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Hydrogen Peroxide Activates p70^{S6k} Signaling Pathway*

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We investigated a possible role of reactive oxygen species (ROS) in p70^{S6k} activation, which plays an important role in the progression of cells from G_0/G_1 to S phase of the cell cycle by translational up-regulation of a family of mRNA transcripts that encode for components of the protein synthetic machinery. Treatment of mouse epidermal cell JB6 with H_2O_2 generated extracellularly by glucose/glucose oxidase led to the activation of p70^{S6k} and p90^{Rsk} and to phosphorylation of p42^{MAPK}/p44^{MAPK}. The activation of p70^{S6k} and p90^{Rsk} was dose-dependent and transient, maximal activities being in extracts treated for 15 and 30 min, respectively. Further characterization of ROS-induced activation of p70^{S6k} using specific inhibitors for p70^{S6k} signaling pathway, rapamycin, and wortmannin revealed that ROS acted upstream of the rapamycin-sensitive component FRAP/RAFT and wortmannin-sensitive component phosphatidylinositol 3-kinase, because both inhibitors caused the inhibition of ROS-induced p70^{S6k} activity. In addition, Ca^{2+} chelation also inhibited ROS-induced activation of p70^{S6k}, indicating that Ca^{2+} is a mediator of p70^{S6k} activation by ROS. However, down-regulation of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive protein kinase C (PKC) by chronic pretreatment with TPA or a specific PKC inhibitor Ro-31-8220 did not block the activation of p70^{S6k} by ROS, indicating that the activation of TPA-responsive PKC was not required for stimulation of p70^{S6k} activity by H_2O_2 in JB6 cells. Exposure of JB6 cells to platelet-derived growth factor or epidermal growth factor led to a rapid increase in H_2O_2, phosphorylation, and activation of p70^{S6k}, which were antagonized by the pretreatment of catalase. Taken together, the results suggest that ROS act as a messenger in growth factor-induced p70^{S6k} signaling pathway.

Addition of mitogens to quiescent cells leads to activation of a number of specific protein kinases. Among the kinases affected are two families of growth factor-regulated serine/threonine kinases that phosphorylate the 40 S ribosomal protein S6 *in vitro*. One of these, referred to as p90^{Rsk} (1, 2), has been shown to lie on a signaling pathway that includes p21^{Ras}, raf-1, MEK1, and mitogen-activated protein kinase (MAPK)[1] and is a direct target of MAPK (3). The other family consists of two enzymes termed p70^{S6k} and p85^{S6k} (4, 5) which represent two isoforms of the same kinase that are encoded by a common gene and are identical except for a 23-amino acid extension at the amino terminus of p85^{S6k} (6, 7). Unlike p90^{Rsk}, p70^{S6k} and p85^{S6k} have been shown to reside on a novel p21^{Ras}-independent mitogenic signaling pathway (8, 9) that bifurcates at the level of the receptor from the p21^{Ras} pathway (9, 10). The p70^{S6k} is cytoplasmic, whereas the amino-terminal extension of p85^{S6k} targets it to the nucleus (11). The major substrate of the kinase in both compartments of the cell appears to be the 40 S ribosomal protein S6 (12), whose multiple phosphorylation in the cytosol has been implicated in the selective translational up-regulation of a family of mRNA transcripts that contain polypyrimidine tract at their 5′ transcriptional start site (13, 14). Consistent with this finding, inhibition of p70^{S6k} activation by microinjection of neutralizing antibodies into cells (15) or by treatment of cells with the inhibitors of p70^{S6k}, the immunosuppressant rapamycin (16–18), severely impedes cell cycle progression.

Although p70^{S6k} is activated by numerous stimuli, including growth factors, cytokines, phorbolesters, oncogenic products, Ca^{2+}, inhibitors of protein synthesis (7, 19), and hormones such as angiotensin II (20), the signal transduction pathway that mediates p70^{S6k} is poorly understood. This pathway bifurcates at a growth factor receptor docking site that is distinct from that of the p21^{Ras}/MAPK pathway (9). Many studies, including point mutational analysis of platelet-derived growth factor (PDGF) receptor (10), and the effect of various mutants of phosphatidylinositol 3-kinase (PI3K) (21) and specific inhibitor wortmannin for PI3K on p70^{S6k} activity (10, 22), have suggested that PI3K is an upstream mediator of p70^{S6k} activity. Recent studies favor a model in which protein kinase B lies below PI3K and upstream of p70^{S6k} (23, 24), although its function as a regulator for p70^{S6k} signaling pathway has been challenged (25, 26). Protein kinase B is activated through a wortmannin-sensitive phosphorylation. Recently, the kinase responsible for phosphorylation has been characterized and has been designated phosphoinositide-dependent protein kinase PDK1 (27). Rapamycin inhibits the activity of FKBP12-rapamycin-associated protein (FRAP/RAFT) kinase by binding to its cognate binding protein, FK506-binding protein

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; PI3K, phosphatidylinositol 3-kinase; FKBP, FK506-binding protein; FRAP, FK506-rapamycin-associated protein; RAFT, rapamycin and FKBP12 target; PKC, protein kinase C; MEM, modified Eagle's medium; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxy-methyl ester; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; G/GO, glucose/glucose oxidase; DCF, 2′,7′-dichlorofluorescein.

(FKBP12), thus inhibiting $p70^{S6k}$ stimulation (28). FRAP/RAFT appears to lies either on downstream of PI3K or on a parallel pathway, because TPA-induced activation of $p70^{S6k}$ is insensitive to specific inhibitor for PI3K, wortmannin, which blocks growth factor-induced stimulation of PI3K and $p70^{S6k}$, but still blocked by rapamycin (29). However, the precise mechanism by which FRAP or PI3K regulates $p70^{S6k}$ signaling pathway remains to be elucidated. This uncertainty appears to be due to the complex nature of the $p70^{S6k}$ activation mechanism, which requires multiple hierarchical phosphorylation by several protein kinases (29, 30). Recently, one of them has been identified as phosphoinositide-dependent protein kinase PDK1 which activates $p70^{S6k}$ through phosphorylation at Thr^{229} (27).

Emerging evidence suggests that reactive oxygen species (ROS) at low concentration may function as signaling intermediators of cellular responses (31). Recently, the production of ROS has been reported in a wide variety of cell types, including vascular smooth muscle cells (32), chondrocytes (33, 34), and fibroblasts (35), and is stimulated by a variety of stimuli, including cytokines such as transforming growth factor interleukin-1 (36) and tumor necrosis factor (33, 37), peptide growth factors PDGF (32, 33), epidermal growth factor (EGF) (38), and basic fibroblast growth factor (33), and G-protein-coupled receptors such as angiotensin II (39) and lysophosphatidic acid (40, 41). The increase in production of ROS following various external stimuli has been associated with various cellular processes, such as cell proliferation (32) or apoptosis (42, 43). However, the mechanisms through which ROS act are still poorly understood. Recently, ROS have been shown to stimulate signaling pathways implicated in growth factor and cytokine effects through activating their important components, such as MAPK, extracellular-regulated protein kinase (32, 44), and c-Jun NH_2-terminal kinase (34), and transcription factors such as NF-κB (43, 45) and AP-1 (33). In addition, ROS have been observed to induce the anchorage-independent growth in soft agar of mouse epidermal cell JB6 (46), the competence-related proto-oncogenes *c-fos* and *c-myc* in JB6 (47), and the phosphorylation of ribosomal protein S6 *in vitro* (48). These observations prompted us to investigate a possible involvement of ROS in the growth factor-stimulated $p70^{S6k}$ and $p90^{Rsk}$ activation pathway, with particular focus on the differential regulation of both pathways by ROS. Our findings support a pivotal role of ROS for $p70^{S6k}$ signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cell Extracts—Balb/c mouse epidermal cells (JB6) were grown at 37 °C in modified Eagle's medium supplemented with 8% fetal bovine serum in a humidified 5% CO_2. 80% confluent cells were made quiescent by culturing for 24 h in modified Eagle's medium (MEM) containing no bovine serum. Serum-starved cells were treated at 37 °C with following stimuli: 0.33 unit/ml glucose/glucose oxidase or 500 nM TPA for 15 min, and 5 ng/ml PDGF or 50 nM EGF for 20 min. Alternatively, cells were pretreated for the indicated times at 37 °C with serum-free MEM containing 2 mM EGTA, 2, 10, and 20 μM BAPTA-AM, 30, 300, and 3000 unit/ml *Aspergillus niger* catalase, 500 nM wortmannin, 5 nM rapamycin, or 5 μM PKC inhibitor Ro-31-8220 before stimulation. In some experiments, cells were pretreated for 24 h with 5 μM TPA. The controls were carried out by incubating the cells for the corresponding period in serum-free MEM containing dimethyl sulfoxide instead of the agents. Following stimulation, the cells were rinsed twice with a 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, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride and then extracted in the same buffer containing 1% Nonidet P-40. Cell extracts were collected with a plastic scraper, homogenized, and cleared by centrifugation at 4 °C for 15 min at 15,000 $\times$ *g*. Protein concentration was measured by the method of Bradford, with bovine serum albumin as the standard. Aliquots of the supernatant were frozen in liquid nitrogen and stored at −70 °C.

Immunoprecipitation and S6 Kinases Activity Assay in Vitro—$p70^{S6k}$ and $p90^{Rsk}$ were immunoprecipitated by incubating 20 μg (total 200 μl) of protein/assay of cell extract to an antibody directed to the carboxyl-terminal 18 residues of $p70^{S6k}$ and the carboxyl-terminal 21 residues of $p90^{Rsk}$ for 2 h incubation at 4 °C, respectively. 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, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 and once with a dilution buffer containing 50 mM MOPS (pH 7.0), 1 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM *p*-nitrophenyl phosphate, and 1% Nonidet P-40. S6 kinase ($p70^{S6k}$ and $p90^{Rsk}$) activity was assayed using the S6 peptide as substrate by incubating the immunoprecipitated S6 kinases for 30 min in 25 μl of reaction mixture containing 50 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM *p*-nitrophenyl phosphate, 0.1% Nonidet P-40, 0.6 μM protein kinase inhibitor, and 12 μM ATP (plus 0.75 μCi of [γ-^{32}P]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-^{32}P]ATP was eliminated by three 10-min washes in 5% phosphoric acid, and phosphorylated S6 peptide bound to the paper was counted. The assays were carried out in duplicate. The results were calculated as units of S6 kinase activity/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 $^{32}P_i$ into S6 peptide/min under the assay condition.

Immunoblotting—Cell lysates were boiled in Laemmli sample buffer for 3 min. Cell lysates containing 50 μg of total protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 15% slab gels for the determination of $p70^{S6k}$ and $p42^{MAPK}/44^{MAPK}$, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in PBS containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with anti-$p70^{S6k}$ or anti-$p42^{MAPK}/44^{MAPK}$ antisera. The membranes were then washed with PBS containing 0.1% Tween 20 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).

Measurement of Intracellular H_2O_2—Cells were grown on coverslips for 2 days and serum-free MEM for 1 day. The serum-starved cells were stabilized in serum-free MEM without phenol red for at least 30 min and stimulated with 5 ng/ml PDGF for different times. Sometimes, cells were preincubated with *A. niger* catalase (300 or 3000 unit/ml) for 20 min before treatment with PDGF or EGF for 5 min, respectively. For the last 10 min of stimulation, 5 μM H_2DCFDA was added to monitor intracellular H_2O_2. 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 three independent experiments, and dichlorofluorescein fluorescence intensities of treated cells were compared with those of unstimulated cells (36, 38).

Measurement of Intracellular Catalase Activity—Catalase activity from nonstimulated or stimulated JB6 cell lysates was assayed by a slightly modified method of Aebi (49). Briefly, serum-starved JB6 cells (1×10^7 cells/ml) were incubated with *A. niger* catalase (3000 unit/ml) for the indicated times. After incubation, cells were washed twice in PBS, trypsinized, and homogenized in extraction buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Cell extracts were cleared by centrifugation at 15,000 $\times$ *g* for 15 min at 4 °C. Catalase activity was assayed using H_2O_2 (10 mM) as a substrate by incubating the catalase in the supernatants for 30 s in 1 ml of reaction mixture containing 50 mM sodium phosphate and potassium phosphate (pH 7.0). Activity was monitored as the decrease in absorbance following decomposition of H_2O_2 at 240 nm. Assays were carried out in duplicates, and the results were expressed as units of catalase activity/μg of protein lysate.

RESULTS AND DISCUSSION

Activation of $p42^{MAPK}/p44^{MAPK}$ and $p90^{Rsk}$ in Response to H_2O_2—Recently, peptide growth factors such as PDGF, basic fibroblast growth factor, and EGF have been demonstrated to produce ROS. One of the targets of ROS appears to be $p42^{MAPK}/p44^{MAPK}$ (3) which is upstream kinase of $p90^{Rsk}$. On the other hand, an earlier observation revealed that exposure of mouse epidermal cells JB6 to ROS could activate a protein kinase in

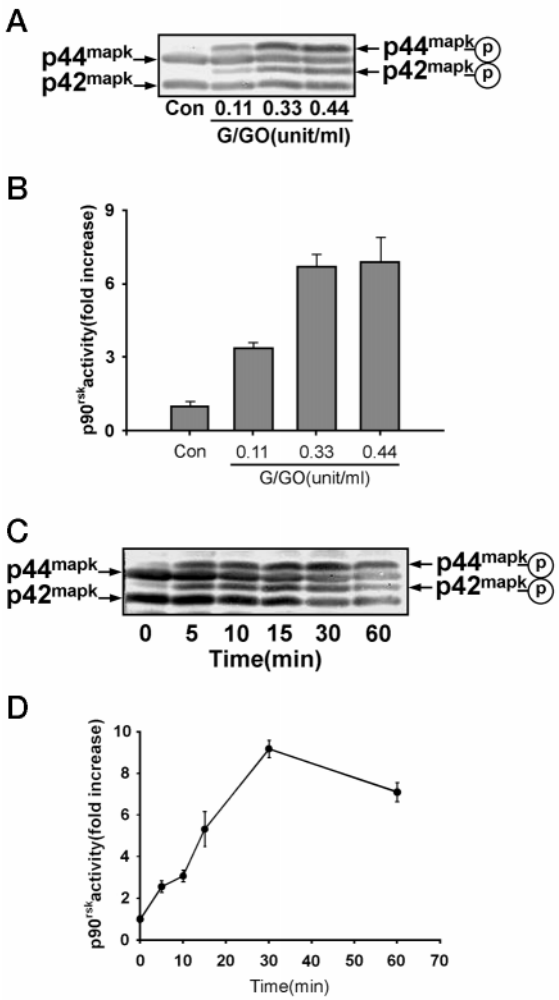

FIG. 1. **Activation of MAPK and $p90^{Rsk}$ in response to H_2O_2 induced by G/GO in JB6 cells.** *A*, serum-starved JB6 cells were stimulated with the indicated concentrations of G/GO for 15 min. Cells were then harvested. The changes of MAPK activity in G/GO-stimulated cell lysates were monitored as slower migration of a family of bands on SDS-PAGE and immunoblot analysis as described under "Experimental Procedures." *B*, $p90^{Rsk}$ in the cell lysates was immunoprecipitated by an antibody directed to the carboxyl-terminal 21 residues of $p90^{Rsk}$ and assayed for S6 kinase activity as described under "Experimental Procedures." The $p90^{Rsk}$ activity was expressed relative to that of unstimulated cells. *Con*, unstimulated cells. *C*, to examine the time course of MAPK, serum-starved JB6 cells were incubated for different times with G/GO (0.33 unit/ml). Detailed experimental conditions are described under "Experimental Procedures." *D*, $p90^{Rsk}$ in the cell lysates stimulated with G/GO (0.33 unit/ml) was assayed to determine time course. The results shown represent the means ± S.E. of three determinations.

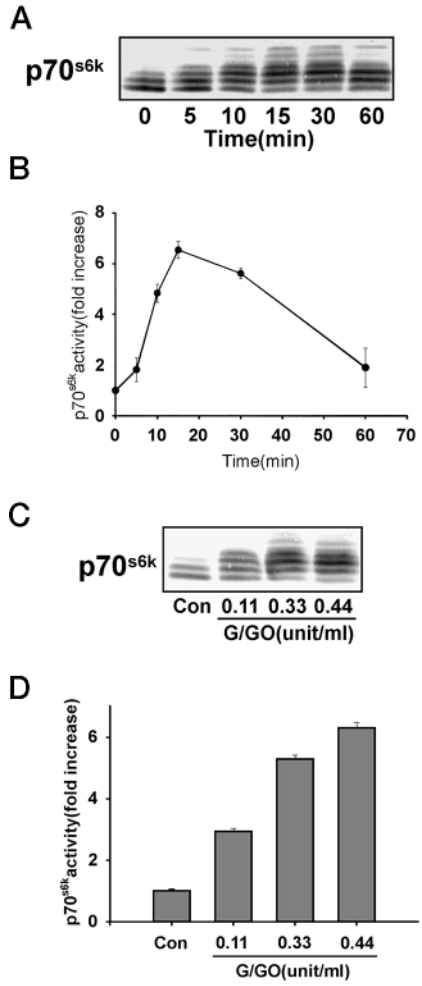

FIG. 2. **Effect of H_2O_2 generated by G/GO on $p70^{S6k}$ in JB6 cells.** *A*, extracts (50 μg of total protein) from quiescent cells or cells stimulated with G/GO (0.33 unit/ml) were subjected to SDS-PAGE and immunoblot analysis using specific antibody against the $p70^{S6k}$ as described under "Experimental Procedures." *B*, $p70^{S6k}$ in the cell lysates was immunoprecipitated by an antibody directed to the carboxyl-terminal 18 residues of $p70^{S6k}$ and assayed for S6 kinase activity as described under "Experimental Procedures." The $p70^{S6k}$ activity was expressed as the fold of increase over control value. *C*, serum-starved JB6 cells were incubated for 15 min with different concentrations of G/GO, and cell lysates were subjected to Western blot analysis. *D*, $p70^{S6k}$ activity in the cell lysates stimulated with different concentrations of G/GO was assayed after immunoprecipitation. The results shown represent the means ± S.E. of three separate experiments. *Con*, unstimulated cells.

cellular extracts which phoshorylated ribosomal protein S6 *in vitro* (48). These studies led to an assumption that $p90^{Rsk}$, the downstream kinase of $p42^{MAPK}/p44^{MAPK}$, might be responsible for the induction of S6 phosphorylation *in vitro*. To examine the possibility, JB6 cells were arrested in G_0, and the $p42^{MAPK}/p44^{MAPK}$ phosphorylation and $p90^{Rsk}$ activity were measured after treatment of the cells with ROS generated extracellularly by glucose/glucose oxidase (G/GO) producing H_2O_2. As shown in Fig. 1, G/GO treatment led to a dose-dependent phosphorylation of $p42^{MAPK}/p44^{MAPK}$ and activation of $p90^{Rsk}$, as measured by its slower migration on Western blots of one-dimensional SDS-PAGE and immune complex kinase assay (Fig. 1, *A* and *B*), respectively. The increase in $p42^{MAPK}/p44^{MAPK}$ phosphorylation following ROS treatment was followed by the activation of $p90^{Rsk}$. The increase in $p90^{Rsk}$ activity was transient after active oxygen treatment, with a peak of S6 phosphorylation occurring after 30 min. A slow inactivation of $p90^{Rsk}$ then ensued within 60 min (Fig. 1*D*). However, the increase in $p42^{MAPK}/p44^{MAPK}$ phosphorylation was apparent as early as 5 min after the H_2O_2 treatment and sustained for more than 30 min (Fig. 1*C*). This time discrepancy indicated that the activation of $p42^{MAPK}/p44^{MAPK}$ preceded activation of $p90^{Rsk}$ located in the downstream position. Taken together, these data demonstrate conclusively that H_2O_2 activates $p42^{MAPK}/p44^{MAPK}$ and $p90^{Rsk}$ in mouse epidermal cell JB6. Consistent with the data presented herein, $p42^{MAPK}/p44^{MAPK}$ activation in response to H_2O_2 has also been reported for other cell types (32, 44).

H_2O_2-induced $p70^{S6k}$ Activation—The kinetics of $p90^{Rsk}$ activation in JB6 cells following ROS exposure was not exactly consistent with that of total S6 kinase activation in cellular extracts (48), suggesting involvement of another family of S6 kinase, $p70^{S6k}$, which is activated by virtually all mitogenic

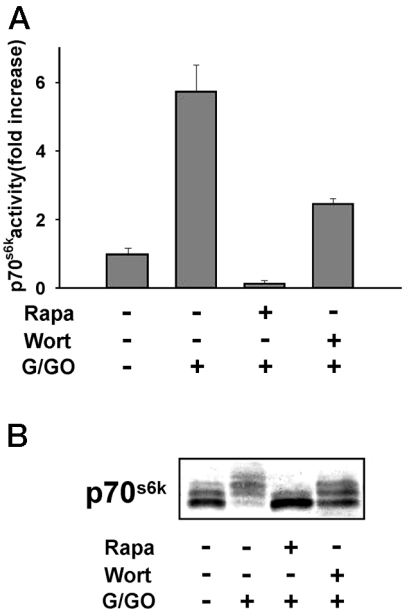

FIG. 3. **Effect of rapamycin or wortmannin on $p70^{S6k}$ activity in G/GO-stimulated JB6 cells.** JB6 cells were incubated in the absence or presence of G/GO (0.33 unit/ml) for 15 min following pretreatment for 30 min with either 5 nM rapamycin or 500 nM wortmannin. Cell lysates were subjected either to immunoprecipitation assay for $p70^{S6k}$ activity (*A*) or to Western blot analysis for $p70^{S6k}$ phosphorylation (*B*) as described in the legend to Fig. 2.

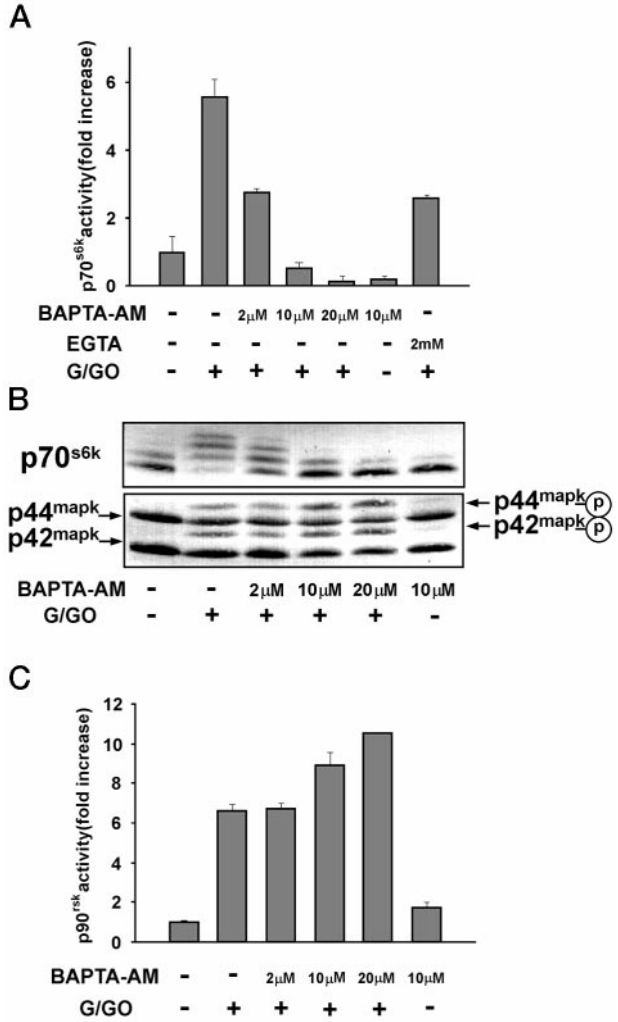

FIG. 4. **Ca^{2+} requirement for the activation of $p70^{S6k}$, $p42^{MAPK}$/$p44^{MAPK}$, and $p90^{Rsk}$ by H_2O_2.** Cells were pretreated with EGTA (2 mM) to chelate extracellular Ca^{2+} for 30 min or BAPTA-AM (2, 10, and 20 μM) for 15 min and then incubated with G/GO (0.33 unit/ml) for 15 min. Both kinase assay ($p70^{S6k}$ and $p90^{Rsk}$, *A* and *C*) and Western blot analysis ($p70^{S6k}$ and $p42^{MAPK}$/$p44^{MAPK}$; *B*) were carried out as described above. The results shown represent the means ± S.E. of three separate experiments.

stimuli, including growth factors, cytokines, phorbol esters, and oncogenic products (7). Indeed, treatment of mouse epidermal JB6 cells with G/GO caused a rapid phosphorylaton and activation of $p70^{S6k}$, as judged by the slower migration of a family of bands on SDS-PAGE and by immune complex kinase assay, respectively (Fig. 2). The S6 phosphorylation increased dose-dependently (Fig. 2, *C* and *D*). The maximal activity found in the extracts of cells treated for 15 min was 6–7-fold higher than basal levels. Within 60 min, the activity and phosphorylation decreased substantially (Fig. 2, *A* and *B*). These results are similar to those found with growth factors or serum-treated various cell lines (50). Taken together, these data indicate that H_2O_2 activates $p70^{S6k}$ as well as $p42^{MAPK}$/$p44^{MAPK}$/$p90^{Rsk}$.

Effect of Rapamycin and Wortmannin on H_2O_2-stimulated $p70^{S6k}$ Activity—Using a variety of receptor mutants (9, 10), a constitutively activated PI3K (21), and PI3K inhibitors wortmannin and LY294002, PI3K has been shown to be a signaling component that proximately exists in receptor tyrosine kinase on $p70^{S6k}$ signaling pathway (10, 22). Another crucial regulator of $p70^{S6k}$ is FRAP/RAFT, the direct target of rapamycin in mammals (28). FRAP/RAFT is a large molecular weight protein a homologous to PI3K with rapamycin-sensitive protein kinase activity (28). Immunosuppressant rapamycin inhibits an upstream kinase FRAP/RAFT near $p70^{S6k}$ through formation of a complex with FKBP12. Because of pivotal roles of PI3K and FRAP/RAFT in the regulation of $p70^{S6k}$ in response to growth factors (21, 51), their involvement in the stimulation of $p70^{S6k}$ by H_2O_2 were examined using selective inhibitors of $p70^{S6k}$, rapamycin and wortmannin. Pretreatment of the cells with 5 nM rapamycin resulted in $p70^{S6k}$ activities below the control levels, and wortmannin pretreatment inhibited the H_2O_2-stimulated increase in $p70^{S6k}$ activity by about 70% (Fig. 3*A*). The inhibitory effects of both agents were paralleled by an increase in the mobility of the kinase, as assessed by the gel mobility immunoblotting (Fig. 3*B*). Under these conditions, both agents had no effect on $p90^{Rsk}$ activation and $p42^{MAPK}$/$p44^{MAPK}$ phosphorylation (data not shown), as expected from a previous report (16). The inhibitor studies indicated that FRAP and PI3K were required for the stimulation of 70^{S6k} by H_2O_2. This was consistent with data that showed the involvement of PI3K and FRAP/RAFT in growth factor-induced 70^{S6k} signaling pathway (21, 51).

Differential Calcium Requirements for H_2O_2-induced $p70^{S6k}$ and MAPK/$p90^{Rsk}$ Activation—Previous studies have shown that incubation of several cells with compounds that increased intracellular calcium (the ionophore A23187, thapsigargin) stimulated $p70^{S6k}$ (20) and that PDGF-induced $p70^{S6k}$ activation was ablated by Ca^{2+} chelation (26), indicating a possible role of Ca^{2+} in regulation of $p70^{S6k}$. We therefore examined involvement of Ca^{2+} in H_2O_2 activation of $p70^{S6k}$. Although incubation of JB6 cells with extracellular Ca^{2+} chelator EGTA (2 mM) inhibited the H_2O_2-stimulated $p70^{S6k}$ activity by about 60% (Fig. 4*A*), the cell-permeant Ca^{2+} chelator BAPTA-AM inhibited the activity dose-dependently with ≥100% inhibition occurring at 10 μM BAPTA-AM (Fig. 4*A*). Chelation of extracellular Ca^{2+} with EGTA only partially affected the H_2O_2-stimulated $p70^{S6k}$ activity, further supporting the thesis that activation of $p70^{S6k}$ by H_2O_2 was dependent on intracellular Ca^{2+} rather than extracellular Ca^{2+}. The inhibition by Ca^{2+}

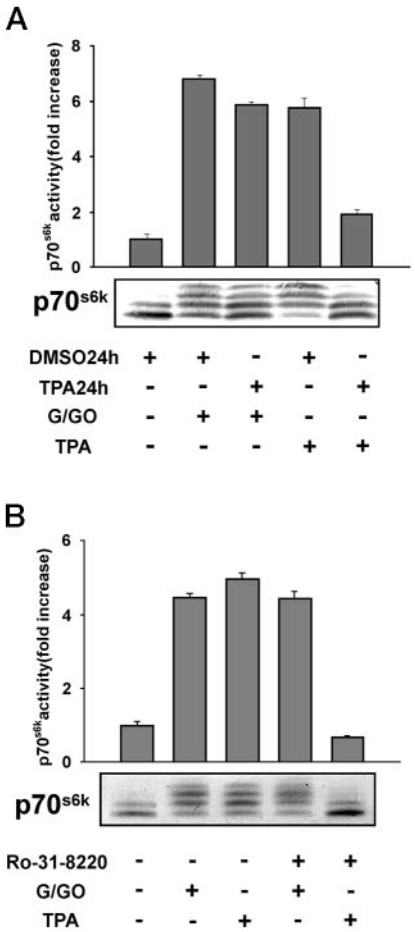

FIG. 5. **Effect of down-regulation of PKC on H_2O_2 stimulation of $p70^{S6k}$ in JB6 cells.** After pretreatment either with 5 μM TPA for 24 h (*A*) or 5 μM Ro-31-8220 for 20 min (*B*), cells were stimulated with 500 nM TPA or G/GO (0.33 unit/ml) as described "Experimental Procedures." Cell lysates were subjected either to Western blot analysis for $p70^{S6k}$ phosphorylation or immunoprecipitation assay for $p70^{S6k}$ activity. The results shown represent the means ± S.E. of three determinations. *DMSO*, dimethyl sulfoxide.

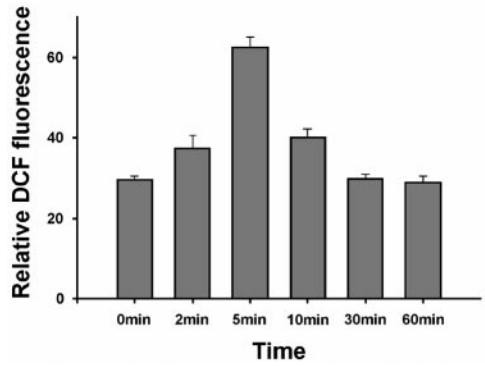

FIG. 6. **Production of H_2O_2 in JB6 cells by PDGF.** JB6 cells were grown on coverslips for 2 days and serum-free MEM for 1 day. The serum-starved cells were stabilized in serum-free MEM without phenol red for at least 30 min and stimulated with 5 ng/ml PDGF for the indicated times. ROS generation was measured by DCF fluorescence as described under "Experimental Procedures." Thirty cells were randomly selected from three independent experiments. Data shown represent relative fluorescence intensities of treated cells to those of unstimulated cells.

chelator on H_2O_2-induced activation of $p70^{S6k}$ was accompanied with inhibition of $p70^{S6k}$ phosphorylation, as monitored with collapse of slow migration of a family of bands into a single band (Fig. 4*B*). In comparison, preincubation of JB6 cells with EGTA or BAPTA-AM did not inhibit $p90^{Rsk}$ activity (Fig. 4*C*). Furthermore, BAPTA-AM stimulated the activation of $p90^{Rsk}$ by H_2O_2 in a dose-dependent manner (Fig. 4*C*), similar to the results obtained by $p42^{MAPK}/p44^{MAPK}$ mobility shift (Fig. 4*B*). These results indicated that H_2O_2 stimulated $p70^{S6k}$ signaling pathway by increasing intracellular Ca^{2+} level, and intracellular Ca^{2+} does not play a substantial role in regulating $p42^{MAPK}/p44^{MAPK}$ and $p90^{Rsk}$ in JB6 cells. Consistent with this result, we noted that exogenous H_2O_2 or H_2O_2 produced endogenously by growth factors caused increase in cytosolic $[Ca^{2+}]$.[2] Similarly, H_2O_2 stimulation of Ca^{2+} release has also been observed in Rat-2 fibroblast (40).

Effects of PKC on H_2O_2-induced $p70^{S6k}$ Activation—PKC comprises a large family of multiple isoforms that exhibit distinct properties, including sensitivities to calcium and the phorbol ester family of tumor promoters. The isoforms are divided into three subgroups; conventional, Ca^{2+}-responsive PKC, the novel, Ca^{2+}-unresponsive PKC, and the atypical, Ca^{2+}- and TPA-unresponsive PKC (52). Recently, $p70^{S6k}$ has been shown to be regulated by TPA-responsive (16, 29) and -unresponsive PKC isoforms (53, 54). In addition, it has been reported that PKC isoforms, including α, βI, and γ of conventional PKC, δ and ϵ of novel PKC, and ζ of atypical PKC, are tyrosine phosphorylated and catalytically activated by treatment of cells with H_2O_2 (55). To further characterize the effect of H_2O_2 on $p70^{S6k}$, we focused on the TPA-responsive PKC isoforms. As shown in Fig. 5, PKC activity was down-regulated by chronic pretreatment with 5 μM TPA for 24 h and then stimulated with H_2O_2 or TPA. The activation and phosphorylation of $p70^{S6k}$ by H_2O_2 was not significantly inhibited by this pretreatment, although PKC was not completely but significantly down-regulated by TPA pretreatment as evidenced by a slight activation of $p70^{S6k}$ activity over the activity of quiescent cells (Fig. 5*A*). Nevertheless, because of the uncertain specificity of the TPA down-regulation, the study was extended by using a specific PKC inhibitor Ro-31-8220, which acts as an ATP-competitive inhibitors of PKC and has been extensively used for studying the role of PKC in cell signaling (56, 57). Preincubation of the cells with Ro-31-8220 completely inhibited the stimulation of $p70^{S6k}$ by TPA, suggesting that the conditions used were sufficient to down-regulate TPA-responsive PKC isoforms (Fig. 5*B*). However, Ro-31-8220 down-regulation of PKC did not attenuate the phosphorylation and activation of $p70^{S6k}$ by H_2O_2 compared with that induced by TPA (Fig. 5*B*). Despite the fact that various PKC isoforms are phosphorylated and activated in response to H_2O_2 (55) and that the TPA-responsive PKC activate $p70^{S6k}$ in other cell types (16, 29) and in mouse epidermal cell JB6, our results did not support a role of TPA-responsive PKC in the regulation of $p70^{S6k}$ by H_2O_2. We could not, however, rule out the possibility that other PKC isoforms less sensitive or nonsensitive to Ro-31-8220 could contribute to the H_2O_2 stimulation of $p70^{S6k}$.

Generation of H_2O_2 in JB6 Cell by PDGF or EGF and Its Effect on $p70^{S6k}$ Activity—By measuring the intracellular generation of ROS with oxidation of the peroxide-sensitive fluorophore 2′,7′-dichlorofluorescein (DCF), we then examined whether ROS could be generated in JB6 cells by PDGF or EGF stimulation. Microfluorometric study with laser scanning confocal microscopy revealed that exposure of quiescent JB6 cells to PDGF-AB isoform (5 ng/ml) or EGF (50 nM) resulted in a rapid increase in DCF fluorescence by 2-fold (Fig. 6). The increase in DCF fluorescence by PDGF was transient with a maximal 2-fold increase over the basal levels at 5 min and then

[2] G.-U. Bae, K.-S. Ha, H.-W. Lee, and J.-W. Han, unpublished observations.

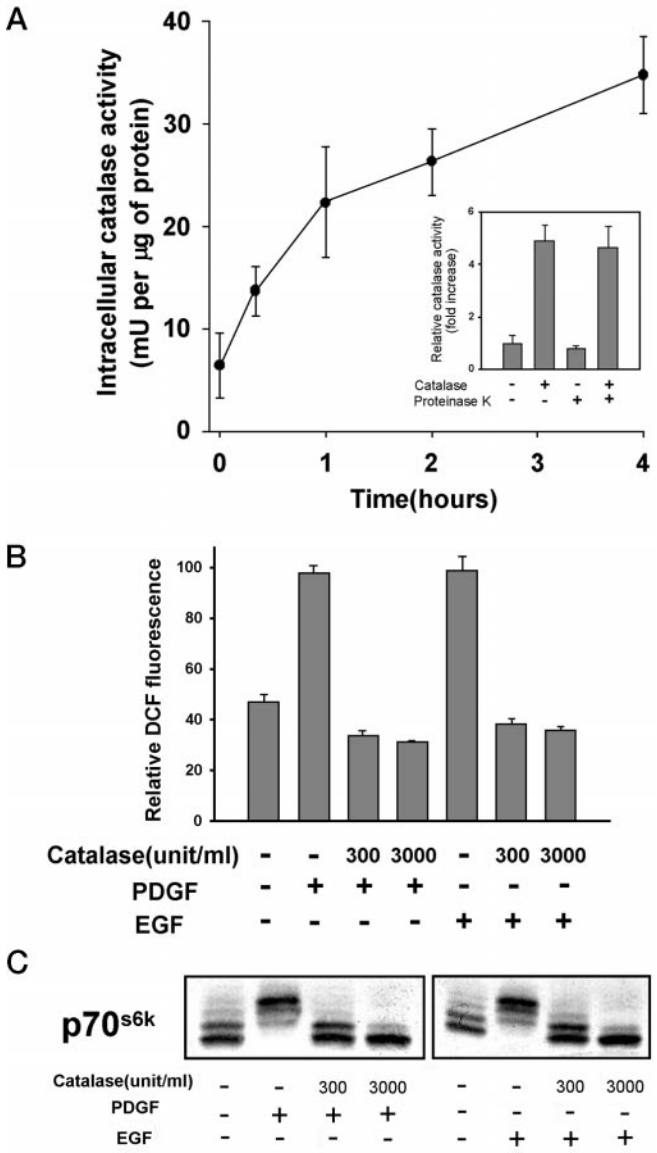

FIG. 7. **Uptake of catalase into JB6 cells and its effect on H_2O_2 generation and $p70^{S6k}$ activation by PDGF or EGF.** *A*, time-dependent intracellular catalase activity following exogenous treatment of catalase to JB6 cells. Serum-starved JB6 cells (1×10^7 cells/ml) were incubated with catalase (3000 unit/ml) for various times. Changes of catalase activity in the cell lysates were monitored as described under "Experimental Procedures." *Inset*, cells incubated with extracellular catalase (3000 units/ml) for 2 h were collected and then exposed to proteinase K (1 mg/ml) in solution for 20 min. After 20 min, cells were quickly settled down and washed with PBS. The cells were subsequently lysed in extraction buffer. Residual catalase activity was measured as described above. The catalase activity in the cell lysates loaded with exogenous catalase was expressed as folds of increase over that of unloaded cells. Specific activity of catalase in the unloaded cells was 5.8 ± 0.4 milliunits/μg protein. The results shown represent the means ± S.E. of three determinations. *B*, inhibitory effect of catalase on H_2O_2 production by growth factors. Cells were preincubated with catalase (300 or 3000 units/ml) for 20 min before treatment with PDGF (5 ng/ml) or EGF (50 nM) for 5 min. ROS generation was measured by DCF fluorescence as described under "Experimental Procedures." Thirty cells were randomly selected from three independent experiments. Data shown represent relative fluorescence intensities of treated cells to those of unstimulated cells. *C*, inhibitory effect of catalase on PDGF- or EGF-stimulated $p70^{S6k}$ activity. Serum-starved JB6 cells (1×10^6 cells/ml) were preincubated in the absence or presence of *A. niger* catalase (300 or 3000 units/ml) for 20 min before treatment with PDGF (5 ng/ml) or EGF (50 nM) for 20 min. Extracts (50 μg of total protein) from quiescent or growth factor-stimulated cells were subjected to SDS-PAGE and immunoblot analysis using specific antibody against the $p70^{S6k}$ as described under "Experimental Procedures."

returned to the basal levels within 30 min (Fig. 6). The time course of DCF fluorescence by PDGF was similar to that by EGF (data not shown). To further characterize the role of H_2O_2 in PDGF- or EGF-induced $p70^{S6k}$ activation in JB6 cells, we reduced H_2O_2 intracellularly generated by PDGF or EGF with catalase. Catalase has been shown to be taken up across the plasma membrane of vascular smooth muscle cells by an unknown mechanism (32). To decrease cytosolic concentration of H_2O_2, JB6 cells were preincubated with catalase (3000 units/ml), and preincubation of catalase led to time-dependent increase in catalase activity (Fig. 7*A*). The maximal activity found in the extracts of the cells treated for 4 h was about 5-fold over the basal levels. The increase in catalase activity was not brought about by its nonspecific binding to the outer membrane of JB6 cells, because the enzymatic activity of catalase-loaded cells was resistant to proteinase K treatment (Fig. 7*A*, *inset*). The PDGF- or EGF-stimulated increase in DCF fluorescence was abolished by the preincubation with catalase, suggesting that the increase in DCF fluorescence was mainly due to H_2O_2 and that the catalase activity taken up into JB6 cells was sufficient to decrease H_2O_2 intracellularly generated by PDGF or EGF (Fig. 7*B*). We next investigated the effect of catalase on PDGF or EGF stimulation of $p70^{S6k}$. Treatment of quiescent JB6 cells with PDGF or EGF led to a rapid phosphorylation of $p70^{S6k}$, as evidenced by a slower migration of a family of bands on SDS-PAGE and immunoblot analysis. However, preincubation of the cells with catalase severely impeded PDGF or EGF stimulation of $p70^{S6k}$, indicating involvement of H_2O_2 in PDGF- or EGF-induced $p70^{S6k}$ activation in JB6 cells (Fig. 7*C*).

In summary, we identified the $p70^{S6k}$ signaling pathway as a novel pathway regulated by ROS. Exposure of mouse epidermal cell JB6 to extracellular H_2O_2 produced by G/GO was sufficient to activate $p70^{S6k}$. ROS activation of $p70^{S6k}$ required Ca^{2+}, PI3K, and FRAP/RAFT but not TPA-responsive PKC. Furthermore, stimulation of endogenous H_2O_2 production by growth factor PDGF or EGF also stimulated $p70^{S6k}$. Using catalase, we showed that H_2O_2 mediated activation of $p70^{S6k}$ by PDGF or EGF. Thus, our studies identified a serine/threonine kinase with a demonstrated role in cell proliferation that was regulated by H_2O_2. Recently, ROS has been shown to have a carcinogenic potential and is associated with tumor promotion (58, 59). Also, it acts as essential intracellular second messengers for several cytokines and growth factors (32, 33, 36–38), thus emphasizing the importance of this pathway in growth control. An identification of H_2O_2 as a regulator of $p70^{S6k}$ contributes to an expanding list of molecules on the $p70^{S6k}$ signaling pathway that have pivotal roles in growth control and oncogenesis.

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