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rac1 Regulates a Cytokine-Stimulated, Redox-Dependent Pathway Necessary for NF-κB Activation

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The signal transduction pathway leading to the activation of the transcription factor NF-κB remains incompletely characterized. We demonstrate that in HeLa cells, transient expression of a constitutively active mutant of the small GTP-binding protein rac1 (V12rac1) leads to a significant increase in NF-κB transcriptional activity. In addition, expression of a dominant-negative rac1 mutant (N17rac1) inhibits basal and interleukin 1β-stimulated NF-κB activity. Gel shift analysis using nuclear extract prepared from HeLa cells infected with a recombinant adenovirus encoding N17rac1 (Ad.N17rac1) showed reduced levels of cytokine-stimulated DNA binding to a consensus NF-κB binding site. We demonstrate that rac proteins function downstream of ras proteins in the activation of NF-κB. In addition, V12rac1 stimulation of NF-κB activity is shown to be independent of the ability of rac proteins to activate the family of c-jun amino-terminal kinases. In an effort to further explore how rac proteins might regulate NF-κB activity, we demonstrate that expression of V12rac1 in HeLa cells or stimulation with cytokine results in a significant increase in intracellular reactive oxygen species (ROS). Treatment of cells with either of two chemically unrelated antioxidants inhibits the rise in ROS that occurs following V12rac1 expression as well as the ability of V12rac1 to stimulate NF-κB activity. These results suggest that in HeLa cells, rac1 regulates intracellular ROS production and that rac proteins function as part of a redox-dependent signal transduction pathway leading to NF-κB activation.

Activation of the transcription factor NF-κB is stimulated by a variety of agents, including cytokines, viruses, phorbol esters, and UV light (reviewed in references 2 and 43). In unstimulated cells, NF-κB is thought to consist of a cytoplasmic complex of homodimeric or heterodimeric Rel-related proteins bound to a member of the IκB family of inhibitor proteins. Agents that stimulate NF-κB result in the phosphorylation and degradation of the IκB inhibitory subunit. Previous studies have suggested that the activation of NF-κB may be redox dependent (16, 28, 37, 40, 44, 50). In particular, micromolar doses of exogenous H_2O_2 stimulate NF-κB activation (28), while chemical antioxidants inhibit the activation of NF-κB by a variety of exogenous stimuli (reviewed in reference 39). Furthermore, agents that stimulate NF-κB, including cytokines, UV light, and phorbol esters, appear capable of increasing intracellular reactive oxygen species (ROS) (25, 27, 46). Nonetheless, relatively little is known regarding the putative pathway in which, for instance, cytokine stimulation would lead to an increase in ROS levels and subsequent NF-κB activation.

The small GTP-binding protein rac1 belongs to the ras superfamily of proteins. Previous studies have established a role for rac proteins in cytoskeletal reorganization and in particular membrane ruffling (34). Recently, rac1 has also been shown to be required for serum-stimulated DNA synthesis in fibroblasts (31) as well as to cooperate with ras proteins in transformation (33). Analysis of downstream effectors suggests that rac proteins regulate the activity of the serine/threonine c-jun amino-terminal kinases (JNKs) (6, 29, 31). In addition, in phagocytic cells, rac proteins are involved in the assembly of the neutrophil NADPH oxidase system (1, 20). Following ligand stimulation of neutrophils, the NADPH oxidase complex is assembled and is responsible for transferring electrons from NADPH to molecular oxygen with the subsequent production of superoxide anions (O_2^-) (reviewed in reference 4). The superoxide generated is then rapidly dismutated spontaneously or enzymatically to hydrogen peroxide.

A variety of evidence suggests that nonphagocytic cells are capable of producing ligand-stimulated ROS (11, 25, 27, 30, 42, 46, 47). Indeed, many of the components of the NADPH oxidase system appear to exist in a variety of cell types (18). It has recently been demonstrated that in fibroblasts, both growth factor- and cytokine-stimulated ROS production occurs through a rac-dependent pathway (46). As such, one function of rac proteins may be to regulate redox-dependent signal transduction pathways. To further test this hypothesis, we sought to explore the ability of rac proteins to regulate the activity of the redox-dependent transcription factor NF-κB.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assays. HeLa cells were obtained from American Type Culture Collection (Rockville, Md.) and cultured in minimum essential medium containing 10% heat-inactivated fetal calf serum. Twenty-four hours prior to transfection, cells were seeded at an approximate density of $2.5 \times 10^4/cm^2$. For transfections, Lipofectamine (Gibco-BRL) was used according to the manufacturer's protocol. For chloramphenicol acetyltransferase (CAT) assays, cells were harvested 48 h after transfection and 20 μg of cellular lysate was used. CAT activity was assessed by the phase extraction method as previously described (10, 41). Results of CAT assays are representative of at least three separate experiments using at least two separate DNA preparations. In some experiments, in which either the constitutively active or the dominant-negative rac1 gene was used, cells were cotransfected with a β-galactosidase reporter gene (pSV-βgal) to normalize for any differences in transfection efficiency. No qualitative differences in the results were obtained after normalization (data not shown). Absolute levels of CAT activity varied from one experiment to the next, so results are expressed as means ± standard deviations of triplicate determinations from a single representative experiment. For CAT assays, treatment with interleukin 1β (IL-1β) (Upstate Biotech) was done for 12 h prior to harvest, while treatment with the antioxidants *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) (Sigma) was done for the final 36 h.

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Plasmids. The expression vectors pEXV-V12rac1 and pEXV-N17rac1, encoding the constitutively active and dominant-negative myc epitope-tagged rac1 cDNAs, have been previously described (34), as have the NF-κB wild-type (3X-κB-CAT) and mutant (3X-mutκB-CAT) reporter genes (9). Expression vectors containing the wild-type sek (SAPK/ERK kinase) and dominant-negative sek [sek(KR)] as well as the epitope-tagged JNK (HA-JNK) were a generous gift of Silvio Gutkind (National Institutes of Health). The V12ras expression vector has been previously described (10), as has the codon 61-activated mutant of Cdc42, L61Cdc42 (6).

Adenovirus construction and infection. Adenovirus Ad.N17rac1, containing the epitope-tagged dominant-negative rac1 cDNA, was constructed by homologous recombination in 293 cells using the adenovirus-based plasmid JM17 (26). Recombinant plaques were screened by the PCR and confirmed by dideoxy sequencing. A single positive adenovirus plaque containing the N17rac1 cDNA and a deletion in the E1 region was amplified in 293 cells and purified on a double cesium gradient as previously described (12). Infections were carried out with the indicated multiplicity of infection (MOI) for 24 h, after which the virus-containing medium was aspirated and replaced with fresh medium. The E1-deleted adenovirus *dl*312 (17), which lacks a cDNA insert, was used as a control for adenovirus infection.

DCF and JNK assays. Forty-eight hours after transfection, cells were incubated for 5 min with Hanks buffered saline (Gibco-BRL) lacking phenol red and supplemented with 5 μg of 2′,7′-dichlorodihydrofluorescin diacetate (DCF) (Molecular Probes) per ml. Images were obtained with a Leica Laser confocal scanning microscope (model TCS-4D) as previously described (30, 47). Fluorescence was quantitated on a 0 to 255 arbitrary gray scale and represents the fluorescent intensity (mean ± standard deviation) of approximately 60 random cells obtained from three separate fields on each of two separate plates. JNK assays were performed as previously described (6) using the truncated ATF2 (amino acids 1 to 96) as a substrate.

Western blot (immunoblot) analysis. Forty-eight hours after transfection, cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Thirty micrograms of protein extract was resolved on an SDS–12% polyacrylamide gel and then transferred to a nitrocellulose filter. The filter was then cut, and exogenous rac1 protein was detected via the myc epitope tag (9E10; Santa Cruz Labs). In some cases, simultaneous measurement of tubulin levels was used to confirm equal protein loading (CP06; Calbiochem). Immunocomplexes were visualized by enhanced chemiluminescence (Tropix).

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared by previously described methods (42). Binding reactions were done for 30 min and used 2.5 μg of nuclear extract in 10 mM Tris (pH 7.5)–50 mM NaCl–1 mM EDTA–10% glycerol–1 μg of poly(dI:dC)–1 mM dithiothreitol. The NF-κB consensus sequence 5′-AGT TGA GGG GAC TTT CCC AGGC-3′ was end labeled to a specific activity of 10^6 dpm/pM, and approximately 10^5 dpm was used per reaction. DNA-protein complexes were resolved on a 6% polyacrylamide gel.

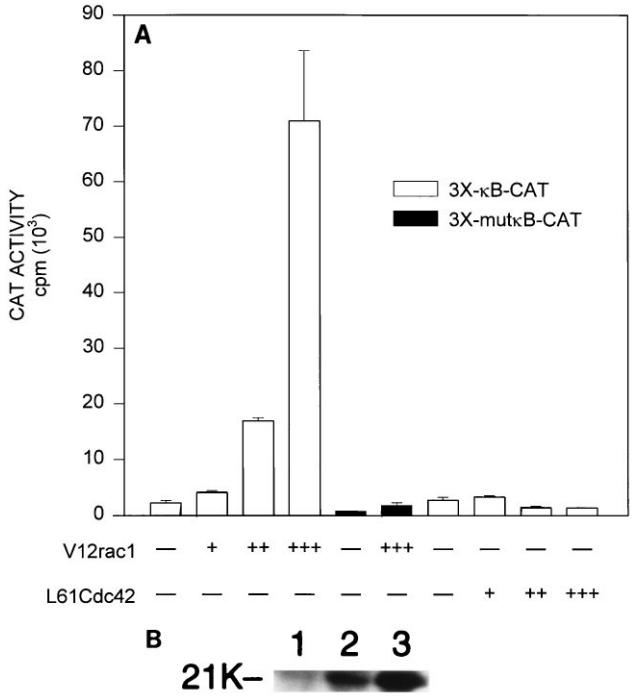

FIG. 1. Expression of V12rac1 increases NF-κB activity. (A) HeLa cells were plated 24 h prior to transfection, and individual wells of a six-well plate were transfected with a total of 3.5 μg of DNA. Cells were harvested 48 h after transfection. Each well received 0.5 μg of the reporter construct 3X-κB-CAT or, where indicated, the mutant 3X-mutκB-CAT reporter construct. In addition, cells were transfected with a total of 3.0 μg of plasmid DNA of which rac1 or activated Cdc42 expression vector composed either 0.5 (+), 1.5 (++), or 3.0 (+++) μg. To maintain equal amounts of total DNA, the appropriate amount of empty expression vector was used as filler DNA. Results were expressed as means ± standard deviations of triplicate determinations from one representative experiment. (B) Western blot analysis of the level of V12rac1 expression. Lysates from cells transfected with a total of 3.0 μg of DNA, with the amounts of V12 rac1 being 0.5 (lane 1), 1.5 (lane 2), and 3.0 (lane 3) μg, were used. An antibody recognizing the myc epitope was used to detect the level of V12rac1 expression.

RESULTS

Stimulation of NF-κB activity by V12rac1. In an attempt to explore the role of rac proteins in NF-κB activation, we cotransfected HeLa cells with an expression vector encoding a constitutively active mutant of rac1, V12rac1, along with a CAT reporter plasmid containing three tandem NF-κB binding sites (3X-κB-CAT). As seen in Fig. 1A, compared with the expression vector alone (pEXV), increasing ratios of the V12rac1 expression vector to reporter plasmid resulted in increasing NF-κB activity. A similar reporter plasmid (3X-mutκB-CAT) containing a single-base-pair substitution in the consensus NF-κB site showed essentially no stimulation with V12rac1 expression. The increase in NF-κB activity seen with increasing amounts of V12rac1 DNA correlated with a proportional increase in rac1 protein expression (Fig. 1B). rac1 stimulation of NF-κB transcriptional activity did not appear to occur through an autocrine mechanism, since neither supernatant obtained from V12rac1-transfected cells nor direct cocultures of V12rac1-expressing cells had any effect on the activity of the NF-κB reporter construct (data not shown). The ability of V12rac1 to stimulate NF-κB activity was not shared by other small GTP binding patterns. In particular, transfection of the activated form of the closely related Cdc42 resulted in no appreciable increase in NF-κB activity (Fig. 1A).

The dominant-negative mutant N17rac1 inhibits NF-κB activation by IL-1β. Previous studies have demonstrated that a serine-to-asparagine substitution at position 17 of rac proteins leads to a protein that exhibits properties of a dominant-negative mutant (34). In order to further assess the role of rac proteins in NF-κB activity, HeLa cells were cotransfected with an NF-κB reporter gene along with a dominant-negative rac1 plasmid (N17rac1) or an expression plasmid lacking an insert (pEXV). Thirty-six hours after transfection, the cytokine IL-1β was added where indicated. As demonstrated in Fig. 2, compared with cells transfected with the expression vector alone, cells transfected with the N17rac1 vector had reduced basal and cytokine-stimulated NF-κB activity. Qualitatively similar results were obtained when tumor necrosis factor alpha was used as a stimulant (data not shown).

In an effort to confirm these results, using an independent assay of NF-κB activation, we infected cells with a replication-deficient recombinant adenovirus encoding N17rac1 (Ad.N17rac1). Expression of the epitope-tagged N17rac1 could be readily ascertained following infection of HeLa cells with Ad.N17rac1 but not in mock-infected cells or in cells infected with a control adenovirus (*dl*312) (Fig. 3A).

Nuclear extracts were prepared from uninfected, *dl*312-infected, and Ad.N17rac1-infected HeLa cells and used for an EMSA. As seen in Fig. 3B, in uninfected and *dl*312-infected cells, IL-1β stimulation resulted in an appreciable increase in NF-κB binding. In contrast, in cells infected with Ad.N17rac1, the levels of basal and cytokine-stimulated NF-κB binding

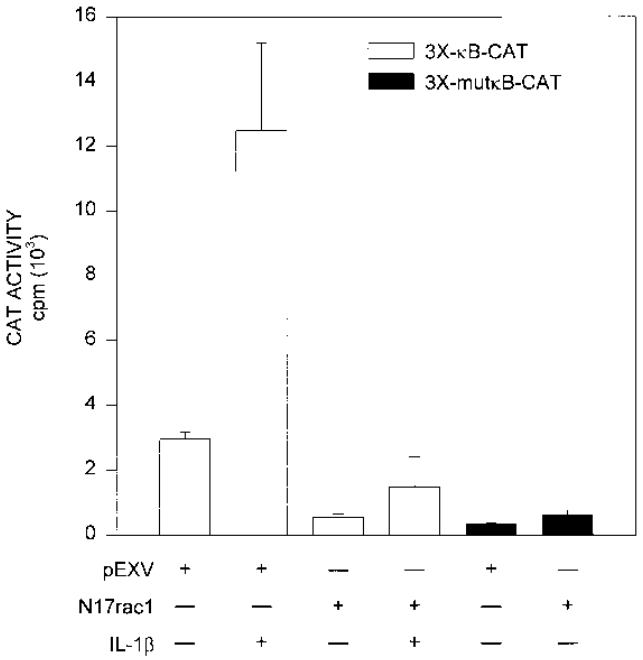

FIG. 2. Transfection of N17rac1 inhibits NF-κB activity. HeLa cells were transfected with 3.5 μg of DNA containing 0.5 μg of either the wild-type 3X-κB-CAT or mutant 3X-mutκB-CAT reporter construct and 3.0 μg of either the expression vector (pEXV) alone or the same expression vector containing the N17rac1 cDNA. Results are means ± standard deviations from triplicate determinations of one representative experiment.

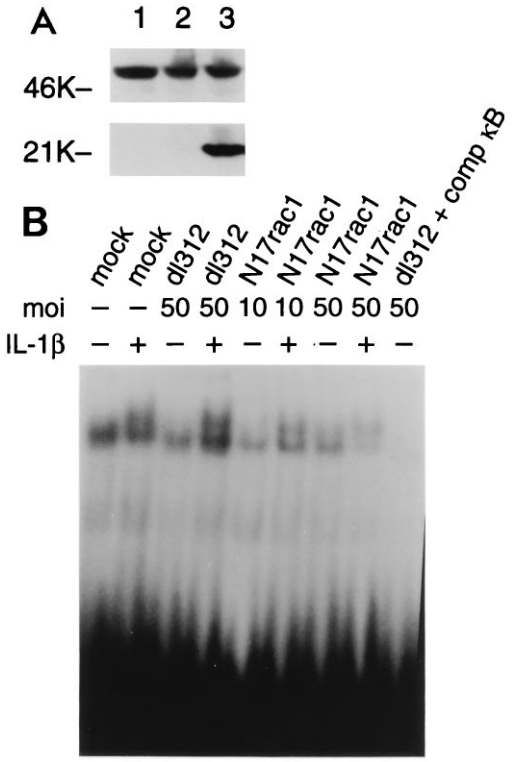

FIG. 3. Infection with Ad.N17rac1 inhibits NF-κB DNA binding. (A) Western blot analysis of lysates from mock-infected cells (lane 1) and HeLa cells infected with an MOI of 50 of the control adenovirus *dl*312 (lane 2) or with an MOI of 50 of Ad.N17rac1 (lane 3). Nitrocellulose filters were probed with an anti-myc antibody (lower panel), which recognizes the epitope-tagged form of rac1, or with an antitubulin antibody (top panel) to confirm equal protein loading. (B) EMSA of mock-infected, *dl*312-infected, or Ad.N17rac1-infected HeLa cells. Cells were harvested 48 h after infection with the indicated MOI. IL-1β (100 U/ml) was added 30 min prior to harvest. A 40-fold excess of unlabeled specific oligonucleotide (comp κB) was added where indicated.

were inhibited. The degree of inhibition was a function of the MOI of Ad.N17rac1, with an MOI of 50 leading to a greater inhibition than an MOI of 10. Western blot analysis confirmed that over this range, a higher MOI resulted in a proportional increase in N17rac1 expression (data not shown).

N17rac1 inhibits ras stimulation of NF-κB. Previous studies have demonstrated a role for ras proteins in NF-κB activation by both UV light and cytokines (8, 9). In a variety of signal transduction pathways, rac appears to function downstream of ras (13, 33, 34). In an effort to test whether rac acts downstream of ras in the activation of NF-κB, we assayed whether N17rac1 could inhibit the ability of an activated ras (V12ras) expression vector to stimulate NF-κB activity. As seen in Fig. 4, transfection of the ras expression plasmid alone resulted in an approximately eightfold increase in the NF-κB transcriptional activity. The increase seen with V12ras expression was blocked by N17rac1 in a dose-dependent fashion. These results are therefore consistent with a pathway in which rac functions downstream of ras.

V12rac1 stimulates NF-κB independently of JNK activation. Expression of V12rac1 has been recently shown to activate the JNKs (6, 29, 31). Evidence suggests that activation of NF-κB requires phosphorylation of the IκB subunit (2, 43), although the cellular kinase responsible for site-specific phosphorylation is unknown. If the JNKs were capable of phosphorylation of Iκ-B, we thought it possible that activation of JNKs might provide a link between rac proteins and NF-κB activation. To test this hypothesis, we employed conditions under which the activity of the JNKs is inhibited. It is generally believed that rac protein activates JNKs through a sequential pathway in which sek kinase lies immediately upstream of the JNKs (7, 35). Expression of a dominant-negative sek, sek(KR), has previously been shown to be capable of inhibiting JNK activation in other cell types (49). However, as seen in Fig. 5A, by use of an NF-κB-dependent reporter gene, cotransfection of sek(KR) had little effect on the ability of either V12rac1 or IL-1β to stimulate NF-κB transcriptional activity. To confirm that under these conditions expression of sek(KR) inhibited JNK activation, we used an in vitro kinase assay of immunoprecipitated epitope-tagged JNK and a truncated ATF2 as a peptide substrate. As seen in Fig. 5B, transfection of sek(KR) effectively inhibited JNK activity stimulated by the presence of V12rac1 or IL-1β. These results suggest that in HeLa cells, the activation of the JNK family is not required for NF-κB activation.

V12rac1 regulates ROS generation in HeLa cells. Another potential mechanism by which rac proteins could regulate NF-κB activity is through the production of ROS. In an effort to assess the level of intracellular ROS, cells were loaded with the fluorophore DCF, which fluoresces upon interaction with H_2O_2 (30, 47). Compared with cells transfected with the expression vector alone (Fig. 6A), cells transfected with the V12rac1 plasmid had a significant increase in basal DCF fluorescence (Fig. 6B). A qualitatively similar rise in DCF fluorescence intensity was noted in control (pEXV)-transfected cells 10 min after stimulation with IL-1β (100 U/ml) (Fig. 6C). In cells transfected with the N17rac1 expression vector, the rise in ROS seen after IL-1β stimulation was markedly reduced (Fig. 6D). Treatment of HeLa cells with two chemically unrelated antioxidants, PDTC and NAC, resulted in a reduction of

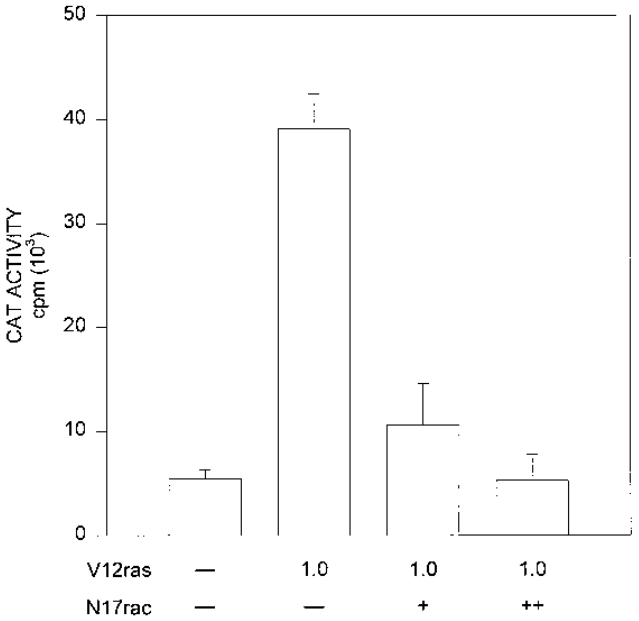

FIG. 4. N17rac1 inhibits ras stimulation of NF-κB activity. HeLa cells were transfected with a total of 3.5 μg of DNA. Each sample received 0.5 μg of the NF-κB-dependent reporter construct 3X-κB-CAT and, where indicated, 1.0 μg of an activated ras (V12ras) plasmid and either 0.5 (+) or 1.0 (++) μg of the N17rac1 plasmid. Results are expressed as means ± standard deviations of a single representative experiment performed in triplicate.

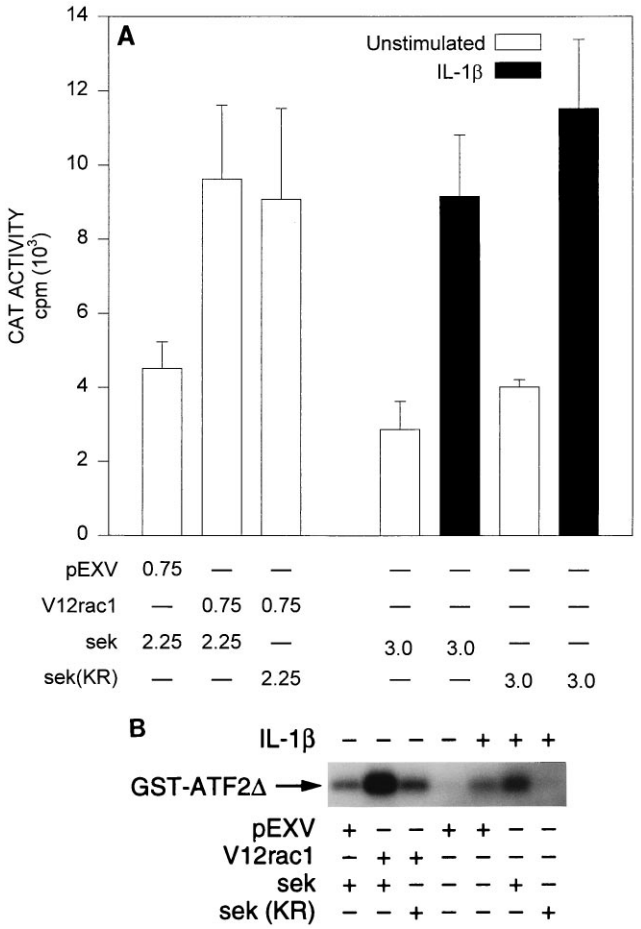

FIG. 5. V12rac1 stimulates NF-κB independently of JNK activation. (A) HeLa cells were transfected with 0.5 μg of the 3X-κB-CAT reporter and the indicated amounts of pEXV, V12rac1, sek, and sek(KR) expression vectors. The data are means ± standard deviations from triplicate determinations of one representative experiment. (B) JNK activity from lysates transfected under the same conditions as above except that 1 μg of an epitope-tagged JNK expression vector (HA-JNK) was added. The cells were stimulated with IL-1β (100 U/ml) for 30 min prior to being harvested. The truncated transcription factor ATF2Δ was used as a substrate for an in vitro kinase reaction.

V12rac1-stimulated DCF fluorescence (Fig. 6E), confirming that the rise in DCF fluorescence was due to an increase in intracellular ROS levels. Both PDTC and NAC have also been previously reported to inhibit NF-κB activation (39).

Antioxidants inhibit V12rac1 stimulation of NF-κB. In an effort to assess the role of ROS in rac1 stimulation of NF-κB, we treated cells with various doses of the cell-permeant antioxidants PDTC and NAC. As seen in Fig. 7A, the ability of V12rac1 to stimulate an NF-κB reporter plasmid was inhibited in a concentration-dependent fashion by both antioxidants. Qualitatively similar results were obtained with gel shift experiments. Compared with control (pEXV)-transfected cells, V12rac1-transfected cells had a significant increase in NF-κB DNA binding (Fig. 7B). These results support our earlier observations regarding V12rac1 stimulation of an NF-κB reporter gene (Fig. 1A). The increase in NF-κB binding seen with V12rac1 was inhibited by treatment with the antioxidant PDTC. Similar EMSA results were obtained with NAC treatment of cells expressing V12rac1 (data not shown).

DISCUSSION

The results presented here suggest a role for rac1 in the signal transduction pathway leading to NF-κB activation. We demonstrate that activated rac1 mutants increase and dominant-negative rac1 mutants inhibit NF-κB transcriptional activity and DNA binding. The ability of rac1 to stimulate NF-κB appears to be separate from the activation of the family of JNKs. In addition, we demonstrate that V12rac1 activates ROS production in cells and that rac proteins are necessary for the rise in ROS which occurs following cytokine stimulation. Treatment of cells with antioxidants reduces the levels of V12rac1-stimulated ROS and rac1-stimulated NF-κB activation. These results suggest that rac1 regulates the intracellular redox state and provide support for a sequential pathway linking small GTP-binding proteins, ROS production, and the activation of NF-κB.

The concept that ROS may regulate gene expression appears to extend throughout evolution. Bacteria respond to a rise in ROS levels by the coordinated expression of gene products responsible for bacterial survival. Regulation of this process occurs by direct oxidation of the *oxyR* and *soxS* transcription factors (45). Similarly, hydrogen peroxide appears to regulate the hypersensitive response in plants (24). Indeed, salicyclic acid appears to function in plants at least in part by binding and inactivating the peroxide-scavenging enzyme catalase, leading to a rise in H_2O_2 and to activation of gene expression (5). In mammalian cells, an increase in ROS levels has been detected following stimulation by cytokines (25, 27) or growth factors (25, 42, 46, 47). Recent evidence suggests that a rise in H_2O_2 is necessary for some aspects of platelet-derived growth factor signal transduction (47).

It is currently unclear exactly how rac proteins regulate the level of ROS in nonphagocytic cells. It is tempting to speculate that there exists a NADPH oxidase enzyme complex whose activity may be regulated by rac proteins and stimulated by

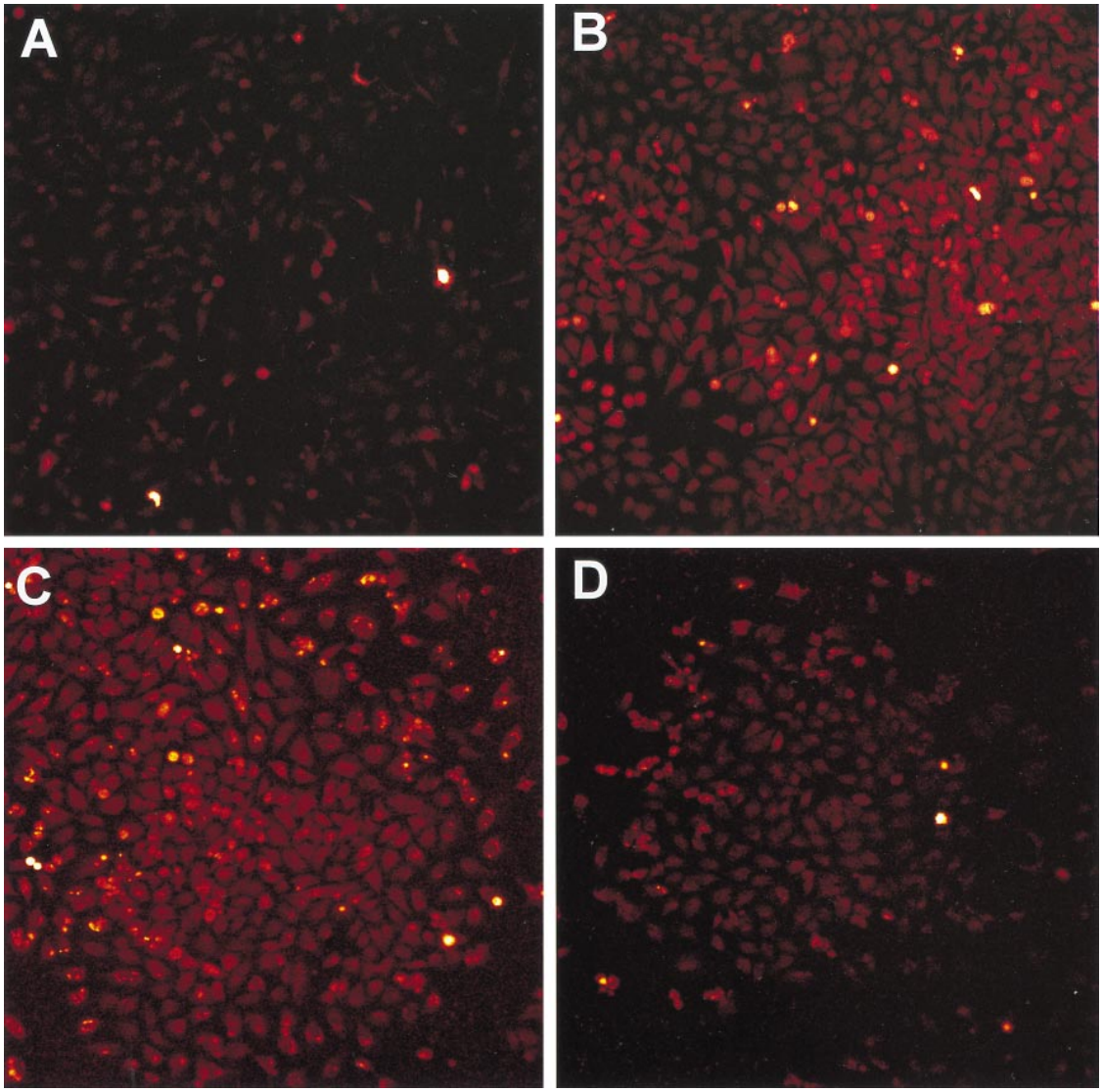

FIG. 6. Regulation of intracellular ROS levels by rac proteins. HeLa cells were plated in 6-cm-diameter dishes and transfected with 5 μg of the empty expression vector pEXV (A and C), the V12rac1 expression vector (B), or the N17rac1 expression vector (D). Levels of fluorescence under basal conditions (A and B) or 10 min after IL-1β (100 U/ml) stimulation (C and D) were determined by using the peroxide-sensitive fluorophore DCF and imaged on a confocal laser scanning fluorescence microscope. (E) DCF fluorescence was quantitated in control (pEXV)-transfected cells, V12rac1-transfected cells, and V12rac1-transfected cells treated for 1 h with antioxidant PDTC (180 μM) or NAC (5 mM). Results represent the fluorescence intensity (means ± standard deviations) of 60 random cells.

growth factors or cytokines. This hypothesis is supported by the existence in nonphagocytic cells of many of the protein components of the NADPH oxidase (18), the presence of NADH or NADPH oxidase activity in membrane fractions (11, 21), and the ability to inhibit the rise in ROS seen with cytokine or growth factor stimulation with pharmacological inhibitors of the neutrophil NADPH oxidase (11, 25). Under such circumstances, similar to what occurs in the neutrophil, rac proteins may directly act via protein-protein interaction to organize the ROS enzyme system. Alternatively, other sources of rac-regulated ROS generation may exist. In particular, recent evidence suggests a role of rac proteins in arachidonic acid metabolism,

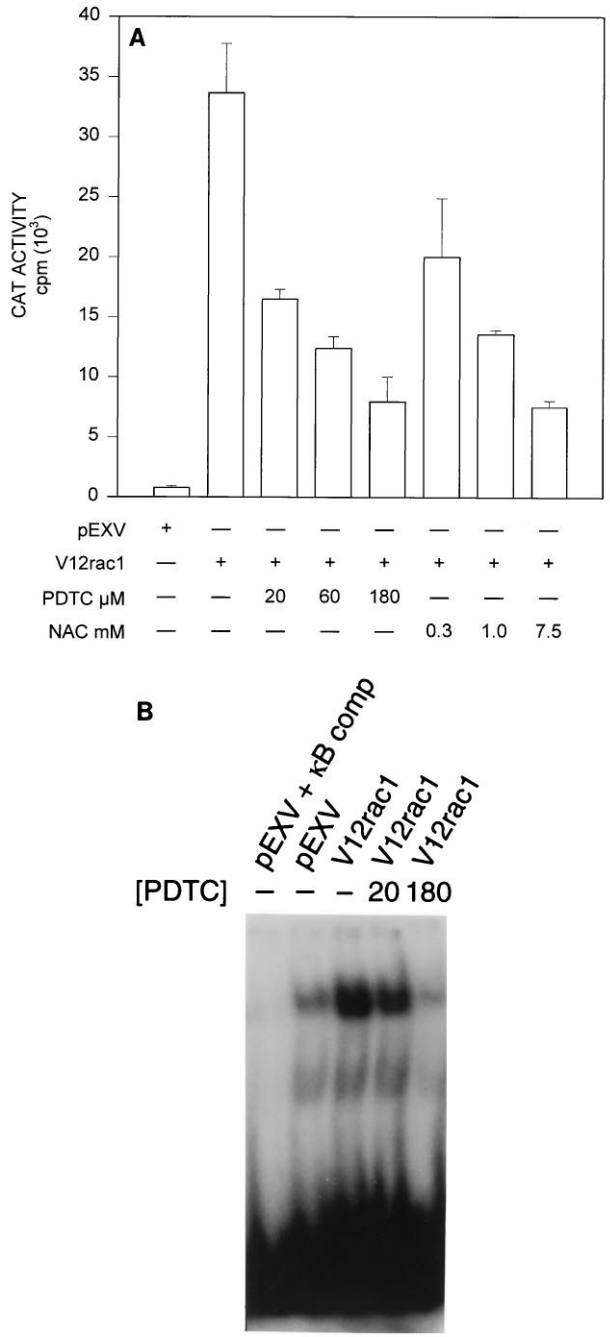

FIG. 7. Antioxidants inhibit V12rac1 stimulation of NF-κB. (A) HeLa cells were transfected with 0.5 μg of the reporter construct 3X-κB-CAT and 3.0 μg of either the V12rac1 expression vector or the expression vector alone. Where indicated, the cells were treated with the listed concentration of NAC or PDTC for 36 h prior to being harvested. (B) Gel shift assays of nuclear extracts of HeLa cells transfected with the empty expression vector (pEXV) or the V12rac1 expression vector. Where indicated, cells were treated with either 20 or 180 μM PDTC for 1 h prior to being harvested. Specific binding to a consensus NF-κB oligonucleotide was determined by adding a 40-fold excess of unlabeled probe (comp κB).

a process known to result in the production of ROS (32). As such, it may be interesting to test the effects of inhibitors of phospholipase A_2 or lipoxygenases on V12rac1-stimulated NF-κB activity. In addition, the recently reported crystal structure of the GDP dissociation inhibitor (GDI) for the bovine α-isoform of Rab suggests a structural organization closely related to that of flavin adenine dinucleotide-containing oxidases (36). This raises the possibility that other GDI-like molecules which interact with ras family members may possess ligand-stimulated oxidase activity.

Previous studies have demonstrated a role for both ROS and ras proteins in NF-κB activation (8, 9). It remained unresolved in what way, if at all, small GTP-binding proteins and ROS interacted or whether they represented separate pathways leading to the activation of NF-κB. The observations described here are consistent with the notion that small GTP-binding proteins in some fashion act to regulate the level of intracellular ROS. As such, these results provide evidence for a sequential pathway in which a cytokine or other stimuli lead to activation of ras, then rac, and subsequently to the generation of ROS.

Activation of rac1 is thought to result in the subsequent sequential activation of a series of protein kinases, including MEK kinase (MEKK), sek kinase, and JNK. Given our finding that rac1 regulates NF-κB activation, it is tempting to speculate that one or more of the rac1-regulated kinases besides JNK may be involved in the site-specific phosphorylation of the IκB inhibitory subunit. Such a hypothesis is supported by the recent observation that MEKK can regulate NF-κB activation (15).

It remains unclear by what mechanism a rac1-regulated rise in ROS could effect the activation of NF-κB. Given that many kinases (3) and phosphatases (14) are redox sensitive, it is possible that ROS act downstream of rac1 and function in the activation of a rac1-regulated kinase, such as MEKK. Alternatively, recent evidence suggests that ROS can directly inhibit the GTPase activity of small GTP-binding proteins (23). A reactive cysteine at position 118 of the ras protein has been mapped as the redox-sensitive site (22). In this case, the rac1-regulated increase in ROS could potentially function upstream of rac1 by inhibiting ras or rac GTPase activity and thus serve to maintain the small GTP-binding proteins in the active configuration.

Alternatively, the rise in ROS could function to activate NF-κB through an independent pathway, perhaps by directly affecting a phosphatase which may be critical to keeping the IκB subunit in a dephosphorylated state. Such a hypothesis is supported by the observation that the phosphatase inhibitors calyculin A and okadaic acid can activate NF-κB even in the presence of antioxidants (48), suggesting that phosphatase inhibitors may act downstream of ROS.

The abnormal production of ROS has been linked to many disease processes, including coronary artery disease, arthritis, and even cancer (for a review, see reference 19). Large clinical studies are under way to evaluate the use of antioxidants in the prevention of a multitude of chronic diseases. It is generally assumed that antioxidants function to scavenge ROS produced by phagocytic cells. Given the growing realization that nonphagocytic cells may employ ROS production in a stress response pathway leading to NF-κB activation, it is reasonable to consider that additional targets of antioxidant therapy exist. As a more detailed understanding of the pathway leading to ROS production in nonphagocytic cells emerges, it may be possible to specifically interrupt the intracellular production of ROS, which in turn may be of benefit in chronic inflammatory and disease states.

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