RESEARCH COMMUNICATION

Regulation of reactive-oxygen-species generation in fibroblasts by Rac1

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In a variety of non-phagocytic cell types, there is a marked increase in intracellular levels of reactive oxygen species (ROS), including superoxide and H_2O_2, after ligand stimulation. We demonstrate that in NIH 3T3 cells transient expression of constitutively activated forms of the small GTP-binding proteins Ras or Rac1 leads to a significant increase in intracellular ROS. An increase in intracellular ROS is also demonstrated after growth factor [platelet-derived growth factor (PDGF) or epidermal growth factor (EGF)] or cytokine [tumour necrosis factor-α (TNF-α) or interleukin (IL)-1β] stimulation of NIH 3T3 cells. Expression of a dominant negative allele of Rac1 inhibits the rise in ROS seen after Ras expression or after stimulation by either growth factors or cytokines. These results provide the first demonstration of the pathway by which ligand stimulation of ROS occurs in non-phagocytic cells and suggest that the family of Ras-related small GTP-binding proteins may function as regulators of the intracellular redox state.

INTRODUCTION

Although reactive oxygen species (ROS) are conventionally viewed as toxic by-products of cellular metabolism, a growing body of evidence suggests that they may regulate signal transduction in both plant [1,2] and animal cells (reviewed in [3]). In mammalian cells, a variety of extracellular stimuli, including cytokines [4,5] and growth factors [6–10], produce a transient burst of ROS. Inhibition of this rise in intracellular ROS by either chemical or enzymic scavengers has been shown to inhibit platelet-derived growth factor (PDGF)-stimulated signal transduction [9] as well as cytokine-stimulated gene transcription [11–13].

The pathway by which ROS are generated is poorly characterized except in certain specialized cell types. Activation of phagocytic cells by a host of different agonists leads to the assembly of an NADPH oxidase enzyme complex composed of at least four proteins (reviewed in [14,15]). This enzyme system is responsible for transferring electrons from NADPH to molecular oxygen with the subsequent generation of superoxide anions (O_2^-). The O_2^- generated is rapidly dismutated spontaneously or enzymically to H_2O_2. In neutrophils, the activity of the NADPH oxidase system is regulated by the small GTP-binding protein Rac2 [16], whereas in macrophages, the NADPH oxidase appears to be regulated by Rac1 [17]. Biochemical evidence suggests the existence in non-phagocytic cells of a cytokine- or growth-factor-activated ROS-generating system that is antigenically distinct but functionally similar to the neutrophil NADPH oxidase [4,5,7,10,18]. Indeed, many but not all of the components of the NADPH oxidase system appear to be expressed in a variety of other cell types, including fibroblasts [19].

Given the known role of both ROS and small GTP-binding proteins in signal transduction, we reasoned that Rac proteins may function to regulate the level of ROS in non-phagocytic cells. In this report, we provide evidence that the small GTP-binding proteins Ras and Rac1 function to regulate the production of intracellular ROS. Given the previous evidence that ROS may function as intracellular second messengers, these results suggest that ROS may act to regulate the activation of downstream effectors of Ras and Rac proteins.

MATERIALS AND METHODS

Cell culture and transfection

NIH 3T3 cells (American Tissue Culture Collection) were grown in Dulbecco's modified essential medium supplemented with 10% (v/v) calf serum/100 units/ml penicillin/100 μg/ml streptomycin. At a time of 24 h before transfection, cells were plated (in 6 cm-diam. dishes) at a density of 1×10^5 cells. The following day, cells were incubated with a total of 5 μg of DNA and 12.5 μl of lipofectamine (Gibco–BRL) in a final volume of 3.5 ml of serum and antibiotic-free medium. After 5 h the transfection mix was aspirated, and cells were returned for the next 36 h to medium containing 10% (v/v) calf serum. At a time of 24 h before assessing ROS levels, cells were placed in medium containing 0.5% (v/v) calf serum. Analysis of transfection efficiencies using a reporter plasmid demonstrated that this procedure resulted in reproducible transient transfection efficiencies equal to or greater than the manufacturer's stated efficacy of approximately 80% (results not shown).

Plasmids

The constitutively activated Rac1 (V12Rac1) and the dominant-negative Rac1 (N17Rac1) plasmids have been previously described [20]. Both constructs were in the mammalian expression vector pEXV; therefore the expression vector alone was used as a control. The activated H-Ras plasmid, V12Ras, containing a position 12 glycine-to-valine substitution, and the c-Myc expression vector were genomic clones and were a generous gift of C. V. Dang (Johns Hopkins University, Baltimore, MD, U.S.A.).

Abbreviations used: DCFDA, 2′-7′-dichlorofluorescin diacetate; ROS, reactive oxygen species; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetyl-L-cysteine; DPI, diphenylene iodonium; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TNF-α, tumour necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; JNK/SAPK, c-Jun N-terminal/stress-active protein kinase.

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Measurement of intracellular ROS

To assay for ROS generation, cells were starved overnight in medium containing 0.5 % (v/v) serum and then placed in minimal essential medium lacking Phenol Red in the presence or absence as indicated of PDGF-AB (5 ng/ml), epidermal growth factor (EGF; 100 ng/ml), tumour necrosis factor-α (TNF-α; 100 ng/ml) or interleukin (IL-1β; 100 ng/ml). After 5 min, the medium was quickly aspirated and replaced with Hanks buffered saline lacking Phenol Red (Gibco–BRL) to which 2′-7′-dichlorofluorescin diacetate (DCFDA; 5 μg/ml) was added immediately before use. Dishes were sealed with parafilm and returned to 37 °C for an additional 5 min, at which time they were imaged on a Leica confocal microscope as previously described [9]. Solutions of DCFDA (Molecular Probes) were made fresh daily in 100 % ethanol and were maintained during the experiment in sealed, foil-wrapped vials. Growth factors and cytokines were obtained from Upstate Biologicals, the antioxidants pyrrolidine dithiocarbamate (PDTC) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma, and diphenylene iodonium (DPI) was purchased from Toronto Research. Quantification of the levels of DCFDA fluorescence was assessed on a relative scale from 0–256 units. Baseline values for unstimulated cells averaged from 0–10 units, whereas Ras- or Rac1-transfected cells, as well as ligand stimulation, generally resulted in DCFDA fluorescence of 30–100 units. Values represent means $\pm$ S.D. of DCFDA fluorescence from 60 random cells averaged from three separate transfections. Statistical analysis was performed using an unpaired Student's *t* test with values of $P < 0.05$ considered significant.

Western blotting

Extracts from cells were prepared 72 h after transfection. Equal amounts (25 μg) of protein lysate were loaded on an SDS/4–20 % polyacrylamide gel. Samples were transferred to a nitrocellulose filter, after which the filter was cut and probed with either an anti-Ras antibody (Santa Cruz) or an anti-tubulin antibody (Oncogene Science) and was observed using enhanced chemiluminescence (Tropix), as previously described [9].

RESULTS

The transient transfection of an expression plasmid encoding a constitutively activated Ras protein (V12Ras) in NIH 3T3 cells (Figure 1A) significantly increased intracellular ROS. To measure intracellular ROS concentration, NIH 3T3 cells were incubated with the fluorophore DCFDA. The level of DCFDA fluorescence has been previously used as a sensitive, albeit indirect, measure of intracellular ROS [8,9,21]. The level of fluorescence seen with V12Ras expression was not attained in untransfected cells, cells transfected with random genomic DNA (results not shown), or cells transfected with a plasmid encoding the c-*myc* gene (Figure 1B). Quantitative measurements in these and similar experiments demonstrated that V12Ras expression led to a 10-fold or greater increase in DCFDA fluorescence. A similar increase in intracellular ROS was observed (Figure 1C) after transient transfection of an expression vector encoding the constitutively activated form of Rac1 (V12Rac1).

In some pathways, including those necessary for transformation, Rac1 functions downstream of Ras proteins [20,22,23]. In an effort to assess whether a similar hierarchy exists for the generation of ROS, we co-transfected cells with a V12Ras expression plasmid with or without a plasmid encoding a dominant-negative form of Rac1 (N17Rac1). Expression of N17Rac1 effectively inhibited the ability of V12Ras to increase ROS levels [V12Ras (1 μg) + pEXV (4 μg): DCFDA mean fluorescence 95 ± 9; V12Ras (1 μg) + N17Rac1 (4 μg): DCFDA mean fluorescence 14 ± 3]. Western-blot analysis demonstrated that the ability of N17Rac1 to block V12Ras-induced increase in DCFDA fluorescence was not mediated through a direct effect on the level of Ras protein expression (Figure 2). Therefore these results are consistent with a pathway for ROS generation in which Rac1 lies downstream of Ras.

To confirm that the increase in DCFDA fluorescence observed after transient transfection of Ras or Rac1 was actually due to a rise in ROS, cells were treated for 90 min with two chemically unrelated cell-permeant antioxidants. At a time of 3 days after transfection, cells were briefly incubated with either PDTC or NAC. As seen in Table 1, both PDTC and NAC reduced the intensity of the V12Rac1-induced increase in DCFDA fluorescence in a concentration-dependent fashion.

Previous studies in other cell types have established that DPI, a potent flavoprotein inhibitor, blocks the rise in ROS seen following angiotensin II or fibroblast growth factor-stimulation [5,7]. As seen in Table 1, a brief 30 min pre-treatment with DPI also inhibits the rise in ROS stimulated by V12Rac1 expression. This suggests that, similar to the NADPH oxidase complex in phagocytic cells, an FAD-binding protein regulated by Rac1 may regulate ROS levels in fibroblasts.

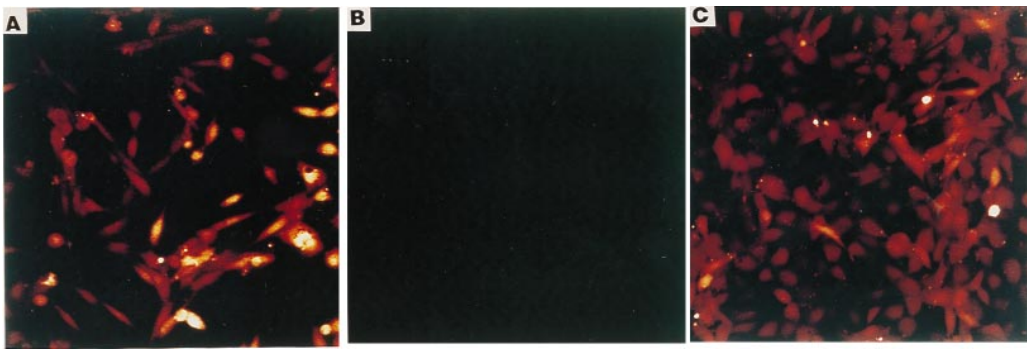

Figure 1 Expression of constitutively activated forms of Ras or Rac1 leads to increased intracellular ROS

NIH 3T3 cells were transfected with 5 μg of expression plasmid encoding V12Ras (**A**), c-Myc (**B**) or V12Rac1 (**C**). At a time of 3 days after transfection, cells were loaded with the peroxide-sensitive fluorophore DCFDA and were imaged by laser confocal microscopy.

Table 1 Levels of DCFDA fluorescence in V12Rac1-transfected cells

Treatment	DCFDA fluorescence
None	45 ± 11
NAC (2 mM)	28 ± 2*
NAC (10 mM)	8 ± 2*
PDTC (20 μM)	15 ± 2*
PDTC (100 μM)	6 ± 1*
DPI (10 μM)	8 ± 4*

* $P < 0.01$ compared with untreated cells

Table 2 Effect of N17Rac1 on ligand-stimulated DCFDA fluorescence

N.D., not done.

	DCFDA fluorescence				
Stimulus ...	None	EGF	PDGF-AB	TNF-α	IL-1β
pEXV (5 μg)	4 ± 1	77 ± 20	84 ± 22	79 ± 18	72 ± 10
N17Rac1 (5 μg)	N.D.	11 ± 4*	12 ± 4*	10 ± 2*	13 ± 6*

* $P < 0.01$ compared with stimulated control (pEXV)-transfected cells

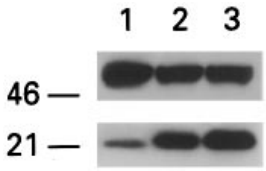

Figure 2 Effect of cotransfection of a dominant-negative Rac1 plasmid on the level of Ras protein expression

Levels of Ras protein (lower panel) from 25 μg of protein lysate obtained from NIH 3T3 cells transfected with vector (pEXV) DNA alone (5 μg; lane 1), V12Ras DNA (1 μg) with vector DNA (4 μg) (lane 2), or V12Ras DNA (1 μg) with N17Rac1 DNA (4 μg) (lane 3). To confirm equal protein loading, the top half of the filter (upper panel) was probed with an anti-tubulin antibody. The positions of molecular-mass markers (kDa) are indicated on the left-hand side.

We next sought to explore the role of Rac1 in ligand-stimulated ROS generation. Stimulation of NIH 3T3 cells with PDGF, EGF, TNF-α or IL-1β produced a marked increase in DCFDA fluorescence within 5 min of growth factor or cytokine stimulation (Table 2). As such, the generation of ROS, similar to the activation of small GTP-binding proteins, represents an early, shared aspect of the signalling pathway triggered by multiple extracellular ligands. The levels of intracellular ROS seen with growth factor or cytokine stimulation were in general similar in magnitude to those observed after expression of constitutively active mutants of Ras or Rac1 proteins.

In an effort to elucidate further the pathway by which ligand-stimulated ROS generation occurs in fibroblasts, NIH 3T3 cells were transfected with a plasmid encoding a dominant-negative Rac1 (N17Rac1). As seen in Table 2, compared with control transfected cells, cells transfected with the N17Rac1 plasmid had a marked reduction in either growth factor- (PDGF or EGF) or cytokine- (TNF-α, IL-1β) stimulated ROS generation. Therefore we conclude that Rac proteins are necessary for the ligand-stimulated generation of ROS.

DISCUSSION

The family of Ras-related small GTP-binding proteins is thought to play a pivotal role in multiple signal-transduction pathways. In particular, Rac proteins have been previously shown to possess several seemingly unrelated functions, including regulation of superoxide production through the neutrophil NADPH oxidase system, modulation of cell growth and transformation and reorganization of the actin cytoskeleton (reviewed in [24,25]). Given that the generation of ROS has been shown to play an essential role in growth factor and cytokine stimulation [9,11], we thought that it was possible that Rac or related proteins may regulate ROS in non-phagocytic cells. In the present paper we demonstrate that expression of constitutively active mutants of Ras or Rac1 leads to a rise in intracellular ROS in fibroblasts. In addition, in NIH 3T3 cells the increase in ROS following growth factor and cytokine stimulation requires Rac1. As such, the data presented here support the hypothesis that ROS may serve as intracellular second messengers and suggest that small GTP-binding proteins regulate redox-sensitive signal-transduction pathways.

Given that we demonstrate a Rac1-regulated rise of ROS in response to a host of ligands, it is tempting to speculate that H_2O_2 may participate in downstream signal transduction. Previous studies have demonstrated that exogenous H_2O_2 activates mitogen-activated protein kinase (MAPK) [9,26,27] as well as c-Jun N-terminal/stress-active protein kinase (JNK/SAPK) [27,28]. It is generally believed that the activation of MAPK is regulated by Ras proteins (reviewed in [24,25]), whereas Rac1 appears to regulate JNK/SAPK [29–31]. We have previously demonstrated that inhibition of the rise in H_2O_2 after PDGF stimulation inhibits MAPK activation [9]. As such, it would be interesting to test whether JNK/SAPK activation by growth factors or cytokines requires the generation of H_2O_2.

The effects of ROS are generally believed to be mediated by covalent modification of critical protein sulphydryl residues. As such, the intracellular redox state has been postulated to regulate a wide range of protein functions, from the activity of tyrosine phosphatases [32] to the ability of transcription factors to bind to DNA [33–36]. The results presented here suggest that ligand stimulation of cells may activate two separate but interrelated pathways. One well characterized pathway leads to the modification of protein function by phosphorylation of critical tyrosine residues. The other pathway, also controlled by small GTP-binding proteins, presumably would result in the generation of ROS and lead to the modification of protein functions by the oxidation–reduction of critical cysteine residues.

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