

# Rapamycin Inhibits Constitutive p70<sup>s6k</sup> Phosphorylation, Cell Proliferation, and Colony Formation in Small Cell Lung Cancer Cells

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## Abstract

The serine/threonine kinase p70<sup>s6k</sup> was found to be constitutively phosphorylated in H 69, H 345, and H 510 small cell lung cancer cells as judged by the retarded electrophoretic mobility of both isoforms of this kinase. Pretreatment of H 69, H 345, and H 510 cells with the potent immunosuppressant rapamycin led to p70<sup>s6k</sup> dephosphorylation in a concentration-dependent manner; half-maximum and maximum effects were achieved at 0.3 and 3 nM rapamycin, respectively. Rapamycin inhibited growth of H 69, H 345, and H 510 cells in liquid culture at similar concentrations to those required for inducing dephosphorylation of p70<sup>s6k</sup>. Furthermore, rapamycin markedly reduced the basal colony forming ability of H 69, H 345, and H 510 cells in semisolid media. Thus, constitutively phosphorylated/active p70<sup>s6k</sup> plays an important role in promoting the growth of small cell lung cancer cells. Furthermore, the rapamycin-sensitive p70<sup>s6k</sup> pathway may provide a novel target for therapeutic intervention in small cell lung cancer.

## Introduction

SCLC<sup>2</sup> constitutes 25% of all pulmonary cancers and is characterized by a very low 2-year survival, despite initial sensitivity to radio- and chemotherapy (1). Thus, novel therapeutic strategies are needed, and these could arise from defining the factors and intracellular signaling pathways that stimulate the proliferation of SCLC (2). A variety of neuropeptides, hormones, and cytokines promote clonal growth of certain SCLC cell lines and have been proposed to act as autocrine/paracrine growth factors for these cells (2–5). However, the signal transduction pathways involved in autocrine/paracrine SCLC cell growth remain poorly understood. In this context, the phosphorylation and subsequent activation of the p70/p85 kDa S6 kinases, collectively referred to as p70<sup>s6k</sup>, is of considerable interest because this pathway is a highly conserved element in the cellular response to growth factors and neuropeptides (6–9). Recent evidence suggests that activation of p70<sup>s6k</sup> may regulate a wide array of cellular processes involved in the mitogenic response including protein synthesis, translation of specific mRNA species, and progression from G<sub>1</sub> to S phase of the cell cycle (6, 8, 9). The immunosuppressant rapamycin, which is a highly potent inhibitor of p70<sup>s6k</sup> phosphorylation and activation by all known stimuli, has emerged as a useful tool to elucidate the cellular function of p70<sup>s6k</sup> (6–8, 10–13). The role of the rapamycin-sensitive p70<sup>s6k</sup> pathway in the growth of SCLC cells is unknown. Here, we demonstrate that p70<sup>s6k</sup> is constitutively phosphorylated in the SCLC cell lines H 69, H 345, and H 510. Rapamycin induced p70<sup>s6k</sup> dephosphorylation, inhibited proliferation in liquid culture, and reduced colony formation in semisolid medium in these SCLC cell lines. Our results identify the rapamycin-sensitive p70<sup>s6k</sup>

pathway as a potential novel target for therapeutic intervention in SCLC.

## Materials and Methods

**Cell Culture.** SCLC cell lines H 69, H 345, and H 510 were generously donated by Dr. A. Gazdar (Bethesda, MD) and purchased from the American Type Culture Collection. Stocks were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 10% CO<sub>2</sub>:90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA medium (RPMI 1640 supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol, 30 nM selenium, and 0.25% BSA).

**p70<sup>s6k</sup> Mobility Shift Assay.** Cultures of H 69, H 345, and H 510 SCLC cells grown in HITESA were washed four times in RPMI 1640 and then incubated in RPMI 1640 for 2 h. Aliquots of 3 × 10<sup>6</sup> cells were subsequently incubated for 20 min with various concentrations of rapamycin in fresh RPMI 1640, as indicated in the figure legends. Cells were then lysed in 2× SDS-PAGE sample buffer. Activation of p70<sup>s6k</sup> was determined by the appearance of slower migrating forms in SDS-PAGE as a result of phosphorylation on threonines 229 and 389 and serine 404 (11, 14). SDS-PAGE gels were transferred to Immobilon membranes, and Western blot analysis was performed using a rabbit polyclonal antibody that recognized both the p85αI and the p70αII isoforms of p70<sup>s6k</sup> (6); immunoreactive bands were visualized using ECL.

**Growth Assay.** SCLC cells, 3–5 days after passage, were washed twice in RPMI 1640 and resuspended in HITESA. Cells were resuspended at a density of 5 × 10<sup>4</sup> cells in 1 ml HITESA in the presence or absence of rapamycin at the concentrations indicated in duplicate. At various times, the cell number was determined using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension through 19- and 21-gauge needles.

**Clonogenic Assay.** SCLC cells, 3–5 days after passage, were washed and resuspended in HITESA. Cells were then disaggregated by two passes through a 19-gauge needle into an essentially single-cell suspension as judged by microscopy. The cell number was determined using a Coulter counter, and 10<sup>4</sup> cells were mixed with HITESA containing 0.3% agarose in the absence or presence of rapamycin, HGF, or galanin at the concentrations indicated and layered over a solid base of 0.5% agarose in HITESA in the absence or presence of rapamycin, HGF, or galanin at the same concentrations in 33-mm dishes. The cultures were incubated in humidified 10% CO<sub>2</sub>:90% air at 37°C for 21 days and then stained with the vital stain nitro blue tetrazolium. Colonies of >120 μm in diameter (16 cells) were counted using a microscope.

**Materials.** Rapamycin was obtained from Calbiochem-Novabiochem, Ltd. (Nottingham, United Kingdom). Recombinant human HGF was generously provided by Dr. E. Gherardi (Imperial Cancer Research Fund, Cambridge, United Kingdom). Anti-p70<sup>s6k</sup> affinity-purified rabbit polyclonal antibody used for Western blotting was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL reagent was obtained from Amersham Corp. (Buckinghamshire, United Kingdom). All other reagents were of the purest grade available.

## Results and Discussion

Activation of p70<sup>s6k</sup> by mitogens can be determined by the appearance of slower migrating forms in SDS-PAGE (6, 8). This results from phosphorylation of p70<sup>s6k</sup> on Thr 229 and 389 and Ser 404, which are not basally phosphorylated in quiescent cells (11, 14). The

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<sup>2</sup> The abbreviations used are: HGF, hepatocyte growth factor; SCLC, small cell lung cancer.

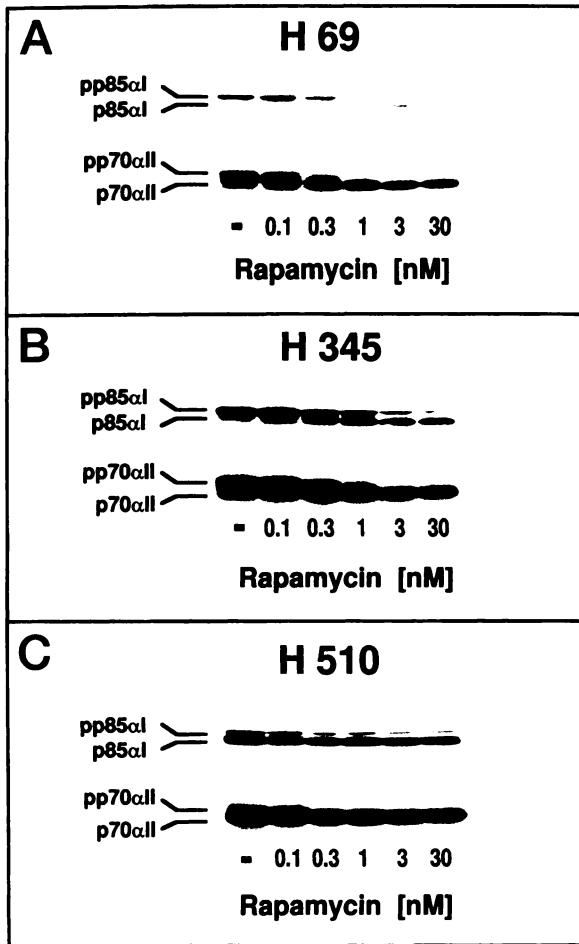


Fig. 1. Constitutive phosphorylation of p70/p85<sup>S6K</sup> in H 69, H 345, and H 510 SCLC cells. Cultures of H 69 (A), H 345 (B), or H 510 cells (C) were treated with various concentrations of rapamycin as indicated for 20 min. Control cells received an equivalent amount of solvent (-). The cells were subsequently extracted in 2× SDS sample buffer. p70/p85<sup>S6K</sup> mobility shift assays were performed as described in "Materials and Methods." The results shown in each case are representative of three independent experiments. The positions of the hypophosphorylated p70<sup>S6K</sup> isoforms (p70αII and p85αI) and the slower migrating phosphorylated p70<sup>S6K</sup> isoforms (pp70αII and pp85αI) are indicated.

phosphorylation of these sites is prevented or reversed by treatment with rapamycin, a potent inhibitor of p70<sup>S6K</sup> activation (6–8, 10–13).

p70<sup>S6K</sup> exists in two isoforms, a p85 (αI) nuclear isoform and a p70 (αII) cytoplasmic isoform encoded by two distinct transcripts differing in their 5' ends and derived from the same gene by alternative splicing (15). To examine whether p70<sup>S6K</sup> is constitutively phosphorylated/activated in SCLC cells, cultures of the cell lines H 69, H 345, and H 510 were incubated in the absence or presence of various concentrations of rapamycin. Cells were lysed, and the lysates were analyzed by immunoblotting using an antibody that recognizes both isoforms of the enzyme. Both p70<sup>S6K</sup> and p85<sup>S6K</sup> exhibited a retarded electrophoretic mobility characteristic of the phosphorylated forms of these isoenzymes (Fig. 1). These slowly migrating forms were prominent in lysates from H 69 and H 345 cells and to a lesser degree in those from H 510 cells. Indeed, incubation of H 69, H 345, and H 510 SCLC cells with phorbol 12,13-dibutyrate, a potent stimulus of p70<sup>S6K</sup> (6), did not result in a further decrease in the mobility of the enzyme from H 69 and H 345 SCLC cells and revealed only a slightly enhanced mobility shift in both isoforms from H 510 SCLC cells (data not shown).

Treatment with rapamycin induced a striking dephosphorylation of p70<sup>S6K</sup> and p85<sup>S6K</sup>, as demonstrated by the increase in the electrophoretic mobility of both isoforms in all three SCLC cell lines (Fig. 1). The effect of rapamycin was concentration dependent; half-maximum and maximum effects were achieved at 0.3 and 3 nM rapamycin, respectively (Fig. 1). Dephosphorylation of p70<sup>S6K</sup> by rapamycin in all three SCLC cell lines was first visible after 2.5 min and reached a maximum 5 min after the addition of rapamycin to the cells (data not shown). These results indicate that the rapamycin-sensitive p70<sup>S6K</sup> is constitutively phosphorylated/activated in the H 69, H 345, and H 510 SCLC cell lines.

Rapamycin can block progression of certain cell lines through the G<sub>1</sub> phase of the cell cycle via inhibition of p70<sup>S6K</sup> activation (6, 8). We, therefore, examined the effect of rapamycin on the proliferation of H 69, H 345, or H 510 SCLC cells in liquid culture. The cells were incubated in HITESA in the absence or presence of rapamycin, and cell numbers were determined over a period of up to 17 days. In the

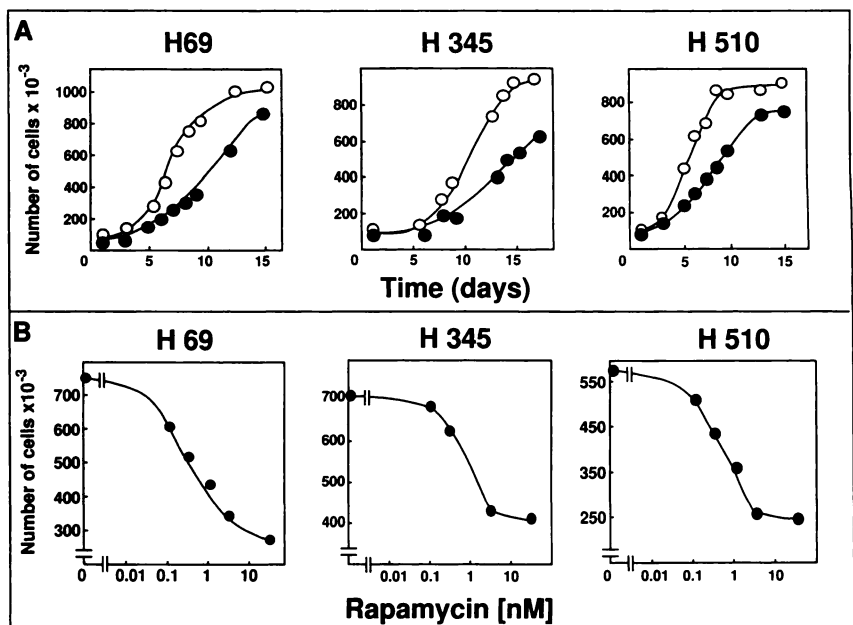


Fig. 2. Rapamycin inhibits the growth of H 69, H 345, and H 510 SCLC cells in liquid culture. A, cultures of H 69 (left panel), H 345 (middle panel), or H 510 (right panel) cells were incubated at a density of  $5 \times 10^4$  cells in 1 ml HITESA (○) or 1 ml HITESA containing 20 nM rapamycin (●). Each point represents the mean of three determinations and is representative of at least two independent experiments. B, cultures of H 69 (left panel), H 345 (middle panel), or H 510 (right panel) cells were incubated at a density of  $5 \times 10^4$  cells in 1 ml HITESA containing various concentrations of rapamycin, as indicated. Cells were counted after 7 days (H 510 cells), after 9 days (H 69), or after 11 days (H 345 cells), respectively. Each point represents the mean of two determinations and is representative of at least two independent experiments.

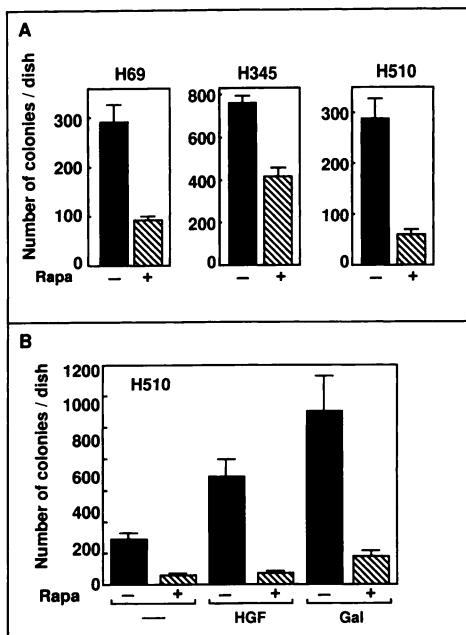


Fig. 3. Rapamycin inhibits the colony-forming ability of H 69, H 345, and H 510 SCLC cells. *A*, single-cell suspensions of H 69, H 345, or H 510 cells were plated at a density of  $1 \times 10^4$  cells/dish in agarose medium containing HITESA in the absence (-) or presence (+) of 20 nM rapamycin (Rapa +, ▨) and incubated for 3 weeks as described in "Materials and Methods." Columns, mean number of colonies formed on five separate dishes; bars, SE. In all cases, a representative of two independent experiments is shown. *B*, single-cell suspensions of H 510 cells were plated at a density of  $1 \times 10^4$  cells/dish in agarose medium containing HITESA and 10 ng/ml HGF (+) or 30 nM galanin (Gal +) in the absence (-) or presence (+) of 20 nM rapamycin (Rapa +, ▨) and incubated for 3 weeks as described in "Materials and Methods." Columns, mean number of colonies formed on five separate dishes; bars, SE. In all cases, a representative of two independent experiments is shown.

presence of rapamycin, cell numbers were reduced in all three SCLC cell lines by a maximum of 55% in H 69, H 345, and H 510 cells, respectively (Fig. 2). The effect of rapamycin on SCLC cell growth in liquid culture was concentration dependent, reaching half-maximum and maximum effects between 0.3–1 and 3–10 nM, respectively (Fig. 2B). These results are in good agreement with the data obtained in the mobility shift assays for p70/p85<sup>S6K</sup> activity in Fig. 1. Thus, the constitutively phosphorylated rapamycin-sensitive p70<sup>S6K</sup> is likely to participate in sustaining the growth of H 69, H 345, and H 510 SCLC cells.

Tumor and transformed cells, including SCLC, are able to form colonies in agarose medium. Indeed, there is a positive correlation between cloning efficiency of the cells and the histological involvement and invasiveness of the tumor in specimens taken from SCLC (16). Consequently, we determined the effect of rapamycin on the ability of SCLC cells to form colonies in semisolid medium. As shown in Fig. 3A, rapamycin markedly inhibited the formation of colonies by H 69, H 345, and H 510 SCLC cells. The colony-forming ability of SCLC cells can be further enhanced by neuropeptide or polypeptide growth factors such as galanin and HGF, respectively (17, 18). To examine the effect of rapamycin on the colony growth stimulated by HGF and galanin, we chose H 510 SCLC cells because in this cell line, p70<sup>S6K</sup> was only partially phosphorylated in the basal state (Fig. 1). As shown in Fig. 3B, rapamycin strikingly inhibited colony formation of H 510 cells in response to either HGF or galanin

by 90 and 84%, respectively. Thus, phosphorylation of p70<sup>S6K</sup> appears to contribute to both basal and growth factor-stimulated colony formation of H 510 SCLC cells in semisolid medium.

In this study, we demonstrate, for the first time that: (a) the pathway(s) leading to rapamycin-sensitive p70<sup>S6K</sup> phosphorylation/activation is constitutively active in H 69, H 345, and H 510 SCLC cells; and (b) rapamycin inhibits SCLC cell multiplication in liquid culture and markedly reduces the basal and also the growth factor-stimulated colony forming ability of these cells. Thus, our results suggest that the constitutively active rapamycin-sensitive p70<sup>S6K</sup> contributes to basal and growth factor-stimulated proliferation of SCLC cells.

Conventional treatment of SCLC does not succeed in preventing the very aggressive clinical course of this tumor (1). Therefore, there is great interest in developing novel approaches to the treatment of SCLC. The results presented in this study suggest that inhibitors of p70<sup>S6K</sup> activation, such as the immunosuppressant rapamycin, could constitute novel antiproliferative agents for SCLC.

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