Activation of Noncanonical NF-κB Signaling by the Oncoprotein Tio*

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NF-κB transcription factors are key regulators of cellular proliferation and frequently contribute to oncogenesis. The herpesviral oncoprotein Tio, which promotes growth transformation of human T cells in a recombinant herpesvirus saimiri background, potently induces canonical NF-κB signaling through membrane recruitment of the ubiquitin ligase tumor necrosis factor receptor-associated factor 6 (TRAF6). Here, we show that, in addition to Tio-TRAF6 interaction, the Tio-induced canonical NF-κB signal requires the presence of the regulatory subunit of the inhibitor of κB kinase (IKK) complex, NF-κB essential modulator (NEMO), and the activity of its key kinase, IKKβ, to up-regulate expression of endogenous cellular inhibitor of apoptosis 2 (cIAP2) and interleukin 8 (IL-8) proteins. Dependent on TRAF6 and NEMO, Tio enhances the expression of the noncanonical NF-κB proteins, p100 and RelB. Independent of TRAF6 and NEMO, Tio mediates stabilization of the noncanonical kinase, NF-κB-inducing kinase (NIK). Concomitantly, Tio induces efficient processing of the p100 precursor molecule to its active form, p52, as well as DNA binding of nuclear p52 and RelB. In human T cells transformed by infection with a Tio-recombinant virus, sustained expression of p100, RelB, and cIAP2 depends on IKKβ activity, yet processing to p52 remains largely unaffected by IKKβ inhibition. However, long term inhibition of IKKβ disrupts the continuous growth of the transformed cells and induces cell death. Hence, the Tio oncoprotein triggers noncanonical NF-κB signaling through NEMO-dependent up-regulation of p100 precursor and RelB, as well as through NEMO-independent generation of p52 effector.

The family of NF-κB transcription factors plays a pivotal role in a multitude of physiological and pathological processes, ranging from tissue homeostasis to inflammation and cancer. Exacerbated NF-κB activation has been associated with a wide range of diseases, including hematopoietic malignancies (1), and accompanies leukemia and lymphoma induced by human T cell leukemia virus type 1, Kaposis sarcoma-associated herpesvirus, and Epstein-Barr virus, respectively (2).

Commonly, a canonical or classical NF-κB pathway is distinguished from a noncanonical or alternative pathway. Canonical NF-κB signaling is induced in response to a wide variety of stimuli, including proinflammatory cytokines, T cell receptor engagement, and exposure of Toll-like receptors to microbial components. Intracellular signaling proceeds through a broad range of adaptor molecules, including members of the tumor necrosis factor receptor-associated factor (TRAF) family, especially TRAF2 and TRAF6. In addition to their adaptor function, these TRAFs possess an intrinsic ubiquitin E3-ligase activity that contributes to their crucial role in NF-κB activation. The point of convergence for canonical NF-κB signaling is the inhibitor of κB kinase (IKK) complex composed of two related kinases, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator (NEMO) or IκKγ. Mechanistically, polyubiquitin-mediated oligomerization of NEMO likely provides the platform for enzymatic activation of IKKα and, in particular, IKKβ. Major substrates of the activated IKK complex are the inhibitors of κB (IκBs). They are phosphorylated at two distinct serine residues and thereby marked for Lys48-linked ubiquitination and proteasomal degradation. This leads to the liberation and nuclear translocation of canonical NF-κB dimers, including the prototypical p50-p65 complex (p50-RelA). Noncanonical NF-κB activation is initiated through distinct tumor necrosis factor receptor superfamily members. Their stimulation inhibits the constitutive turnover of the NF-κB-inducing kinase (NIK). Subsequently, NIK-activated IKKα homodimers phosphorylate the NF-κB2 precursor molecule p100, which is associated with RelB. Phosphorylation induces ubiquitination and partial degradation of p100 to p52. Resulting p52-RelB NF-κB dimers translocate to the nucleus to activate gene transcription (3).

The two NF-κB pathways are generally assigned to different biological functions. Canonical signaling dominates early transcription of proinflammatory and antiapoptotic genes, although the delayed noncanonical responses govern cellular differentiation and organ development. This implies that the diverse NF-κB dimers activated by these pathways are involved in different transcriptional programs. However, although more than 100 NF-κB-regulated genes have been identified, their assignment to individual NF-κB dimers or pathways is still fragmentary. The poor amenability of this subject is based on the multitude of potential dimers, their interactions with divergent κB sites and other transcriptional regulators, and the dynamic

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2 The abbreviations used are: NF-κB, nuclear factor κ light chain enhancer of activated B cells; TRAF, tumor necrosis factor receptor-associated factor; IKK, inhibitor of κB kinase; NEMO, NF-κB essential modulator; IκB, inhibitor of κB; NIK, NF-κB-inducing kinase; SFK, Src family kinase; Tio, two in one; Hsp, heat shock protein; cIAP2, cellular inhibitor of apoptosis 2; PBL, peripheral blood lymphocyte; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LTR, long terminal repeat; IFN, interferon; ACHP, 2-amino-6-(2-(cyclopropylmethylamino)-6-hydroxypyridinyl)-4-(4-piperidinyl)-3-pyrindinecarbonitrile.
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control of NF-κB signaling (5). Another layer of complexity is added by the receptors triggering NF-κB activity; they usually elicit signaling to additional transcription factors as well as cross-talk between the canonical and noncanonical pathways (6). A prominent example for the dynamic reciprocal regulation is the induction of p100 (NF-κB2) and RelB, the regulator and effector molecules of the noncanonical pathway, by canonical NF-κB proteins (7, 8). Thus, addressing any of the biological NF-κB functions requires dissection of both the canonical and the noncanonical pathways.

Herpesvirus atelivirus induces T cell malignancies in New World primates other than its natural host, the spider monkey, and transforms simian T cells in culture (9). The closely related herpesvirus atelivirus infects other than its natural host, the spider monkey, and transforms simian T cells in culture (9), the closely related herpesvirus atelivirus infects primates other than its natural host, the spider monkey, and transforms simian T cells in culture (9). Its transforming potential depends on the presence of the oncogenes tip30 and tip11. The function of Tip30 is the induction of p100 (NF-κB2) and RelB, the regulator and effector molecules of the noncanonical pathway, by canonical NF-κB proteins (7, 8). Thus, addressing any of the biological NF-κB functions requires dissection of both the canonical and the noncanonical pathways.

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ELISA kit according to the manufacturer’s instructions (Bender MedSystems, BMS204). Results are presented as mean of three independent experiments ± S.D.

Flow Cytometry—For surface staining, cells were incubated with a phycoerythrin-Cy5-coupled CD14 antibody (ImmunoTools) in FCM buffer (phosphate-buffered saline; 5% fetal calf serum, 0.01% NaN₃) for 1 h, washed twice in FCM buffer, and then resuspended in phosphate-buffered saline for measurement. To determine viability after IKKβ inhibitor treatment, cells were harvested every 24 h and stained with 10 μg/ml propidium iodide. Flow cytometry was performed on a FACSCalibur™ (BD Biosciences).

Oligonucleotide Pulldown—For oligonucleotide pulldown experiments, nuclear extracts were prepared with the nuclear/cytosol fractionation kit (Biovision) according to the manufacturer’s protocol. Protein concentrations were determined with the Bio-Rad protein assay. Nuclear extracts (200 μg) were incubated with 50 pmol of 5’-biotinylated probe; controls for binding specificity were incubated additionally with 500 pmol of unbiotinylated competitor oligonucleotide. Pulldown conditions and buffers are described elsewhere (22). Probes were generated by annealing 5’-biotin-labeled oligonucleotides HIV-LTR-forward (5’-GGGACTTTCGGGACCTTCCGGGACTTTCC-3’) with HIV-LTR-reverse (5’-GGAAAGTCCCGGAAGAATCCCGGAAACCC-3’) and 5’-biotin-labeled IFNβ-forward (5’-GGGAAATCCGGGAATTCGGGAATTCCGGGGACTTTCC-3’) with IFNβ-reverse (5’-GGAAATTCGGGAAATTCGGGGACTTTCCGGGAATTCCGGGGACTTTCC-3’). Oligonucleotides were obtained from Biomers.

NF-κB Inhibitor Treatment—IKKβ inhibitor (AChP; Calbiochem) was added to 2 × 10⁵ Jurkat T cells 4 h after transfection at the indicated concentrations. Cells and supernatants were harvested and processed for luciferase assay or ELISA 48 h after transfection. Transformed PBLs were dispensed into 24-well plates at 2 × 10⁶ cells/well. Inhibitor was added at the indicated concentrations. For time-response measurements, fresh inhibitor was added every 24 h.

RESULTS

Tio Activates Endogenous NF-κB-Dependent Target Genes—The viral oncoprotein Tio was shown to activate NF-κB in dependence on TRAF6 interaction, but independent of SFK binding within a pcDNA3.1 background (19). To confirm the NF-κB-inducing capacity of Tio within pEF1 constructs, Jurkat T cells were co-transfected with an NF-κB-specific luciferase reporter (Fig. 1A). Wild-type Tio as well as the phosphorylation site mutant Y136F and the mutant defective in SFK binding (mSH3b) strongly induced NF-κB activity. In contrast, abrogation of TRAF6 binding rendered the mutant mT6b and the double mutant mT6b-mSH3b inert. We next investigated whether Tio affected the expression of the endogenous, NF-κB-regulated cIAP2 and IL-8 genes (23, 24). Compared with a vector-transfected control, Tio strongly induced cIAP2 expression. Induction was abrogated upon mutation of the TRAF6 binding site, whereas mutation of the NF-κB activation domain of Tio (Tyr136) phosphorylation sites resulted in an intermediate cIAP2 expression that correlated with reduced expression levels of these mutants (Fig. 1B). Tio-transfected cells secreted high amounts of IL-8. This effect was abolished by interruption of Tio-TRAF6 interaction and was independent of Tyr136 phosphorylation of Tio. However, secretion of IL-8 was also diminished when Tio was not able to interact with SFKs (Fig. 1C).

Tio-mediated NF-κB Activity Depends on NEMO—Because the IKK complex is a point of convergence for canonical NF-κB-inducing stimuli, we asked whether its regulatory subunit, NEMO, was required for Tio-mediated NF-κB activity. Parental NEMO-carrying (NEMO⁺) and NEMO-deficient (NEMO⁻) Jurkat T cells, both stably transduced with an NF-κB-driven CD14 reporter, were used. These cells were transfected with Tio and NEMO expression constructs and analyzed for CD14 surface expression by flow cytometry (Fig. 2A). In NEMO⁺ cells, expression of Tio led to an increase of CD14 on the cell surface, whereas overexpression of NEMO alone did not. After co-transfection of Tio and NEMO plasmids, expression of CD14 was comparable with
that in Tio-transfected cells (Fig. 2A, upper panel). In the NEMO− cell line, however, expression of Tio alone did not lead to increased CD14 surface expression, neither did transfection of a NEMO-encoding plasmid. But upon co-transfection of Tio and NEMO constructs, CD14 surface expression was restored through Tio relied on an intact canonical IKK complex.

Tio Induces p100/p52 Processing Independent of NEMO—After transfection of NEMO− cells with any of the Tio constructs, we observed an efficient processing of p100 to its active form, p52 (Fig. 3A, four left lanes). This induction depended on Tio-TRAF6 interaction but was independent of SFK association (four right lanes). Thus, up-regulation of p100 and RelB expression through Tio relied on an intact canonical IKK complex.

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FIGURE 2. Induction of an NF-κB-responsive CD14 reporter by pEF1-Tio in the presence or absence of NEMO. A, Jurkat T cells (NEMO− and NEMO−), stably transfected with an NF-κB-driven CD14 reporter, were transfected with Tio and NEMO expression constructs, alone or in combination. Vector-transfected cells served as a negative control. CD14 surface expression was detected 48 h after transfection with a phycoerythrin-Cy5-labeled CD14 antibody by flow cytometry. B, NEMO− Jurkat T cells were transfected with Tio or Tio mutants (mT6b, Y136F, mSH3b, mT6b-mSH3b) alone or in combination with a NEMO expression plasmid. Whole cell lysates were prepared 48 h after transfection and analyzed by immunoblotting for expression of endogenous cIAP2. Expression of FLAG-Tio and NEMO was verified. Hsp90α/β expression served as a loading control.

FIGURE 3. Processing of p100 to p52 and expression of endogenous RelB in presence and absence of NEMO. A, NEMO− Jurkat T cells were transfected with Tio or mutant expression plasmids. NEMO was reconstituted by co-transfection of a NEMO expression construct. Immunoblot analysis was performed 48 h after transfection for endogenous p100/p52 and RelB. Tio expression was confirmed with a FLAG antibody, and NEMO was detected by a specific antiserum. Hsp90α/β expression served as loading control. B, experiment analogous to A was performed in the absence of NEMO. A NEMO-transfected sample served as positive expression control.
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Noncanonical NF-κB Activation by the Tio Oncoprotein—To evaluate Tio-mediated activation of the noncanonical NF-κB pathway, we assessed Tio-induced nuclear localization and DNA binding capability of p52 and RelB in NEMO⁺ and NEMO⁻ Jurkat T cells. Fractionation experiments revealed that p52 and RelB efficiently translocated to the nucleus upon expression of Tio, independent of NEMO and, thus, independent of the transcriptional up-regulation of p100 and RelB (Fig. 4, input controls). Biotinylated oligonucleotides derived from the HIV LTR region and the IFNβ promoter, which both bind p52-RelB with high affinity (25), specifically precipitated Tio-induced nuclear p52 and RelB, even in the NEMO-negative context (Fig. 4).

Tio Induces Stabilization of NIK—The central regulator of noncanonical NF-κB activity is the NF-κB-inducing kinase, NIK. Constitutive turnover of NIK ensures quiescence of the alternative pathway in unstimulated cells. We therefore investigated whether endogenous NIK was stabilized in Jurkat T cells expressing Tio. Immunoprecipitated endogenous NIK could be detected only in the presence of Tio (Fig. 5). This stabilization of NIK was independent of Tio-TRAF6 and Tio-SFK interactions and could be observed in the presence as well as in the absence of NEMO (Fig. 5A). Next, expression of NIK was analyzed in PBLs transformed with a Tio-recombinant virus. Stabilization of endogenous NIK could be detected in three independent cell lines (1763 YYYY, 1765 YYYY, and 1766 YYYY) (Fig. 5B). Thus, Tio likely induces noncanonical NF-κB activity through stabilization of NIK.

IKKβ Inhibition Represses Tio-induced NF-κB Activity in Transformed Human T Cells—To address the influence of the NF-κB signal on the growth of virus-transformed PBL lines, we employed the IKKβ inhibitor, ACHP, a compound that inhibits growth of multiple myeloma cells and induces cell death in adult T cell leukemia cells (26–28). First, the efficacy of ACHP on Tio-mediated NF-κB signaling in Jurkat T cells was confirmed. To this end, Tio-expressing cells transfected with an NF-κB-specific reporter were treated with increasing concentrations of ACHP (Fig. 6). NF-κB reporter activity was strongly reduced by ACHP, even at the lowest concentration applied (Fig. 6A). Likewise, IL-8 secretion of these cells was diminished by more than 70% (Fig. 6B), whereas Tio expression remained unaffected (Fig. 6C). Hence, the IKKβ inhibitor ACHP efficiently counteracted Tio-induced NF-κB activity.

Subsequently, the virus-transformed PBL lines were treated with the ACHP inhibitor, and their viability was monitored (Fig. 7). In all virus-transformed cell lines treatment induced dose- and time-dependent cell death, whereas cell viability of an inhibitor-treated Jurkat control was not affected (Fig. 7A). After 4 days, 30–50% of transformed PBLs treated with 2.5 μM ACHP were still viable, whereas less than 10% had survived incubation with 10 μM ACHP (Fig. 7B). These results confirm the notion that Tio-induced canonical NF-κB signal is required to maintain the transformed phenotype of human PBLs.

To analyze NF-κB target gene expression, the transformed cell lines were treated with 2.5 μM ACHP for 48 h and then assessed for expression of p100/p52, RelB, and...
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FIGURE 6. Effects of IKKβ inhibition on Tio-induced NF-κB reporter activity and IL-8 expression in Jurkat T cells. Jurkat T cells were transfected with Tio expression plasmid and subsequently incubated with the indicated concentrations of IKKβ inhibitor, ACHP, starting 4 h after transfection. Cells were harvested 48 h after transfection. Three independent experiments were performed. A, co-transfection of an NF-κB reporter. NF-κB activity is depicted as relative response ratio with reference to the average of untreated Tio-transfected cells, vec, vector. B, determination of IL-8 levels in the supernatants by ELISA. C, protein expression of Tio verified using a FLAG-antibody. Detection of Hsp90α/β expression served as loading control.

cIAP2. Expression of the Tio oncogene and of Hsp90α/β confirmed cell viability under these conditions. IKKβ inhibitor treatment drastically reduced p100 expression levels in all three cell lines tested, whereas down-regulation of RelB and cIAP2 was less prominent, but nevertheless readily observed (Fig. 8A). In contrast to p100, the levels of p52 were only marginally affected by ACHP, resulting in a strong reduction of the p100/p52 expression ratio (Fig. 8B). Thus, p100 processing and hence noncanonical NF-κB signaling were still active when canonical signaling was inhibited in virus-transformed human T cells.

DISCUSSION

Previously we have reported that the viral oncoprotein Tio recruits TRAF6 and thereby activates NF-κB (19). The identification of NEMO as another essential cofactor now confirms that Tio utilizes the canonical pathway to induce NF-κB-regulated genes. Inhibition of IKKβ, the kinase assigned to canonical signaling, severely impaired the proliferation of human T cells transformed by a recombinant virus expressing Tio. Thus, NF-κB activation is required to maintain the permanent growth of these T cells. In analogy, the Tio-related oncoprotein StpC depends on its TRAF2 binding site to support viral transformation (12). These findings are in agreement with NF-κB representing a key pathological feature in various lymphoid malignancies, including lymphoproliferative disorders associated with human T cell leukemia virus type I, Kaposi sarcoma-associated herpesvirus, and Epstein-Barr virus (29).

In accordance with the activation of the canonical pathway, cellular target genes of Tio-induced NEMO-dependent NF-κB activity include p100 and RelB, the major NF-κB proteins of noncanonical signaling. In addition, Tio drives the processing of p100 to p52, a hallmark for the activation of this pathway. Processing is independent of NEMO expression and of the ability of Tio to bind TRAF6 or Src family kinases. Tio expression further results in the nuclear translocation and DNA binding activity of p52 and RelB. Thus, independent of canonical signaling, Tio triggers an additional, as yet unknown mechanism, including the stabilization of NIK, to activate the noncanonical pathway. The relevance of this feature for Tio-dependent viral growth transformation of human T cells remains to be established. An essential role of noncanonical signaling in T cell transformation by herpesvirus saimiri is suggested by abrogation of StpC-mediated NF-κB activation upon overexpression of kinase-inactive NIK (30). However, canonical and noncanonical NF-κB activity in StpC-expressing cells remains to be differentiated. In contrast, processing of p100 to p52 is established for the Tax protein of human T cell leukemia virus type I (31), vFLIP of Kaposi sarcoma-associated herpesvirus (32), and LMP1 of Epstein-Barr virus (33). Of note, the noncanonical pathway is constitutively activated by genetic mutations in multiple myeloma (34, 35) and likely contributes to Notch1-induced T cell leukemia (36). Although different mechanisms are employed, this conserved pattern suggests a functional relevance of noncanonical signaling in lymphoid malignancies in general.

Analyses as to the role of noncanonical signaling in oncogenesis require the discrimination from effects mediated by canonical NF-κB signaling. In the immune system, the canonical and noncanonical NF-κB pathways have been assigned to inflammatory and developmental stimuli, respectively. However, extensive cross-talk largely hampers the dissection of specific genetic programs (6). This is exemplified by p100, which is transcriptionally up-regulated in response to canonical signals; in its newly appreciated role as a functional IκB, p100 is resistant to canonical IKKβ signals but may be processed to release canonical p50/p65 dimers in response to noncanonical IκKα activation (37). Genetically modified mouse models were intensely studied to resolve the differential functions of NF-κB proteins and their pathway-specific regulators. They confirmed the complexity of immune regulation through NF-κB (38) and even revealed unexpected antiinflammatory effects in epithelial cells (39). Given the restricted amenability of this intricate
regulatory network to conventional methods, a systems biology approach was suggested to resolve the circuitry in NF-H9260B signaling (6). As a consequence, target genes that distinguish between individual NF-H9260B pathways or even dimers as well as their relevance for lymphoid malignancies remain to be defined.

NEMO-independent activation of the noncanonical NF-H9260B pathway by Tio was demonstrated by the induced binding of p52 and RelB to oligonucleotides derived from the HIV LTR and the IFN/H9252 promoter. However, the luciferase reporter gene we used did not respond to Tio expression in the absence of NEMO. A lack of binding to the sequences in the reporter is unlikely, given the broad spectrum of kB sites recognized by p52-RelB dimers (25). Rather, Tio-induced noncanonical dimers may bind DNA, but turn into activators only in cooperation with additional transcription factors and co-activators (5) that may not be recruited to the reporter construct. Promoter elements preferentially binding individual NF-kB dimers have been postulated (40), and a unique type of binding site has been described for p52-RelB dimers (41). However, more recent reports challenge the existence of differential NF-kB sites (25, 42). Taken together, specificity of gene regulation may not be determined by the sequence of the NF-kB binding motif within a promoter, but rather by flanking elements recruiting additional transcription factors and by interactions of the bound NF-kB dimers with other transcriptional regulators. Such a context-specific cofactor dependence would allow the integration of multiple upstream signals at a single promoter site. This model also accommodates the recently identified dualism of transcriptional activation by canonical NF-kB p65; one set of p65-regulated promoters depends on direct contact with the Mediator complex, whereas another set of promoters responds to p65 independent of Mediator recruitment (43). Further analyses into the NEMO-independent activation of p52 and RelB by Tio can be instrumental in defining transcriptional NF-kB specificity in the absence of any apparent cross-talk.

Noncanonical NF-kB signaling is initiated by membrane-bound receptors, but downstream events had hardly been defined. Only recently, stabilization of the constitutively active NIK has been identified as a mechanism to transduce signals for p100 processing (4, 44). Thereby, NIK represents the central regulator of the noncanonical p52/RelB pathway but can simultaneously relieve canonical dimers from inhibitory p100 (37). We now describe the viral oncoprotein Tio, an integral membrane protein, as an inducer of p52 and RelB DNA binding activity and a stabilizer of NIK. A Tio double mutant, devoid of interaction sites for TRAF6 and Src family kinases, supports this activity without triggering a canonical
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NF-κB signal. Thus, studying the mechanism of p52 generation as well as the composition of NF-κB dimers induced by Tio, and especially by the double mutant, promises further insights into the pathway of noncanonical NF-κB signaling.

In summary, like many lymphoid tumor cells, human T cells transformed to permanent growth by a recombinant herpesvirus express the viral oncoprotein Tio depend on the presence of active NF-κB. To provide this activity, Tio triggers not only the canonical pathway, but also a NEMO-dependent and a NEMO-independent route to noncanonical NF-κB signaling. Taken together, these findings establish Tio as a tool to study upstream mechanisms as well as target gene regulation by noncanonical NF-κB dimers that may be relevant in lymphoid malignancies.

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