Dbi and the Rho GTPases Activate NFκB by IκB Kinase (IKK)-dependent and IKK-independent Pathways*

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Marta S. Cammarano and Audrey Minden‡

From the Department of Biological Sciences, Columbia University, New York, New York 10027

Dbi is a guanine nucleotide exchange factor that activates the Rho family GTPases Cdc42, Rac, and Rho. Dbi and all three GTPases are strong activators of transcription factor NFκB, which has been shown to have an important role in Dbi-induced oncogenic transformation. Here we show that although Dbi activation of NFκB requires Cdc42, Rac, and Rho, the different GTPases activate NFκB by different mechanisms. Whereas Rac stimulates the activity of the IκB kinase IKKβ, Cdc42 and Rac activate NFκB without activating either IKKα or IKKβ. Like Dbi, Rac activation of IKKβ is mediated by the serine/threonine kinases NIK but not MEKK. This differs from Rac activation of the JNK pathway, which was previously shown to be mediated by MEKK. The pathway leading from Rho and Cdc42 to NFκB is more elusive, but our results suggest that it involves an IKKα/IKKβ-independent mechanism. Finally, we show that the signaling enzymes that mediate NFκB activation by Dbi and the Rho GTPases are also necessary for malignant transformation induced by oncogenic Dbi.

The Rho family of GTPases, including members of the Cdc42, Rac, and Rho subfamilies, function as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state (1). Guanine nucleotide exchange factors (GEFs) catalyze the activation of the GTPases by exchanging GDP for GTP. Dbi is a GEF that acts both in vivo and in vitro as an exchange factor for Cdc42, Rho, and Rac (2, 3). Dbi contains a Dbl homology domain that is required for GEF activity (4) adjacent to a pleckstrin homology domain that is most likely responsible for proper localization at the membrane (5). Dbi is a representative prototype of a growing family of proto-oncogenes that contain Dbl homology/pleckstrin homology elements. Activated forms of the Dbl family members are associated with a variety of neoplastic pathologies (2, 3, 6). It is generally thought that the activation of Rho family GTPases may be responsible for their potent transformation capabilities.

† To whom correspondence should be addressed: Dept. of Biological Sciences MC 2460, Columbia University, Sherman Fairchild Center, Rm. 813, 1212 Amsterdam Ave., New York, NY 10027. Tel.: 212-854-5632; Fax: 212-856-8246; E-mail: agm24@columbia.edu.

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* The abbreviations used are: GEF, guanine nucleotide exchange factor; IKK, IκB kinase; NIK, NFκB-inducing kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; JNK, c-Jun N-terminal kinase; IKKα, IKKβ, NFκB, nuclear transcription factor-κB; TNF, tumor necrosis factor; PAK, p21-activated kinase; HA, hemagglutinin; GST, glutathione S-transferase; Luc, luciferase.

Indeed, Cdc42, Rac, and Rho were each shown to contribute to distinct aspects of Dbi-induced transformation (7). Rho proteins have also been shown to be necessary for transformation by other oncogenes including Ras (8–12).

The Rho family GTPases were originally identified as proteins that have important roles in regulating the organization of the actin cytoskeleton and the formation of focal adhesions (13–18). Later the GTPases were also found to activate signal transduction pathways that lead to the regulation of gene expression. Cytoskeletal organization and the regulation of gene expression are both likely to contribute to the cellular changes involved in cell growth and oncogenic transformation. Expression of constitutively active mutants of Rac and Cdc42 in many different cell types results in stimulation of the JNK (also known as stress-activated protein kinase) (19–21) and p38 pathways (22, 23), which in turn regulate expression of specific genes. All three GTPases also regulate other signaling pathways such as the pathway leading to activation of the serum response factor (24). The signaling pathway by which Rac and Cdc42 activate JNK has been well characterized. JNK activation by Rac and Cdc42 was shown to be mediated by the mitogen-activated protein kinase kinase kinase MEKK, which phosphorylates the mitogen-activated protein kinase kinase JNKK (also known as SEK1 or MKK4 (25–27)). JNKK in turn phosphorylates and activates JNK. Besides MEKK, other mitogen-activated protein kinase kinase kinases such as the mixed lineage kinases have also been shown to mediate JNK activation in response to the GTPases (28).

More recently, the GTPases and some of their GEFs, including Dbl, have been shown to activate nuclear transcription factor-κB (NFκB) (29, 30). A major function of NFκB is the regulation of genes involved in immune and inflammatory responses (for review, see Ref. 31). NFκB is also capable of protecting cells against apoptosis (32–37) most likely by activating antiapoptotic genes (38). NFκB may also control cell cycle regulatory genes such as cyclin D1 (39–41) and has been found to be required for oncogenic transformation by a number of oncogenes (33, 42–46).

The signaling pathway by which NFκB is activated by cytokines such as TNFα or interleukin 1 is well characterized. In unstimulated cells, NFκB is usually found in the cytoplasm sequestered by a group of regulatory proteins known as IκBs (IκBα, -β, and -ε) (31). Exposure of cells to TNFα or interleukin 1 results in phosphorylation of IκBα on two critical serines. This targets IκB for ubiquitination-dependent degradation by the proteosome complex and leads to the release and subsequent translocation of NFκB to the nucleus where it can regulate the expression of target genes (31). A large multiprotein complex containing two catalytic subunits, IKKα and IKKβ, is rapidly stimulated by interleukin 1 and TNFα (47–50). IKKα

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Signaling Pathways Leading from Dbl to NFκB

and IKKβ can form homodimers or heterodimers in vitro, and purified recombinant forms of each can directly phosphorylate IκBα and IκBβ at the proper sites (49). In addition, the IKK complex contains a regulatory subunit, IKKγ, that appears to bind IKKα–IKKβ as a dimer (51). The protein kinase NIK has been shown to phosphorylate and activate the IKKα and is thought to mediate IKK activation in response to stimuli such as TNFα (52) and the expression of the C/EBP-2 protein kinase (53). MEKK has also been shown to phosphorylate and activate IKK when overexpressed (54, 55), and it has been proposed to mediate IKK and NFκB activation by the Tax transactivator protein of human T cell leukemia virus 1 (54).

Less is known about the signaling pathway by which Dbl and the Rho family GTPases activate NFκB. Here we show that the three GTPases, Cdc42, Rac, and Rho, activate NFκB by different pathways. Whereas Rac activates NFκB by a pathway that depends on IKKβ, Cdc42 and Rho activate NFκB in the absence of IKK stimulation. The Rac-dependent pathway requires NIK and the Rac effector PAK but does not require MEKK. Dbl requires both branches of the pathway for full activation of NFκB.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pRK5-Myc-tagged Dbl (amino acids 495–826) was a gift from A. Hall and has been described previously (56). pEGFP-C1-hPAK1 (amino acids 83–149) (57) were gifts from J. Chernoff and have been described previously (55). pLPC-IKKα (amino acids 1–225) was a gift from J. Chernoff and has been described previously (23). SRαMEKK1 and dominant negative SRαMEKK1(K322M) have been described previously (58). pCMVM2-JNK and GST-c-Jun have been described previously (23). PAK1(T423E) and pPAK1 were a gift from R. Prywes. pEXVRhoV14, pEXVRacN17, and pCMVCdc42N17 have been described previously (23). pLPC-IKKα is a gift from J. Chernoff and has been described previously (55). pCDNA3-NIK wild type and pCDNA3-NIK KK-AA were from R. Pestell. C3 transferase expression vector was a gift from C. Seven. pEGFP-C1-hPAK1 (amino acids 83–149) (57) were gifts from J. Chernoff. pRCβ-actin HA-IKKβ, pEBG-IKKβ, pRCβ-actin HA-IKKα, GST-IKKβ, GST-IKKα(S-A), and pCMV-IKKβ(88-SS-AA) were gifts from A. Lin and have been described elsewhere (59). pEGFP-C1-hPAK1 (amino acids 83–149) (57) were gifts from J. Chernoff. pPAK1 was a gift from R. Prywes.

**Cell Lines and Transfections**—All cell lines were maintained at 37 °C in 5% CO₂ and cultured in Dulbecco’s modified Eagle’s medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. HeLa and 293 cells were cultured in 10% fetal bovine serum; NIH3T3 cells were cultured in 10% bovine calf serum. Transient transfections into HeLa and NIH3T3 were carried out using the LipofectAMINE method (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells were plated at a density of 3.7 × 10⁵/3.5-cm² diameter dish and were starved 24 h after transfection in 0.2% serum. 293 cells were transfected using a standard calcium phosphate precipitation method.

**Dual Luciferase Assays**—Luciferase assays were carried out in both HeLa and NIH3T3 cells with similar results. However, because the basal levels of luciferase activity were lower in HeLa cells, only these results are shown in the figures. In both cases, cells were transfected as
Dbl regulates IKKβ kinase activity. A, cells were transfected with 0.5 μg of GST-IKKβ expression vector together with empty vector, Dbl (0.5 μg), or MEKKΔ (0.5 μg) expression vectors. IKK activity was assayed after normalizing for IKK expression by Western blot. Either GST-IκBα-(1–54) (top panel) or GST-IκBα(S32T/S36T) (middle panel) were used as substrates. The segments of the autoradiograms that contain the phosphorylated substrates are shown on the top and middle panels. The level of IKKβ present in the extracts used for the kinase assay was assessed by Western blot using anti-HA antibody and is shown in the bottom panel. B, cells were transfected with 4 μg of HA-IKKα expression vector together with either empty vector, NIK, MEKKΔ, or Dbl expression vectors (8 μg). IKK activity was measured as described above for IKKβ. The level of IKKα present in the extracts used for the kinase assay was assessed by Western blot using an anti-HA antibody and is shown in the bottom panel.

described above and harvested 48 h after transfection. Luciferase assays were carried out using the dual luciferase kit (Promega). Firefly luciferase reporter constructs (200 ng of the pBIIX-Luc) were transfected together with 50 ng of the Renilla luciferase reporter plasmid pRL-TK as an internal control. Cells were lysed in 150 μl of passive lysis buffer (Promega), and 7.5 μl of lysate was assayed for firefly and Renilla luciferase activity according to the manufacturer’s instructions. Transfection efficiencies were corrected through normalization of the firefly luciferase activity to the activity obtained from the Renilla Luciferase. All experiments were performed at least three times, and the results averaged. Statistical analyses were performed using the Student t test with significant differences established as p < 0.05.

Purification of Recombinant GST Fusion Proteins—GST-IKKβ (pEBG-IKKβ) expression vectors. Both vectors gave identical results. For IKKα assays, 293 cells were used instead of NIH3T3 cells because IKKα was poorly expressed in NIH3T3 cells, and we could not get sufficient expression for immune complex kinase assays in these cells. In both cases, cells from each transfection were lysed in M2 buffer (60) 48 h after transfection. Approximately 100 μg of cell extracts was incubated with either anti-HA monoclonal antibody and protein A-Sepharose (for isolation of HA-IKKα) or glutathione-agarose beads (Sigma) (for isolation of GST-IKKα and incubated 2 h to overnight at 4 °C. The immune complexes were washed twice in M2 buffer (58) and twice in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2) and incubated at 30 °C in 30 μl of kinase buffer containing 20 μM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM dithiothreitol, 50 μM Na3VO4, 20 μM ATP, and 5 μCi of [γ-32P]ATP. Approximately 2 μg of GST-IκBα wild type or S32T/S36T fusion protein was used as substrate in each reaction. Reactions were stopped after 30 min by denaturation in SDS loading buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, and substrate phosphorylation was visualized by autoradiography. For PAK1 autophosphorylation assays, Myc-tagged PAK was immunopurified from cell lysates using anti-Myc antibody. Immune complex kinase assays were carried out as described above for IKK in the absence of substrate, and the reaction was stopped after 20 min. PAK phosphorylation was then examined by SDS-polyacrylamide gel electrophoresis and autoradiography. JNK assays were performed as described previously (23).

**Western Blots**—Cells were harvested in M2 buffer (58), and equal amounts of cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore Corp.). The membrane was immunoblotted with the appropriate antibody. The following antibodies were used: mouse monoclonal anti-HA 12CA5 (Roche Molecular Biochemicals), anti-GST mouse monoclonal antibody (Sigma), mouse monoclonal anti-Myc 9E10 (Santa Cruz Biochemicals), mouse monoclonal anti-FLAG (Eastman Kodak Co.), rabbit polyclonal anti-IKKα antibody (Santa Cruz Biochemicals), and rabbit polyclonal anti-MEKK antibody (Santa Cruz Biochemicals). Immunocomplexes were visualized by the enhanced chemiluminescence detection method (Amersham Pharmacia Biotech).

**Focus Formation Assays**—Focus formation assays in NIH3T3 cells were carried out as described previously (23).

### RESULTS

**Dbl Activation of NFκB Is Mediated by the Rho Family GTPases**—To examine activation of NFκB, HeLa cells were transfected with the pBIIX-Luc reporter (which contains two NFκB sites and a fos minimal promoter upstream of the luciferase gene) together with either empty vector or vector containing oncogenic Dbl. Luciferase activity was measured 48 h after transfection. As seen in Fig. 1A, oncogenic Dbl activation of pBIIX-Luc was completely blocked by the super-repressor IκBα (S-A) (61), indicating that NFκB activation by Dbl is likely to be mediated by IκB phosphorylation (Fig. 1A). To see whether Dbl activation of NFκB requires the Rho family GTPases, two inhibitors were used. The first was a PAKR expression vector. PAKR contains the regulatory domain of PAK2, which specifically binds to activated Rac and Cdc42 (59). PAKR serves as an inhibitor of Cdc42 and Rac by titrating out the activated forms of the GTPases therefore blocking their ability to activate downstream effectors. The other inhibitor was a C3 transferase expression vector. C3 transferase specifically inhibits Rho activity (15, 17). As seen in Fig. 1A, NFκB activity induced by Dbl is significantly blocked by expression of both PAKR and C3 transferase, suggesting that Cdc42 and/or Rac as well as Rho are necessary for its activation of NFκB. When both PAKR and C3 transferase were used together, the inhibition was even greater, suggesting that a pathway activated by Rac/Cdc42 cooperates with a Rho-activated pathway to activate NFκB. Although dominant negative Rac and Cdc42 also have an inhibitory effect on NFκB activation by Dbl (Fig. 1A), PAKR is considered to be a more reliable inhibitor of endogenous Rac and Cdc42 in these assays because the N17 mutants are thought to function by binding to the GEFs and forming a rather stable complex that could titrate out the exchange factors (62). An inhibitory effect could therefore be attributed to titration of the Dbl protein. PAKR in contrast should specifically inhibit the activities of endogenous Rac and Cdc42 rather than Dbl. Dominant negative Cdc42 and Rac have different effects on NFκB activation by Dbl. This may reflect a different binding affinity of the different dominant negative mutants to Dbl, or it may reflect the fact that both of these mutants were expressed at different levels as shown in Fig. 1A.

**Dbl Activation of NFκB Is Blocked by Dominant Negative IKKβ**—Dominant negative mutants of IKKα and IKKβ were analyzed for their abilities to block Dbl activation of NFκB. These constructs were transfected together with oncogenic Dbl expression vector and the pBIIX-Luc reporter construct. Although dominant negative IKKβ significantly blocked Dbl activation of NFκB, dominant negative IKKα had very little effect (Fig. 1B). Furthermore, when expressed together with suboptimal doses of IKKβ, Dbl could synergize with IKKβ to stimulate NFκB activity (Fig. 1C).

**Signaling Pathways Leading from Dbl to NFκB**

![Image](90x541 to 257x729)
**Fig. 3.** The three Rho GTPases Rac, Rho, and Cdc42 activate NFκB by different mechanisms. A, cells were transfected with 200 ng of pBIX-Luc plasmid together with 0.1 μg of Myc-Cdc42V12, M2-RacV12, or Myc-RhoV14 expression vector along with either empty vector or 0.1 μg of an expression vector containing dominant negative IκB and 50 ng of the internal control pRL-TK vector. Luciferase activity was measured and normalized to the Renilla luciferase activity. The fold activation refers to the value of luciferase activity obtained in the presence of the activators compared with the activity obtained in their absence. Data are shown as the mean ± S.E. B, cells were transfected with 0.5 μg of GST-IKKβ vector together with 0.25 μg of M2-RacL61, Myc-RhoV14, or Myc-Cdc42V12. IKKβ activity was assayed as described in Fig. 2, and IκB phosphorylation is shown in the top panel. The IKKβ expression level in the extracts used for the kinase assay is shown in the middle panel as detected by Western blots probed with anti-GST antibody. Western blots of the Rho GTPases probed with antibodies directed against the epitope tags are shown in the bottom panel. C, cells were transfected with 0.25 μg of HA-IKKβ vector together with 0.5 μg of Dbl or RacL61 in the absence or presence of 1 μg of the PAK autoinhibitory domain (PAK1-(83–149)) or with 0.5 μg of wild type NIK. IKKβ activity was assayed as described above. Shown in the bottom panel are IKKβ expression levels in a Western blot that was performed by probing the proteins immunoprecipitated from the same amount of extracts used in the kinase assay with an anti-HA antibody. D, cells were transfected with 0.5 μg of GST-IKKβ expression vector along with empty vector or 0.5 μg of Dbl or constitutively active Myc-PAK1 (PAK1(T423E)). IKKβ activity was assessed as described above, and IκB phosphorylation is shown in the top panel. A Western blot showing IKKβ expression levels in the extracts used for the kinase assay as detected with an anti-GST antibody is shown in the bottom panel. As a positive control, PAK1(T423E) (PAK1TE) autophosphorylation was examined by immune complex kinase assay using anti-Myc antibody, and PAK1 expression levels were examined by probing Western blots with anti-Myc antibody. WT, wild type.

Dbl Stimulates IKKβ Activity—Because the IKKs form a large complex that binds many proteins, a dominant negative IKKβ might therefore have a rather global effect in that it may titrate other important signaling molecules. To examine the role of the IKKs in more detail, we looked at the induction of the IKK enzymatic activity in response to oncogenic Dbl. To determine whether Dbl activates IKKβ, an in vitro kinase assay was carried out. In this assay pEGB-IKKβ (a eukaryotic expression vector containing GST-tagged IKKβ) was transfected with either empty vector or oncogenic Dbl. MEKKδ, an activated form of MEKK that has previously been shown to be a strong activator of IKKβ (55), was used as a positive control. After transient expression, GST-IKKβ expression levels were analyzed by Western blot and quantitated. Equal amounts of GST-IKKβ were then purified from cell lysates using glutathione-agarose-conjugated beads and assayed for the ability to phosphorylate bacterially expressed IκBα in the presence of [γ-32P]ATP. IκBα phosphorylation was analyzed after SDS-polyacrylamide gel electrophoresis and autoradiography. Dbl stimulated IKKβ activity to levels comparable with MEKKδ (Fig. 2A). As expected, IKKβ that was activated by Dbl or MEKKδ was not able to phosphorylate IκBα(S32T/S36T). The GST-IκBα(S32T/S36T) mutant is a very poor IκK substrate because the phospho-acceptor sites (serine 32 and serine 36) are replaced by threonine residues (63) (Fig. 2A, middle lane). Using a similar assay, we found that in contrast to IKKβ, Dbl could not activate IKKα (Fig. 2B), whereas NIK, which was used as a positive control, activated IKKα, and MEKKδ activated IKKα weakly.

Rac, but Not Cdc42 and Rho, Activates IKKβ—Because Dbl activation of NFκB appears to be mediated by IKKβ and the Rho family GTPases, we were interested in determining whether the Rho GTPases Cdc42, Rac, and Rho activate NFκB by an IKK-dependent pathway. All three GTPases activated the pBIX-Luc promoter —6–10-fold (see Fig. 3A). Immune complex kinase assays were carried out to see whether the GTPases could also activate IKK. Surprisingly, we found that only activated Rac stimulated IKKβ activity, whereas activated RhoA only activated the kinase minimally, and activated Cdc42 did not activate the kinase at all (Fig. 3B). None of the GTPases activated IKKα (data not shown). This suggests that although Rac can activate NFκB via activation of IKK, Cdc42 and Rho may activate NFκB by an IKK-independent pathway. A well-known target for Rac is the serine/threonine kinase PAK. PAK1 was recently shown to activate NFκB but not IKK (64). To determine whether PAK is required for Rac activation of IKK, IKKβ and activated Rac vectors were transfected along with either empty vector or the PAK1 autoinhibitory domain (PAK1-(83–149)), which is known to block endogenous PAK activity (57). As shown in Fig. 3C, the PAK1 autoinhibitory domain completely blocked Rac activation of IKKβ, indicating that PAK is necessary for IKKβ activation by Rac. The PAK autoinhibitory domain also blocked oncogenic Dbl activation of IKKβ. These results suggest that PAK1 is necessary for NFκB...
activation by Dbl and Rac. However, an activated PAK1 mutant, PAK1(T423E), was not sufficient to activate IKK on its own (see Fig. 3D), suggesting that although PAK is necessary for IKK activation, it is not sufficient. Likewise, we were not able to observe NFκB activation in response to activated PAK1 using luciferase reporter assays (data not shown), although the activated PAK1 had considerable kinase activity when assayed for autophosphorylation (Fig. 3D) and myelin basic protein phosphorylation (data not shown).

**Fig. 4.** Dbl and Rac activation of IKKβ requires NIK but not MEKK. A, cells were transfected with 200 ng of pBIIX-Luc reporter together with either 0.1 μg of Dbl (open bars) or RacV12 (filled bars) along with either empty vector or increasing amounts (0.5 or 1 μg) of a dominant negative forms of NIK (NIKAA) or MEKK (MEKKα(K432M)). Total luciferase activity in the lysates from cells transfected with Dbl or RacV12 and empty vector is taken as 100%. Data are shown as the mean ± S.E. *, a significant decrease relative to Dbl or Rac alone (p < 0.05). B, cells were transfected with 0.25 μg of HA-IKKβ vector together with either empty vector, 0.5 μg of Dbl, or 0.5 μg of RacL61 vectors and 0.5 or 1 μg of dominant negative NIK or MEKKα expression vectors. IKKβ activity was detected as described in Fig. 2. Levels of expression of IKKβ were visualized by Western blot using an anti-HA antibody after immunoprecipitation as previously described and are show in the middle panel. The bottom panel shows expression of the dominant negative MEKKΔ protein in whole cell extracts as detected using an anti-MEKK antibody. C, to test the activity of dominant negative MEKKΔ cells were transfected with 0.25 μg of M2-JNK vector together with either empty vector or 0.5 μg of RacL61 vector and 0.5 μg of dominant negative MEKK expression vector. After transient expression, JNK activity was assessed by immune complex kinase assays using GST-c-Jun as a substrate. Phosphorylated c-Jun is shown in the top panel. The total level of JNK in the cell lysates as assessed by probing with anti-M2 antibody is shown in the bottom panel. MEKKΔ-K-M, MEKKΔ(K432M); WT, wild type; NIKAA, NIK(KK-AA).

Our results suggest that PAK1 is necessary for IKK activation by Rac, although it is not sufficient to activate IKK on its own. Taken together, our results suggest a signaling pathway where Dbl activates Rac and PAK, which in turn activates NIK, either directly or indirectly, and most likely in cooperation with other signaling enzymes. NIK in turn phosphorylates IKKβ, leading to IkB phosphorylation and degradation followed by nuclear translocation of NFκB. It should be noted that our results differ somewhat from those of Frost et al. (64), who showed that Rac does not activate IKKβ and that activated PAK can stimulate NFκB activity, albeit in the absence of IKKβ activation. These results may be explained by the fact that different cell types and a different constitutively active PAK1 mutant were used in the two studies.

Our results suggest that PAK1 is an important mediator of IKKβ activation by Dbl and Rac. The PAK1 autoinhibitory domain, which is quite specific for PAK (57), completely inhibited IKKβ activation by Rac and Dbl. However, we have found that activated PAK1 is not sufficient on its own to activate IKKβ or the NFκB luciferase reporter strongly. Likewise, we have not seen NFκB activation in response to other members of the PAK family (data not shown). The results from our study suggest that although PAK is necessary for IKKβ activation by Rac, it is not sufficient. This suggests that other factors may cooperate with PAK to activate IKKβ. One possibility is that reactive oxygen may be involved in this pathway. Reactive oxygen was previously shown to have an important role in the signaling pathway leading from Rac to NFκB activation (65). Alternatively, other Rac/Cdc42 effectors such as mixed lineage
kinase, which was shown to activate NFκB, may have a role in
IKK activation by Rac (66).

It is interesting that Rac activates NFκB via NIK rather
than MEKK. Although dominant negative MEKK was shown
to block NFκB activation by Rac in COS cells (30), we did not
see any inhibition by dominant negative MEKK in HeLa or
NIH3T3 cells. Consistent with this finding, by using MEKK-
null cells, Xia et al. (67) have recently reported that although
MEKK is necessary for JNK activation by proinflammatory
stimuli, it is dispensable for NFκB activation by the same
signals. Our results suggest a model in which Rac activates two
diverging signaling pathways. One pathway is mediated by
MEKK and leads to JNK activation (23). Although MEKK is
required for the activation of JNK by Rac, Rac has not been
shown to stimulate MEKK activity on its own. Thus, although
when overexpressed MEKK can stimulate NFκB activity (54,
55), Rac most likely does not stimulate MEKK activity suffi-
ciently to allow it to activate NFκB. Instead, we propose that a
second pathway exists in which Rac activates PAK, which, in
turn activates NIK either directly or indirectly. Activation of
NIK in turn leads to NFκB activation most likely by phospho-
rylating IKKβ.

In contrast to Rac, we have found that Cdc42 and Rho acti-
vate NFκB without activating either IKKβ or IKKα. Although
we cannot completely rule out the possibility that IKKα and
IKKβ activation by Rho and Cdc42 is too weak to be detected in
our assays, even expression of high levels of Cdc42 and Rho did
not produce noticeable IKK activity. Thus, our results strongly
suggest that these two GTPases can trigger an IKK-independent
pathway leading to NFκB activation. Recently, UV irradiation
was also shown to activate NFκB by an IKK-independent mechanism (68, 69). In the case of Rho and Cdc42, however,
this result is quite surprising because activation of NFκB by
Rho and Cdc42 does appear to require IκB phosphorylation as
assessed by experiments with the IκB super-repressor. Our
results suggest that a kinase other than IKKα or IKKβ may
phosphorylate IκB and thereby activate NFκB in response to
Rho and Cdc42. It should be noted to this regard that dominant
negative IKKβ did partially inhibit Cdc42 and Rho activation of
the NFκB luciferase reporter (data not shown). The most likely
explanation for this is that dominant negative IKKβ binds to
and titrates a Rho and Cdc42 activated kinase and thereby
indirectly inhibits NFκB activation by the GTPases. Alterna-
tively, it could act by titrating IKKγ or an IKKγ-related pro-
tein, which could potentially be part of an IKKα/β-independent
kinase complex that phosphorylates IκB. Interestingly, Cdc42
but not Rho activation of NFκB was partially blocked by dom-
inant negative NIK (data not shown). This suggests that al-
though it does not activate IKKβ, Cdc42 still requires NIK
activity to activate NFκB. These data are consistent with re-
cent work done with NIK knockout mice that suggests that
NIK may have a role in NFκB activation that is independent of
IKKα/β activity in response to some extracellular stimuli (70).
The exact role for NIK in the NFκB pathway thus still remains
to be fully clarified.

Understanding the signaling pathways activated by Dbl is
especially important because Dbl is a potent oncogene. All
three Rho family GTPases, Cdc42, Rac, and Rho, have been
shown to contribute to different aspects of oncogenic transfor-
mation by Dbl (7). The NFκB pathway is particularly relevant
to studying Dbl-induced oncogenesis because NFκB was re-
cently shown to be one of the factors that is important in this
process (46). Here we show that all the enzymes that we found
to be involved in NFκB activation by Dbl, PAK1, NIK, IKKβ,
and IκB are all necessary for focus formation induced by onco-
genic Dbl. The mechanism by which NFκB regulates transfor-
mation in response to Dbl is not known, although NFκB was
shown to regulate transformation by Ras by promoting cell
survival (42). Elucidating how the NFκB pathway contributes
to oncogenesis by other oncogenes such as Dbl will be critical
to understanding the signaling pathways that control cell
growth and proliferation.

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FIG. 5. Focus formation by oncogenic Dbl requires enzymes in the
NFκB signaling pathway. A, NIH3T3 cells were transfected with oncogenic Dbl
(200 ng) together with 1 μg of either empty vector, IκBα/β(SS-AAA),
NIK/IKKα (NIKAA), or dominant negative PAK1. Cells were grown for 2 weeks
following transfection. The plates were then stained with crystal violet and pho-
tographed. Representative plates are shown. B, foci were counted, and the num-
ber of foci is indicated as the percentage of foci induced by Dbl. Dbl produced ~500
foci/μg of transfected Dbl DNA. The results are an average of three independent
experiments and are presented as ± S.E.
