]. In the present work, we explore how the multiresponsive IL-6 promoter is modulated by the proinflammatory cytokine TNF and what the underlying mechanisms for promoter stimulation might be. Briefly, the regulation of expression of the *IL-6* gene is adapted to its key function, namely a systemic alarm signal that recruits diverse host defence mechanisms in order to limit tissue injury. The IL-6 promoter behaves as a sophisticated biosensor for environmental stress, surveys immunological homeostasis, and is induced by a plethora of chemical or physiological compounds, including bacterial endotoxins, viruses (HIV, human T-cell leukemia virus), or inflammatory cytokines such as TNF and IL-1. Characterization of the human IL-6 promoter revealed a highly conserved control region of 300 bp upstream of the transcriptional initiation site that contains most, if not all, of the elements necessary for its induction by a variety of stimuli commonly associated with acute inflammatory or proliferative states. Electrophoretic mobility shift assays, as well as promoter deletion and point mutation analysis, revealed the presence and functional involvement of an NF-κB-binding element between positions −73 and −63, a multiple response element consisting of CRE followed by a binding site for the CCAAT enhancer-binding protein (C/EBPβ or NF-IL6) between −173 and −145, and an activator protein-1 site located between −283 and −277 [22, 23].

The cytokine TNF elicits a large number of biological activities varying from cell proliferation and cell differentiation to cell death and is also an important mediator of inflammation and cellular immune responses [24–26]. After binding to its membrane-bound 55-kDa receptor, TNF initiates cell death mechanisms and signaling pathways leading to activation of the transcription factor NF-κB and of several MAPK pathways (p38, ERK and Jun N-terminal kinase) effecting gene expression [27, 28]. Previously, we showed that these activation steps only require induced clustering of the intracellular domains of the TNF receptor or of their respective 'death domains' [29, 30]. In the mouse fibrosarcoma cell line L929, NF-κB plays a crucial role in *IL-6* gene expression mediated by TNF [20, 22]. NF-κB is typically a dimer between p50 and the transactivation subunit p65 (RelA). In unstimulated cells, NF-κB resides in the cytoplasm bound to its inhibitor IκB, from which it is released after cell stimulation [31]. NF-κB then migrates into the nucleus, where it effects the expression of numerous target genes [32]. Today, the mechanism most widely studied for inducible NF-κB activation is phosphorylation of IκBs. Several inducers converge to IκB kinase activation, which subsequently causes phosphorylation, ubiquitinylation, and degradation of IκB family members as well as nuclear translocation of NF-κB [33–39]. Alternative pathways that cause NF-κB activation have also been identified [40–46]. In recent years, the relevance of additional regulatory mechanisms controlling the transcriptional activity of NF-κB in the nucleus has gained increasing attention.

ROLE OF TNF IN NUCLEAR NF-κB ACTIVATION

We found that, in addition to TNF-induced cytoplasmic NF-κB activation and nuclear DNA binding, the TNF-activated p38 and ERK MAPK pathways contribute to transcriptional activation of the IL-6 promoter by modulating the transactivation capacity of the NF-κB p65 subunit [22, 47]. A rapid *in vivo* phosphorylation of the p65 subunit was observed in response to TNF and/or IL-1 in various cell lines [47–59]; the p65 κB subunit itself, however, was not found to be a substrate for phosphorylation by TNF-induced MAPK. Besides inducible phosphorylation, both p65 and p50 are constitutively phosphorylated. Taken together, the current data indicate that TNF can induce phosphorylation of both transactivation and RHD of p65, but the contribution of each phosphorylation event to TNF-induced p65 transactivation may be cell type-specific. Detailed phosphorylation studies of the NF-κB homologue *dorsal* in *Drosophila* and x-ray crystallography of p50/p65 dimer bound to DNA demonstrate that five conserved serines, though distributed over the primary structure of RHD, are rather close in the tertiary structure (near the PKA motif at position Ser276) and may form an end point to multiple kinase pathways [60–64]. A schematic overview summarizing the various inducible pathways leading to p65/*dorsal* phosphorylation is given in Fig. 1 [47–60].

TNF SIGNALING TO THE IL-6 ENHANCEOSOME

Another aspect of nuclear regulation of NF-κB activity relies on its interactions with chromatin-modifying cofactor complexes [65–68]. One of the major questions in eukaryotic transcriptional regulation concerns the way in which the transcription machinery gains access to promoter DNA, wrapped in an amalgam of histones and proteins known as "chromatin" [69–71]. Current research indicates that reversible modifications of chromatin (acetylation, phosphorylation, methylation, ubiquitinylation, ADP-ribosylation, glycosylation) affect relaxation or tightening of protein/DNA interactions [72]. This process originates from transcription factors which, after DNA binding, recruit cofactor complexes, exerting various enzymatic activities (i.e.

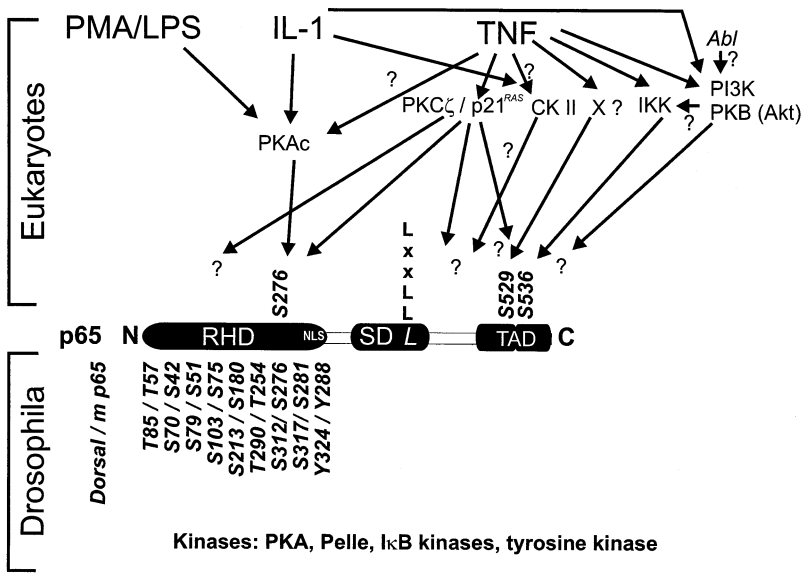

FIG. 1. Schematic overview summarizing the various inducible pathways leading to p65/*dorsal* phosphorylation. Abl, Abelson tyrosine kinase; CK II, casein kinase II; IKK, IκB kinase complex; NLS, nuclear localization signal; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PMA/LPS, phorbol-12-myristate 13-acetate/lipopolysaccharide; SD, synergy-specific domain; TAD, transactivation domain.

acetyltransferase, deacetylase, methyltransferase, kinase, ubiquitin–ligase, etc.) [73–80]. The latter, in turn, modify histones/nucleosomes as well as transcription factors, resulting in local and transient chromatin relaxation, which attracts additional protein complexes (i.e. ATP-dependent chromatin-remodeling complexes, RNA polymerase holoenzyme, kinases, and complexes involved in cell cycle control, proliferation, or differentiation) [79, 81–83]. The complete pattern of chromatin "tags" residing around the promoter enhanceosome probably includes a unique message for further downstream signaling events [72]. Unraveling this chromatin vocabulary is of crucial importance to understanding many DNA template-based processes as well as the origin of various diseases. Chromatin-modifying complexes have indeed been associated with the control of cell growth and differentiation, whereas aberrant activities frequently result in transformation and tumorigenesis [84–91]. Transcription factor-selective and signal-specific cofactor and/or HAT/histone deacetylase recruitment have now become a prime focus of investigation [92–98]. In this respect, our findings support a molecular model for synergistic transcription, in which the cointegrator CBP/p300 may be recruited to and engaged onto the multiresponsive IL-6 promoter by multiple protein–protein interactions with the DNA-binding transcription factors activator protein-1, CREB, C/EBP, and NF-κB, sequentially arranged along the promoter sequence. The ultimate switch for gene induction is achieved by cytoplasmic activation of NF-κB, e.g. in response to TNF, which subsequently binds to the IL-6 promoter sequence and engages the available CBP/p300 for transcriptional activation [23]. Similar observations were recorded with other natural NF-κB-driven promoters, such as the IL-8, E-selectin, and HIV-1 promoters [23, 99–101]. Whether this engagement relies on conformational changes of CBP by interaction with NF-κB [102] and/or is the result of concomitant phosphorylations of either NF-κB or CBP, e.g. by TNF induction, is at present not clear [52, 53, 92, 103]. Zhong and colleagues have proposed an allosteric molecular model in which phosphorylation of the RHD induces intramolecular conformational changes of the N- and C-termini of p65, which may expose an entry platform for CBP interaction. p65–CBP interactions have now been mapped at the N- and C-termini of p65, including a synergism-specific domain (p65 AA322–458) [53, 104–107]. The latter domain revealed a single copy of the motif Leu-Gly-Ala-Leu-Leu and conforms to the consensus LxxLL, which is the signature motif present in CBP and CBP-interacting proteins; it forms a short amphipathic helix, providing the structural basis for protein–protein interactions [107, 108]. An initial interaction between CBP and the RHD of p65 may be further stabilized by simultaneous interaction of the LxxLL motif in p65 (AA449–453) and CBP (AA358–362). Further, we cannot exclude that other factors corecruited to the IL-6 promoter may also contribute to (part of) the acetylation process and/or chromatin-remodeling activities: involvement of p300/CBP-associated factor, SRC-1, p160, bcl-3, activator-recruiting cofactor coactivators or Groucho and/or p202 corepressors has also been demonstrated in NF-κB transactivation [68, 109–117]. These findings suggest that, by analogy with the coregulator exchange in nuclear receptor functions [118], transcriptional activity of NF-κB may be regulated by a balance of corepressor and/or coactivator interactions that could be modulated by signal-induced modification of these proteins [116]. At present, acetylases are known to modify, in addition to histones, a variety of other proteins, including transcription factors (p53, E2F1, erythroid Kruppel-like factor (EKLF), T-cell factor (TCF), GATA1, HMGI(Y), ACTR, TFIIF, TFIIEβ, HIV1-tat), nuclear acetylases (CBP/p300, p300/CBP-associated factor), shuttling import factors (importin-α), and α-tubulin, which regulate many different functions such as DNA recognition, protein–protein interaction, and protein stability [119, 120]. In view of the synergy of NF-κB-driven

gene transcription in response to sustained HAT activity [23], one might expect acetylation of the NF-κB p50/p65 subunits as well. However, we and others have so far not been able to demonstrate significant *in vivo* acetylation of NF-κB [121, 122].

Since p38 and ERK signaling were not found to be responsible for TNF-induced p65 phosphorylation, we analyzed their possible role in cofactor-dependent activities. Remarkably, p38 and ERK inhibitors were able to specifically revert p65-engaged HAT activity, whereas glucocorticoids were not able to abrogate HAT activity so as to repress p65 transactivation. Similarly, although cross-talk of histone acetylation with caspase [123] and methylation [124–127] activities has been described, we were unable to demonstrate any modulation of p65-recruited HAT activity after addition of caspase (zDEVD-fmk) or methylation (azacytidine) inhibitors. Cross-talk of MAPK signaling and histone acetylation has recently been further substantiated [128–130]. p38 MAPK have also been shown to phosphorylate TATA-binding protein, which affects its binding to DNA and various protein–protein interactions such as p65:TATA-binding protein and/or CBP/p300:TATA-binding protein [131]. In addition, although no specific TNF-dependent phosphorylation of CBP/p300 has been reported so far, phosphorylation-dependent control of CBP/p300 transactivation and/or HAT capacity by PKA, ERK, p90 ribosomal 56 kinase (p90rsk), CaMIV, and cyclin-dependent kinases is now well documented [74, 132–137]. Other targets likely sensitive to MAPK-dependent regulation and liable to contribute to gene transactivation are the SWI/SNF chromatin-remodeling complex [138], p300/CBP-associated factor [139], nucleosomes [77, 140–142], locus control regions [143], $p21^{Waf/Cip1}$ [144, 145], and cell-cycle components [146–148]. Finally, the RNA polymerase carboxy-terminal domain and the RNA synthesis process itself have also been found to be modulated by MAPK [149–151].

NEGATIVE MODULATION OF TNF-INDUCED IL-6 GENE INDUCTION

In addition to gene activation, repression mechanisms are also required to guarantee tight control of IL-6 expression. Silencing of NF-κB-dependent gene activity was observed with RBP-Jκ, which competes with NF-κB for DNA binding at the IL-6-κB motif [152]. One may thus postulate that the *IL-6* gene promoter is dynamically regulated at the NF-κB site by an equilibrium of a coactivator complex (including CBP/p300) interacting with NF-κB and a corepressor complex containing histone deacetylase-1 associated with RBP-Jκ [153, 154]. In the absence of activated NF-κB, the IL-6-κB site is occupied by the RBP-Jκ corepressor complex, which may shield the RNA polymerase holoenzyme from CBP effects mediated by activator protein-1, CRE, and C/EBP. However, deacetylase inhibitor experiments with trichostatin A and studies with point-mutated IL-6 promoter variants having lost RBP-Jκ binding without affecting NF-κB suggest only a minor role of this repressor complex in promoter regulation in response to sustained HAT activity [23].

Glucocorticoids are also known to counteract NF-κB-dependent gene induction. Gene repression by glucocorticoids was reported to result from removal of the activating factor NF-κB from the promoter DNA by increased levels of the inhibitor molecule IκB [155, 156]. However, we and others have not found any evidence in favor of this mechanistic model [157–159]. Moreover, using 'dissociated compounds', which discriminate between gene activation and transrepression, we have clearly demonstrated that NF-κB-driven genes can be fully repressed in the absence of IκB activation [160]. Others have postulated that gene repression results from competition between different activating transcription factors for a limited amount of commonly required nuclear cofactors, e.g. CBP/p300 [118, 161, 162]. In contrast to this hypothesis, we found, after overproduction of nuclear cofactors, that gene repression still occurs and, moreover, remains highly specific for transactivation by NF-κB p65. Taken together, we feel that the second hypothesis does not provide a valid working model to explain glucocorticoid-mediated gene repression. We rather believe that interference of the glucocorticoid receptor with direct contacts between p65 and the TATA box environment is the basis for specific gene repression [163–168]. Whether this relies on direct steric hindrance [169] or on interference of glucocorticoids with nuclear signaling cascades affecting protein–protein interactions is not yet clear [131, 165, 170–172]. Similar conclusions were reached for gene repression by the peroxisome proliferator-activated receptor-α [173].

CONCLUSIONS

In order to further understand *IL-6* gene regulation, it will be necessary to determine how CBP/p300 can simultaneously integrate functions of various transcriptional activators present in the IL-6 promoter [65, 174, 175]. Two valuable working models are currently available. Studies with the interferon-β enhanceosome show a strict stereospecific requirement for optimal CBP recruitment and transcriptional synergism promoting a looping cofactor model with multiple transcription factor interactions [73, 121, 176, 177]. Alternatively, a coactivator sequestration model has been proposed in which multiple transcription factors compete for limited amounts of CBP and become opponents to mediate CBP effects [178, 179]. Along the same line, accessory proteins may selectively regulate CBP access for specific transcription factors [180, 181]. So far, our experimental data with the IL-6 promoter are in favor of the first model, since for optimal CBP synergistic activities all factors are required; nevertheless, the unique role of p65 in CBP engagement in the IL-6 promoter context remains to be further explored. To unravel the transcriptional activation of the IL-6 promoter in response to TNF at a detailed molecular level, it remains to be

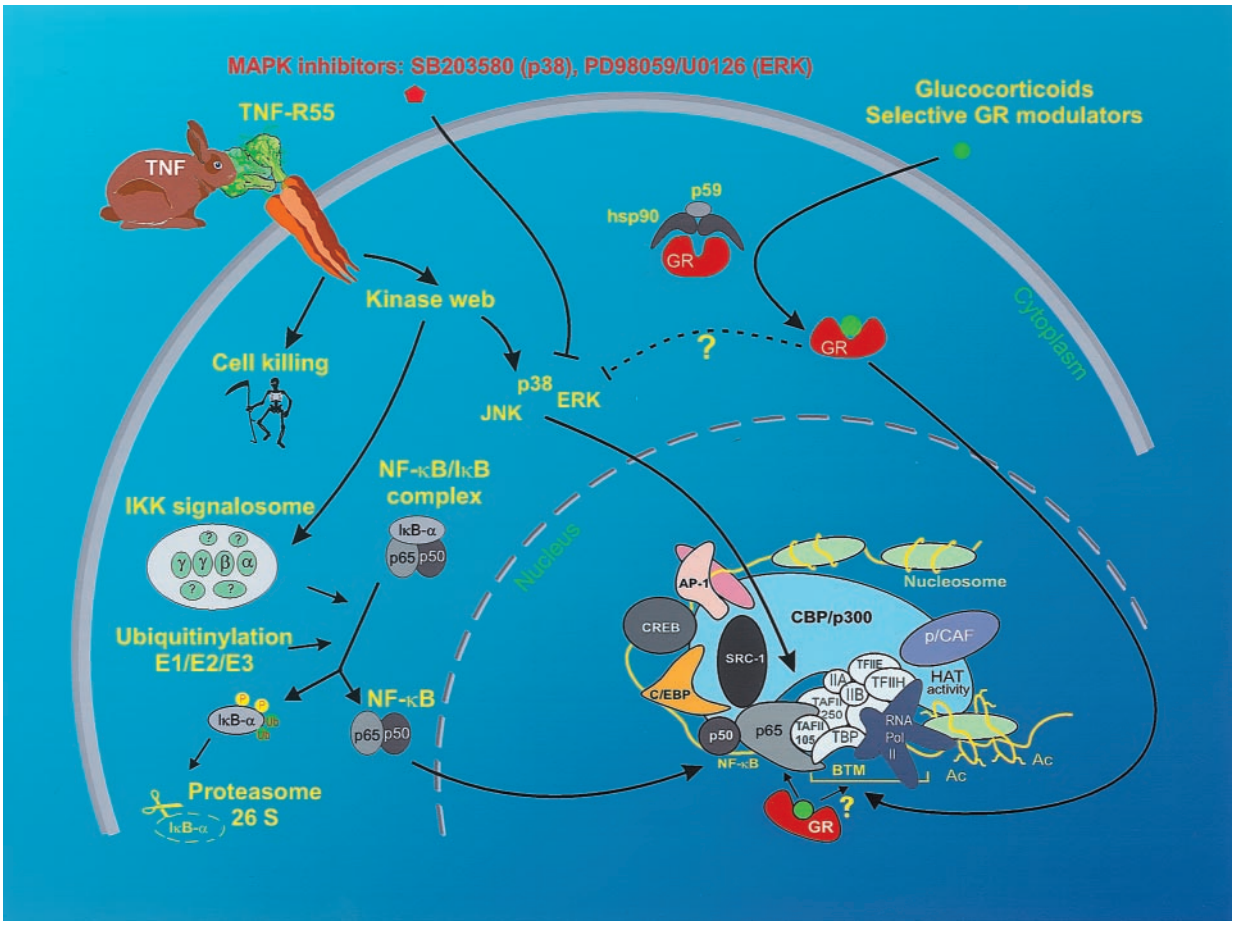

FIG. 2. Two-sided activation model for *IL-6* gene expression in response to TNF. AP-1, activator protein-1; C/EBP, CCAAT enhancer-binding protein; GR, glucocorticoid receptor; JNK, Jun N-terminal kinase; SRC, steroid receptor coactivator; TBP, TATA-binding protein; hsp90, heat-shock protein 90; BTM, basal transcription machinery; and Ac, acetylated lysine.

established how nucleosomes remodel at the IL-6 promoter after inflammatory stimuli and how this process is affected/driven by protein modification events (acetylation, phosphorylation) of various components of the IL-6 enhanceosome [182–185]. In conclusion, our results support a two-sided model for *IL-6* gene expression in response to TNF. In this model, IL-6 promoter-bound transcription factors recruit CBP and cofactors which, after activation and phosphorylation of NF-κB p65 by TNF, are transformed into a transcriptionally competent enhanceosome. This coincides with concomitant enhanceosome-activating signals delivered by MAPK, which actually results in transcriptional stimulation. This two-sided activation model, also including the possible repressive mechanisms by glucocorticoids, is illustrated in Fig. 2. Our findings suggest that the p65–CBP interface may be an attractive and potential target for therapeutic inhibition in cancer [186], viral replication [187, 188], or inflammatory disorders [189, 190] such as atopy [191], bowel [192, 193], or pulmonary diseases [194, 195], rheumatoid arthritis [196], and septic shock [197]. The first pharmacological compounds that selectively interfere with nuclear NF-κB signaling have recently been reported and described to inhibit gut inflammation [55, 198, 199]. Unlike conventional salicylates and sulfasalazines that interfere with IκB degradation [200, 201], mesalamine and helenalin specifically block nuclear NF-κB function by preventing phosphorylation and inducing alkylation, respectively. Finally, chromium(VI) has also been shown to interfere in nuclear competence of NF-κB in response to TNF by selectively inhibiting p65/CBP interaction rather than DNA binding [57].

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