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Perspective: Expanding Role of Cyclin Dependent Kinases in Cytokine Inducible Gene Expression

Allan R. Brasier
Departments of Internal Medicine, Sealy Center for Molecular Medicine, and the Institute for Translational Sciences, University of Texas Medical Branch, Galveston, TX-77555-1060

Abstract

The Positive Transcriptional Elongation Factor b (P-TEFb), a heterodimer of CDK9 and Cyclin T1, is widely implicated in control of basal gene expression. Here, P-TEFb is involved in transitioning paused RNA polymerase II to enter productive transcriptional elongation mode by phosphorylating negative elongation factors and Ser^2 of the heptad repeat in the RNA Pol II COOH terminal domain (CTD). This perspective will examine recent work in two unrelated inducible signaling pathways that illustrate the central role of P-TEFb in mediating cytokine inducible transcription networks. Specifically, P-TEFb has been recently discovered to play a key role in TNF-inducible NF-κB activation and IL-6-inducible STAT3 signaling. In these signaling cascades, P-TEFb forms protein complexes with the activated nuclear RelA and STAT3 transcription factor in the cellular nucleoplasm, an association important for P-TEFb's promoter targeting. Studies using siRNA-mediated knockdown and/or selective CDK inhibitors show that P-TEFb plays a functional role in activation of a subset of NF-κB-dependent targets and all STAT3-dependent genes studied to date. Interestingly, cytokine inducible genes that are sensitive to P-TEFb inhibition share an induction mechanism requiring inducible RNA Pol II recruitment. Chromatin immunoprecipitation studies have preliminarily indicated that this recruitment is dependent on CDK enzymatic activity. The potential of inhibiting P-TEFb as an anti-inflammatory therapy in innate immunity and systemic inflammation will be discussed.

Keywords

NF-κB; cyclin dependent kinases (CDK); PTEF-b; STAT3; inflammation

Enhanced transcription is a major mechanism underlying metazoan gene expression that allows the cell to rapidly respond to developmental, hormonal or inflammatory stimuli, and still produce phenotypic memory of the stimulus. Transcription is a coordinated response involving several distinct, inter-dependent processes, including preinitiation complex formation, chromatin enhanceosome formation/coactivator recruitment, de-repression and transcriptional elongation. Preinitiation complex formation refers to the process where a basal promoter is bound by a multiprotein complex of TATA box binding protein, kinases, and ubiquitin ligases.[1] In some promoters, hypophosphorylated RNA polymerase II is also bound; in this form, RNA Pol II produces a series of short, "unproductive", transcripts that are truncated and rapidly degraded. Enhanceosome formation is linked to inducible gene expression, and is a process initiated by binding activated sequence specific transcription factors and their associated coactivators, such as p300/CBP or the Mediator complex.[1, 2] These coactivators contain histone acetyltransferase activity that modify repressive

*to whom correspondence should be addressed: Allan R. Brasier, MD, MRB 8.128, 301 University Blvd., Galveston, TX 77555-1060, Phone (409)-772-2824, arbrasie@utmb.edu.

nucleosomes and allow the upstream coactivators to productively couple with the basal/pre-initiation complex. In highly inducible genes, RNA Pol II recruitment occurs coincident with inducible transcription factor binding.[3] Subsequently, RNA Pol II is relieved of repression, and acquires its processive form, producing full-length, spliced polyadenylated transcripts that encode the target protein, a process referred to as transcriptional elongation. The relative contributions of these steps in any inducible genetic network is not fully understood.

Because of its link with many pathological conditions, inducible gene expression in response to cytokine cascades has been intensively studied. Cytokines are secreted proteins that function as hormonal mediators of inflammation, innate responses to cellular infection, hepatic acute phase response and adaptive immunity.[4, 5] Upon binding cognate cell surface receptors, signaling cascades are activated that affect the responding cell to induce genes that encode homeostatic proteins, adhesion molecules important in leukocyte activation or recruitment, and further amplify the inflammatory signal by secreting downstream cytokine cascades. This perspective will focus on recent work that has illuminated the critical of the positive transcription elongation factor b (P-TEFb), in cytokine inducible expression in two distinct cytokine signaling pathways. Understanding this process at the molecular level may lead to potential new therapeutics that can be used to modify the host inflammation, augment immune responses, or permit more rapid adaptive immune responses.

P-TEFb and Transcriptional Elongation

It has been observed for some time that RNA Pol II is paused on the 5' end of promoters.[6] Here, RNA Pol II is complexed with the 5,6-dichloro-1-β-D ribofuranosyl-benzimidaole (DSB)-sensitive factor complex (DRIF) and negative elongation factor (NELF). In the process of gene activation, RNA Pol II is phosphorylated at Ser^5 in the repeated YSDPTSPS motif ("heptad repeats") in the COOH terminal domain (CTD) by the preinitiation complex subunit TFIIH, an event that signals its transition to mode sufficient for promoter clearance and transcript elongation [1]. Studies have shown phosphorylation of SPT4 and 5 (components of the DRIF complex) and NELF releases these proteins from Pol II. Concomitantly, phosphorylation of the Ser^2 residue of the heptad repeats allows Pol II to enter processive mode where full-length transcripts are produced.[7]

The homologous proteins CDKs -7, -8 and -9 all share RNA Pol II CTD kinase activity. Of these, CDK9 has emerged as one of the major CTD kinases that phosphorylates Ser^2 in the heptad repeat. CDK9 is a 43 kDa protein first identified by PCR amplification of a mouse library using degenerate primers to amplify genes homologous with the cdc2 NH2 terminal kinase domain.[8] Sequence analysis showed CDK9 had a highly homologous PITALRE sequence to the cdc2 PSTAIRE motif in the NH2 terminal β sheet. Like other CDKs, CDK9 associates with cyclins (Ccns)-T, -K and –E; this association is important for CDK9 kinase activity. Most of the CDK9 in the cell is associated with nuclear CcnT1, found distributed primarily in the nucleus and associated with nuclear speckles and the splicing factor, SC35.[9, 10] The complex of CDK9-Ccn T1 is homologous to the *Drosophila melanogaster* positive transcriptional elongation factor (P-TEFb7). P-TEFb is a kinase whose activity is not detectably regulated through the cell cycle.

Biochemical fractionation studies have shown that nuclear P-TEFb exists in two functionally distinct complexes. Half of nuclear P-TEFb is found as an inactive complex associated with HEXIM1 and 7SK snRNA.[11] Tandem affinity isolation and mass spectrometry have shown that P-TEFb also associates with the bromodomain containing protein, termed Brd4.[12] Functional assays using siRNA mediated Brd4 knockdown have indicated that Brd4 is required for P-TEFb induced HIV LTR transcriptional elongation. Currently it is thought

that the bromodomain of Brd4 may be required for P-TEFb activity through its ability to associate with acetylated lysines and thereby facilitates its chromatin access or binding. In addition, Brd4 may bind components of the Mediator complex, including TRAPs-230, -170, -100 and -80.[12] The Mediator binding function of Brd4 in PTEFb function has not yet been addressed.

Mechanisms underlying P-TEFb Activation

Despite its name, the kinase activity of CDK9 is not regulated in a cell cycle-dependent manner, but rather via its association with specific proteins (Figure 1). Work on the mode of regulation of P-TEFb in activation of the HIV TAR sequence has shown that the activation occurs in several distinct steps. Activated P-TEFb can be produced by dissociation of HEXIM1 and 7SK snRNA.[13] First, CDK undergoes autophosphorylation, an event that allows its associated CcnT1 to bind TAT via release of an intermolecular association.[14] The concept that there are cellular kinases that activate CDK9 is intriguing, but their identities have not yet been described. Additionally CDK9 is inducibly acetylated, a phenomenon whose effects on P-TEFb activity are currently unclear or controversial. One group has reported that the histone acetyltransferase activity of p300/CBP acetylates CDK9 on Lys^{44} in the ATP binding domain, enhancing its kinase activity toward the RNA Pol II CTD.[15] By contrast, the GNAT histone acetylases, P/CAF and GCN5, acetylate Lys^{44} and Lys^{48} in the ATP binding domain of CDK9 resulting in inhibition of kinase activity and altered nuclear distribution into insoluble nuclear matrix.[16] One explanation for these discordant results may be that other sites on CDK9 whose modification that affect the response to CDK9 acetylation on Lys^{44}.

Although the majority of CDK9 is complexed with CcnT1 in nuclear speckles, a small fraction of CDK9 is found in an apparently uncomplexed form in the cytoplasm. Inhibition of nuclear exportin, CRM1, using leptomycin B leads to the nuclear accumulation of CDK9, leading to the conclusion that a fraction of the “free” (non Ccn-complexed) CDK9 is dynamically undergoing cytoplasmic-nuclear shuttling.[9] An important unresolved issue is what controls physiological P-TEFb activation and/or partitioning into activated Brd4, or inactive HEXIM1-7SK snRNA complexes. Previous work has shown that P-TEFb primarily plays a role in expression of genes containing a TATA box 17, however its mechanism for recruitment to these promoters is not fully understood. Recent work has indicated that P-TEFb association with nuclear inducible transcription factors play an important role in P-TEFb recruitment in cytokine cascades.18, 19 These include the TNF-induced NF-κB and the IL-6 induced STAT3 signaling pathways, discussed separately in detail below.

The TNF-induced NF-κB pathway

TNF is released by tissue resident macrophages upon encountering infecting viral or bacterial products and activates neighboring cells by binding a ubiquitously expressed receptor, the TNFRI. Employing systematic microarray analyses of the TNF-induced transcriptional response, our laboratory has shown the transcription factor nuclear factor – κB (NF-κB) is responsible for mediating a significant fraction of the genomic response.[20–23] The transcriptional activator subunits of the NF-κB family are complexed and inactivated in the cellular cytoplasm by binding inhibitors of NF-κBs, IκBs, a class of ankryin repeat domain-containing proteins that block nuclear translocation and inhibit DNA binding.24 NF-κB activation involves RelA liberation from its cytoplasmic inhibitors, allowing it to translocate into the nucleus and activate target genes (Figure 2).

NF-κB is controlled by distinct regulatory pathways, termed the “canonical”, “noncanonical” and “cross-talk” pathways, that are activated by selective stimuli and involve liberating RelA from distinct cytoplasmic complexes.[25, 26] Of these, the canonical

pathway is the major pathway induced by cytokines;27 the noncanonical and cross-talk pathways are induced by RNA virus infection or lymphokines and, for brevity, are not further discussed here.[28–30] Recent work seeking to understand the canonical pathway has shown that it consists of separable "modules" by which we refer to signaling subpathways whose action are to control either nuclear translocation or transcriptional activation. Specifically, TNF stimulation controls release of RelA sequestered by IκBα via an IκB kinase (IKK)-IκBα module, whereas licensing of transcriptional activation of RelA by Ser^{276} phosphorylation via an reactive oxygen species (ROS)-catalytic subunit of protein kinase A (PKAc) module.[19, 31]

TNFα binding to TNFRI transduces intracellular signals by inducing submembranous complex formation with adapter signaling proteins that interact with the death domain of the TNFRI cytoplasmic tail. These adaptor proteins include the TNF receptor associated factor (TRAFs) -2, -6 and receptor interacting protein [RIP32], proteins with ubiquitin ligase activity responsible for downstream activation of MAP3Ks, particularly the TGFβ associated kinase-1 (TAK1), a process that occurs via a Lys^{63}-linked ubiquitination step.[33] Subsequently, the activated submembranous complex recruits and activates IKK [34], the first committed step in NF-κB translocation, by TAK1-initiated phosphorylation.[33]

The IKK is a multi-subunit kinase complex [35], whose core is composed of two highly homologous serine-threonine kinases, IKK-α and β, associated with a regulatory subunit, IKKγ.[36–38] Although IKKβ plays the major catalytic role, the activation and behavior of the IKK is determined by the associated regulatory IKKγ subunit. IKKγ plays multiple roles in IKK activation through its ability to organize the assembly of IKKs into the activated high molecular weight complex,[34, 39] bind ubiquitinated signaling adapters,[40, 41] recruit the IκBα inhibitor into the activated IKK complex where it becomes phosphorylated marking IκBα for degradation,[39] and to serve as an adapter molecule to recruit upstream kinases that phosphorylate the catalytic subunits.[40, 42, 43] Moreover, we have found that IKKγ is alternatively spliced in an exon that encodes for its self-association and oligomerization domains, a feature that allows distinct IKK complexes to selectively couple to distinct activating cascades.[26, 44] Through these activities, IKKγ forms a molecular bridge between IKK, its upstream activators, and its substrate.

TNF induced IKK activation is mediated by phosphorylation at Ser residues in the conserved IKK α/β activation loop, producing autophosphorylation.[45] Activated IKKβ then phosphorylates IκBα on Ser residues 32 and 36 in its NH2 terminal regulatory domain,[27] making it a substrate for proteolysis through the 26S proteasome and calpain pathways.[35, 46] Liberated from its IκBα inhibitor, RelA rapidly enters the nucleus.

Systematic microarray studies conducted by our laboratory have revealed significant complexity in the genomic response.[22, 47] For example, time series analyses in TNF stimulated cells have found that expression of NF-κB-dependent genes is nonsynchronous, separable by the timing of peak gene expression, with each expression group encoding distinct biological functions [21, 22, 25]. This characteristic allows coordinated evoluation of cellular biological responses to TNF activation.[22, 25]

RelA Ser^{276} phosphorylation is necessary for transcriptional competency via P-TEFb

Although NF-κB translocation is necessary for target gene expression, it is not sufficient [48]. Recent work has shown that site-specific phosphorylation of NF-κB is required for transcriptional activation. RelA is inducibly phosphorylated on a number of Ser residues; Ser^{276} and Ser^{536} are thought to be the most important in regulating transcriptional activity.

[19, 31, 49, 50] In this regard, we have defined a TNF-induced regulatory pathway mediated by intracellular ROS as a second messenger that is required for NF-κB dependent gene expression. Here, inhibition of ROS formation blocks Ser^{276} phosphorylation and NF-κB dependent gene expression without affecting NF-κB translocation.[31, 48] This pathway is dependent on the catalytic subunit of protein kinase C (PKAc), a kinase that co-purifies with IκBα[49] and whose activity is induced by TNF.31 Because inhibition of ROS formation or siRNA mediated PKAc knockdown prevents TNF-induced RelA Ser^{276} phosophorylation without affecting its nuclear translocation, we have interpreted these findings to indicate that the ROS-PKAc transcriptional activating module is a separate pathway whose function is also necessary for NF-κB dependent gene expression (Figure 2).

Phosphorylation at Ser^{276} in the mid molecule of RelA is thought to reduce intermolecular NH2 and COOH terminal interactions, allowing phospho-Ser^{276} RelA to complex with p300/CBP coactivators,[51] resulting in RelA acetylation[52] and form a stable enhanceosome on endogenous gene targets in ChIP assays.[31]

We have recently discovered that RelA Ser^{276} phosphorylation also allows complex formation with P-TEFb, a process that results in phospho-RelA to associate with both transcriptional coactivators as well as bridge with the transcriptional elongation machinery.[19] Nondenaturing co-immunoprecipitation experiments show that RelA inducibly associates with P-TEFb, in a manner that is absolutely dependent on RelA Ser^{276} phosphorylation. This association results in inducible CDK9 binding to the downstream genetic targets of NF-κB, including *IκBα*, *Groβ* and *IL-8* genes. Because we could selectively disrupt Ser^{276} phosphorylation without affecting NF-κB translocation using antioxidants, we used these agents to test the effect of Ser^{276} phosphorylation on activation of downstream genes. These findings revealed the surprising result that a subset of genes, *Groβ* and *IL-8*, were absolutely dependent on phospho- Ser^{276} RelA, but others, *IκBα*, were not (Figure 2).

To more selectively test the role of CDK9 interaction, CDK9 was downregulated by siRNA mediated transfection. The same subset of genes, *Groβ* and *IL-8* were significantly inhibited, whereas the phospho-Ser^{276} RelA-independent genes were not affected. Interestingly only the genes requiring inducible RNA Pol II recruitment as a mechanism in their promoter activation were inhibited by CDK9 downregulation. These data suggest that P-TEFb is involved in the activation of a subset of NF-κB-dependent genes whose mechanism of activation involves Pol II recruitment.

Role of CDK9 in IL-6 signaling via the Jak-STAT3 pathway

IL-6 is a ubiquitously expressed anti-inflammatory cytokine that plays a major role in hepatic acute phase response and vascular inflammation.[5] IL-6 signaling in target cells is initiated by its binding to the IL-6Rα receptor, an event that triggers ligand-mediated oligomerization with the ubiquitously expressed transmembrane gp130 β-subunit, and subsequent formation of a hexameric IL-6·IL-Rα·gp130 high-affinity complex [Figure 3, and ref. (53)]. Receptor ligation induces conformational changes in the cytoplasmic domains of gp130 that bring Janus tyrosine kinases (JAKs) into close proximity. This molecular crowing results in trans-autophosphorylation, an event that initiates IL-6 signaling.54 JAK1, in turn, phosphorylates gp130 on the docking sites for the signal transducers and activators of transcription (STAT); STAT isoforms -1 and -3 are then recruited, where they, too, become phosphorylated.55

Of the signaling pathways downstream of the IL-Rα·gp130 complex, STAT3 appears to play a major role. Tyr phosphorylated STATs-1 and -3 form intermolecular SH2–SH3 domain interactions, an event that is coupled to its nuclear translocation and transcriptional activation of target genes.[54, 55] Analyses of complex formation with STAT3-dependent

transcriptional enhancers have shown that STAT3 undergoes additional post-translational modifications that permits interactions with co-factors and co-activators.[56]

As with the NF-κB pathway, STAT3 associates with the p300/CBP coactivator, whose actions are to open chromatin structure, allowing other chromatin-modifying proteins to bind to DNA and activate transcription.[57, 58] The p300/CBP association requires both the NH2-terminal modulatory domain and the COOH-terminal transactivation domain of STAT3.[56, 57] Studies from our laboratory first described two novel acetylation sites on the STAT3 NH2 terminus at Lys^{49} and Lys^{87} that are required to stabilize the STAT3-p300/CBP complex through an additional interaction mediated by the modified STAT3 NH2 terminus.[56]

By several experimental techniques, we and others have found that activated nuclear STAT3 binds CDK9.[18, 59] Importantly co-immunoprecipitation experiments from our laboratory indicate that both phospho-Tyr^{705} STAT3 and Ac Lys^{87} STAT3 are associated with P-TEFb.[18] These observations indicate that the transcriptionally active form of STAT3 is complexed with P-TEFb, and that this complex forms in the nucleoplasm prior to DNA interaction. Interestingly, STAT3 appears to directly contact CDK9 in the P-TEFb complex via its NH2 and COOH terminal domains.[18, 59] This interaction is apparently distinct from how NF-κB complexes with P-TEFb as prior mapping studies indicate that RelA binds the cyclin box of CcnT1.[60] These findings indicate that multiple sites for transcription factor interaction exist on P-TEFb.

Chromatin immunoprecipitation (ChIP) assays using primers selective for the upstream and coding regions of STAT3-dependent γ-fibrinogen (FBN) show that P-TEFb is not uniformly concentrated on the 5' regulatory site of the γ- FBN promoter. Interestingly a greater abundance of PTEFb is found in the coding region of the gene.[18] These findings suggest additional roles of P-TEFb in inducible expression outside of initial Ser^2 CTD phosphorylation, such as those described in the heat shock response in *Drosophila* where P-TEFb plays an important role in 3'RNA processing.[61] Whether 3' mRNA processing is mediated by P-TEFb in mammalian gene expression, and if so, if it plays a role in cytokine inducible gene expression will require further work.

siRNA mediated knockdown of CDK9 confirms the functional role of P-TEFb in STAT3-dependent gene expression of the endogenous γ- *FBN* gene[52] and *p21waf*.[59] As observed for the NF-κB-dependent genes, activation of the STAT3-dependent genes involves recruitment of Pol II. In this system, ChIP analysis of the effects of CDK9 inhibitor show that Pol II recruitment is itself dependent on functional CDK activity.[18] These studies further indicate that the IL-6-STAT3 signaling pathway can be affected by modulating P-TEFb action. The question whether P-TEFb is a general transcriptional enhancer necessary for activating all STAT3-dependent genes, or whether only specific subsets of STAT3-dependent genes require P-TEFb has not been fully explored.

Implications for modulation of inflammation

Glucocorticoids are potent anti-inflammatory hormones that act to inhibit TNF-inducible gene expression. Work from the Yamamoto laboratory has discovered that the activated glucocorticoid receptor may antagonize NF-κB activation by blocking P-TEFb recruitment.[62] This study also indicated that heterogenous mechanisms are involved in NF-κB-dependent gene expression with the IL-8 gene being sensitive to the inhibitory actions of glucocorticoids, whereas IκBα is not. These findings are remarkably similar to those we reported after antagonizing NF-κB·P-TEFb complex formation using antioxidants to inhibit phospho-Ser^{276} formation, which selectively inhibited *IL-8*, but not *IκBα*, gene expression.[63] We interpret these findings to suggest that both glucocorticoids and antioxidants exert some

of their anti-inflammatory actions by disrupting the NF-κB·P-TEFb interaction. The design of more potent or selective inhibitors of this association may be useful clinically to selectively disrupt subnetworks of NF-κB induced inflammation.

Similarly, disruption of STAT3 signaling may have specific applications in modulating vascular inflammation, a process where IL-6 may play a central role.[5] Selective disruption of STAT3·P-TEFb may have significant effects on atherosclerosis and aortic remodeling in cardiovascular disease.

Summary

In this perspective, I have highlighted recent work illustrating the concept that P-TEFb plays a central role in mediating cytokine inducible gene expression. Although the bromodomain containing Brd4 protein appears to be important for P-TEFb activation, our studies also show that P-TEFb is targeted to specific inducible genes by nucleoplasmic association with activated NF-κB and STAT3 transcription factors. Currently we think P-TEFb forms complexes with NF-κB via distinct molecular contacts than those used for STAT3 interaction. A number of interesting questions are currently unresolved. What is the role of the nucleocytoplasmic shuttling form of CDK9? What are the full spectrum of substrates for the P-TEFb complex; and how does CDK9 phosphorylation affect their activity? Does P-TEFb play a role outside of simply inducing transcriptional elongation, for example, in 3'RNA processing? Is P-TEFb is a universal enhancer for STAT3 dependent transcription, or does it play a role in a subset of STAT3-dependent target genes? Further investigation into these questions and examination of the effect of disruption of this complex in inflammatory disease will be illuminating.

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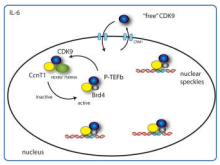

Figure 1. P-TEFb states in the resting cell

Shown is a schematic view of the nuclear states of activated P-TEFb. Approximately half of nuclear P-TEFb is inactive state associated with HEXIM1 and 7SK RNA. The component that is actively involved in RNA Pol II dependent transcription is associated with Brd4. A small fraction of CDK9 is undergoing active nuclear-cytoplasmic shuttling mediated by CRM1-dependent nuclear export. Abbreviations: Brd4, bromodomain 4; P-TEFb, positive transcriptional elongation factor-b.

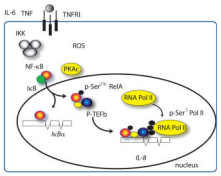

Figure 2. Role of P-TEFb NF-κB-dependent transcription

Schematic view of the TNF signaling pathway. Two pathways are activated downstream of liganded TNF, each required for NF-κB dependent activation. IKK mediates RelA translocation by phosphorylation of the IκBα inhibitor; PKAc responds to ROS generation to induce Ser^{276} phosphorylation. Phospho-Ser^{276} RelA is competent for P-TEFb association, a complex that activates *IL-8* and other cytokine genes. This process involves RNA Pol II recruitment. By contrast, unphosphorylated RelA activates IκBα expression. Abbreviations: IKK, IκB kinase; TNFRI, TNF receptor-I.

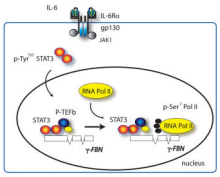

Figure 3. Role of P-TEFb in STAT3-dependent transcription

Schematic view of IL-6 signaling pathway. Tyrosine phosphorylated STAT3 is induced to form a complex with P-TEFb via association with CDK-9. Abbreviations: IL-6Rα, IL-6 receptor-α; gp130, glycoprotein 130 (transducin); JAK, janus kinase.