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Rac1 Regulates Interleukin 1-induced Nuclear Factor κB Activation in an Inhibitory Protein κBα-independent Manner by Enhancing the Ability of the p65 Subunit to Transactivate Gene Expression*

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Caroline A. Jefferies and Luke A. J. O'Neill‡

From the Department of Biochemistry and Biotechnology Institute, Trinity College, Dublin 2, Ireland

We have examined the involvement of Rac1 in nuclear factor κB (NFκB) activation by interleukin 1 (IL1). IL1 induced a rapid and sustained activation of Rac1 in the thymoma cell line EL4.NOB-1. Transient transfection with dominant negative RacN17 inhibited IL1-induced κB-dependent reporter gene expression but not IκBα degradation, whereas constitutively active RacV12 potentiated κB-dependent reporter gene expression in response to IL1 but had no effects on its own. Using porcine aortic endothelial cells stably transfected with RacV12 or RacN17 under the control of an inducible promoter, we confirmed that RacV12 did not affect IκBα degradation, nor did RacN17 inhibit the IL1-induced response. RacV12 was also unable to induce nuclear translocation of NFκB. These effects suggested a role for Rac1 in p65-mediated transactivation of NFκB, independent of IκBα regulation. In support of this we found that IL1 activated a pathway leading to increased p65 transactivation activity and that RacV12 alone could drive this response in both cell systems. Additionally, RacN17 inhibited IL1-driven p65-mediated transactivation. From data using specific inhibitors of p38 and p42/p44 kinases we propose that both p38 and p42/p44 lie downstream of Rac1 on the IL1 pathway leading to enhanced transactivation by p65.

The proinflammatory cytokine interleukin 1 (IL1)[1] is a crucial mediator of both inflammatory and immune responses. The involvement of IL1 in the pathogenesis of inflammatory diseases such as rheumatoid arthritis has led to intensive studies on how IL1 signals are transduced in target cells. Although significant advances have been made in this area recently, particularly with respect to activation of the transcription factor nuclear factor κB (NFκB), several aspects of IL1 signaling remain to be elucidated fully (1). A number of different lines of evidence suggest a role for GTP-binding proteins in IL1 signaling events in cells (2–4). In particular, a role for the small G protein Rac1 (a member of the Rho subfamily of G proteins) in IL1 signaling has been proposed. Although Rac1 was originally described for its effects on the cytoskeleton in cells, more recently it has been shown to play a role in other signaling events (5, 6). Most notably, Rac1 has been suggested to regulate mitogen-activated protein kinase (MAPK) pathways in cells, in particular the stress-activated protein kinase pathways, p38 and c-Jun NH_2-terminal kinase (JNK) (7–9). The proposal that Rac1 may play a role in IL1 signaling came from studies demonstrating that a dominant negative mutant of Rac1 (RacN17) inhibited the activation of both p38 and JNK MAPK pathways by IL1 (7–9), although more recently a role for Rac1 in JNK activation by IL1 has been disputed (10).

In addition, Rac1 has been shown to play a role in activation of NFκB, a ubiquitous transcription factor that regulates the expression of many genes up-regulated by IL1 (11, 12). The best characterized form of NFκB exists in resting cells as a dimer of two proteins, the subunits p50 (which binds the κB motif) and p65 or RelA (which is required for transactivation of gene expression). This heterodimer is complexed to the inhibitory subunit IκBα which, upon stimulation, is phosphorylated and subsequently degraded. NFκB is then free to enter the nucleus and bind to its consensus sequence on target genes (13, 14). Regulation of IκB degradation and the subsequent release of NFκB is a crucial control point in the pathway. However, recent results suggest that an additional IκB-independent pathway is activated, which results in enhanced transactivation potential of NFκB once it is bound to its consensus sequence (15, 16). Activation of these pathways has been shown to result in increased phosphorylation of the p65 (RelA) subunit of NFκB and to promote interaction of p65 with the coactivator protein p300/CBP (17–19). The upstream kinases regulating these events have yet to be identified conclusively, although recent evidence suggests that p38 and p42/44 MAPK pathways may play a role in regulating NFκB transactivation in response to stimulation with both IL1 and TNF-α (15, 16). In addition, several reports indicate that casein kinase II and protein kinase A may be involved in events leading to enhanced phosphorylation of the p65 subunit of NFκB (17, 20). Where Rac1 might participate in either pathway culminating in IκB phosphorylation or p65-mediated transactivation has not been investigated. Evidence for a role for Rac1 in NFκB function is based on the ability of a constitutively active mutant of Rac1 (RacV12) to drive a κB-dependent reporter gene. In addition, dominant negative RacN17 in the same studies inhibited IL1β-stimulated NFκB DNA binding, possibly by inhibiting the generation of reactive oxygen species (ROS) (11, 12).

We have found that the involvement of Rac1 in NFκB activation in response to IL1 appears to be independent of IκBα degradation or nuclear translocation and DNA binding of NFκB. Instead, our data clearly point to a role for Rac1 downstream of these events at the level of enhancing the NFκB transactivating potential of its p65 subunit once bound to its consensus sequence. We therefore propose that IL1 initiates

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‡ To whom correspondence should be addressed. Tel.: 353-1-608-2439; Fax: 353-1-677-2400; E-mail: laoneill@tcd.ie.

[1] The abbreviations used are: IL, interleukin; NFκB, nuclear factor κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; CBP, cAMP-responsive element binding protein; IκB, inhibitory protein κB; TNF, tumor necrosis factor; ROS, reactive oxygen species; PAE, porcine aortic endothelial; AP, activated protein; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; PAK, p21-activated kinase.

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two pathways in the NFκB system, the well characterized one leading to IκB phosphorylation, and the second, requiring Rac1, which regulates p65-mediated transactivation of gene expression.

MATERIALS AND METHODS

Cell Culture and Reagents—EL4.NOB-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded at 1×10^6 ml^{-1} for experiments and pretreated with inhibitors prior to stimulation with IL1α as indicated in the figure legends. Porcine aortic endothelial (PAE) cells were grown in F-12 nutrient mixture (Ham's F-12; Sigma) containing 15% fetal calf serum, 2 mM L-glutamine, 50 μg/ml gentamycin, and 500 nM puromycin at 37 °C in a humidified atmosphere of 5% CO_2. PAE cells stably transfected with either RacV12 (V12Rac-PAE) or RacN17 (N17Rac-PAE) were grown as above but with the addition of 0.1 mM hygromycin B. 24 h prior to the induction of Rac1 mutants, cell lines were cultured in serum-free medium containing 0.2% fetal calf serum, 0.1% fatty acid-free bovine serum albumin, 2 mM L-glutamine, 50 μg/ml gentamycin, 500 nM puromycin, and 0.1 mM hygromycin B. Expression of RacV12 and RacN17 was induced by the addition of 15 mM filter-sterile isopropyl-β-D-thiogalactopyranoside to the starvation medium for the time periods indicated in the figure legends. Human recombinant IL1α was a kind gift from Prof. J. Saklatvala (Kennedy Institute of Rheumatology, U. K.). The pyridinyl imidazole SB203580 was kindly provided by Peter Young, Smithkline Beecham Pharmaceuticals, King of Prussia, PA. PD98059 (2′-amino-3′-methoxyflavone) was a kind gift from Alan Saltiel, Parke-Davis Research Division, Warner Lambert Company, Ann Arbor, MI. All inhibitors were prepared in dimethyl sulfoxide.

Plasmid Constructs—The pEF expression vector encoding myc-tagged constitutively active RacV12 and dominant negative RacN17 and the AP1 reporter plasmid (AP1 chloramphenicol acetyltransferase, AP1-CAT) were all kind gifts from Dr. D. Cantrell (ICRF, London) and have been described elsewhere (21). GST-PAK (residues 1–252) was also a kind gift from Dr. Cantrell. The κB-luciferase reporter gene (pGL3–5xκB-luc) was a kind gift from Dr. R. Hofmeister (Universität Regensgurg, Regensburg, Germany). Gal4-p65$^{1-551}$ plasmid encoding the full p65 subunit (amino acids 1–551) fused to the DNA binding domain of Gal4 was obtained from Dr. Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and has been described previously (22, 23). The Gal-luciferase reporter gene was purchased from Stratagene. IκBctag was constructed by cloning IκBα into the pcDNA3 expression vector, which contained a sequence encoding the SV5 Pk tag, and was a kind gift from Prof. R. T. Hay (University of St. Andrews, Scotland). All plasmids were purified using an endotoxin-free protocol (Wizard® PureFection DNA Purification, Promega, Madison, WI).

Affinity Precipitation of Active Rac1 using GST-PAK—EL4.NOB-1 cells (1×10^7) were stimulated for various time points with IL1. Activation was terminated by washing cells with ice-cold phosphate-buffered saline followed by lysis in 1 ml of lysis buffer (25 mM HEPES, pH 7.5, 1% Nonidet P-40, 0.25% deoxycholate, 10% glycerol, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na_3VO_4, 2 μg/ml aprotonin) containing 10 μg of GST-PAK and incubated for 1 h at 4 °C. Cell lysates were cleared by centrifugation and supernatants incubated with 30 μl of glutathione agarose beads for 60 min at 4 °C. The bead pellet was washed three times with lysis buffer and finally resuspended in 30 μl of Laemmli sample buffer. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis, and associated active Rac1 was detected by Western blot analysis using an anti-Rac-specific antibody (Upstate Biotechnology, Lake Placid, NY).

Transient Transfection and Reporter Gene Assays—EL4.NOB-1 cells (1.4×10^7) were transfected with plasmids as described in the figure legends in a final volume of 1.2 ml using DEAE- dextran (24). PAE cell lines were transfected using Fugene (Roche Diagnostics Ltd., East Sussex, U. K.) according to the manufacturer's recommendations. After a period of recovery (16–18 h) cells were treated as indicated in the figure legends. To assay luciferase activity, cells were lysed using passive lysis buffer (Promega) and luciferase activity determined using standard procedures. All experiments were done in triplicate, and luciferase activity was normalized to protein concentration as determined by the method of Bradford (25). Cell lysates for assessing the activity of CAT were prepared by repeated freeze-thaw cycles and enzyme activity determined as described previously (26).

Immunoprecipitation and Western Blot Analysis—EL4.NOB-1 cells were treated as described in the figure legends, and treatment was terminated by the addition of 5 ml of ice-cold phosphate-buffered saline. Cells were lysed on ice (30 min) in buffer containing 25 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4, and 0.5% Nonidet P-40. Lysates were cleared by centrifugation, and after preclearing for 30 min with protein A-insoluble (Sigma), Rac1 was immunoprecipitated with 4 μg of mouse monoclonal anti-Rac (Upstate Biotechnology) for 60 min at 4 °C. The immune complexes were precipitated by incubation with protein A-Sepharose for 60 min at 4 °C, and PAK association was detected using a polyclonal anti-PAK antibody (Santa Cruz). For Western blot analysis of IκBα degradation and expression of myc-tagged constructs, total cell lysates were prepared using radioimmune precipitation buffer (27). Equivalent amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose or polyvinylidene difluoride membranes; after incubation with primary antibodies as indicated (1 h at room temperature), blots were incubated with the appropriate peroxidase-conjugated secondary antibody (45 min at room temperature). Visualization was by enhanced chemiluminescence according to manufacturer's recommendations (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described by Osborn *et al.* (28) from cells (5×10^6) treated as described in figure legends. Nuclear extracts (4–8 μg of protein) were incubated (30 min at room temperature) with 10,000 cpm of double-stranded [γ-^{32}P]ATP NFκB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Incubations were performed in the presence of 2 μg of poly(dI·dC) as nonspecific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 4% glycerol, and 100 μg/ml nuclease-free bovine serum albumin. DNA-protein complexes were resolved on native (5%) polyacrylamide gels that were subsequently dried and autoradiographed.

RESULTS

Rac1 Is Activated after IL1 Stimulation—Although previous evidence points to Rac1 playing an important role in IL1-induced signaling it has not been shown conclusively that stimulation of cells with IL1 activates the small G protein. To address this question we used a murine thymoma cell line, EL4.NOB-1, which is strongly responsive to IL1 (27) and employed a technique that relies on the fact that only in its active state will Rac1 bind its downstream effector PAK1 (29). We therefore used a GST-PAK fusion protein (residues 1–252), which contained the crucial domain essential for Rac1 binding, in GST pull-down experiments followed by anti-Rac immunoblot analysis to assess the level of Rac1 activation in our cells. As shown in Fig. 1*A*, virtually no PAK-associated Rac1 was detected in unstimulated cells (*lane 1*). After stimulation with IL1, however, the amount of associated Rac1 increased, with Rac1 being activated as early as 5 min (*lane 2*) and activation increasing up to 60 min (*lane 4*). We confirmed this result by immunoprecipitating endogenous Rac1 in our cells and tested for PAK1 association by immunoblot analysis. Fig. 1*B* shows that IL1 stimulation of cells for 15 min results in increased association of endogenous PAK1 with Rac1, indicating increased activation of the G protein. This method also confirms that the effector with which Rac1 associates after IL1 activation is PAK1, which has been shown previously to regulate p42/p44 MAPK activation (30).

Dominant Negative RacN17 Inhibits IL1-induced κB-dependent Reporter Gene Expression—To investigate the role of Rac1 in IL1-induced activation of NFκB we used two mutants of Rac1, constitutively active RacV12 and dominant negative RacN17 (5). These mutants, which have been characterized extensively, have point mutations in the GDP/GTP binding site which prevent GTP hydrolysis or GDP exchange, respectively. Fig. 2 demonstrates the effect of IL1 in cells transiently co-transfected with a NFκB-dependent reporter gene, κB-luciferase, and a plasmid encoding dominant negative RacN17. Treatment of cells for 3 h with 10 ng/ml IL1 increased κB-luciferase activity 10-fold (Fig. 2*A*). This effect was inhibited in cells

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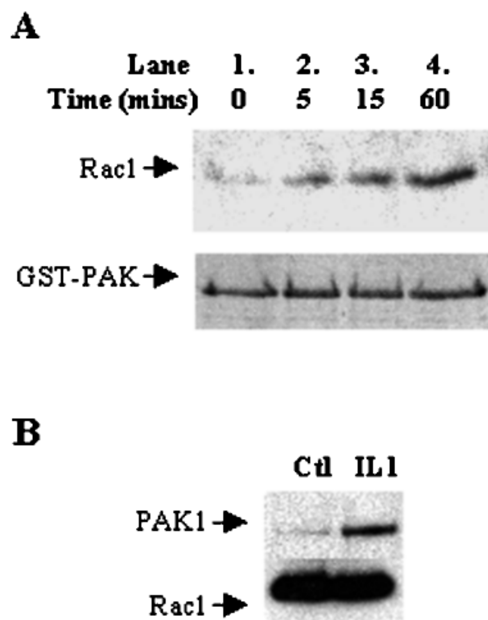

FIG. 1. **IL1 stimulation of EL4.NOB-1 cells activates Rac1.** *A*, activated Rac1 was affinity purified from EL4.NOB-1 (2×10^7) cell lysates (stimulated with IL1 (10 ng/ml) for various time points as indicated) using GST-PAK and detected by Western blot analysis using an anti-Rac antibody (Upstate Biotechnology). After transfer, gels were stained to show that equivalent amounts of GST-PAK were added to each sample (*lower panel*). Identical results were observed in a further experiment. *B*, total Rac was immunoprecipitated from EL4.NOB-1 cells (1×10^7) stimulated with IL1 (10 ng/ml) as indicated and PAK association determined by Western blotting using an anti-PAK antibody. Blots were stripped using 50 mM glycine buffer (pH 2) and reprobed using the anti-Rac antibody to demonstrate that equal levels of Rac were immuoprecipitated in both samples (*lower panel*).

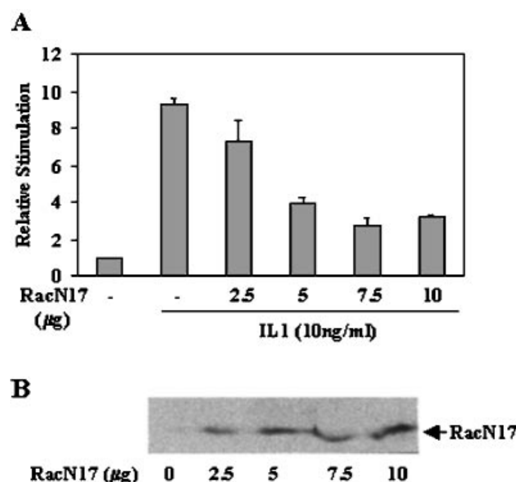

FIG. 2. **Effect of RacN17 on NFκB-dependent transcription.** *A*, EL4.NOB-1 cells (1.4×10^7) were transiently transfected with the NFκB-dependent reporter gene κB-luciferase (2.5 μg) and increasing amounts of plasmid encoding RacN17 (0–10 μg). The total amount of plasmid transfected in each case was kept constant by adding the appropriate amounts of relevant empty vector plasmid. After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 3 h) and extracts assayed for luciferase activity. Results are expressed as fold increase compared with unstimulated control samples (mean ± S.D. of triplicate determinations) and are representative of at least three separate experiments. *B*, myc-tagged RacN17 expression was detected by Western blot analysis of whole cell lysates following transfection of cells with increasing amounts of plasmid encoding myc-tagged RacN17 (0–10 μg) using a monoclonal antibody that recognized the myc epitope. The band detected was at a molecular mass of 22 kDa as would be expected for myc-tagged RacN17.

cotransfected with increasing amounts of RacN17 with 10 μg of plasmid reducing the effect of IL1 by 70%. This effect correlated with the level of expression of RacN17 in the cells as judged by Western blot analysis using an anti-myc antibody, which recognized epitope-tagged RacN17 (Fig. 2*B*).

Constitutively active RacV12 has been shown previously to activate NFκB in rabbit synovial fibroblasts and HeLa cells (11, 12). In our system cotransfection with RacV12 had no effect on κB-luciferase activity but did potentiate the IL1-driven response by 2-fold (Fig. 3*A*). In contrast, transfection of cells with RacV12 activated an AP1-driven reporter gene, *AP1-CAT*, without the need for additional stimuli. IL1 alone had only a marginal effect on this response but potentiated the effect of RacV12 (Fig. 3*B*). Expression of myc-tagged RacV12 in transfected cells was detected by Western blot analysis using an anti-myc antibody (Fig. 3*C*).

Rac1 Does Not Participate in the Pathway to IκBα Degradation Induced by IL1—A crucial regulatory control point on the pathway to NFκB activation is the phosphorylation, ubiquitination, and subsequent degradation of IκB. We therefore examined the effect of constitutively active RacV12 and dominant negative RacN17 on IL1-induced IκB degradation. For this we used a tagged version of IκBα (IκBctag) which, when expressed in cells, can be distinguished from endogenous IκBα in Western blot analysis because of its higher molecular weight. Cells were cotransfected with expression plasmids encoding IκBctag and either RacV12 or RacN17 to ensure that the effects on IκBα degradation could be analyzed on transfected populations of cells. After stimulation with IL1 (10 ng/ml, 30 min) the ability of either RacV12 or RacN17 to induce or inhibit IκBctag degradation, respectively, was analyzed by Western blot analysis using an antibody that recognized IκBα. As shown in Fig. 4*A*, stimulation with IL1 resulted in degradation of both endogenous and tagged IκBα (compare *lane 2* with *lane 1*). The expression of RacV12 had no effect on IκBctag degradation (*lane 3*), nor did it enhance the effect of IL1 (*lane 4*) as had been seen on κB-luciferase (Fig. 3*A*). Furthermore, and most importantly, RacN17 did not inhibit IL1-induced degradation of IκBctag (*lane 6*) as would be expected if Rac1 was involved in regulating this crucial regulatory step on the pathway to NFκB activation.

We next analyzed the effects of constitutively active RacV12 on nuclear translocation and DNA binding of NFκB as determined by electrophoretic mobility shift assay on RacV12-transfected cells. As shown in Fig. 4*B*, IL1 induced strong activation of NFκB (compare *lane 2* with *lane 1*). However, transfection of cells with RacV12 (*lanes 3* and *4*) or RacN17 (*lanes 5* and *6*) had no effect on NFκB DNA binding as judged by electrophoretic mobility shift assay, either on their own or in IL1-treated cells.

These results were confirmed using PAE cells that were stably transfected with epitope-tagged constitutively active RacV12 (V12Rac-PAE) or dominant negative RacN17 (N17Rac-PAE) under the control of an isopropyl-β-D-thiogalactopyranoside-responsive promoter. Expression of these constructs was detected readily using an anti-Rac antibody because of their higher molecular weight compared with endogenous Rac1, and expression of either was found to occur from 6 h (not shown) and was maximal at 24 h postinduction (Fig. 4*C*). Comparison of *lanes 1* and *3* in Fig. 4*D* shows that induction of RacV12 expression was unable to induce IκBα degradation, nor was it able to enhance the DNA binding activity of NFκB (Fig. 4*E*). RacN17 induction similarly did not affect IL1-induced IκBα degradation (Fig. 4*D*, compare *lanes 7* and *8*) or DNA binding activity of NFκB (Fig. 4*E*, *lanes 7* and *8*). The lack of effect of RacV12 or RacN17 was also evident at earlier induction times (data not shown).

Rac1 Is Required for Increased Transactivation Potential of p65 in Response to IL1—Because RacN17 inhibited IL1-in-

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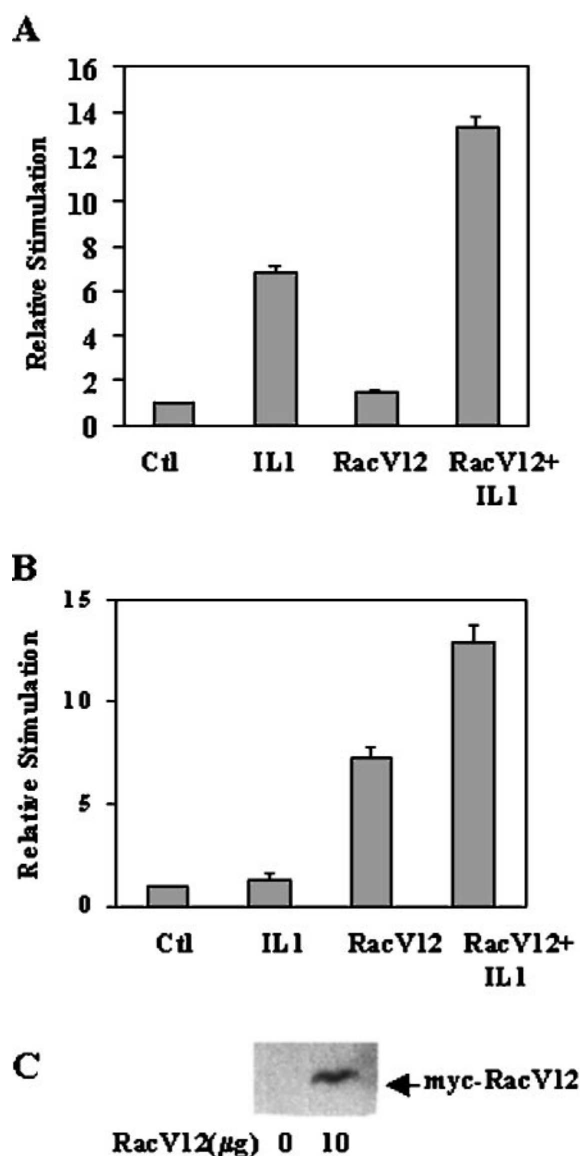

FIG. 3. **Effect of RacV12 on κB-dependent gene expression.** *A*, EL4.NOB-1 cells (1.4×10^7) were transiently transfected with κB-luciferase (2.5 μg) and RacV12 (10 μg). After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 3 h) and extracts assayed for luciferase activity. Results are expressed as fold increase compared with unstimulated control samples (mean ± S.D. of triplicate determinations) and are representative of at least three separate experiments. *B*, cells were transiently transfected with AP1-CAT reporter gene (10 μg) and RacV12 (10 μg). After recovery, cells (1×10^6/ml) were stimulated with IL1 (10 ng/ml, 24 h) and extracts assayed for CAT activity. Results are expressed as fold increase over unstimulated control for three separate experiments (mean ± S.E. of four individual experiments). *C*, myc-tagged RacV12 expression was detected by Western blot analysis as described in the legend to Fig. 1. The band detected was at a molecular mass of 22 kDa as would be expected for myc-tagged RacV12.

duced κB-dependent reporter gene expression but not IκBα degradation and because RacV12 potentiated κB-dependent reporter gene expression in response to IL1 but had no effects on its own, our results indicated a role for Rac1 in transactivation by NFκB. To investigate this possibility we cotransfected EL4.NOB-1 cells with the p65 subunit of NFκB fused to the DNA binding domain of Gal4 (Gal4-$p65^{1-551}$) and a Gal4-responsive reporter gene, Gal-luciferase (22, 23). The advantage of this assay is that Gal4-$p65^{1-551}$ is exclusively nuclear and is regulated independently of IκB thus allowing the effects of various stimuli or genes of interest on transactivation by p65 to be studied. Fig. 5*A* demonstrates how IL1 treatment (10 ng/ml, 5 h) increased p65-mediated transactivation 2.5-fold over control in keeping with results observed in L929sA cells following stimulation with TNF-α (15). Cotransfection of EL4.NOB-1 cells with constitutively active RacV12 also more than doubled transactivation by p65 in the absence of further stimulation. IL1 did not increase the effect of RacV12 further. Similarly, both IL1 stimulation and the induction of RacV12 expression in V12Rac-PAE cells increased the ability of p65 to drive transactivation (Fig. 5*A*). Although IL1 stimulation of noninduced V12Rac-PAE cells gave rise to only a 1.5-fold increase over control levels, induction of RacV12 resulted in a 2-fold stimulation. Addition of IL1 to induced V12Rac-PAE cells caused a slight increase in the response. Importantly, cotransfection of EL4.NOB-1 cells with increasing amounts of plasmid encoding dominant negative RacN17 resulted in dose-dependent inhibition of enhanced transactivation potential induced by IL1 (Fig. 5*B*). Transfection with 5 μg of plasmid reduced the transactivation potential of p65 to basal levels. Furthermore, plasmid amounts greater than 5 μg lowered the response below basal, indicating a role for Rac1 in the basal signal (Fig. 5*B*). We were unable to test the N17Rac-PAE cells in this assay because IL1 was unable to drive transactivation in noninduced cells (not shown). Prolonged exposure of blots from N17Rac-PAE cells revealed a low level of constitutive expression of RacN17 in noninduced cells (Fig. 5C, *lane 1*), which provided a possible explanation for the lack of effect of IL1 on noninduced cells. In comparison, the V12Rac-PAE cells, under the same conditions, did not show this basal level of expression of RacV12 (Fig. 5*C*, *lane 2*).

p42/p44 and p38 MAPK Are Involved in Enhanced p65-mediated Transactivating Activity in Response to IL1 and RacV12—Recent reports have demonstrated an involvement of both p42/p44 and p38 MAPK pathways in NFκB transactivation in response to TNF stimulation (15). We therefore tested the involvement of these kinases in regulating transactivation using specific inhibitors of each of these pathways, the MEK1 inhibitor PD98059 (33) and the p38-specific inhibitor SB203580 (34, 35). Fig. 5*D* demonstrates how treatment of cells with both PD98059 and SB203580 inhibited transactivation by p65 in response to both IL1 and RacV12. With respect to both IL1- and RacV12-induced increase in transactivation activity, PD98059 reduced the effect to basal levels indicating that p42/p44 MAPK lies downstream of both IL1 and Rac1 in events leading to enhanced transactivation activity of p65. In addition SB203580 inhibited both responses by at least 50%, suggesting that p38 MAPK is also involved in regulating these pathways. Our result indicates that p42/p44 and, to a lesser extent, p38 MAPK mediate the effects of IL1 and Rac1 on enhanced p65 transactivation activity.

DISCUSSION

In this study we provide evidence that Rac1 does not lie on the IL1-induced signaling pathway leading to IκBα phosphorylation and degradation, but instead participates in a second process required for NFκB function, namely the ability of the p65 subunit of NFκB to transactivate gene expression. Our data indicate that there are two separate signals activated by IL1 in the NFκB system. First, NFκB becomes activated by phosphorylation and degradation of IκBα by the signalsome resulting in the subsequent release of NFκB. The second signal enhances the transactivating potential of NFκB, acting on the complex once it is bound to its consensus sequence. Using the two well characterized mutants of Rac1, constitutively active RacV12 and dominant negative RacN17, our results clearly show a role for Rac1 in the latter of these two pathways activated by IL1. Transfection of EL4.NOB-1 cells with RacV12

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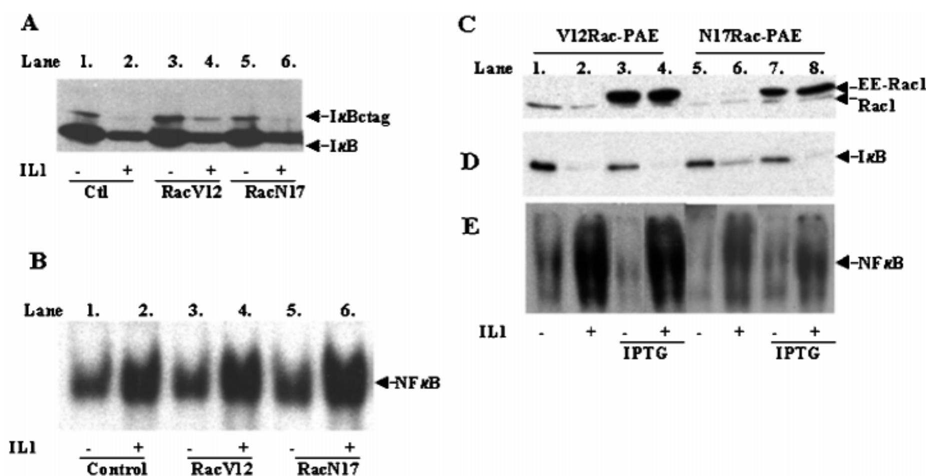

FIG. 4. **Effect of Rac1 on IκBα degradation and DNA binding activity of NFκB.** *A*, EL4.NOB-1 cells (1.4×10^7) were transfected with plasmids encoding IκBctag (10 μg) and either RacV12 or RacN17 (10 μg) as indicated. After 18-h recovery, cells (1×10^6/ml) were stimulated as indicated with IL1 (10 ng/ml, 30 min), and the effect of the Rac1 mutants on IL1-induced degradation of both endogenous and tagged IκBα (IκBctag) was determined by Western blot analysis using an antibody that recognized IκBα. As expected, endogenous IκBα was detected at 38 kDa, and expression of IκBctag was detected at 40 kDa. *B*, the effect of RacV12 and RacN17 on DNA binding ability of NFκB was assessed by electrophoretic mobility shift assay on cells transfected with either RacV12 (10 μg) or RacN17 (10 μg) after stimulation with IL1 (10 ng/ml, 1 h) as indicated. Nuclear extracts were prepared and incubated with radiolabeled κB-dependent probe (30 min, room temperature). DNA-protein complexes are shown. All results are representative of three separate experiments. *C*, V12Rac-PAE and N17RacPAE cells were serum starved for 24 h and expression of the mutants induced (for 24 h) as indicated. Expression of the EE-tagged constructs was detected using an anti-Rac antibody. The effect of RacV12 and RacN17 expression on IL1-induced endogenous IκBα degradation (*D*), and DNA binding ability of NFκB (*E*) was determined as for *A* and *B*.

was unable to drive κB-dependent reporter gene activity, although it potentiated the IL1 response, indicating the need for additional signals in order to see the effects of Rac1 on NFκB-mediated reporter gene expression. This was not the case with the AP1 reporter system, where RacV12 alone drove the response, possibly via activation of JNK or p42/p44 MAPK pathways. Adding IL1, which was only marginally effective on its own, potentiated the Rac1 response. A similar result has been described using JNK activation as a readout (10), which may explain this result.

The key additional signal required for the κB-luciferase response is most likely IκBα phosphorylation and degradation. Unlike IL1, expression of RacV12 in either EL4.NOB-1 or PAE cells did not drive this response, and furthermore RacN17 did not block the effect of IL1. In addition, RacV12 alone was unable to induce nuclear translocation and DNA binding of NFκB. In EL4.NOB-1 and PAE cells, however, RacV12 enhanced the transactivating potential of p65 in the absence of IL1, and importantly RacN17 inhibited IL1-induced transactivation by p65. Taken together these results strongly indicate a role for Rac1 in the pathway leading to enhanced transactivation by p65 but not IκBα phosphorylation and degradation.

Previous studies in HeLa cells and rabbit synovial fibroblasts have proposed that Rac1 mediates NFκB activation via a redox-dependent pathway involving ROS (11, 12). Although Rac1 has been shown to regulate ROS production in a number of different systems, this ability has been demonstrated to be highly cell type-specific (36, 37). Indeed, recent studies in lymphocytes have clearly shown no role for Rac1 in ROS-dependent activation of NFκB, supporting our view that in our system Rac1 lies on an alternate pathway regulating NFκB activation (38). We have been unable to find a role for ROS in NFκB activation by IL1 in EL4-NOB-1 (39).

A key question concerns how Rac1 might enhance p65 transactivating activity. Recent studies have pointed to the involvement of both p42/p44 and p38 MAPK pathways in p65 function in response to TNF-α stimulation (15). Using specific inhibitors of p42/p44 and p38 MAPK pathways (PD98059 and SB203580, respectively), our results also indicate a role for these MAPK pathways downstream of IL1. We have shown previously that IL1 activates both p42/p44 and p38 MAPK in these cells and that this response is blocked by their respective inhibitors (24). Recently it has been shown that SB203580 inhibits phosphorylation of TATA-binding protein, preventing interaction with p65 and thereby blocking transactivation. This provides a possible mechanism for the effect of SB203580 in our studies, implying that activation of p38, via a pathway involving Rac1, leads to TATA-binding protein phosphorylation, promoting transactivation by p65.

Previous work in our laboratory using the T cell distal element of the IL2 promoter found that neither p42/p44 nor p38 MAPK pathways were involved in IL1-induced activation of this element as determined using the CAT reporter gene linked to the T cell distal element (24). This site, although capable of binding NFκB, is not a canonical κB site. It binds additional (but as yet unidentified) factors, and studies have clearly shown differences in how the T cell distal element and the NFκB element are regulated in response to IL1 (40). Our results here clearly indicate that both p42/p44 and p38 MAPK are involved in regulating NFκB transactivation by p65, and hence κB-linked gene expression. In addition it appears that both p42/p44 and p38 MAPK lie downstream of Rac1 on the pathway leading to p65-mediated transactivation of NFκB as PD98059 and SB203580 inhibited RacV12-driven transactivation by p65. Although a role for Rac1 in IL1-mediated activation of p38 MAPK has been demonstrated previously (41), the involvement of Rac1 in p42/p44 activation in response to IL1 has yet to be shown. Our results indicate that Rac1 lies upstream of p42/p44 MAPK and is in keeping with a report that has shown that the downstream effector of Rac1, PAK, can regulate p42/p44 activation via a Raf-independent pathway (42). As well as demonstrating Rac1 activation by IL1, our results demonstrate that the downstream effector with which Rac1 associates after IL1 stimulation is PAK1. Our results therefore support a role for IL1 and Rac1, possibly via PAK1 activation, in regulating p42/p44 and p38 activation and indicate that Rac1, via the p42/p44 and p38 MAPK pathway, is critically involved in regulating the transactivation potential of

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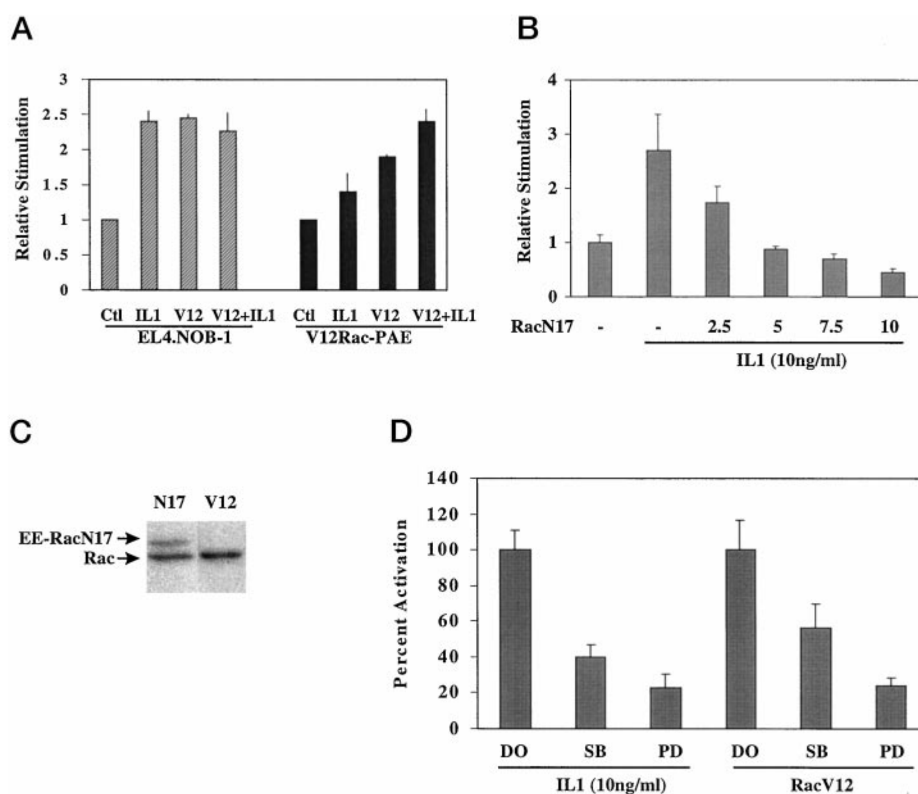

FIG. 5. **Effect of Rac1 on p65-mediated transactivation of NFκB.** *A*, EL4.NOB-1 cells (1.4×10^7) and V12Rac-PAE (4×10^4) were transfected with Gal-luciferase reporter plasmid (5 μg and 350 ng, respectively) and Gal4-p65$^{1-551}$ (2.5 μg and 350 ng, respectively). In addition, EL4.NOB-1 cells were cotransfected with RacV12 (10 μg) as indicated. RacV12 expression was induced 6 h post-transfection in V12Rac-PAE cells, and both cell types were stimulated with IL1 (10 ng/ml, 6 h) 18 h later. *B*, EL4.NOB-1 cells (1.4×10^7) were transfected with Gal-luciferase reporter plasmid (5 μg) and expression plasmids encoding Gal4-p65$^{1-551}$ (2.5 μg) and increasing amounts RacN17 (0–10 μg) as indicated. Cells were allowed to recover for 16–18 h after which they were stimulated with IL1 (10 ng/ml, 6 h) and cell extracts prepared and assayed for luciferase activity. *C*, the expression of EE-RacN17 and EE-RacV12 in noninduced N17Rac-PAE and V12Rac-PAE cell lines, respectively, was detected by Western blotting using an anti-Rac antibody. *D*, EL4.NOB-1 cells were transfected as in *A*, and after recovery they were incubated in complete medium containing 0.5% fetal calf serum. Cells were pretreated with inhibitors or vehicle control (dimethyl sulfoxide) as indicated (1 h, 37 °C): *DO*, dimethyl sulfoxide; *PD*, PD98059 (30 μM); SB203580, 30 μM SB203580. Subsequent to this they were stimulated with IL1 (10 ng/ml, 6 h) as indicated and lysates assayed for luciferase activity. Results (mean ± S.D. for triplicate determinations) in *A* and *B* are represented as fold increase compared with unstimulated controls; in *C* they are shown relative to response to IL1 or RacV12. In all cases results are representative of at least three separate experiments.

NFκB in response to IL1.

Several reports have demonstrated that upon stimulation with either TNF-α or IL1 the p65 subunit of NFκB becomes phosphorylated on multiple serines thus potentially acting to enhance p65 transactivating potential (17, 18). Although p38 and p42/p44 may be involved, these are unlikely to phosphorylate p65 directly because of the lack of consensus sites for phosphorylation. The kinase(s) directly responsible for phosphorylating p65 have yet to be identified, although a role for casein kinase II has been proposed as it has been shown to phosphorylate the transactivation region found in the COOH-terminal domain of p65 (20). Recently, an as yet unidentified kinase has been shown to regulate phosphorylation of the transactivation domain of p65 on serine 529, regulating the transactivational activity of NFκB (18). In addition, recent work demonstrated that protein kinase A is involved in events leading to the phosphorylation of serine 276 in the Rel homology domain of NFκB (17). The NH_2-terminal Rel homology domain is crucial for regulating the binding of p65 to its consensus sequence. This domain has also been shown recently to play an important role in regulating transactivation signals in response to TNF-α stimulation, with protein kinase Cζ (activated by Ras) shown to play a role in regulating phosphorylation of this domain on a site other than serine 276 (43). In addition to p65 subunit phosphorylation, interaction with the coactivator p300/CBP has been shown to enhance NFκB transcriptional activity. p300/CBP is constitutively associated with RNA polymerase II, and interaction with p65 via its COOH-terminal transactivation domains results in increased transcriptional activity of NFκB which is enhanced after phosphorylation of the Rel homology domain by protein kinase A (17, 19). We would speculate that Rac1 is required for some or all of these events in our system via the activation of both p38 and p42/p44 MAPK pathways. As mentioned above, TATA-binding protein would be another possible target for p38 here.

How IL1 may mediate Rac1 activation is as yet unclear, although a recent report indicates that Rac1 may associate with the IL1 receptor complex (44). Our data clearly support this observation in that we provide direct evidence for Rac1 activation by IL1 and the subsequent interaction between Rac1 and PAK1. Furthermore, the intracellular domain of the IL1 receptor has been shown to associate with the p85 regulatory domain of phosphatidylinositol 3-kinase (which has previously been shown to activate Rac1 (31, 45)) via a potential phosphotyrosine motif on the receptor (32). Whether phosphatidylinositol 3-kinase interaction with IL1 type I receptor results in Rac1 activation in response to IL1 stimulation remains to be elucidated.

In conclusion, our results indicate that IL1 mediates the activation of two separate signaling pathways that, combined, regulate the activity of the transcription factor NFκB. We have demonstrated a role for Rac1 in IL1-induced enhancement of NFκB transactivation potential independent of both IκB degradation and nuclear translocation and DNA binding of NFκB. Furthermore, both p38 and p42/44 MAPK pathways are re-

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quired for p65-dependent transactivation of NFκB mediated by both IL1 and Rac1.

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