

Hydrogen peroxide mediates Rac1 activation of S6K1

Gyu-Un Bae^{a,1}, Yong Kee Kim^{b,1}, Hyoung-Keun Kwon^a, Jong Woo Park^a, Eun Kyung Lee^a,
Se Jin Paek^a, Wahn Soo Choi^c, In Duk Jung^d, Hoi Young Lee^d, Eun-Jung Cho^a,
Hyang Woo Lee^a, Jeung-Whan Han^{a,*}

^aCollege of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea

^bCollege of Medicine, Kwandong University, Gangneung 210-701, South Korea

^cCollege of Medicine, Konkuk University, Chungju 380-701, South Korea

^dCollege of Medicine, Konyang University, Nonsan 320-711, South Korea

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Abstract

We previously reported that hydrogen peroxide (H_2O_2) mediates mitogen activation of ribosomal protein S6 kinase 1 (S6K1) which plays an important role in cell proliferation and growth. In this study, we investigated a possible role of H_2O_2 as a molecular linker in Rac1 activation of S6K1. Overexpression of recombinant catalase in NIH-3T3 cells led to the drastic inhibition of H_2O_2 production by PDGF, which was accompanied by a decrease in S6K1 activity. Similarly, PDGF activation of S6K1 was significantly inhibited by transient transfection or stable transfection of the cells with a dominant-negative Rac1 (Rac1N17), while overexpression of constitutively active Rac1 (Rac1V12) in the cells led to an increase in basal activity of S6K1. In addition, stable transfection of Rat2 cells with Rac1N17 dramatically attenuated the H_2O_2 production by PDGF as compared with that in the control cells. In contrast, Rat2 cells stably transfected with Rac1V12 produced high level of H_2O_2 in the absence of PDGF, comparable to that in the control cells stimulated with PDGF. More importantly, elimination of H_2O_2 produced in Rat2 cells overexpressing Rac1V12 inhibited the Rac1V12 activation of S6K1, indicating the possible role of H_2O_2 as a mediator in the activation of S6K1 by Rac1. However, H_2O_2 could be also produced via other pathway, which is independent of Rac1 or PI3K, because in Rat2 cells stably transfected with Rac1N17, H_2O_2 could be produced by arsenite, which has been shown to be a stimulator of H_2O_2 production. Taken together, these results suggest that H_2O_2 plays a pivotal role as a mediator in Rac1 activation of S6K1. © 2004 Elsevier Inc. All rights reserved.

Keywords: S6K1; Rac1; H_2O_2

Introduction

Activation of cell growth and proliferation requires an accelerated rate of protein synthesis, which is regulated in part by intracellular activation of several signaling protein kinase cascades [1]. Recent studies using molecular approaches have demonstrated that ribosomal protein S6 kinase 1 (S6K1) plays an important role in linking growth

and proliferation signals to the regulation of protein synthesis and cell size [2–5]. S6K1 phosphorylates multiple sites of the 40 S ribosomal protein S6 resulting in a specific increase in the translation of a subset of mRNAs containing a polypyrimidine tract in their 5'-untranslated region (5' TOP mRNAs) [6,7]. About 20% of the total mRNA in the cell consists of this class of mRNAs which encodes many components of the protein synthetic apparatus [6,8]. Understanding the regulation of S6K1 will have major implications not only for the control of normal cellular growth, but also for cases of abnormal growth such as cancer. In fact, the S6K1 gene has been shown to be amplified and overexpressed in primary tumors [9]. Consistent with this finding, inhibition of S6K1 activation by microinjection of neutral-

* Corresponding author. College of Pharmacy, Sungkyunkwan University, #300 Chonchon-dong, Jangan-gu, Suwon 440-746, South Korea. Fax: +82 31 290 5403.

E-mail address: jhhan551@skku.edu (J.-W. Han).

¹ The first two authors contributed equally to this work.

izing antibodies into cells [2], or by treatment of cells with the immunosuppressant rapamycin [3,10,11], severely suppresses cell cycle progression. Recently, analogues of the S6K1 signaling pathway antagonist rapamycin have been tested in pre-clinical and phase I studies, demonstrating preliminary evidence of antitumor effects in renal cell carcinoma and non-small-cell lung cancer [12].

Although S6K1 is activated by numerous stimuli, including growth factors, cytokines, phorbol esters, oncogenic products, Ca^{2+} , inhibitors of protein synthesis [13,14], and hormones such as angiotensin II [15], the signal transduction pathway that mediates S6K1 is poorly understood. Many studies, including point mutational analysis of platelet-derived growth factor (PDGF) receptor [16], the effect of various mutants of phosphatidylinositol 3-kinase (PI3K) [17], and specific inhibitor wortmannin for PI3K on activity [18,19], have suggested that PI3K is an upstream mediator of S6K1 activity. Rapamycin inhibits the activity of FKBP12-rapamycin-associated protein (FRAP) kinase by binding to its cognate binding protein, FK506 binding protein (FKBP12), thus inhibiting S6K1 stimulation [20]. The Ser/Thr protein kinase activity of FRAP is essential for the phosphorylation of S6K1 *in vitro* and *in vivo* [21,22]. Regulation of S6K1 requires at least two upstream signal inputs. While the FRAP pathway is believed to sense the availability of amino acids and cellular energy levels, PI3K pathway transduces mitogenic signals, and two pathways converge on S6K1 [21–23]. The nutrient-sensing mechanism of FRAP has recently been revealed to involve a protein complex containing the regulatory associated protein of mTOR (Raptor) [24,25], and potentially, the tuberous sclerosis complex proteins TSC1/TSC2 [26].

There is emerging evidence that reactive oxygen species (ROS) at low concentration may function as a signaling intermediary of cellular responses [27]. The generation of ROS by external stimuli is associated with various cellular processes, such as cell proliferation [28] or apoptosis [29]. ROS have been shown to stimulate signaling pathways implicated in growth factor and cytokine effects through activating their important components, such as transcription factors, NF- κ B [30] and AP-1 [31], and especially also to stimulate ERK1/2 [27,32,33]. Recently, we have identified hydrogen peroxide (H_2O_2) as a critical mediator for mitogenic activation of S6K1 signaling [28]. An acute increase of cellular H_2O_2 is required for PDGF or EGF stimulation of S6K1. In addition, it has been demonstrated that ultraviolet-induced activation of S6K1 is mediated by hydrogen peroxide production [34]. Pharmacological inhibitor studies showed that FRAP, PI3K, and Ca^{2+} , but not TPA-response PKC, might play a role as downstream mediators of H_2O_2 in H_2O_2 activation of S6K1 in mouse epidermal cell JB6 [28]. However, upstream pathways that can lead to H_2O_2 production required for S6K1 activation were poorly characterized.

Rac1 and Cdc42, Rho family members, have been shown to be involved in Ras-induced cellular transformation [35–

38]. Small GTPases are usually regulated by specific guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GTP for GDP. Gain-of-function mutations in Vav (a Rho family GEF) also result in oncogenesis [39,40]. Recent studies favor a model in which Rac1 lies below PI3K [41,42] and upstream of $\text{p}70^{\text{S6K}}$ [43,44]. Interestingly, activated Cdc42/Rac1 potently stimulates S6K1 *in vivo* [43]. The model suggests that membrane recruitment of S6K1 by activated Cdc2/Rac1 allows phosphorylation and activation of S6K1 by a membrane bound kinase. Although the signaling pathways used by the small GTPases to elicit cellular transformation seem to involve the activation of a variety of kinases, reactive oxygen intermediates and transcription factors [45], the precise mechanism by which Cdc2/Rac1 regulates S6K1 signaling pathway, remain to be elucidated. It has recently been demonstrated that in fibroblast, both growth factor- and cytokine-stimulated ROS production occurs through a Rac1-dependent pathway [46]. If H_2O_2 production indeed lies downstream of Rac1, it would provide a molecular link between Rac1 activation of S6K1 and cell growth/proliferation. In this study, we investigate the putative pathway of Rac1– H_2O_2 –FRAP–S6K1. Our data provide strong evidence that Rac1 activates S6K1 through H_2O_2 production.

Materials and methods

Reagents

Rapamycin and PDGF were purchased from Sigma. Anti-catalase antisera was purchased from Calbiochem (San Diego, CA). Anti-S6k1 and -GST antisera were purchased from Santa Cruz Technology (Santa Cruz, CA). [γ - ^{32}P] ATP was from Amersham (Piscataway, NJ, USA). All reagents from commercial sources were of analytical grade.

Cell culture and preparation of cell extracts

NIH-3T3 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (complete medium) in a humidified 5% CO_2 . Alternatively, serum-starved cells were pretreated for 30 min at 37°C with serum-free DMEM containing rapamycin (5 nM) before PDGF stimulation. Empty vector or Rac1 mutant genes stable-transfected Rat2 cells were kind gift from J.H. Kim (Korea University, Korea) and are described elsewhere [47]. Cells were plated at 1.5×10^6 per 60 mm dish for 24 h, were made quiescent by culturing 36 h, in DMEM containing no serum. Serum-starved cells were then treated at 37°C with 5 ng/ml PDGF for indicated times. After incubation, cells were rinsed twice with an ice-cold wash buffer solution containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamide, and 0.1 mM phenylmethylsul-

fonyl fluoride, and then extracted in the same buffer containing 0.1% Nonidet P-40. Cell extracts were collected with a plastic scraper, homogenized, and cleared by centrifugation at 4°C for 15 min at 12,000 rpm. Aliquots of the supernatant were frozen in liquid nitrogen and stored at –70°C.

Immunoprecipitation and S6K1 activity assay

S6K1 were immunoprecipitated by incubating 20 µg (total 200 µl) of protein/assay of cell extract to an antibody directed to the C-terminal 18 residues of S6K1 for 2 h incubation at 4°C. Immunoprecipitation was facilitated by the addition of protein A-Sepharose (20 µl) for 30 min at 4°C on a shaking plate. The beads were then washed twice at 4°C with an extraction buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM PMSF, and 0.1% Nonidet P-40, and once with a dilution buffer containing 50 mM MOPS (pH 7.0), 1 mM DTT, 5 mM MgCl₂, 10 mM *p*-nitrophenyl phosphate, and 0.1% Nonidet P-40. S6K1 activity was assayed using the S6 peptide as a substrate by incubating the immunoprecipitate for 30 min in 25 µl of reaction mixture containing 50 mM MOPS (pH 7.0), 5 mM MgCl₂, 1 mM DTT, 10 mM *p*-nitrophenyl phosphate, 0.1% Nonidet P-40, 0.6 µM PKI, and 12 µM ATP (plus 0.75 µCi [γ -³²P] ATP). The reaction was terminated by the addition of 10 µl of 100 mM EDTA (pH 7.0). Following a brief centrifugation, the supernatant was spotted on P-81 paper. Unincorporated [γ -³²P] ATP was eliminated by 10 min wash three times in 5% phosphoric acid, and phosphorylated S6 peptide bound to the paper was counted. The assays were carried out in duplicates. The results were calculated as units of S6 kinase activity per mg of protein lysate and expressed as the fold of increase over the control value. One unit of activity represents the transfer of 1 pmol of ³²Pi into S6 peptide per min under the assay condition.

Measurement of intracellular reactive oxygen species (ROS)

NIH-3T3 cells were grown on coverslips for 2 days and serum-free DMEM for 1 day. The serum-deprived cells were stabilized in serum-free DMEM without phenol red for at least 30 min and stimulated with PDGF or arsenite for indicated times. Sometimes, cells were transiently transfected on 60 mm dish by the SuperFect™ method using catalase DNA or preincubated with LY294002 (25 µM) for 30 min before treatment with PDGF or arsenite. Empty vector or Rac1 mutant genes stable-transfected Rat2 cells were grown on coverslips for 2 days. The proliferating cells were stabilized in complete DMEM without phenol red for at least 30 min. Sometimes, cells were preincubated with *Aspergillus niger* catalase (300 unit/ml) for 20 min. For the last 5 min of stimulation, 5 µM DCF was added to monitor intracellular H₂O₂. Then, the cells were immediately

observed with a laser scanning confocal microscope (Carl Zeiss LSM 410). The images of samples excited by a 488-nm argon laser were filtered by a long pass 515 nm filter. Thirty cells were randomly selected from each experiment and the mean relative fluorescence intensity of each group of cells was then measured by Karl Zeiss vision system (KS400, version 3.0).

Expression plasmid and transfection

The pCAT10 plasmid containing the human catalase cDNA was obtained from American Type Culture Collection. pEF6/V5-His-TOPO TA Mammalian Expression Kit was obtained from Invitrogen Corporation (San Diego, CA). The human fibroblast catalase cDNA from pCAT10 was equipped with oligonucleotide linkers using PCR. The primer sequences for catalase are as follows: sense, TGCAGTGTCTGCACAGCAAACC, antisense, TACACGGATGAACGCTAAGCTTCG. The sequence of PCR amplification was one cycle of denaturation at 95°C for 5 min followed by annealing at 56°C for 30 s and extension at 72°C for 1 min. This cycle was followed by 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C, repeated 38 times. The PCR reaction was sampled every five cycles from cycle 25 to 40, inclusively. The samples were separated on a 2% agarose gel, stained with ethidium bromide, and compared for intensity. The resulted 1.68-kb pair PCR product was ligated in frame into the pEF6/V5-His-TOPO to obtain the final recombinant human catalase plasmid. GST-tagged pEBGs expressing the Rac1N17-GST and Rac1V12-GST were each kindly provided by J. K. Chung (Department of Biological Sciences, Korea Advanced Institute of Science and Technology). NIH-3T3 cells were transiently transfected with empty vector or protein expressed plasmids using SuperFect™ transfection system (Qiagen). Twenty-five microliters of SuperFect transfection reagent was added to 5 µg of plasmid and incubated at room temperature for 10 min. After adding 1 ml of complete medium, DNA and transfection reagent mix were added to 1 × 10⁵ cells per well and incubated for 3 h at 37°C in a humidified 5% CO₂. Cells were washed with PBS and grown in complete medium for 48 h before kinase assays.

Immunoblotting

NIH-3T3 cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate, and 1 mM NaF). Proteins were separated on SDS-polyacrylamide gel electrophoresis (15%) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in PBS containing 0.1% Tween 20 (PBS-T) and 5% (w/v) dry skim milk powder, and incubated overnight with anti-catalase, -S6K1 and -GST. The membranes were then

washed with PBS-T and incubated for 2 h with an anti-rabbit secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

Cell growth assay

Cell growth was assessed with the sulforhodamine B (SRB) protein dye assay 48 h after the cell seeding at 5×10^4 cells/well in 6-well plates in DMEM containing 0.5% FBS [45]. Forty-eight hours later, synchronized cells were treated with 5 ng/ml PDGF or 10% FBS. The cells were then fixed by incubating with 1 ml of 10% trichloroacetic acid at 4°C for 1 h, followed by five washes with distilled water. After completely air-drying the plate, 0.4% SRB solution in 1% glacial acetic acid was added at room temperature for 30 min to stain the cells. Subsequently, the plate was washed five times with 1% glacial acetic acid and allowed to air-dry overnight. Tris-HCl (1 ml, 10 mM) was then added to each well to dissolve the SRB bound to cellular protein, which was measured by absorbance at 490 nm using an EL 808 ultra microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The absorbance is proportional to the number of cells attached to the culture plate. Therefore, the results of SRB represent the proliferative effect of Rac1 on Rat2 cell lines.

Results and discussion

Overexpression of recombinant catalase inhibits production of H_2O_2 and activation of S6K1 by PDGF

Recently, we showed that H_2O_2 was able to mediate the activation of S6K1 in mouse epidermal cells JB6. In deed, elimination of H_2O_2 intracellularly generated by PDGF or EGF through preincubation of the cells with catalase was accompanied by the inhibition of S6K1 activity by growth factors [28]. To obtain more direct evidence for the potential involvement of H_2O_2 in S6K1 signaling, we examined the effect of catalase overexpressed in NIH-3T3 cells on the production of H_2O_2 and the activation of S6K1 by PDGF. NIH-3T3 cells were transiently transfected with recombinant catalase and the production of H_2O_2 and the activation of S6K1 by PDGF were assayed. Overexpression of catalase was evidenced unambiguously by immunoblot analysis (Fig. 1B). Microfluorometric studies with laser-scanning confocal microscopy revealed that exposure of quiescent cells to PDGF resulted in a significant increase of DCF fluorescence. The PDGF-induced increase of DCF fluorescence was completely abolished by catalase overexpression (Fig. 1A), indicating that the predominant species of ROS produced was H_2O_2 , and the amount of overexpressed catalase was enough to eliminate the H_2O_2 intracellularly produced by PDGF. In parallel, transient transfection of the cells with recombinant catalase attenuated the PDGF

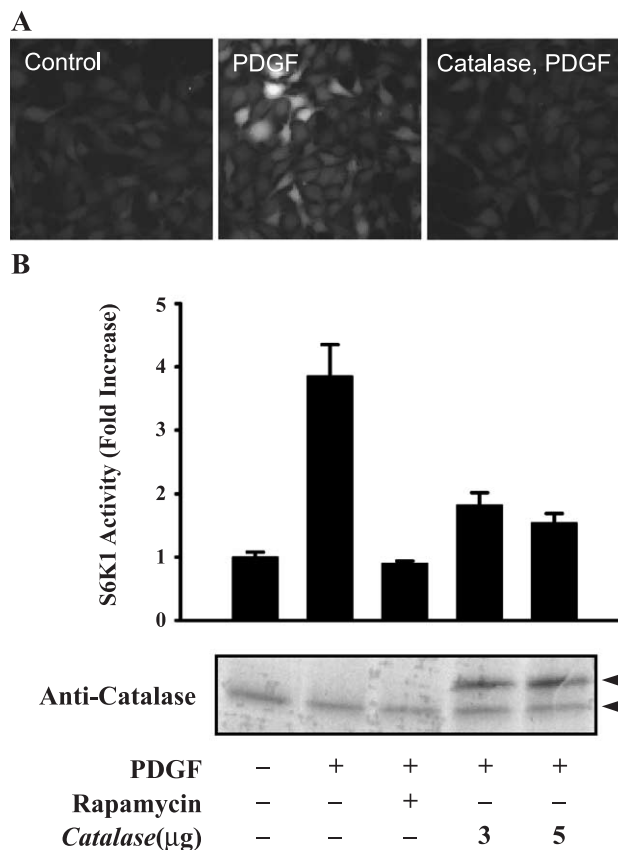


Fig. 1. Effect of catalase overexpression on PDGF-induced S6K1 activity and H_2O_2 production in NIH-3T3 cells. (A) NIH-3T3 cells were transfected with 3 μg of a plasmid expressing catalase or cognate empty vector. Cells were cultured in DMEM supplemented with 10% FBS until 80% confluency, after which they were incubated overnight in DMEM containing no serum. Cells were washed with DMEM without phenol red and treated with PDGF (5 ng/ml) for 10 min. ROS generation was measured by DCF fluorescence as described under Materials and methods. (B) NIH-3T3 cells transfected with either empty vector or catalase gene were serum starved for 24 h followed by pretreatment for 30 min with 5 nM rapamycin and stimulation with PDGF (5 ng/ml) for 20 min. After preparing total cell lysates, S6K1 was immunoprecipitated by an antibody directed to the C-terminal 18 residues of S6K1, and the precipitates were assayed for S6 kinase activity as described under Materials and methods. The results shown represent means \pm SE of three separate experiments. The expression level of catalase was detected by Western analysis of 50 μg of the respective cell lysate using anti-catalase antibody. Endogenous catalase and His-tagged catalase are indicated by the lower and upper arrow, respectively.

activation of S6K1, comparable to the effect of rapamycin on the S6K1 activity (Fig. 1B), suggesting that H_2O_2 mediates PDGF activation of S6K1 in NIH-3T3 cells.

Rac1 activation of S6K1 is mediated by H_2O_2

It has been reported that Rho family G proteins activates various signaling kinases including S6K1 [43,48] and ERK1/2 [49] in fibroblasts. In addition, PDGF-induced production of H_2O_2 has been demonstrated to be mediated by Rac1 [41]. These studies led to an assumption that H_2O_2 might mediate Rac1 activation of S6K1. To examine the

possible link between Rac1 and S6K1 activation, we first confirmed the effect of Rac1 on the activation of S6K1 by PDGF using transient transfection of NIH-3T3 cells with dominant negative Rac1 (Rac1N17) or constitutively active Rac1 mutant (Rac1V12). Consistent with previous report, overexpression of Rac1N17 significantly inhibited the phosphorylation and activation of S6K1 by PDGF as judged by the slower migration of a family of bands on SDS-PAGE (Fig. 2A). In contrast, Rac1V12 was able to activate S6K1 in the absence of PDGF. To further confirm whether PDGF activation of S6K1 requires Rac1 signaling, we examined the effect of stable-transfected Rac1 mutants on S6K1 activation in Rat2 cells by PDGF. It should be noted that expression of Rac1N17 decreased both the basal and PDGF-stimulated activity of S6K1, whereas a constitutively active Rac1 mutant (Rac1V12) led to an increase in basal activity of S6K1. However, PDGF stimulation of Rac1V12 express-

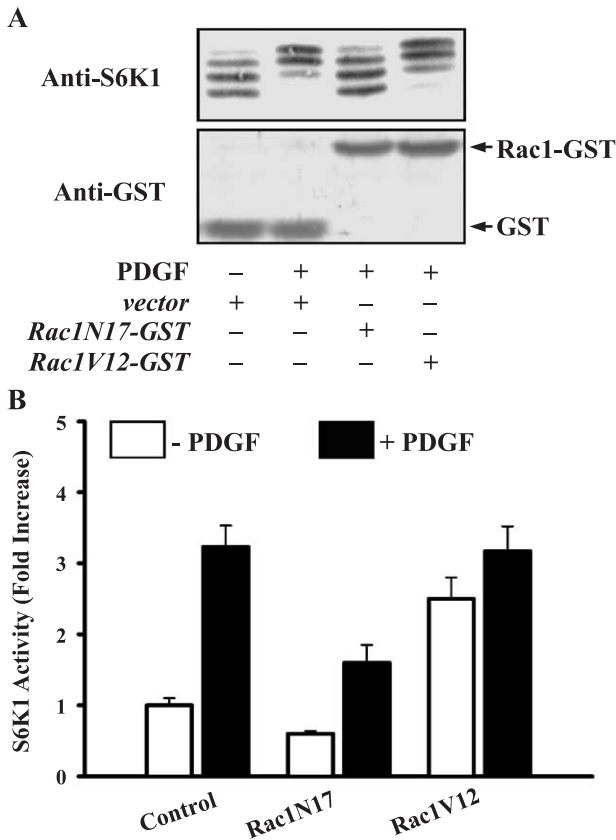


Fig. 2. Effect of Rac1 mutants on PDGF-induced S6K1. (A) NIH-3T3 cells were transiently transfected with GST fusions of Rac1N17, Rac1V12, or the control GST vector pEBG. Cells were starved for 24 h, then stimulated with PDGF (5 ng/ml) for 20 min. Whole cell lysates of the transfects were monitored as slower migration of a family of bands on SDS-PAGE and immunoblot analysis with S6K1 or anti-GST antibody as described under Materials and methods. (B) Rat2 cells were each stably transfected with Rac1N17, Rac1V12, or the expression vector alone (control). Where indicated, the cells were stimulated with PDGF (5 ng/ml) as described Materials and methods. Cell lysates were subjected to immunoprecipitation assay for S6K1. The results shown represent means \pm SE of three separate experiments.

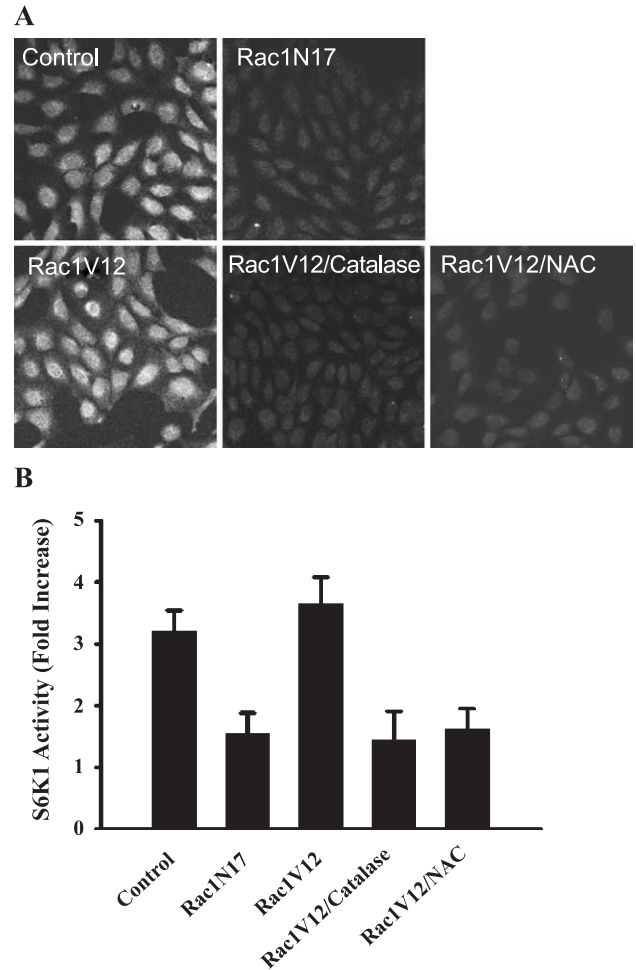


Fig. 3. Requirement of H_2O_2 for Rac1 activation of S6K1. (A) DCF fluorescence was measured with a confocal laser-scanning microscope after incubation of Rat2 cells stable-expressing Rac1N17, Rac1V12 in the presence of PDGF (5 ng/ml) as described under Materials and methods. Alternatively, cells were incubated with *Aspergillus niger* catalase (300 U/ml) or NAC (10 mM) for 30 min before PDGF stimulation; control represents cells expressing empty vector in the presence of PDGF (5 ng/ml). (B) After preparing total cell lysates, the lysates were subjected to immunoprecipitation assay for S6K1 as in Fig. 1B. The results shown represent means \pm SE of three separate experiments.

ing cells caused no more increase in S6K1 activity as compared with control cells (Fig. 2B). Taken together, these results indicate that the Rac1 signaling is required for the activation of S6K1 by PDGF.

To determine whether the Rac1–S6K1 link is mediated by H_2O_2 , we then examined the level of H_2O_2 production in cells expressing Rac1 mutants. The intracellular level of H_2O_2 was markedly reduced by about 4-fold in cells stably transfected with dominant negative mutant Rac1N17, compared with control cells stimulated with PDGF. However, the intracellular level of H_2O_2 in Rac1V12-expressing cells was similar to that of control cells (Fig. 3A), indicating that Rac1 signaling is necessary for the production of H_2O_2 . We then measured the activity of S6K1 in these cells. S6K1 activity

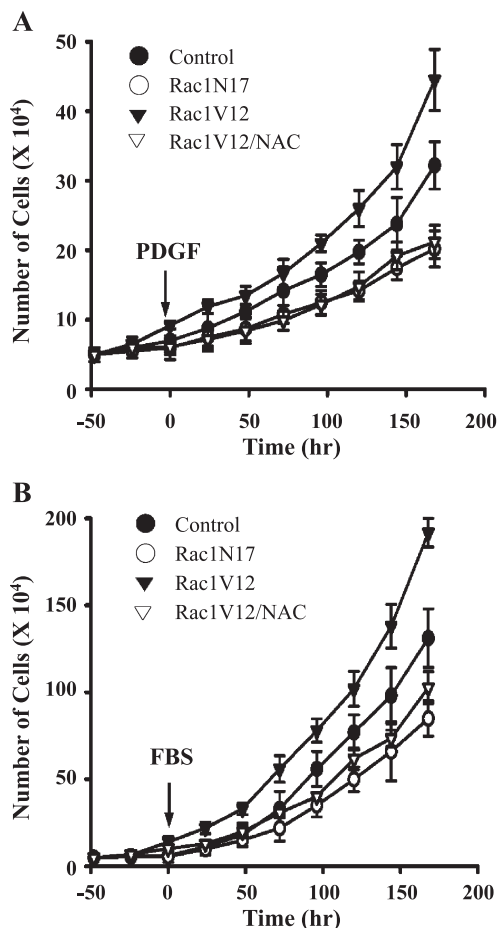


Fig. 4. Effects of Rac1 mutants on the growth of Rat2 cells. (A) Rac1N17 (○), Rac1V12 (●) or empty vector (▼) stable-transfected Rat2 cells were seeded at 5×10^4 cells/well in 6-well plates in DMEM containing 0.5% FBS. Forty-eight hours later, cells were treated with 5 ng/ml PDGF, or (B) 10% FBS. Cells were then incubated further for the indicated times, at which the number of cells was determined by SRB assay, as described under Materials and methods. Alternatively, Rac1V12-overexpressing cells were incubated with NAC (10 mM) for 30 min before PDGF of FBS stimulation. Data presented are the mean \pm SE of triplicate determinations from three experiments.

was good accordance with the level of H_2O_2 ; S6K1 activity was inhibited by overexpression of Rac1N17 as compared with control cells, whereas the activity in the cells transfected with Rac1V12 was comparable to that of control cells (Fig. 3B). In addition, treatment of the Rac1V12-transfected cells with either catalase or *N*-acetyl-L-cysteine (NAC) caused a reduction in the intracellular level of H_2O_2 , which was accompanied by the inhibition of S6K1 activity (Fig. 3B). These results strongly indicate that Rac1– H_2O_2 signaling appears to be a prerequisite for the activation of S6K1.

Rac1-mediated production of H_2O_2 is required for cell growth

As it has been demonstrated that S6K1 signaling mediates cell growth and proliferation [2–5], we next

examined whether Rac1– H_2O_2 signaling, an upstream signal of S6K1, could regulate cell proliferation. As shown in Fig. 4A, growth rate of the cells transfected with Rac1V12 was considerably higher than that of control cells transfected with empty vector. And moreover, the cells were able to grow before PDGF stimulation. On the other hand, Rac1N17 significantly inhibited PDGF-mediated proliferation of Rat2 cells as compared with the control cells. In addition, elimination of H_2O_2 by treatment of Rac1V12-overexpressing Rat2 cells with NAC led to a decrease in growth rate, comparable to that of Rat2 cells

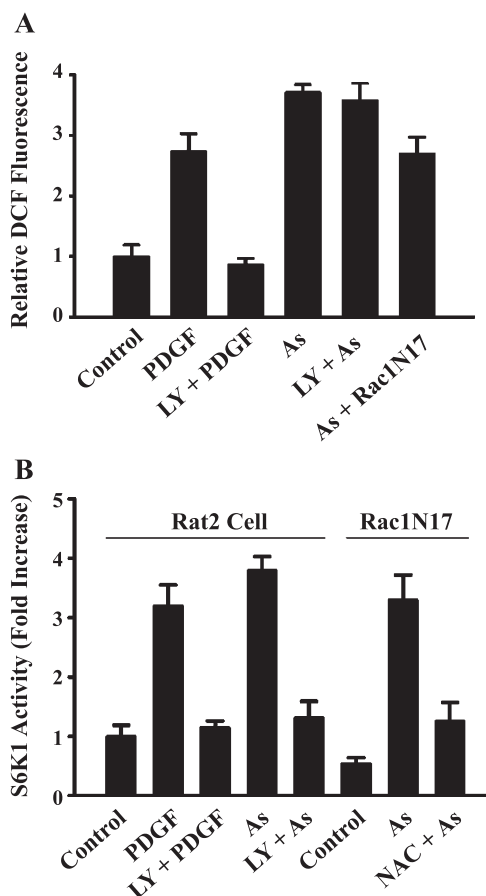


Fig. 5. Differential involvement of PI3K/Rac1 in H_2O_2 production. (A) After pretreatment with 25 μ M LY294002 (LY) for 30 min, Rat2 cells were treated with PDGF (5 ng/ml) for 10 min or with arsenite (100 μ M) for 60 min. Alternatively, Rat2 cells stably transfected with Rac1N17 were incubated with arsenite (100 μ M) for 60 min. DCF fluorescence was measured with a confocal laser-scanning microscope as described under Materials and methods. Fifty cells were randomly selected, and the fluorescence intensity was then measured by Karl Zeiss vision system (KS400, version 3.0) and averaged. The fluorescence intensities of each group were expressed as fold increase over control value. Data shown represent the means \pm SE of fluorescence intensities of each group. (B) After pretreatment with 25 μ M LY294002, Rat2 cells were stimulated with PDGF (5 ng/ml) or arsenite (100 μ M). Alternatively, Rac1N17-overexpressing cells were incubated with NAC (10 mM) for 30 min before arsenite stimulation. Cell lysates were subjected to immunoprecipitation assay for S6K1. The results shown represent means \pm SE of three separate experiments.

stably transfected with Rac1N17 (Fig. 4A). Consistent with this result, we also observed similar effects in Rac1-expressing Rat2 cells in the presence of serum (Fig. 4B). These results suggest that Rac1 participates in the cell proliferation of Rat2 cells and that Rac1/H₂O₂-mediated S6K1 activation might be closely involved in the process of cell proliferation.

Differential involvement of PI3K/Rac1 in H₂O₂ production

Although it has been suggested that PI3K acts as an upstream molecule of H₂O₂ produced by PDGF [41], this has been disputed, because exposure of cardiomyocytes to H₂O₂ markedly increased PI3K activity and tyrosine phosphorylation of the p85 regulatory subunit [50]. To examine whether PI3K acts as an upstream or downstream molecule of H₂O₂ production, we examined the effect of LY294002, a specific inhibitor of PI3K, on the production of H₂O₂ by PDGF or arsenite. Pretreatment of NIH-3T3 with LY294002 completely blocked the PDGF-induced increase in H₂O₂ production (Figs. 5A, B). Similar effect was also observed with wortmannin, another inhibitor of PI3K (data not shown), indicating that PI3K may act as an upstream molecule of H₂O₂. In contrast, the inhibition of PI3K by LY294002 pretreatment did not affect the level of H₂O₂ produced by arsenite, which has been shown to be a stimulator of H₂O₂ production in JB6 cells [51], indicating that H₂O₂ production by arsenite did not require PI3K activity. Interestingly, arsenite caused an increase in H₂O₂ production in Rat2 cells stably transfected with Rac1N17 which were unable to generate H₂O₂ in the presence of PDGF as shown in Fig. 3A. In parallel, S6K1 activity was correlated with the levels of H₂O₂ production except for arsenite treatment in the presence of LY294002 (Fig. 5B). The arsenite-dependent activation of S6K1 was blocked by LY294002 but not by Rac1N17. The effect of LY294002 is consistent with previous observations [28,50,51]. Taken together, these results suggest that PI3K/Rac1 may be differentially involved in H₂O₂/S6K1 signaling depending on stimuli.

In summary, we identified the H₂O₂ as a critical mediator for Rac1 activation of S6K1. Expression of activated allele of Rac1 was sufficient to generate H₂O₂ and activate S6K1 in stably transfected Rat2 cells. The Rac1 activation of S6K1 required H₂O₂ production, because elimination of H₂O₂ led to inhibition of S6K1 activation by Rac1V12. Consistent with these results, removal of H₂O₂ generated by PDGF also attenuated activation of S6K1. These observations were further confirmed by using dominant negative version of Rac1. Rat2 cells stably transfected with Rac1N17 were not able to produce H₂O₂ in the presence of PDGF. Furthermore, S6K1 in the cells was inactive even in the presence of PDGF. Thus, our study identified S6K1, a serine/threonine kinase, with a demonstrated role in cell proliferation and growth that is regulated by Rac1/H₂O₂ signaling pathway. Recently, ROS has been shown to have a

carcinogenic potential and is associated with tumor promotion [52,53]. Furthermore, it was reported that activation of S6K1 by UV exposure, a major factor in the development of human skin cancer, was mediated by H₂O₂ [34]. ROS also acts as essential intracellular second messengers for several cytokines, growth factors, and chemicals [27,31,51,54–57] and is involved in activation of many signaling pathway, such as p53, NFAT, ERK, and Akt [28,57–59], thus emphasizing the importance of this pathway in growth control. An identification of Rac1/H₂O₂ as a regulator of S6K1 contributes to an expanding list of molecules on the S6K1 signaling pathway that has pivotal role in growth control and oncogenesis.

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