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Constitutive Activation of $p70^{S6k}$ in Cancer Cells

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The mitogen-stimulated serine/threonine kinase $p70^{S6k}$ 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 family of mRNA transcripts which contain polypyrimidine tract at their 5 transcriptional start site. Here, we report that $p70^{S6k}$ was constitutively phosphorylated and activated to various degrees in serum-deprived AGS, A2058, HT-1376, MG63, MCF7, MDA-MB-435S, MDA-MB-231 and MB-157. Rapamycin treatment induced a significant dephosphorylation and inactivation of $p70^{S6k}$ in all cancer cell lines, while wortmannin, a specific inhibitor of PI3-K, caused a mild dephosphorylation of $p70^{S6k}$ in AGS, MDA-MB-435S and MB-157. In addition, SQ20006, methylxanthine phosphodiesterase inhibitor, reduced the phosphorylation of $p70^{S6k}$ in all cancer cells tested. Consistent with inhibitory effect of rapamycin on $p70^{S6k}$ activity, rapamycin inhibited [^{3}H]-thymidine incorporation and increased the number of cells at G_0/G_1 phase. Furthermore, these inhibitory effects were accompanied by the decrease in growth of cancer cells. Taken together, the results indicate that the antiproliferative activity of rapamycin might be attributed to cell cycle arrest at G_0/G_1 phase in human cancer cells through the inhibition of constitutively activated $p70^{S6k}$ of cancer cells and suggest $p70^{S6k}$ as a potential target for therapeutic strategies aimed at preventing or inhibiting tumor growth.

Key words: $p70^{S6k}$, Rapamycin, Proliferation

INTRODUCTION

Activation of tumor cell proliferation requires transcriptional up-regulation of the immediate early genes as well as accelerated rate of translation of various genes, which are regulated by intracellular activation of several signaling protein kinase cascades (Chang and Karin, 2001; Clemens and Bommer, 1999). Among them, two protein kinase cascades, referred to as extracellular signal-regulated kinase (ERK1/2) signaling pathway and $p70^{S6k}$ signaling pathway, have been demonstrated to be responsible for the up-regulation of transcription (Davis, 1993) and translation in response to various growth factors (Morley and Thomas, 1991), respectively. Ligand-mediated dimerization of growth factors triggers the activation of receptor-type tyrosine kinases, resulting in autophosphorylation of tyrosine residues (Avruch *et al.*, 1994; Seger and Krebs, 1995), and these residues then serve as docking sites for the recruitment of downstream signaling mediators (Feig, 1993; Schlessinger, 1993).

One of the signaling pathways bifurcating at the specific docking sites of receptor tyrosine kinase is the ERK1/2 signaling pathway consisting of Grb2/Sos, Ras, Raf-1, MEK1 and ERK1/2 (Avruch *et al.*, 1994). Activated ERK1/2 is responsible for the phosphorylation of a variety of cellular proteins, including downstream kinases and transcription factors that are involved in the transcriptional regulation of the immediate early genes (e.g., *c-myc, c-jun*, and *c-fos*) (Davis, 1993). The other signaling pathway is the $p70^{S6k}$ pathway that bifurcates at a growth factor receptor docking site that is distinct from that of the Ras/ERK1/2 pathway (Ming *et al.*, 1994). Many studies, including point mutational analysis of platelet-derived growth factor (PDGF) receptor (Chung *et al.*, 1994), and the effect of various mutants of phosphatidylinositol 3-kinase (PI3-K) (Weng *et al.*, 1995) and PKB (Downward, 1995; Kitamura *et al.*, 1998), and specific inhibitors for PI3-K and FK506-rapamycin-associated protein (FRAP)

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on $p70^{S6k}$ activity (Cheatham *et al.*, 1994; Chung *et al.*, 1994), have suggested that the $p70^{S6k}$ signaling pathway includes PI3-K, PDK1, PKB and FRAP as upstream mediators of $p70^{S6k}$ activity, although the function of PKB as a regulator for $p70^{S6k}$ signaling pathway has been challenged (Andjelkovic *et al.*, 1996; Conus *et al.*, 1998). The major substrate of the $p70^{S6k}$ appears to be the 40S ribosomal protein S6 (Stewart and Thomas, 1994), whose multiple phosphorylation in the cytosol has been implicated in the selective translational up-regulation of a family of mRNA transcripts which contain polypyrimidine tract at their 5 transcriptional start site (Jefferies *et al.*, 1994a; Jefferies *et al.*, 1994b).

Dysregulation of the $p70^{S6k}$ pathway or ERK1/2 pathway through alterations in any one of several mediators involved in the cascade can lead to cellular transformation (Cowley *et al.*, 1994; Seufferlein and Rosengurt, 1996). Here, we demonstrate that $p70^{S6k}$ is constitutively phosphorylated and activated in various cancer cell lines. Rapamycin, a specific inhibitor for $p70^{S6k}$, induced $p70^{S6k}$ inactivation and G_0/G_1 arrest of cell cycle, resulting in the inhibition of proliferation of tumor cell.

MATERIALS AND METHODS

Cell culture and preparation of cell extracts

AGS stomach cancer cell line and MDA-MB-231, MB-157 mammary gland cancer cell lines were grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). A2058 melanoma, HT-1376 bladder cancer cell line, MG63 osteosarcoma, and MCF7, MDA-MB-435S mammary gland cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented 10% FBS and 1% penicillin/streptomycin. JB6 mouse epidermal cell line is cultured in minimum essential medium (MEM) (Invitrogen) containing 8% FBS and 1% penicillin/streptomycin. All cells were incubated in a humidified 5% CO_2 at 37°C. 80% confluent cells were made quiescent by culturing for 48 h in serum free medium (in the case of JB6, 24 h). Stabilized cells were treated for 30 min at 37°C in phosphate-buffered saline (PBS) containing 5 nM rapamycin, 500 nM wortmannin, or 1.2 mM SQ20006. The controls were carried out by incubating the cells for the corresponding period in PBS containing ethanol (rapamycin) or dimethyl sulfoxide (DMSO) (wortmannin) instead of the agent. 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, 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 12,000 × g. Protein concentration was measured by Bradford method, with BSA 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

$p70^{S6k}$ was immunoprecipitated by incubating 20 μg of protein of cell extract to an antibody directed to the C-terminal 18 residues of $p70^{S6k}$ 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 the extraction buffer and once with a dilution buffer containing 50 mM MOPS, 1 mM DTT, 5 mM $MgCl_2$, 10 mM *p*-nitrophenyl phosphate, and 1% Nonidet P-40. $p70^{S6k}$ activity was assayed using the S6 peptide as substrate by incubating the immunoprecipitated $p70^{S6k}$ for 30 min in 25 μl of reaction mixture containing 50 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 10 mM *p*-nitrophenyl phosphate, 0.1% NP-40, 0.6 μM PKI, 12 μM ATP (plus 0.75 [γ-^{32}P] ATP (Amersham Pharmacia Biotech)). 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. S6 kinase assay was conducted in duplicate. The results are expressed in units of S6 kinase activity per mg of protein lysate. One unit of activity results in the transfer of 1 pmole of $^{32}P_i$ into S6 peptide per min under the assay condition.

Immunoblotting

Cell lysates were boiled in Laemmli sample buffer for 3 minutes. Cell lysates, containing 50 μg of total protein for the determination of $p70^{S6k}$, were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 15% slab gels, and proteins were 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-$p70^{S6k}$ antisera. The membranes were then washed with PBS-T and incubated with for 2 h with an anti-rabbit secondary antibody conjugated to alkaline phosphatase. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

Growth assay

5×10^4 cells were seeded at the 6 cm dish with an

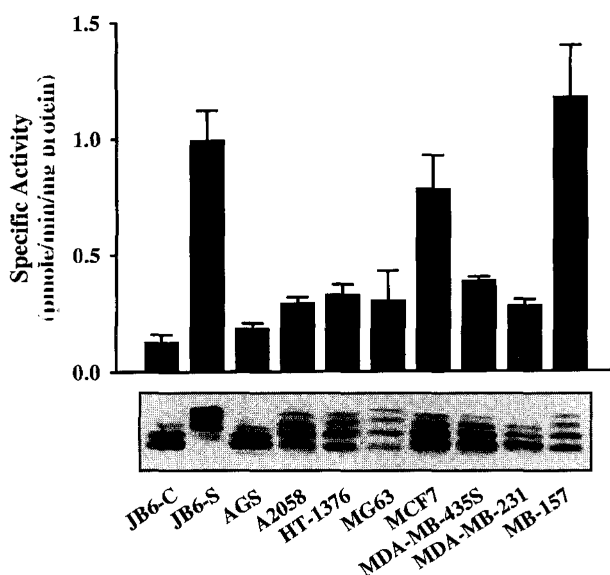

Fig. 1. $p70^{S6k}$ in various human cancer cells are constitutively activated. After serum deprivation of various human cancer cells for 48 h, cell lysates were immunoprecipitated using a specific antibody for $p70^{S6k}$ and assayed for S6 kinase activity as described under "Materials and Methods". The results shown represent the means ± s.e.m. of three independent experiments. The levels of $p70^{S6k}$ phosphorylation in above cell lysates were monitored as slower migration of a family of bands on SDS-PAGE and immunoblot analysis using an anti-$p70^{S6k}$ antibody. In the case of JB6 cell, it was grown in serum-free media for 24 h (JB6-C), and then stimulated with 10% FBS for 15 min (JB6-S). The activity and phosphorylation level of $p70^{S6k}$ in JB6 cell were analyzed by the same methods.

appropriate medium containing 0.5% FBS. After stabilization for 48 h, cell cultures were changed with fresh medium without a serum. Cells were pretreated with 5 nM rapamycin for 30 min, then stimulated with 10% FBS over a period of up to 10 days. At each day, cells were collected and viable cell numbers were counted by hemocytometer by trypan blue (0.4% w/v, Sigma) exclusion method.

[^{3}H]-thymidine incorporation

Cells were seeded at the 24 well plate with 1×10^5 per well. After incubation for 48 h, cells were pretreated with rapamycin for 30 min, stimulated by 10% FBS, added with 0.25 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech) and incubated for 4 h. The cells were washed twice with PBS and trypsinized for 10 min, and transferred to an eppendorf tube. Following the addition of 10% TCA and placing in an ice bath for 30 min, the TCA-insoluble materials were collected and solubilized with 0.2 M NaOH, containing 0.5% sodium dodecyl sulfate for 30 min at 37°C. The amount of [^{3}H]-labelled thymidine in the whole solutions was counted using a liquid scintillation counter.

Flow cytometry

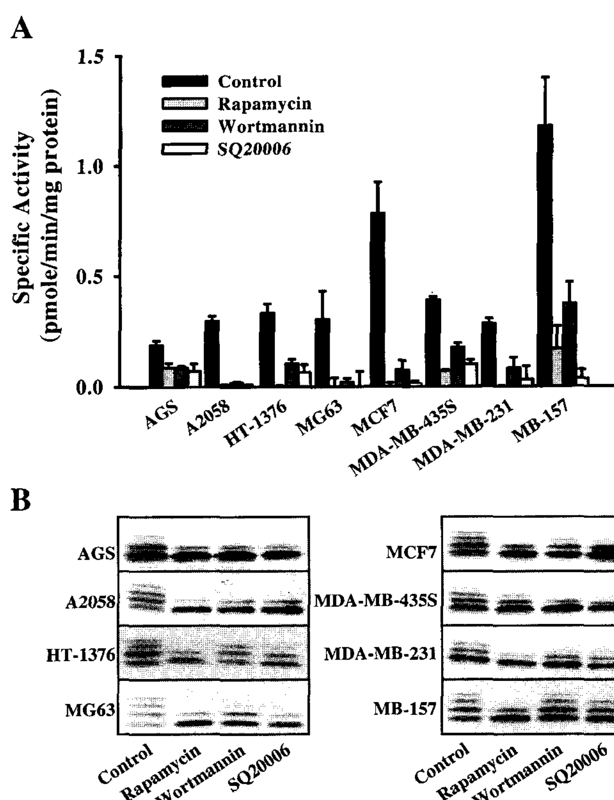

Fig. 2. Constitutively activated $p70^{S6k}$ in various human cancer cells is inhibited by rapamycin, wortmannin, and SQ20006.
(A) Serum-deprived cancer cells were treated with 5 nM rapamycin, a specific $p70^{S6k}$ inhibitor, 500 nM wortmannin, a specific PI3-K inhibitor, and 1.2 mM SQ20006, a $p70^{S6k}$ inhibitor, for 30 min, resceptively. Cell lysates were immunoprecipitated using a specific antibody for $p70^{S6k}$ and assayed for S6 kinase activity as described under "Materials and Methods". The results shown represent the means ± s.e.m. of three independent experiments. (B) The levels of $p70^{S6k}$ phosphorylation in above cell lysates were measured by immunoblot alalysis. Data show a representative blot from three independent experiments.

1×10^5 cells were seeded with an appropriate medium containing 10% FBS. At the 80% confluency, cell cultures were changed with a medium containing 0.5% FBS and incubated for 48 h. The cultures were changed with a serum free medium, and pretreated with rapamycin (5 nM) for 30 min. Following to the stimulation by 10% FBS for 18 h, the cells were trypsinized, fixed with 70% ethanol, and treated with 0.25 μg/ml RNase. Nuclei were stained with 50 μg/ml propidium iodide and the DNA distribution of the cell cycle was analyzed by BRYTE HS system (BioRad) and ModFit LT (Verity Software House, Inc., Topsham, ME).

RESULTS AND DISCUSSION

Constitutive activation of $p70^{S6k}$ in various human cancer cells

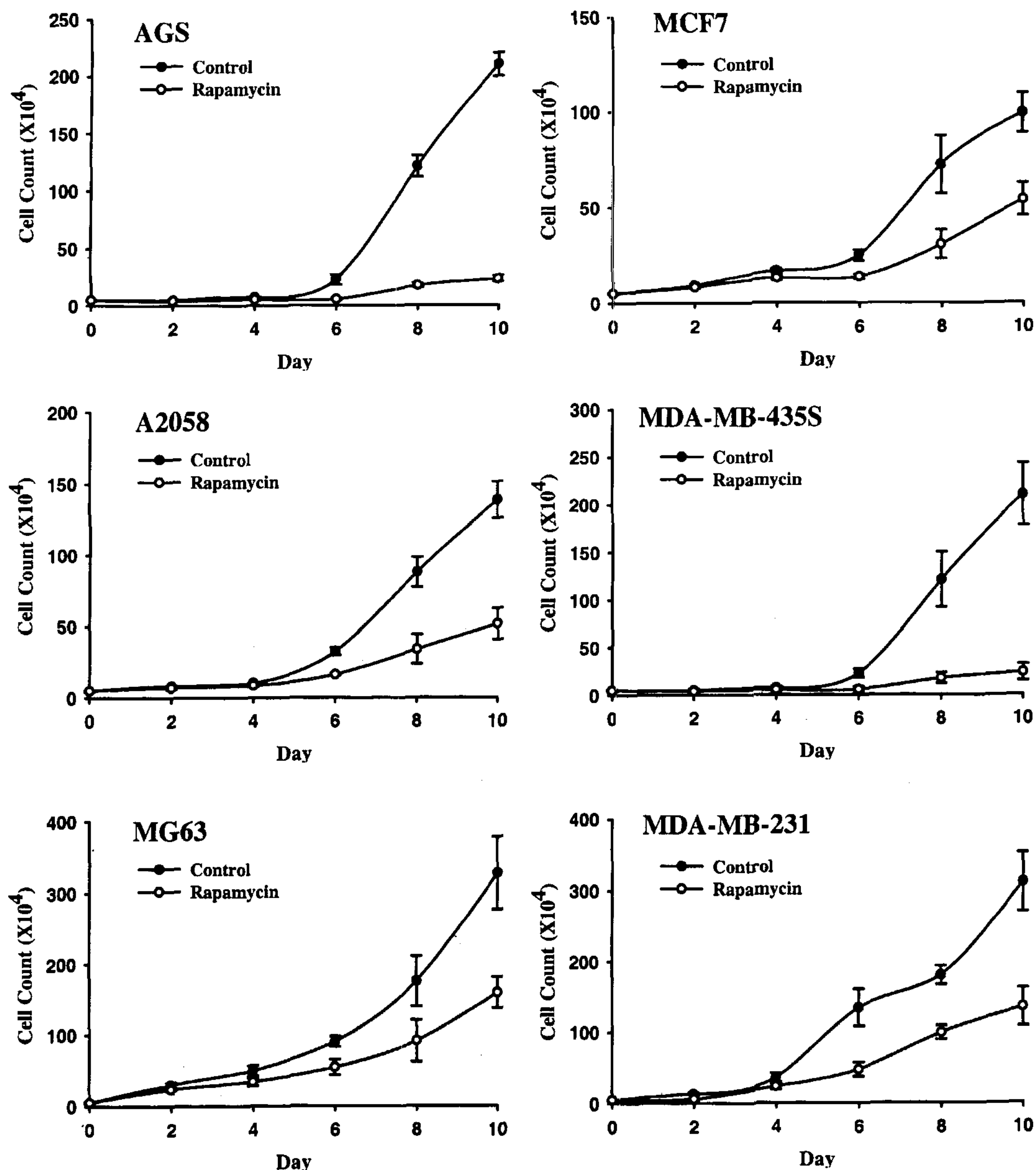

Fig. 3. Rapamycin inhibits the cell growth in various human cancer cells. 5×10^4 cells were stabilized for 24 h in the medium containing 0.5% FBS. After pretreatment with either vehicle or rapamycin (5 nM) for 30 min, each cells was treated with 10% FBS for the indicated times. Over a period of up to 10 days, cell numbers were measured by trypan blue exclusion method. The results shown represent the means ± s.e.m. of three independent experiments.

Previous studies demonstrated that $p70^{S6k}$ play 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. To examine whether $p70^{S6k}$ of various human tumor cells is constitutively phosphorylated and activated, cultures of cancer cell lines were extracted following serum deprivation. As shown in Fig. 1, their $p70^{S6k}$ were highly phosphorylated to various degrees in the absence of serum, as measured by its slower migration on western blots of one-dimensional SDS-PAGE, whereas normal mouse epidermal cell JB6 had a basal form of the $p70^{S6k}$ after serum deprivation for 24 h, which could be fully phosphorylated in response to serum treatment. To further confirm the activity of $p70^{S6k}$ in cancer cells, cell extracts were subjected to the immune complex kinase assay. Consistent with its phosphory-lation, their $p70^{S6k}$ was significantly activated to various degrees in the absence of serum. $p70^{S6k}$ of MCF7 and MB-157 after serum deprivation is more highly pho-sphorylated and activated compared with that of other cancer cell lines. In addition, a constitutively activated form of the $p70^{S6k}$ in

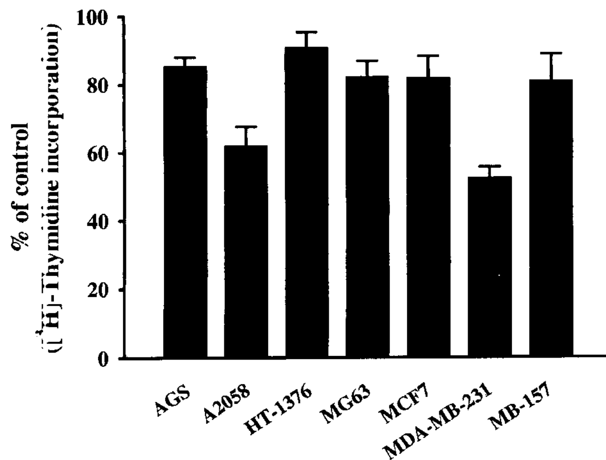

Fig. 4 Rapamycin inhibits the DNA synthesis in human cancer cells. After serum deprivation of various human cancer cells for 48 h, rapamycin were added to the cultures, and further incubated for 4 h in appropriate medium containing 10% FBS and 0.25 μCi of [^{3}H]-thymidine. Results are presented as percentage of cpm of control culture and represented the means ± s.e.m. of three independent experiments.

AGS MCF7 and MB-157 could be further activated by serum stimulation (data not shown). Taken together, the results indicate that p70^{S6k} might play an important role in the activation of cancer cell proliferation.

Inhibition of the constitutively activated p70^{S6k} by its specific inhibitors

We next examined the inhibitory effect of specific inhibitors of p70^{S6k}, including rapamycin, wortmannin and SQ20006, which were previously demonstrated to exert strong inhibition of p70^{S6k} activity (Ferrari *et al.*, 1993; Chung *et al.*, 1994; Cheatham *et al.*, 1994), on their constitutively activated p70^{S6k}. As shown in Fig. 2, rapamycin induced a significant dephosphorylation of p70^{S6k} as demonstrated by the increase in the electro-phoretic mobility of p70^{S6k} in all cancer cell lines, while wortmannin, a specific inhibitor of PI3-K, caused a mild dephosphorylation of p70^{S6k} in AGS, MDA-MB-435S and MB-157. In addition, SQ20006, methy-lxanthine phos-phodiesterase inhibitor, reduced the phos-phorylation of p70^{S6k} in all cancer cells tested. Similarly, the constitutive activation of p70^{S6k} was also inhibited by those, as measured by immunoprecipitation kinase assay. Taken together, the results indicate that p70^{S6k} could be activated through different signaling pathways which are specific for cancer cell types.

Inhibition of the growth of human cancer cells by rapamycin

To determine the possible role of p70^{S6k} in the proliferation of cancer cells, we then examined the effect of inhibition of p70^{S6k} on the growth of cancer cells. As shown in Fig. 3, cell growth was inhibited to various degrees by rapamycin treatment. These results are in good agreement with the inhibitory effects of rapamycin on p70^{S6k} activity observed in Fig. 2. To further analyze the antiproliferative effect of inhibition of p70^{S6k}, its effect on the cell cycle progression was next investigated. Treatment of asynchronous cancer cells with rapamycin resulted in the inhibition of [^{3}H]-thymidine incorporation (Fig. 4), indicating significant inhibition of G_0/G_1-S progression. Further analysis of the effect of rapamycin on the distribution of cell population showed effects similar to the above result (Fig. 5). Rapamycin treatment increased the number of cells at G_0/G_1 phase from 46.7% to 69.8%, whereas the cells at S phase decreased from 36.3% to 13.1% in MCF7. Similar effects were observed in other cancer cell lines, indicating inhibition of the cell cycle at G_0/G_1 phase. Taken together, the results indicate that the antiproliferative activity of rapamycin might be attributed to cell cycle arrest at G_0/G_1 phase in cancer cells through the inhibition of constitutively activated p70^{S6k}.

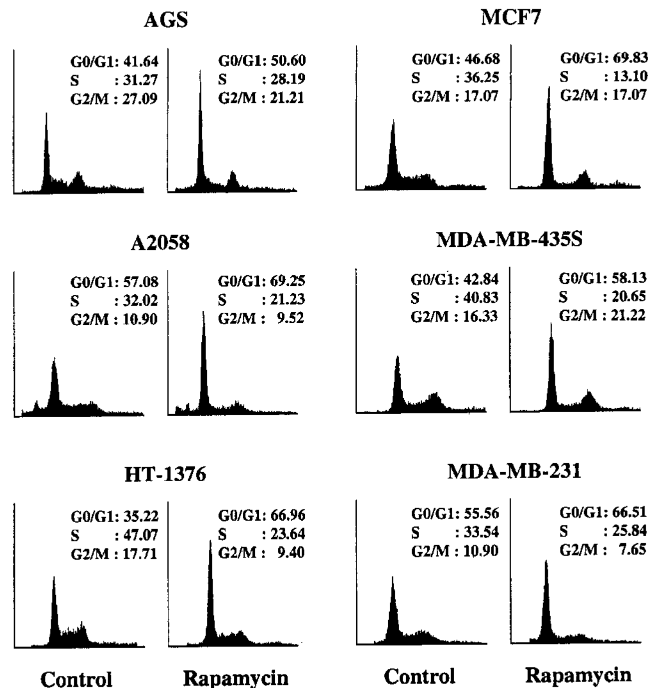

Fig. 5. Rapamycin causes the cell cycle arrest in human cancer cells at the G_0/G_1 phase.
Cells were stabilized in the medium containing 0.5% FBS for 48 h. After pretreatment with either vehicle or rapamycin (5 nM) for 30 min, the cells were stimulated with 10% FBS, and incubated for 18 h. The cells were harvested and fixed in 70% ethanol. After staining with propidium iodide, DNA contents were analyzed by flow cytometry as described under "Materials and Methods". Data show a typical representative of two independent experiments.

Maintenance of a prolonged increased rate of protein synthesis is necessary for the development of tumor

formation. In support of our observations, rapamycin analogues have been tested in pre-clinical and phase I studies which preliminary provided evidences of anti-tumor effects being observed in renal cell carcinoma and non-small cell lung cancer (Seufferlein and Rozengurt, 1996). Furthermore, a specific role of $p70^{S6k}$ in breast cancer has been inferred from the observation that $p70^{S6k}$ gene is amplified and overexpressed in a number of 8% primary breast tumors (Barlund *et al.*, 2000).

In summary, we demonstrate that $p70^{S6k}$ is constitutively phosphorylated and activated in AGS, A2058, HT-1376, MG63, MCF7, MDA-MB-435s, MDA-MB-231 and MB-157. However, the pathway leading to $p70^{S6k}$ activation appear to be different between cancer cell lines tested, because $p70^{S6k}$ is differentially sensitive to its inhibitors, rapamycin, wortmannin and SQ20006. Ramaycin inhibits [^{3}H]-thymidine incorporation and induces G_0/G_1 arrest of cancer cells, leading to the inhibition of cell proliferation. Thus, our findings presented herein provide the possible role of $p70^{S6k}$ in development of tumor formation and suggest $p70^{S6k}$ as a potential target for therapeutic strategies aimed at preventing or inhibiting tumor growth.

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