Hydrogen Peroxide Activates p70S6k Signaling Pathway*

(Received for publication, April 20, 1999, and in revised form, August 5, 1999)

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We investigated a possible role of reactive oxygen species (ROS) in p70S6k activation, which plays an important role in the progression of cells from G1/G0 to G2 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 H2O2 generated extracellularly by glucose/glucose oxidase led to the activation of p70S6k and p90Rsk and to phosphorylation of p42MAPK/P44MAPK. The activation of p70S6k and p90Rsk was dose-dependent and transient, maximal activities being in extracts treated for 15 and 30 min, respectively. Further characterization of ROS-induced activation of p70S6k using specific inhibitors for p70S6k signaling pathway, rapamycin, and wortmannin revealed that ROS act upstream of the rapamycin-sensitive component FRAP/RAFT and wortmannin-sensitive component phosphatidylinositol 3-kinase, because both inhibitors caused the inhibition of ROS-induced p70S6k activity. In addition, Ca2+ chelation also inhibited ROS-induced activation of p70S6k, indicating that Ca2+ is a mediator of p70S6k 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 p70S6k by ROS, indicating that the activation of TPA-responsive PKC was not required for stimulation of p70S6k activity by H2O2 in JB6 cells. Exposure of JB6 cells to platelet-derived growth factor or epidermal growth factor led to a rapid increase in H2O2, phosphorylation, and activation of p70S6k, which were antagonized by the pretreatment of catalase. Taken together, the results suggest that ROS act as a messenger in growth factor-induced p70S6k 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 p90Rsk (1, 2), has been shown to lie on a signaling pathway that includes p21Ras, Raf-1, MEK1, and mitogen-activated protein kinase (MAPK)† and is a direct target of MAPK (3). The other family consists of two enzymes termed p70S6k and p85S6k (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 p85S6k (6, 7). Unlike p90Rsk, p70S6k and p85S6k have been shown to reside on a novel p21Ras-independent mitogenic signaling pathway (8, 9) that bifurcates at the level of the receptor from the p21Ras pathway (9, 10). The p70S6k is cytoplasmic, whereas the amino-terminal extension of p85S6k 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 polyadenylic tracts at their 5’ transcriptional start site (13,14). Consistent with this finding, inhibition of p70S6k activation by microinjection of neutralizing antibodies into cells (15) or by treatment of cells with the inhibitors of p70S6k, the immunosuppressant rapamycin (16–18), severely impedes cell cycle progression.

Although p70S6k is activated by numerous stimuli, including growth factors, cytokines, phorbol esters, oncogenic products, Ca2+, inhibitors of protein synthesis (7, 19), and hormones such as angiotensin II (20), the signal transduction pathway that mediates p70S6k is poorly understood. This pathway bifurcates at a growth factor receptor docking site that is distinct from that of the p21Ras/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 p70S6k activity (10, 22), have suggested that PI3K is an upstream mediator of p70S6k activity. Recent studies favor a model in which protein kinase B lies below PI3K and upstream of p70S6k (23,24), although its function as a regulator for p70S6k 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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(FKBP12), thus inhibiting p70$^{S6k}$ stimulation (28). FRAP/RAFT appears to lie 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 intermediates 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 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$_2$ atmosphere 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 carboxy-terminal 18 residues of p70$^{S6k}$ and the carboxy-terminal 21 residues of p90$^{Rsk}$ for 2 h incubation at 4 °C, respectively. Immunoprecipitation was followed by the addition of protein A-Sepharose (20 µl) for 30 min on a shaking plate at 4 °C. Following a 10-min incubation with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 5% (v/v) dry milk), the beads were then washed twice at 4 °C with an elution buffer containing 50 mM Tris-HCl (pH 7.0), 5 mM MgCl$_2$, 1 mM dithiothreitol, 10 µM p-nitrophenyl phosphate, 0.1% Nonidet P-40, 0.6 µM protein kinase inhibitor, and 12 µM ATP (plus 0.75 µCi of [γ-32P]ATP). The reaction was terminated by the addition of 10 µl of 200 mM EDTA (pH 7.0). Following a brief centrifugation, the supernatant was spotted on P-81 paper. Unincorporated [γ-32P]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 into the assay buffer.

**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 p42MAPK/p44MAPK, 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 milk powder and incubated overnight with anti-p70$^{S6k}$ or anti-p42MAPK/p44MAPK 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$_2$O$_2$**—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 (3000 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$_2$DCFDA was added to monitor intracellular H$_2$O$_2$. Then the cells were immediately observed with a laser scanning confocal microscope (Carl Zeiss, model LSM 410). The images of samples excited by a 485 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 (2000 unit/ml) for the indicated times. After incubation, the 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 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.

**RESULTS AND DISCUSSION**

**Activation of p42MAPK/p44MAPK 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 p42MAPK/p44MAPK (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...
cellular extracts which phosphorylated ribosomal protein S6 in vitro (48). These studies led to an assumption that p90 Rsk, the downstream kinase of p42MAPK/p44MAPK, might be responsible for the induction of S6 phosphorylation in vitro. To examine the possibility, JB6 cells were arrested in G0, 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 H2O2. 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 p80 Rsk then ensued within 60 min (Fig. 1D). However, the increase in p42 MAPK/p44 MAPK phosphorylation was apparent as early as 5 min after the H2O2 treatment and sustained for more than 30 min (Fig. 1C). 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 H2O2 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 H2O2 has also been reported for other cell types (32, 44).

H2O2-induced p70S6k 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...
stimuli, including growth factors, cytokines, phorbol esters, and oncogenic products (7). Indeed, treatment of mouse epidermal JB6 cells with G/GO caused a rapid phosphorylation 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 H2O2 activates p70S6k as well as p42 MAPK/p44 MAPK/p90 Rsk.

Effect of Rapamycin and Wortmannin on H2O2-stimulated p70S6k 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 p70S6k 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 up-stream kinase FRAP/RAFT near p70S6k through formation of a complex with FKBP12. Because of pivotal roles of PI3K and FRAP/RAFT in the regulation of p70 S6k, both agents were examined using selective inhibitors of p70S6k, rapamycin and wortmannin. Pretreatment of the cells with 5 nM rapamycin resulted in p70 S6k 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 H2O2. 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 H2O2-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 Ca2+ chelation (26), indicating a possible role of Ca2+ in regulation of p70 S6k. We therefore examined involvement of Ca2+ in H2O2 activation of p70 S6k. Although incubation of JB6 cells with extracellular Ca2+ chelator EGTA (2 mM) inhibited the H2O2-stimulated p70 S6k activity by about 60% (Fig. 4A), the cell-permeant Ca2+ chelator BAPTA-AM inhibited the activity dose-dependently with >100% inhibition occurring at 10 μM BAPTA-AM (Fig. 4A). Chelation of extracellular Ca2+ with EGTA only partially affected the H2O2-stimulated p70 S6k activity, further supporting the thesis that activation of p70 S6k by H2O2 was dependent on intracellular Ca2+ rather than extracellular Ca2+.
EGTA or BAPTA-AM did not inhibit p90 Rsk activity (Fig. 4A). A result, we noted that exogenous H₂O₂ or H₂O₂ produced endogenous p42MAPK/p44MAPK and p90Rsk in JB6 cells. Consistent with this, p70S6k phosphorylation was observed in Rat-2 fibroblast (40).

Similarly, H₂O₂ stimulation of Ca²⁺ pathway by increasing intracellular Ca²⁺ level, and intracellular Ca²⁺ does not play a substantial role in regulating p42MAPK/p44MAPK and p90S6k in JB6 cells. Consistent with this result, we noted that exogenous H₂O₂ or H₂O₂ produced endogenously by growth factors caused increase in cytosolic [Ca²⁺]. Similarly, H₂O₂ stimulation of Ca²⁺ release has also been observed in Rat-2 fibroblast (40).

Effects of PKC on H₂O₂-induced p70S6k 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, Ca²⁺-responsive PKC, the novel, Ca²⁺-unresponsive PKC, and the atypical, Ca²⁺- and TPA-unresponsive PKC (52). Recently, p70S6k 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 H₂O₂ (55). To further characterize the effect of H₂O₂ on p70S6k, 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 H₂O₂ or TPA. The activation and phosphorylation of p70S6k by H₂O₂ 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 p70S6k activity over the activity of quiescent cells (Fig. 5A). 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 p70S6k by TPA, suggesting that the conditions used were sufficient to down-regulate TPA-responsive PKC isoforms (Fig. 5B). However, Ro-31-8220 down-regulation of PKC did not attenuate the phosphorylation and activation of p70S6k by H₂O₂ compared with that induced by TPA (Fig. 5B). Despite the fact that various PKC isoforms are phosphorylated and activated in response to H₂O₂ (55) and that the TPA-responsive PKC activate p70S6k 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 p70S6k by H₂O₂. We could not, however, rule out the possibility that other PKC isoforms less sensitive or nonsensitive to Ro-31-8220 could contribute to the H₂O₂ stimulation of p70S6k.

Generation of H₂O₂ in JB6 Cell by PDGF or EGF and Its Effect on p70S6k Activity—By measuring the intracellular generation of ROS with oxidation of the peroxide-sensitive fluorophore 2 ',7' -dichlorofluorescin (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 ng/ml) 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...
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The time course of DCF fluorescence by PDGF was similar to that by EGF (data not shown). To further characterize the role of H$_2$O$_2$ in PDGF- or EGF-induced p70$^{S6k}$ activation in JB6 cells, we reduced H$_2$O$_2$ 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$_2$O$_2$, JB6 cells were preincubated with catalase (3000 units/ml), and preincubation of catalase led to time-dependent increase in catalase activity (Fig. 7A). 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. 7A, 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$_2$O$_2$ and that the catalase activity taken up into JB6 cells was sufficient to decrease H$_2$O$_2$ intracellularly generated by PDGF or EGF (Fig. 7B). 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$_2$O$_2$ in PDGF- or EGF-induced p70$^{S6k}$ activation in JB6 cells (Fig. 7C).

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$_2$O$_2$ 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$_2$O$_2$ production by growth factor PDGF or EGF also stimulated p70$^{S6k}$. Using catalase, we showed that H$_2$O$_2$ 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$_2$O$_2$. 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$_2$O$_2$ 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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