Oncoprotein Suppression of Tumor Necrosis Factor-induced NFκB Activation Is Independent of Raf-controlled Pathways*

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Julie L. Hanson‡§, Vasiliki Anest‡§, Julie Reuther-Madrid‡§§, and Albert S. Baldwin‡§§**

From the ¶Lineberger Comprehensive Cancer Center, §Curriculum in Genetics and Molecular Biology, and the ¶¶Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7295

Extensive data indicate that the transcription factor NFκB is activated by signals downstream of oncoproteins such as Ras or breakpoint cluster region (BCR)-ABL. Consistent with this, evidence has been presented that NFκB activity is required for Ras and BCR-ABL to transform cells. However, it remains unclear whether these oncoproteins activate a full spectrum of NFκB-dependent gene expression or whether they may augment or interfere with other stimuli that activate NFκB. The data presented here indicate that BCR-ABL expression in 32D myeloid cells or oncogenic Ras expression in murine fibroblasts blocks the ability of tumor necrosis factor (TNF) to activate NFκB. This suppression of NFκB is manifested by an inhibition of TNF-induced inhibitor of NFκB (IKK) activity and NFκB DNA binding potential but not by blocking TNF-induced nuclear accumulation of NFκB/p65. The inhibition of NFκB is not observed in oncogenic Raf-expressing cells and is not fully restored by the suppression of PI3-kinase or MEK pathways. Oncogenic Ras suppresses the ability of TNF to activate the expression of NFκB-dependent genes, such as iNOS (inducible nitric oxide synthase) and RANTES (regulated on activation normal T-cell expressed and secreted). These studies suggest that the ability of Ras and BCR-ABL to activate NFκB involves an uncharacterized pathway that does not involve classic IKK activity and that suppresses the TNF-induced IKK pathway through a Raf/MEK/Erk-independent mechanism.

Mechanisms controlling gene-specific transcription downstream of oncoprotein-dependent signaling are poorly understood. Although certain transcription factors, including Ets and NFκB proteins, have been shown to be required for transformation in response to oncprotein expression (1–3), it is unclear whether transforming proteins elicit a full transcription factor-dependent response or whether a limited set of transcription factor-regulated genes are induced to control transformation. In fact, based on the broad range of genes regulated by NFκB, it has been hypothesized that its role in oncogenic transformation would involve induction of a limited set of genes to prevent an effective immunological response against neoplastic cells (4).

Small GTPases of the Ras family are important signaling molecules in the regulation of a variety of cellular processes, including growth, differentiation, and survival (5). Growth factors and other external stimuli lead to transient activation of Ras; however, mutations in Ras alleles, which occur in ~30% of human tumors, result in a constitutively active protein (6). Active, GTP-bound Ras signals to a variety of downstream effector pathways. The three most extensively characterized Ras effectors are Raf kinase, phosphatidylinositol 3-kinase (PI3-kinase), and Rap1GDS (7, 8). Activation of Ras and its downstream signal transduction cascades ultimately leads to activation of transcription factors involved in proliferation, differentiation, and apoptosis. Ras also functions to control downstream signaling of other oncoproteins, such as the fusion protein breakpoint cluster region (BCR)-ABL that is associated with acute lymphoblastic and chronic myelogenous leukemia (9–13). Work from our laboratory and others has demonstrated that the transcription factor NFκB is activated by Ras (3, 14–16) and by BCR-ABL in a Ras-dependent manner (17). Inhibition of NFκB blocks Ras-induced transformation in vitro (3) and BCR-ABL-induced tumorigenesis (17).

NFκB is a family of dimeric transcription factors involved in immune and inflammatory responses, cellular growth, differentiation, and apoptosis (18). There are five mammalian NFκB family members: RelA/p65, RelB, c-Rel, p50/p105, and p52/p100. All members share homology in a 300-amino acid region called the Rel homology domain. The Rel homology domain is important for dimerization, nuclear translocation, DNA binding, and binding to the inhibitor of κ B (IκB) family of proteins (18). In unstimulated cells, IκB proteins localize NFκB dimers in the cytoplasm by masking the nuclear localization sequence of NFκB. Activation of NFκB can occur through various stimuli, including cytokine stimulation, bacterial and viral infection, and oncogenic signals (18). Receptor activation of TNFR1, IL-1R, and various Toll-like receptors initiates signal transduction cascades ultimately leading to activation of the IκB kinase (IKK) complex. The IKK complex is composed of three subunits, IKKα, IKKβ, and IKKγ (18). Whereas IKKγ is a regulatory subunit, both IKKα and IKKβ have inducible catalytic activity. Upon activation, the IKK complex phosphorylates IκBα and IκBβ at specific serine residues, which targets IκB for ubiquitination and subsequent degradation by a proteasome-dependent pathway. Once IκB is degraded, the nuclear localization sequence of NFκB is unmasked, allowing nuclear accumulation, DNA binding, and transcriptional activation of target genes (18).

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** To whom correspondence should be addressed. Tel.: 919-966-3652; Fax: 919-966-0444; E-mail: jhall@med.unc.edu.

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Although nuclear accumulation is an important step in NF-κB activation, post-translational modifications on p65 are proposed to be necessary for the transcriptional competence of nuclear NF-κB. For example, phosphorylation of p65 on serine 276 is required for stable interactions with the transcriptional coactivator CBP and to stimulate transcriptional activation of NF-κB target genes (19, 20). Other sites of phosphorylation have also been described that may contribute to the inherent transcriptional activity of NF-κB (21–23). Evidence has also been presented that Akt, which functions downstream of PI3-kinase, can control the transcriptional activation function of the p65 NF-κB subunit through a mechanism dependent on IKK function but in a manner which does promote enhanced DNA binding potential (24, 25). In addition, a number of oncogenes activate NF-κB by increasing transcriptional activation function (26). Consistent with this, we have shown that both oncogenic Ras and Raf activate an NF-κB-dependent reporter gene in mouse fibroblasts without stimulating enhanced DNA binding of NF-κB subunits (3, 14). Similarly, previous work suggests that BCR-ABL activates an NF-κB-dependent reporter but in a manner that did not promote strong DNA binding activity (17).

Here we investigate the ability of BCR-ABL and Ras to modulate cytokine-induced NF-κB-dependent responses. Our studies indicate that in murine myeloid cells and fibroblasts, BCR-ABL and Ras, respectively, strongly suppress TNF-induced NF-κB activation by blocking both IKK activity and DNA binding. Inhibition of the PI3-kinase and MEK/extra-cellular signal-regulated kinase (Erk) pathways could not fully overcome the Ras-induced block on NF-κB activation. Additionally, oncogenic Ras suppresses TNF-induced activation of the NF-κB-dependent genes iNOS and RANTES. These studies suggest that the ability of Ras and BCR-ABL to activate NF-κB involves an uncharacterized pathway that does not involve classic IKK activity and that suppresses the TNF-induced IKK pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Spontaneously immortalized mouse embryo fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cells stably expressing a constitutively active form of H-Ras, H-RasV12, or vector control were obtained by transfection of pZIP-H-RasV12 or pZIPneo with Polyfect (Quiagen). Cells expressing H-RasV12 were selected in medium containing 500 μg/ml G418. Surviving cells were pooled 2 weeks later. NIH 3T3 control and Raf-expressing cells were described previously (3). 32D myeloid cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% Wehi-conditioned medium, and penicillin-streptomycin. Generation of 32D cells expressing BCR-ABL fusion protein p185 was described previously (17). Cells were treated with 10 ng/ml TNF (Promega), 5 ng/ml IL-1β (Sigma), 20 μM PD98059 (Alexis Pharmaceuticals), or 10 μM LY294002 (Alexis Pharmaceuticals) as indicated.

EMSAs and Western Blot Analysis—Preparation of nuclear and cytoplasmic extracts and subsequent electrophoretic mobility shift assays (EMSAs) were performed as previously described (27). Briefly, nuclear extracts were prepared following TNF stimulation and incubated with an α-32P-labeled DNA probe containing an NF-κB consensus from the major histocompatibility complex class I promoter. For supershifts, 1 μl of antibody (described below) was incubated with protein-DNA complexes for 10 min prior to loading gel. DNA-protein complexes were separated on a 5% non-denaturing polyacrylamide gel. The gel was dried, and DNA-protein complexes were visualized by autoradiography. Western blot analysis was performed by preparing nuclear, cytoplasmic, or whole cell extracts and separating proteins by SDS-PAGE. After transferring the separated proteins to nitrocellulose the blots were blocked with 5% milk and then incubated in primary antibody (IκBα, c-Rel, RelB, p50, and p52 (Santa Cruz Biotechnology), p65 (Rockland), phospho-IκBα Ser-32/36, phospho-c-Jun Ser-73, and phospho-Akt Ser-473 (Cell Signalling), phospho-p42/p44 Thr-202/Tyr-204 (New England Biolabs)) for either 1–2 h or overnight. The membranes were then washed in TBST and incubated for 1 h in anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Promega) and washed again. Protein bands were visualized with enhanced chemiluminescence detection (Amersham Biosciences).

Kinase Assay—500 μg of whole cell extracts prepared from cells as previously described (28) was incubated with 25 μl of IKKγ antibody (Upstate) overnight at 4 °C. Protein A-Sepharose beads washed in whole cell extract buffer were added and incubated for 1–2 h at 4 °C. The beads were then washed three times in 1 ml of pulldown buffer and one time in assay dilution buffer (ADB). Next, the beads were incubated at 30 °C for 30 min in 25 μl of 1× ADB plus 4 μg of GST-TxRα, MgCl2, and ATP. 30 μl of 2× sample buffer was added, and the proteins were separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose, and Western blotting for phospho-IκBα serine 32/36 (New England Biolabs) was performed.

Luciferase Assays—Control and H-RasV12-transformed cells were plated at 2 x 104 cells/well in a 24-well plate. 24 h later 100 ng of pGL4-xβ-luciferase, 200 ng of β-actin Lazu, and 200 ng of empty vector or 100 ng of Gal4-luciferase, 50 ng of Gal4-p54, and 350 ng of empty vector DNA was transfected into the cells with Polyfect (Qiagen) according to the manufacturer's protocol. 24 h post-transfection the cells were harvested in 100 μl of M-PER buffer (PIERC) and assayed for luciferase and β-galactosidase activity. All data are represented as luciferase units/β-galactosidase units.

Ribonuclease Protection Assay—Ribonuclease protection assays were performed on a custom template according to the manufacturer's protocol (BD PharMingen). Briefly, 20 μg of total RNA, isolated with Trizol (Invitrogen), was hybridized overnight to biotinylated RNA probes. Unhybridized RNA and biotinylated probes were then digested with RNase A. The protected RNA complexes were then precipitated and resolved on a 4.75% polyacrylamide area sequencing gel. The complexes were then transferred to a nitrocellulose membrane. After UV cross-linking, the membrane was incubated with streptavidin-horseradish peroxidase. Protected complexes were visualized by autoradiography after incubation with luminal/enhancer solution.

RESULTS

BCR-ABL and H-RasV12 Expression Inhibits TNF−induced NFκB Activation—Previous reports indicate that oncogenic Ras and BCR-ABL can activate NFκB transactivation function independent of enhanced DNA binding. However, little is known about how oncoprotein-induced signaling may affect the ability of other stimuli to activate NFκB. To determine whether oncogenic Ras or BCR-ABL have an effect on TNF-induced NFκB activation, immortalized MEFs transformed with H-RasV12 or 32D cells expressing the p185 BCR-ABL fusion protein were examined. Transformed cells (H-Ras or BCR-ABL-expressing) and control cells (MEFs or 32D cells) were stimulated with TNF for 15, 30, or 60 min. Nuclear extracts were prepared, and NFκB DNA binding activity was determined by EMSA. As expected, TNF rapidly induces NFκB DNA binding activity by 15 min in control cells; however, this activation is greatly reduced in both H-RasV12-transformed MEFs (Fig. 1A) and BCR-ABL-expressing 32D cells (Fig. 1B). Super-shift analysis of the MEF control 30' time point revealed that the NFκB DNA binding complex I contains p65 and p50 subunits, while complex II is shifted by the c-Rel antibody (Fig. 1A, right panel).

BCR-ABL transformation is dependent on its kinase function and on its ability to utilize Ras-dependent downstream signaling (9–13). We have previously published that BCR-ABL-induced NFκB activation is dependent on BCR-ABL-induced Ras activation (17). Therefore, in the remaining experiments, we have focused largely on the ability of oncogenic Ras to modulate TNF-induced NFκB activation.

H-RasV12 Blocks TNF-induced IKK Activity—To determine the mechanism by which H-RasV12 inhibits TNF-induced NFκB activation, IκBα phosphorylation and degradation were examined. Control and Ras-transformed MEFs were treated with 10 ng/ml TNF for 5, 15, 30, 45, and 60 min, and whole cell extracts were prepared. These extracts were analyzed for the induction of phosphorylation and degradation of IκBα. As ob-
served in Fig. 2A, Ras-transformed cells exhibit reduced levels of IκBα phosphorylation at serines 32 and 36 in response to TNF treatment. Consistent with this result, Ras-transformed MEFs exhibit significantly reduced IκBα degradation (Fig. 2A, middle panel). Similar results were also obtained in BCR-ABL-transformed myeloid cells (data not shown). To determine whether other downstream signaling components associated with TNF receptor activation are affected in Ras-transformed cells, we examined the phosphorylation status of c-Jun. In control MEFs, TNF-inducible phosphorylated c-Jun levels were detected. Although phosphorylated c-Jun levels are higher in Ras-transformed cells, TNF stimulation further increases...
phosphorylated c-Jun (Fig. 2A, lower panel). These data suggest the TNF-induced c-Jun NH$_2$-terminal kinase signaling pathway is not affected in Ras-transformed MEFs.

Because IxBa phosphorylation and subsequent degradation are controlled by IKK, the activity of IKK was examined in control and Ras-transformed MEFs. IKK assays were performed by treating MEFs with TNF for the indicated times, harvesting whole cell extracts, and immunoprecipitating the IKK complex with an antibody to IKK$\gamma$. The immunoprecipitated complex was then incubated with GST-IxBa as a substrate in an in vitro kinase assay. The proteins were separated by SDS-PAGE, and Western blot analysis for phospho-IxBa Ser-32/36 was performed. As expected, TNF rapidly activates the IKK complex in control MEFs, whereas no activation of the complex by TNF is observed in Ras-transformed MEFs (Fig. 2B). Interestingly, in Western blot analysis of whole cell extracts with antibodies to phospho-IxBa Ser-32/36 (Fig. 2A), minimal phosphorylation is detected in the H-RasV12-transformed MEFs, whereas the results from the kinase assay suggest that IKK activation was completely blocked (Fig. 2B). One possible explanation is that the IKK assay may not be as sensitive as protein immunoblotting for phosphorylated IxBa. Alternatively, it is possible that another IxB kinase activity, not associated with IKK$\gamma$, is activated by TNF and is able to phosphorylate IxBa in vivo.

Currently, it is proposed that IxBa degradation controls the ability of NFkB to accumulate in the nucleus following cytokine treatment. Therefore we examined whether the inhibition of NFkB in BCR-ABL- and Ras-transformed cells results in a defect in nuclear accumulation of NFkB subunits. Whereas TNF induces nuclear accumulation of p65 in 32D cells, nuclear levels of p65 are very high in unstimulated BCR-ABL-transformed 32D cells and remain high following TNF treatment (Fig. 2C, upper panel). Unexpectedly, the ability of TNF to stimulate nuclear accumulation of the p65 subunit appears unaffected in Ras-transformed cells, whereas nuclear accumulation of c-Rel is blocked (Fig. 2D, lower panel). Nuclear levels of RelB and p52 are unaffected by TNF treatment in control and Ras-transformed MEFs. Overall, these data indicate that IKK-directed IxBa phosphorylation is not required for nuclear accumulation of p65 but is required for optimal NFkB DNA binding activity and gene expression. Alternatively, expression of Ras may bypass this requirement. Notably, basal levels of nuclear NFkB p65 subunit are modestly elevated in Ras-transformed cells and even higher in BCR-ABL-transformed 32D cells, suggesting that in the absence of external stimuli, oncprotein-induced signaling pathways promote the nuclear accumulation of the p65 subunit. Together these data indicate that oncogenic Ras and BCR-ABL do not inhibit TNF-induced nuclear translocation of p65; however, H-RasV12 does inhibit TNF-induced c-Rel nuclear translocation.

H-RasV12 Does Not Inhibit IL-1$\beta$-induced NFkB Activation—A variety of different cytokines are known to activate NFkB. To determine whether oncogenic Ras inhibits NFkB activation by other cytokines, control and H-RasV12-transformed MEFs were treated with 5 ng/ml IL-1$\beta$ for 5, 10, 15, or 30 min. Nuclear and cytoplasmic extracts were prepared and analyzed by EMSA and Western blot, respectively. EMSA analysis revealed no defect in IL-1$\beta$-induced NFkB DNA binding activity (Fig. 3, upper panel). Furthermore, IL-1$\beta$-induced IxBa phosphorylation is equivalent in control and H-RasV12-transformed MEFs (Fig. 3, lower panel). These data suggest that oncogenic Ras specifically inhibits TNF-induced NFkB activation but not IL-1$\beta$-induced NFkB activation.

The signaling components involved in TNF and IL-1$\beta$ receptor activation are well established. Although activation of both receptors results in NFkB activation, the signaling molecules that lead to IKK activation are different for these two pathways (29). The differences in these signaling pathways suggest that oncogenic Ras may inhibit a component of the TNF pathway that does not overlap with the IL-1$\beta$ pathway. However, Western blot analysis for TNF signaling components (TNFR1, TRADD, TRAF2, RIP, MEKK3, IKK$\alpha$, IKK$\beta$, and IKK$\gamma$) shows no changes in protein levels between control and Ras-transformed MEFs (data not shown).

Inhibition of TNF-induced NFkB Activation Requires Multiple Ras Effector Pathways—Ras proteins activate a variety of downstream effectors that ultimately lead to activation of transcription factors involved in cellular growth control. The most extensively studied effector pathways of Ras are controlled by PI3 kinase, Raf, and RasGEFs (7, 8). A recent study revealed that normal rat kidney cells expressing an inducible, constitutively active Raf-1 suppressed TNF- and IL-1$\beta$-induced NFkB activation (30), suggesting that Ras inhibition of TNF signaling could be because of the ability of Ras to activate the Raf-1 kinase pathway. To determine whether the effect observed in Ras-transformed murine fibroblasts is due solely to activated Raf, control and Raf-transformed 3T3 cells were treated with TNF, and NFkB DNA binding potential was analyzed by EMSA (Fig. 4). This experiment demonstrates that NFkB DNA binding is not impaired in Raf-transformed 3T3 cells. In fact, the expression of Raf appears to augment the ability of TNF to activate NFkB as measured by EMSA. These data show Ras activation of Raf is not sufficient to inhibit TNF-induced NFkB DNA binding.

To further analyze Ras signaling pathways involved in NFkB inhibition, pharmacological inhibitors of the MEK/Erk and PI3 kinase pathways, PD98059 (PD) and LY294002 (LY), respectively, were used. Cytoplasmic and nuclear extracts of the treated control and Ras-transformed cells were prepared to determine whether the inhibitors have an effect on TNF-induced NFkB DNA binding potential. In control cells the inhibitors have no effect on NFkB DNA binding potential (Fig. 4, B and C, lanes 1–3). Furthermore, the PD and LY compounds alone or in combination (data not shown) have minimal effects on NFkB DNA binding potential in Ras-transformed fibroblasts (Fig. 4, B and C, lanes 10–12). Western blot analysis of cytoplasmic extracts for phospho-p42/p44 clearly demonstrates the effectiveness of the PD compound (Fig. 4B, lower panel). Immunoblotting for phospho-Akt demonstrates that the LY compound is capable of inhibiting TNF-induced phosphorylation of Akt in control MEFs and decreasing basal levels of phospho-Akt in Ras-transformed MEFs. The LY compound, however, does not completely block TNF-induced phosphorylation of Akt (Fig. 4C, lower panel). A slight increase in TNF-induced NFkB DNA binding in Ras-transformed MEFs treated with the LY compound is observed, and further investigation is needed to determine whether complete inhibition of TNF-induced phosphorylation of Akt would further enhance TNF-induced NFkB DNA binding activity in Ras-transformed MEFs. These data demon-
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Previously, we and others have shown that oncogenic Ras as well as other oncoproteins (such as BCR-ABL) activate an NFκB-dependent reporter (3, 14, 16, 17). Consistent with previous reports, Ras functionally activates NFκB in an experimental system. Control and H-RasV12-transformed MEFs were transfected with either a reporter construct containing four NFκB sites upstream of the luciferase gene or Gal4-luciferase along with a Gal4-p65 fusion protein. H-RasV12-transformed MEFs exhibited enhanced luciferase activity of both the NFκB reporter and Gal4-p65, demonstrating H-RasV12 activation of NFκB transcriptional activity (Fig. 5B).

DISCUSSION

This study examines the role of two oncoproteins, oncogenic H-Ras and BCR-ABL, in their ability to regulate the transcription factor NFκB and NFκB-dependent gene expression under TNF stimulation. Previously, we have shown that BCR-ABL expression leads to the activation of an NFκB-dependent promoter and that BCR-ABL-induced tumorigenesis requires NFκB (17). Additionally, we and others have shown that oncogenic Ras activates NFκB functional activity and that Ras-induced transformation requires NFκB (3, 15, 16). However, it remains unclear whether the activation of NFκB by these oncoproteins leads to an NFκB-dependent response similar to that induced by cytokines or whether oncogene expression can alter the NFκB activation pathway induced by cytokines such as TNF. This study reveals that oncogenic H-Ras blocks TNF-induced NFκB DNA binding activity (Fig. 1A) and gene expression (Fig. 5B) in murine fibroblasts. Additionally, an inhibition of TNF-induced NFκB DNA binding activity in BCR-ABL-transformed 32D myeloid cells is observed (Fig. 1B).

The data presented here indicate that the primary inhibition of TNF-induced NFκB activation in Ras-transformed MEFs is through the suppression of IKK activity (Fig. 2B), blocking TNF-induced IκBα phosphorylation and degradation (Fig. 2A). Importantly, Ras-transformed MEFs are not inhibited for IL-1β-induced NFκB activation (Fig. 3). These data suggest that the level of inhibition occurs on a component of the TNF-induced signaling pathway that leads to IKK activation. However, Western blot analysis for TNF signaling components (TNFFR1, TRADD, TRAF2, MEKK3, RIP, IKKα, IKKβ, and IKKγ) demonstrated that there is no difference in levels of these proteins between control and H-RasV12-transformed MEFs (data not shown). Ras or other oncogenic signals may directly inhibit IKK activity by inducing a factor that binds to and inhibits IKK. For example, RKIP (Raf kinase inhibitory protein) has been shown to bind to IKK and inhibit TNF-induced NFκB activation (31). However, an increase in RKIP protein levels or interaction between RKIP and IKKα or IKKβ is not observed in Ras-transformed cells. Additionally, oncogenic signaling may inhibit recruitment of adaptor molecules to the TNF receptor. Future studies are directed toward further elucidating the mechanism of Ras-induced IKK inhibition following cytokine exposure of fibroblasts.

TNF-induced NFκB activation is characterized by IKK-directed IκBα phosphorylation, degradation, and subsequent nuclear translocation of NFκB heterodimers (18). Our study demonstrates that although TNF-induced IκBα phosphorylation...
and degradation are inhibited in Ras-transformed MEFs, inducible p65 nuclear translocation is unaffected (Fig. 2C). Several possibilities may explain this observation. First, TNF may target NFκB to the nucleus independent of IκBα degradation. Second, the p65 that translocates into the nucleus in Ras-transformed cells may not be associated with IκB proteins, and the suppression of DNA binding could reflect an alternative, Ras-induced nuclear mechanism that functions downstream of the release of p65. Finally, this effect could be specific for Ras-transformed cells and may not reflect mechanisms associated with non-transformed cell types. Additional mechanisms of inhibition on nuclear NFκB could include effects on post-translational modifications of NFκB subunits. Although phosphorylation of p65 on serines 276 (20) and 529 (21) has not been shown to be important for DNA binding function, others have shown that phosphorylation of p65 and p50 enhances DNA binding (32–34). Additionally, acetylation of p65 has been shown to both enhance and inhibit DNA binding (35, 36). Alternatively, oncoproteins may induce factors that interact with NFκB and inhibit DNA binding or transactivation function. A recent study (37) demonstrates that the basic helix-loop-helix proteins Twist-1 and -2 interact with p65 and inhibit TNF-induced transactivation of NFκB.

We have also analyzed whether Ras effectors contribute to NFκB inhibition. Although a previous report indicated that induction of Raf-1 expression in normal rat kidney cells blocked the ability of cytokines to activate NFκB (30), our work indicates that Raf expression in NIH3T3 cells does not block NFκB activation in response to TNF treatment and may actually augment this response (Fig. 4A). Consistent with this, inhibition of the signaling pathway downstream of Raf does not overcome the Ras-induced block on NFκB activation (Fig. 3B). Furthermore, inhibition of another Ras effector pathway controlled by PI3-kinase does not completely revert the Ras-induced inhibition (Fig. 4C). Other Ras effector proteins have been identified, and it will be important to determine which of these pathways suppresses NFκB activation in murine fibroblasts and myeloid cells.

Although we did not detect an increase in IKK activity in Ras-transformed MEFs (Fig. 2B), IKK activation was shown to be required for Ras- and Raf-induced NFκB activation in rat liver epithelial cells (RLE) (16). Additionally, Ras- and Raf-transformed RLE cells exhibit significantly higher NFκB DNA binding activity than the parental, non-transformed cells (16). Therefore, the mechanism of Ras-induced NFκB activation could be different in RLE cells versus MEFs. In support of this hypothesis, a recent report (38) showed little to no activation of IKKα or IKKβ in H-RasV12-transformed 3T3 fibroblasts. In the data presented here, the immunoprecipitated IKK complex may differ from the complex immunoprecipitated by Arsur et al. (16). Individual IKK subunits (IKKα and IKKβ) were immunoprecipitated in the RLE cells, whereas the IKK complex associated with IKKγ was immunoprecipitated in this study. It remains possible that the IKK complex, or a qualitatively distinct form of IKK, is involved in Ras-induced NFκB activation in murine fibroblasts. Therefore, the possibility that oncogenic Ras can suppress NFκB at a different signaling or functional juncture in RLE cells as compared with MEFs is of current interest.

How does oncogenic Ras and BCR-ABL activate NFκB while simultaneously suppressing the cytokine-induced pathway? Previously we have shown that inducible activation of oncogenic Ras in Rat-1 fibroblasts leads to a transient activation of NFκB as measured by EMSA (39). It is likely that this response involves IKK based on the inducible increase in NFκB DNA binding activity. However, we have previously published (3) and show here that Ras-transformed murine fibroblasts do not exhibit enhanced NFκB DNA binding activity (Fig. 1), and reporter assays demonstrate that NFκB functional activity is elevated in Ras- and BCR-ABL-transformed cells. Further-

![Image](https://example.com/image.png)

**Fig. 5**. NFκB-dependent gene expression is inhibited in H-RasV12-transformed MEFs. A, 20 μg of total RNA from control and H-RasV12-transformed and p65-/− MEFs treated with TNF for 0, 1, 2, 4 h was analyzed by ribonuclease protection assay as described under “Experimental Procedures.” B, control and H-Ras-transformed MEFs were transfected with plasmids for pGL4-p65 and Gal4-luciferase (left panel) or pGL4-p65 and Gal4-luciferase (right panel). 24 h post-transfection the cells were harvested and assayed for luciferase activity. Data are represented as relative luciferase units (luciferase units/β-galactosidase units).
more, nuclear levels of p65 appear elevated in Ras-transformed cells and are clearly elevated in BCR-ABL cells (Fig. 2, D and C, respectively). These observations raise at least two potential possibilities: (i) the nuclear p65 found in Ras- and BCR-ABL-transformed cells may not be a component of a heterodimeric complex capable of binding to the consensus NF\(\kappa\)B probe, and/or (ii) Ras- and BCR-ABL-induced signals, while promoting IKK-independent nuclear accumulation, target pre-existing nuclear NF\(\kappa\)B to enhance the transactivation potential of the p65 NF\(\kappa\)B subunit. Consistent with this latter point, we have observed that both oncogenic Ras and BCR-ABL activate a Gal4 fusion with the transcriptional activation domain of p65 (3, 17).

It has been hypothesized that because of the role of NF\(\kappa\)B in protecting cells from Ras-induced apoptosis (15), expression of antiapoptotic genes, such as cIAP1 and -2, TRAF1 and -2, XIAP, IEX-1, A1/Bfl-1, or Bel-2, would be controlled by NF\(\kappa\)B in Ras-transformed cells (40). NF\(\kappa\)B has also been shown to be involved in the regulation of cell cycle proteins; therefore, another potential NF\(\kappa\)B-regulated gene in response to oncogenic Ras may be cyclin D1. Although we do observe an increase in Bel-2 and cyclin D1 mRNA levels in Ras-transformed cells, further studies are required to determine the role that NF\(\kappa\)B may play in regulating these genes in a manner induced by Ras expression. Longer term studies will be required to determine whether the form of NF\(\kappa\)B that is activated by oncogenic Ras and potentially by other oncoproteins is qualitatively or functionally distinct from that induced by cytokines.

The data presented here support a model in which Ras transformation in murine fibroblasts and BCR-ABL transformation in myeloid cells initiate an uncharacterized signal transduction pathway that activates NF\(\kappa\)B in a manner distinct from the TFN-induced NF\(\kappa\)B activation pathway (Fig. 6). Although we do not rule out the possibility that the IKKs are involved in oncogenic transformation, activation of NF\(\kappa\)B in Ras-transformed MEFs does not involve IKK-directed phosphorylation of IkBa. Additionally, H-RasV12 and BCR-ABL expression suppresses the ability of TNF to activate NF\(\kappa\)B (Fig. 6). Oncogenic Ras suppresses TNF-induced NF\(\kappa\)B activation at two levels: inhibition of IKK activity and NF\(\kappa\)B DNA binding, resulting in the inhibition of TNF-induced NF\(\kappa\)B-dependent gene expression (Fig. 6).

Collectively, this study highlights unique roles for oncoproteins in differentially regulating NF\(\kappa\)B-dependent genes. Whereas oncogenic Ras increases the transactivation potential of NF\(\kappa\)B in the absence of external stimuli, it suppresses TNF-induced NF\(\kappa\)B activation. We suggest that oncogenic suppression of cytokine-induced NF\(\kappa\)B activation may be important for neoplastic cells to evade TNF-induced host immune response. Consistent with an altered function of oncogenic Ras alleles, it has been recently published (41) that MEFs stably overexpressing wild-type H-Ras are not inhibited for TNF-induced NF\(\kappa\)B activation. One way in which tumor cells can evade the host immune detection is through the down-regulation of major histocompatibility complex (MHC) class I molecules (42, 43). It has been previously reported that oncogenic Ras can inhibit MHC class I expression, leading to an enhanced metastatic phenotype (44, 45). Interestingly, NF\(\kappa\)B has been reported to play a role in MHC class I gene expression (46). Additionally, the ability of NF\(\kappa\)B to induce cytokines and chemokines may lead to an enhanced immunological response against transformed cells. Thus, suppression of one arm of the NF\(\kappa\)B pathway may provide an advantage toward the successful growth of tumor cells.

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