THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 38, Issue of September 17, pp. 26776–26782, 1999
Printed in U.S.A.

p70 S6 Kinase-mediated Protein Synthesis Is a Critical Step for Vascular Endothelial Cell Proliferation*

(Received for publication, May 25, 1999, and in revised form, July 9, 1999)

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In this work, we analyzed the role of the PI3K-p70 S6 kinase (S6K) signaling cascade in the stimulation of endothelial cell proliferation. We found that inhibitors of the p42/p44 MAPK pathway (PD98059) and the PI3K-p70 S6K pathway (wortmannin, Ly294002, and rapamycin) all block thymidine incorporation stimulated by fetal calf serum in the resting mouse endothelial cell line 1G11. The action of rapamycin can be generalized, since it completely inhibits the mitogenic effect of fetal calf serum in primary endothelial cell cultures (human umbilical vein endothelial cells) and another established capillary endothelial cell line (LIBE cells). The inhibitory effect of rapamycin is only observed when the inhibitor is added at the early stages of G_0-G_1 progression, suggesting an inhibitory action early in G_1. Rapamycin completely inhibits growth factor stimulation of protein synthesis, which perfectly correlates with the inhibition of cell proliferation. In accordance with its inhibitory action on protein synthesis, activation of cyclin D1 and p21 proteins by growth factors is also blocked by preincubation with rapamycin. Expression of a p70 S6K mutant partially resistant to rapamycin reverses the inhibitory effect of the drug on DNA synthesis, indicating that rapamycin action is via p70 S6K. Thus, in vascular endothelial cells, activation of protein synthesis via p70 S6K is an essential step for cell cycle progression in response to growth factors.

Blood vessels represent one of the most quiescent tissues of adult mammals. However, in response to the appropriate stimuli, quiescent endothelium can produce new vessels in a process known as angiogenesis (formation of new blood vessels from pre-existing vasculature) (1). This happens in normal situations such as embryonic development and wound healing and during the female reproductive cycle. However, activated blood vessel growth is found in many diseases, such as tumor progression, diabetic retinopathy, and arthritis (2). In the last few years, several studies have led to the discovery of inducers and inhibitors of the angiogenic process. Fibroblast growth factors 1 and 2 (also known as aFGF and bFGF, respectively) and vascular endothelial growth factor are among the inducers, whereas thrombospondin-1, angiostatin, and endostatin are inhibitors. However, the signaling mechanisms regulated by these agents that control the reversible growth arrest state of endothelial cells are not well understood.

Two signaling cascades have emerged as major players in the mitogenic and anti-apoptotic response: the Ras-p42/p44 MAPK[1] cascade and the PI3K-p70 S6 kinase (S6K) cascade. Both pathways are initiated at the level of the plasma membrane after activation of growth factor receptors and integrins. The Ras-p42/p44 MAPK module implicates activation of the low molecular weight GTP-binding protein $p21^{ras}$ and the sequential activation of a series of protein kinases: Raf-extracellular signal-regulated kinase kinase-p42/p44 MAPK (3, 4). In quiescent cells, p42/p44 MAPKs are cytoplasmic, but after stimulation by growth factors, both isoforms translocate to the nucleus, a key step in growth signaling (5). p42/p44 MAPKs phosphorylate several transcription factors, such as members of the Ets family, thereby controlling the expression of cell cycle-regulated genes (6). Cyclin D1 is one of the multiple genes that are positively controlled by the p42/p44 MAPK cascade (7), and its induction is essential for the progression to S phase (8, 9).

Activation of PI3K by growth factors leads to the production of 3′-phosphorylated inositols that act as second messengers for pleckstrin homology domain-containing signaling molecules, such as Akt/PKB (10, 11). Activation of PI3K seems to be required for cell growth but in a cell type- and stimulus-dependent manner. Failure to activate p110 PI3K prevents reinitiation of DNA synthesis in response to platelet-derived growth factor and epidermal growth factor in NIH3T3 cells (12, 13), and the knockout of p85 PI3K results in a lack of B cell proliferation in response to different agonists (14, 15). Expression of a constitutively active PI3K favors DNA replication in 3T3-L1 adipocytes (16), and expression of an inducible form of PI3K is sufficient for cell cycle entry in quiescent fibroblasts (17). Two downstream signaling pathways have been described for PI3K, Akt-GSK3 and p70/p85 S6K, which can be distinguished by their sensitivity to the drug rapamycin (10, 11). p70/p85 S6K are two isoforms of the kinase that controls the ribosomal protein S6 phosphorylation in response to mitogens. They are controlled by FRAP/mTOR, the target of rapamycin. p70/p85 S6K is activated by all mitogenic stimuli, including growth factors, cytokines, and phorbol esters, and plays an essential role in controlling the translation machinery (18, 19).

In this work, we analyzed the contribution of PI3K-p70/p85 S6K in the control of vascular endothelial cell growth. We have found that inhibitors of the PI3K-p70/p85 S6K completely block mitogenesis in these cells and that the mechanism implicates the inhibition of protein synthesis.

* This work was supported by research grants from CNRS, INSERM, Association pour la Recherche contre le Cancer, and the European Community (Contract B104-CT97-2071). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; S6K, S6 kinase; PI3K, phosphatidylinositol 3-kinase; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Materials—Ly294002 was obtained from Alexis, wortmannin and rapamycin from BioMol, and PD98059 from New England Biolabs. Cycloheximide was obtained from Sigma. Cell culture media, FCS, glutamine, and antibiotics were obtained from Life Technologies, Inc. The most commonly used chemicals were purchased from Sigma.

Cells and Culture Conditions—Murine lung endothelial 1G11 cells were obtained from Drs. Alberto Mantovani and Annunciata Vecchi (Instituto Ricerche Farmacologiche Mario Negri, Milan, Italy) (20). They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% inactivated FCS, 50 units/ml penicillin, 50 μg/ml streptomycin sulfate, 150 μg/ml endothelial cell growth supplement (Becton Dickinson), 100 μg/ml heparin (Sigma), 1% nonessential amino acids, and 2 mM sodium pyruvate. Before the incubation with growth factors, cells were depleted for 24 h in a 1:1 mixture of DMEM and Ham's F-12. When indicated, rapamycin or the solvent ethanol was added 15 min before the stimulation with 20% FCS.

Mouse brain capillary endothelial LIBE cells were obtained from Dr. Claesson-Welsh (Ludwig Institute for Cancer Research, Uppsala, Sweden) and were cultivated as previously described (21). Human umbilical vein endothelial cells (HUVEC) were obtained as described previously (22) and were cultivated in EBM-2 supplemented endothelial cell medium (Clonetics, BioWhittaker). The established Chinese hamster lung fibroblast line CCL39 was cultivated in DMEM containing 7.5% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate.

Retroviral Infection and Generation of Wild Type and Rapamycin-resistant p70 S6K-expressing 1G11 Cells—Retroviral supernatants were generated by transient transfection of BOSC23 cells (23) with plasmids pBabe, pBabe-p70 S6K (wild type), and pBabe-p70S6KE_{389}-D_3E (rapamycin-resistant mutant) (24). Both constructions have an amino-terminal Myc tag. Positive clones were selected on the basis of resistance to puromycin (10 μg/ml) and confirmed by Western blot with an antibody against Myc. The studies were performed using different independent clones of 1G11-p70S6K WT and 1G11-p70S6K$E_{389}D_3$E cells.

Determination of DNA Synthesis—1G11 cells were cultured in 24-well plates for 48 h and deprived of growth factors for 24 h in a 1:1 mixture of DMEM and Ham's F-12 medium. Cells were then stimulated with fresh DMEM medium containing 20% FCS and 0.25 μCi/ml [*methyl*-^{3}H]thymidine (Amersham Pharmacia Biotech) (3 mM final concentration). After 20 h of incubation, cells were fixed and washed twice with ice-cold trichloroacetic acid (5%). The precipitated material was solubilized with 0.1 N NaOH, and the incorporated radioactivity was counted by liquid scintillation. When indicated, cells were incubated in presence of thymidine for 24, 36, or 48 h before fixation. Results are expressed as a percentage of the maximal [^{3}H]thymidine incorporation in the presence of FCS alone.

Determination of Protein Synthesis—1G11 cells were seeded in 12-well plates and rendered quiescent by FCS starvation for 24 h. Cells were rinsed with DMEM and [^{3}H]leucine (L-leucine 2,3,4,5-^{3}H-labeled), 2 μCi/ml, 0.8 mM final concentration) was added with or without 20% FCS in 250 μl of DMEM. After 12 h of stimulation, cells were fixed and washed twice with ice-cold trichloroacetic acid (5%). The precipitated material was solubilized with 0.1 N NaOH, and the incorporated radioactivity was counted by liquid scintillation. The lack of effect of the rapamycin on leucine transport was measured by counting the radioactivity in the first trichloroacetic acid wash.

Western Blot Analysis—Cells were washed twice with cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 4 μg/ml aprotinin, 1% Triton X-100) for 15 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × *g* for 5 min at 4 °C. Proteins from cell lysates were separated on acrylamide/bisacrylamide (29:1) SDS gels and electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) in 25 mM Tris-HCl, 0.19 M glycine, 20% ethanol. Membranes were blocked in PBS containing 5% nonfat dry milk for 1 h at 37 °C. The blots were then incubated with polyclonal anti-p70 S6 kinase (Sigma); monoclonal anti-p27 (Transduction Laboratories); monoclonal anti-cyclin D1 (NeoMarkers); polyclonal anti-p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit antiserum Alb-1, which specifically recognizes MAPK phosphatase 1 (MKP1) (25); polyclonal antiphospho-(Ser^{473})-Akt (New England Biolabs); polyclonal anti-Akt (a generous gift from Dr. B. Hemmings, Friedrich Miescher Institut, Basel, Switzerland); monoclonal 9E10 anti-Myc (Roche Molecular Biochemicals); and monoclonal anti-retinoblastoma protein (pRb) (Pharmigen) antibodies in the blocking solution overnight at 4 °C. After washing in PBS, 0.1% Tween 20, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega) or anti-mouse IgG (Jackson Laboratories) in blocking solution for 1 h and revealed with an ECL system (Amersham Pharmacia Biotech).

Where indicated, the p70 S6 kinase and p42/p44 MAPK activities were determined by a mobility shift assay in which, following cell lysis, proteins were separated by SDS-polyacrylamide gel electrophoresis in a 9% gel (acrylamide:bisacrylamide 30:0.3 for p70 S6K) or 12.5% (acrylamide:bisacrylamide 30:0.2 for p42/p44 MAPKs), and Western blotting was performed with anti-S6 kinase antiserum or anti-p42/p44 MAPK antiserum EIB (26).

RESULTS

PI3K-p70 S6K and p42/p44 MAPK Inhibition Abolish DNA Synthesis in 1G11 Endothelial Cells—In order to determine the importance of PI3K activation in the stimulation of endothelial cell proliferation by growth factors, quiescent 1G11 endothelial cells were stimulated with 20% FCS in the absence or presence of two different inhibitors of PI3K, wortmannin (27) and Ly294002 (28), followed by measurement of DNA synthesis. Thymidine incorporation was increased 50-fold in FCS-stimulated cells. Preincubation with wortmannin or Ly294002 strongly inhibited FCS-stimulated thymidine incorporation (Fig. 1*A*). Treatment with wortmannin or Ly294002 specifically prevented PI3K activation, measured by the phosphorylation state of two downstream targets of PI3K, Akt and p70 S6K (Figs. 1*B* and 2*B*). These results indicate that activation of PI3K is essential for G_0/G_1 to S phase progression of 1G11 vascular endothelial cells.

p70 S6K lies downstream of PI3K activation by growth factors (29, 30). Given the effect of PI3K inhibitors on DNA synthesis in endothelial cells, next we studied the effect of rapamycin (a specific inhibitor of p70 S6K activation by inhibition of its activator FRAP/mTOR) on thymidine incorporation stimulated by 20% FCS. In parallel, we evaluated the importance of p42/p44 MAPK activation by using an inhibitor of the MAPK/extracellular signal-regulated kinase kinase 1 (MEK1), PD98059. Preincubation with 50 μM PD98059 before stimulation resulted in 80% inhibition of thymidine incorporation, while preincubation with 10 nM rapamycin completely inhibited the mitogenic effect of FCS (Fig. 2*A*). The same result was obtained by measuring bromodeoxyuridine incorporation in the presence of rapamycin (70% inhibition). Furthermore, the addition of rapamycin to exponentially proliferating 1G11 cells diminished the total cell number by 65% after 4 days in the presence of the drug (data not shown). The inhibition of p70 S6K activity was revealed by the lack of shift in a SDS-polyacrylamide gel (Fig. 2*B*) and by measuring phosphorylation of a S6 peptide (data not shown). The specificity of this inhibition is confirmed by the absence of p42/p44 MAPK inhibition in response to rapamycin or Ly294002, while PD98059 only inhibited p42/p44 MAPK activation (Fig. 2*B*). These results clearly indicate that PI3K-p70 S6K is essential for growth factor mitogenicity in 1G11 endothelial cells. They also confirm that, as seen in other cell systems, P70 S6K is a downstream target of PI3K in vascular endothelial cells.

1G11 is a vascular endothelial cell line obtained from murine lung. To investigate whether the effects of rapamycin are general to vascular endothelial cells, we performed DNA synthesis assays with primary cultures of HUVEC, and a different vascular endothelial cell line, LIBE cells (a mouse brain capillary cell line). Preincubation with rapamycin completely abrogated FCS stimulation of thymidine incorporation in 1G11 and HUVEC cells and caused a 65% inhibition in LIBE cells (Fig. 3). In contrast, preincubation of CCL39 fibroblasts in the presence of rapamycin only slightly inhibited thymidine incorporation by 18%. These results suggest that the inhibitory effect of rapamycin on mitogenicity is a general phenomenon for vascular

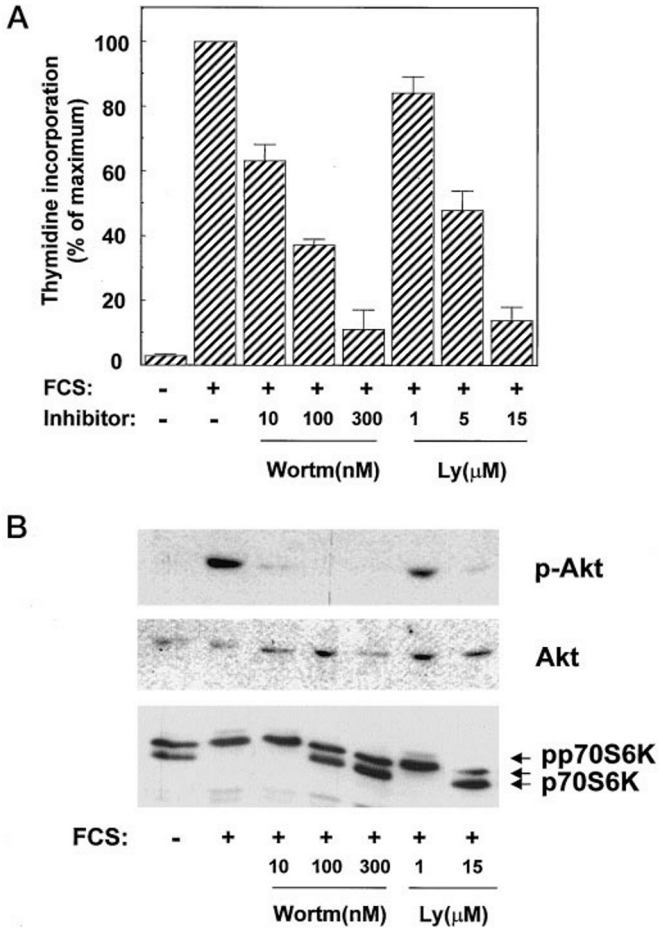

FIG. 1. **PI3K inhibition by wortmannin or Ly294002 prevents serum-induced reinitiation of DNA synthesis in 1G11 cells.** *A*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS in the presence or the absence of the indicated concentrations of wortmannin or Ly294002. After 20 h of incubation, DNA synthesis was measured as described under "Experimental Procedures." Results are an average of four different experiments. *B*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS in the presence or the absence of the indicated concentrations of wortmannin or Ly294002 for 1 h. After this time, cells were lysed, and Western blot was performed using anti-active Akt (p-Akt) and total Akt (Akt) antibodies. The same extracts were loaded on a 9% SDS-polyacrylamide gel (shift-up) and blotted with anti-p70 S6K antibody. Hyperphosphorylated and active forms of p70 S6K (indicated as pp70 S6K) migrated more slowly that hypophosphorylated forms (indicated as p70 S6K). A representative Western blot is shown.

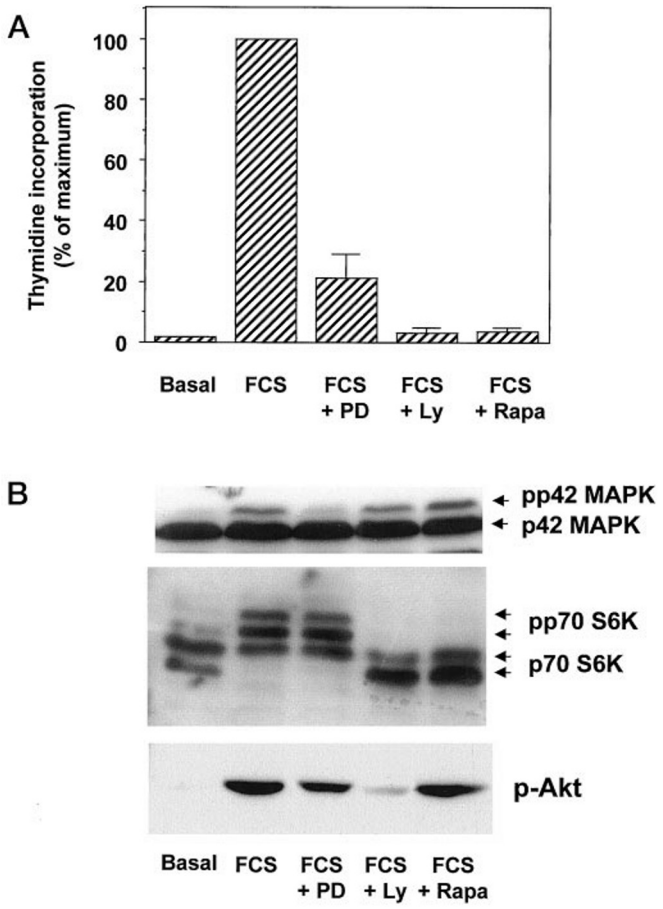

FIG. 2. **PD98059 and rapamycin inhibit serum-induced DNA synthesis in 1G11 cells.** *A*, 1G11 quiescent cells were preincubated for 15 min in the presence or in the absence of 50 μM PD98059, 15 μM Ly294002, or 10 nM rapamycin, and DNA synthesis was measured as described. Results are an average ± S.E. of four independent experiments. *B*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS for 1 h in the presence or absence of 50 μM PD98059, 15 μM Ly294002, or 10 nM rapamycin. Phospho-Akt, p70 S6K, or p42 MAPK was detected by immunoblotting with specific antibodies. Hyperphosphorylated and active forms of p42 MAPK (indicated as *pp42 MAPK*) and p70 S6K (indicated as *pp70 S6K*) migrated more slowly that nonphosphorylated forms. These Western blots are representative of three independent experiments.

endothelial cells.

Rapamycin Blocks 1G11 Endothelial Cells in the G_1 Phase of the Cell Cycle—It has been described that rapamycin abolishes cell proliferation in T cells, whereas it only delays entry of Swiss 3T3 fibroblasts into S phase (31). To address the temporal effect of rapamycin in 1G11 endothelial cells, thymidine incorporation studies were performed for periods of time up to 48 h in the presence or in the absence of rapamycin. As is shown in Fig. 4*A*, rapamycin blocked mitogenicity at all the times assayed (96% of inhibition at 24 h, 87% at 36 h and 77% at 48 h). This indicates that, in vascular endothelial cells, rapamycin blocks cells in G_1 phase rather than delaying entry into the cell cycle.

To confirm that rapamycin affects a critical step during the transition G_0/G_1 to S phase, a time course of rapamycin addition after serum stimulation was performed. As it has been described, rapamycin addition before FCS completely blocked thymidine incorporation (Fig. 4*B*). However, when rapamycin was added after FCS stimulation, a gradual release of the inhibition was observed during the prereplicative phase, resulting in 50% inhibition at 6 h and only 22% when the cells enter S phase (Fig. 4*B*). This result indicates that rapamycin affects an early step in the G_1 phase of the cell cycle and that DNA replication is not sensitive to the inhibition by rapamycin. This block in G_1 was confirmed by the lack of hyperphosphorylation of pRb in cells pretreated with rapamycin and stimulated for 18 h with FCS (data not shown).

Rapamycin Blocks Mitogenesis by Inhibiting Protein Synthesis—The role of p70 S6K in the stimulation of the cellular proliferation is based in its capacity to phosphorylate the ribosomal protein S6 in response to mitogens (18). This phosphorylation leads to an increase in the translation of a subset of messenger RNAs, which are essential for the demands of proliferating cells. Therefore, rapamycin could induce its effect on endothelial cell proliferation by inhibiting growth factor-mediated protein synthesis. To evaluate this possibility, we measured the variations in initial rates of protein synthesis. Serum stimulation of quiescent 1G11 cells resulted in a 40% increase in leucine incorporation (Fig. 5). Interestingly, the addition of rapamycin completely blocked serum-stimulated protein syn-

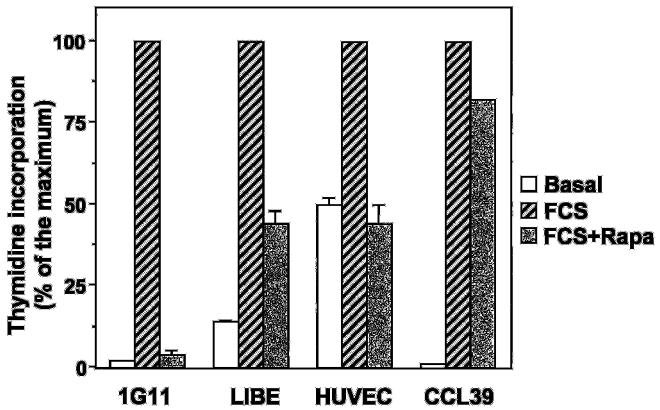

FIG. 3. **Rapamycin inhibits DNA synthesis in different vascular endothelial cell types but not in CCL39 fibroblasts.** 1G11 mouse lung vascular endothelial cells, LIBE mouse brain capillary vascular endothelial cells, primary HUVEC, and CCL39 fibroblasts were depleted of growth factors for 24 h. After this time, cells were stimulated with 20% FCS for 20 h in the presence or absence of 10 nM rapamycin, and DNA synthesis was measured as described. Results are an average of two (CCL39 cells) or five experiments (rest of cells).

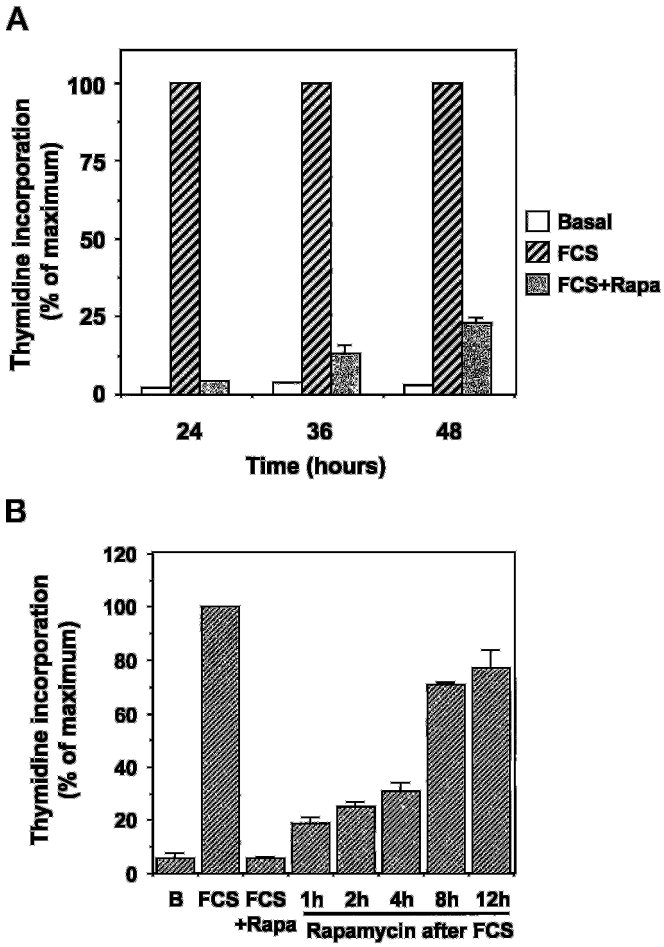

FIG. 4. **The inhibitory action of rapamycin is persistent and affects early in the G_1 phase.** *A*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS in presence of [*methyl*-^{3}H]thymidine for 24, 36, and 48 h with or without rapamycin. Cells were then processed as described. Results are an average ± S.E. of three different experiments. *B*, quiescent 1G11 cells were restimulated with 20% FCS. 10 nM rapamycin was added at time 0 or 1, 2, 4, 8, or 12 h after the addition of FCS. After 20 h of total incubation, DNA synthesis was measured. Results are an average ± S.E. of three independent experiments.

thesis, without having any effect on the basal synthesis rate. We also assayed the effect of Ly294002 on protein synthesis. Ly294002 potently inhibited protein synthesis and even reduced the basal levels by 30% (Fig. 5).

We next investigated whether the effect of rapamycin on thymidine incorporation correlated with protein synthesis inhibition. 1G11 cells were preincubated in the presence of different concentrations of rapamycin (from 0.01 to 10 nM) followed by measurement of p70 S6K activation and the rates of thymidine and leucine incorporation. As observed in Fig. 6*A*, rapamycin inhibited thymidine incorporation stimulated by FCS with the same dose-dependence than protein synthesis. In both cases, the IC_{50} for rapamycin is approximately 0.1 nM with a total inhibition at 1 nM. These effects correlated perfectly with the inhibition of p70 S6K activity as measured by the shift-up assay (Fig. 6*B*). This strict dependence between the rate of DNA synthesis and the rate of protein synthesis was confirmed with the use of a potent inhibitor of protein synthesis, cycloheximide. As is observed in Fig. 6*C*, cycloheximide inhibited leucine and thymidine incorporation with a similar IC_{50} (0.015 μg/ml) for both processes. These results therefore suggest that the block of mitogenesis induced by rapamycin is due to the inhibition of serum-stimulated protein synthesis.

In order to confirm the role of p70 S6K on the stimulation of protein synthesis by growth factors, we analyzed the effect of rapamycin on the synthesis of two proteins expressed in the G_1 phase, cyclin D1 and p21. Treatment of quiescent cells with 20% FCS caused an induction of cyclin D1 for up to 16 h, with a maximum at 12 h (Fig. 7). This increase was completely abrogated by treatment with 10 nM rapamycin. The same result was obtained with the induction of p21, a growth factor-induced inhibitor of the cyclin-dependent kinases (32) (Fig. 7). We also analyzed the effect of rapamycin on the growth factor-mediated degradation of the cyclin-dependent kinase inhibitor p27. As is observed in Fig. 7, the decrease in p27 levels was not affected by treatment with rapamycin.

Cyclin D1 and p21 are induced after long term stimulation, with the maximal expression attained around 12 h. In contrast, MAPK phosphatase 1 is induced by growth factors much earlier than cyclin D1 or p21, with a maximum at 1 h that is maintained until 4 h and thereafter returned to basal levels after 12 h (Fig. 8*A*). Interestingly, rapamycin had no effect on the induction of MAPK phosphatase 1, despite the fact that the drug immediately affected protein synthesis (Fig. 8*B*). Thus, the lack of effect of rapamycin on MAPK phosphatase 1 reflects the selective action of p70 S6K on the translation of different cell cycle-regulated proteins.

Overexpression of a p70 S6K Mutant That Is Resistant to Rapamycin Decreases Rapamycin Sensitivity of 1G11 Cells—The target of rapamycin is FRAP/mTOR, a kinase that regulates p70 S6K. Moreover, FRAP/mTOR also has a direct effect on the initiation of protein synthesis by phosphorylating and inactivating eIF4E-BP1/PHAS-1, a repressor of mRNA translation (33–35). To evaluate whether the effects of rapamycin are driven by p70 S6K inhibition, we isolated 1G11 cells stably expressing either wild type or a rapamycin-resistant mutant form of p70 S6K (p70S6K-$E_{389}D_3E$) (24). Different clones overexpressing the wild type form or the p70S6K-$E_{389}D_3E$ form (Fig. 9*A*) were analyzed for their capacity to reinitiate DNA synthesis in the presence of different concentrations of rapamycin. Rapamycin inhibited thymidine incorporation in clones overexpressing the wild type form with exactly the same dose response as in parental cells (Fig. 9*B*). In contrast, cells overexpressing the rapamycin-resistant form of p70 S6K presented a clear resistance to the inhibition by the drug. The dose-response curves were shifted 1 log rightward for the clones expressing the rapamycin-resistant p70 S6K variant. This re-

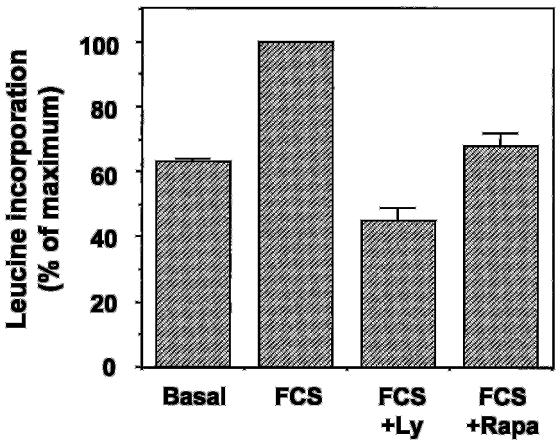

FIG. 5. **Rapamycin inhibits serum-stimulated protein synthesis.** Quiescent 1G11 endothelial cells were stimulated or not (*Basal*) for 12 h with 20% FCS in the presence of [^{3}H]leucine and either no inhibitor, 15 μM Ly294002, or 10 nM rapamycin. Protein synthesis was measured as described under "Experimental Procedures." Results are expressed as a percentage of the maximal [^{3}H]leucine incorporation in the presence of FCS alone and are an average ± S.E. of four different experiments.

sult indicated that the effect of rapamycin is p70 S6K-dependent and is not caused by a direct effect of FRAP/mTOR kinase on protein synthesis or other possible side effects of rapamycin.

DISCUSSION

In this work we have shown that inhibition of p42/p44 MAPK and PI3K-p70 S6K cascades blocks fetal calf serum-induced DNA synthesis reinitiation in vascular endothelial cells. The arrest of proliferation by inhibition of the p42/p44 MAPK pathway is not surprising, knowing the central role played by this cascade in the stimulation of cell proliferation (4). In endothelial cells, it has been described that PD98059 completely inhibits thymidine incorporation stimulated by vascular endothelial growth factor (36–38) and affects the mitogenic effect of bFGF (39). Moreover, repression of p42/p44 MAPK activity in confluent endothelial cells is required for the maintenance of the quiescent state (21). In contrast, much less is known about the role of the PI3K-Akt-p70 S6K module in the control of endothelial cell growth. Our results have shown that two different inhibitors of PI3K, wortmannin and Ly294002, completely block serum-stimulated DNA synthesis in vascular endothelial cells. These inhibitors prevent p70 S6K activation, confirming that PI3K activity is required for activation of p70 S6K but not p42/p44 MAPK. Inhibition of PI3K results in a block of endothelial cell proliferation that correlates with the inhibition of p70 S6K (compare inhibition of thymidine incorporation in wortmannin-treated cells with that of Akt and p70 S6K). However, more interestingly, specific inhibition of p70 S6K activation by rapamycin was found to be sufficient to inhibit DNA synthesis reinitiation. Moreover, only p70 S6K activation and not Akt phosphorylation was found to be sensitive to rapamycin treatment. This result points out that, although Akt activation could have a key role in cell survival on its own, Akt stimulation is not sufficient to trigger DNA replication. This finding, which highlights the key role of p70 S6K in vascular endothelial cell proliferation, was already clearly established for lymphocytes, although the conclusions were more loose in other systems such as fibroblasts. Indeed, when p70 S6K is fully inhibited by rapamycin in CCL39 fibroblasts (Fig. 3 and data not shown), DNA synthesis reinitiation is only minimally affected without effects on protein synthesis. The cause for this difference between fibroblasts and vascular endothelial cells is still unclear.

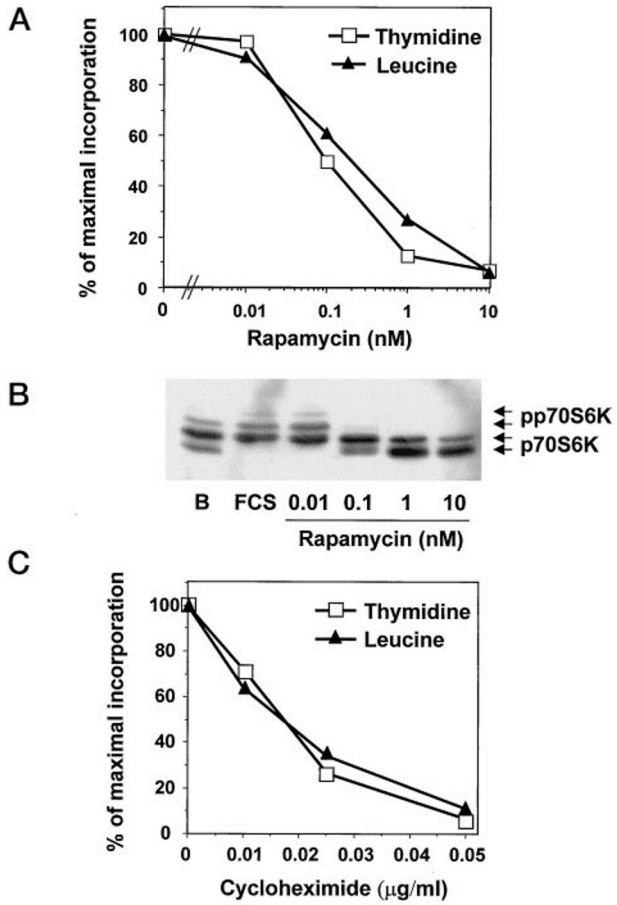

FIG. 6. **Inhibition of DNA synthesis by rapamycin correlates with the inhibition of protein synthesis.** *A*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS in presence of the indicated concentrations of rapamycin. Thymidine or leucine incorporation was measured as described. Results are an average of two different experiments and are expressed as a percentage of the maximal incorporation obtained in presence of FCS alone. *B*, quiescent 1G11 cells were stimulated with 20% FCS for 1 h in the presence of the indicated concentrations of rapamycin. Cells were lysed, and p70 S6K was immunodetected with a specific antibody. The active hyperphosphorylated forms are indicated (*pp70 S6K*). *C*, quiescent 1G11 endothelial cells were stimulated with 20% FCS in presence of the concentrations of cycloheximide indicated. Thymidine or leucine incorporation were measured as described before. Results are expressed as a percentage of the maximal [^{3}H]thymidine or leucine incorporation in the presence of FCS alone and are an average of two different experiments.

Activation of the protein synthesis by growth factors is an essential step for cell cycle progression, and its inhibition causes fibroblast growth arrest in G_0/G_1 (40, 41). PI3K-p70 S6K is one of the most important signaling pathways implicated in the stimulation of protein synthesis. Thus, p70 S6K is responsible for the phosphorylation of the ribosomal 40 S subunit S6 protein. When phosphorylated, polysomes will specifically translate a subset of mRNAs, all characterized by an oligopyrimidine tract at their 5′-untranslated region. These mRNAs are coding for members of the protein synthetic machinery (18, 41–43). Since the IC_{50} for rapamycin was identical for the inhibition of serum-induced DNA synthesis and protein synthesis, the inhibition of protein synthesis by rapamycin is likely to be responsible for the block of DNA synthesis in vascular endothelial cells. It is interesting to note that rapamycin only affects the increase in leucine incorporation induced by growth factors without affecting the basal rate of protein synthesis. Thus, it is possible that rapamycin would only affect the expression of growth factor-controlled gene products. Accordingly, we have found that rapamycin completely prevents the

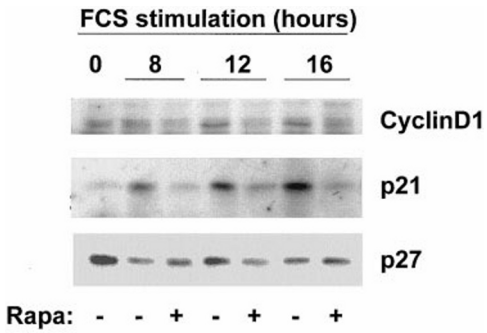

FIG. 7. **Rapamycin blocks cyclin D1 and p21 induction without effect on p27 degradation.** Quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS in the presence or absence of 10 nM rapamycin. After the times indicated, cells were lysed, and Western blots were performed with anti-cyclin D1, anti-p21, and anti-p27 antibodies. A representative Western blot is shown.

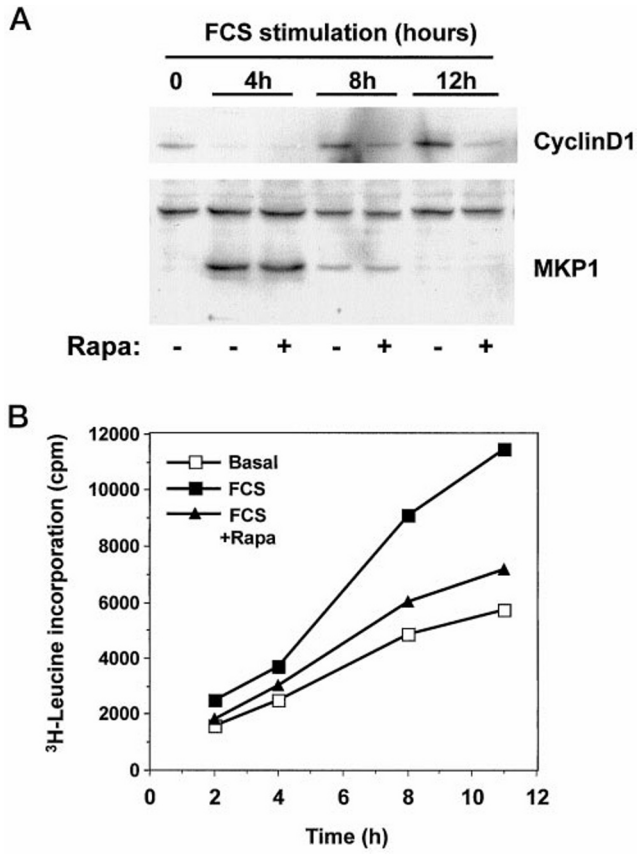

FIG. 8. **Rapamycin does not block synthesis of an early gene protein, MAPK phosphatase 1 (*MKP1*).** *A*, quiescent 1G11 vascular endothelial cells were stimulated with 20% FCS for the indicated periods of time in the presence or the absence of 10 nM rapamycin. Cells were lysed, and Western blot analysis was performed with anti-MAPK phosphatase 1 and anti-cyclin D1 antibodies. A representative Western blot is shown. *B*, quiescent 1G11 cells were stimulated or not (*Basal*) with 20% FCS in the presence or the absence of 10 nM rapamycin and with [^{3}H]leucine. At the times indicated, leucine incorporation was measured as described. A representative experiment is shown.

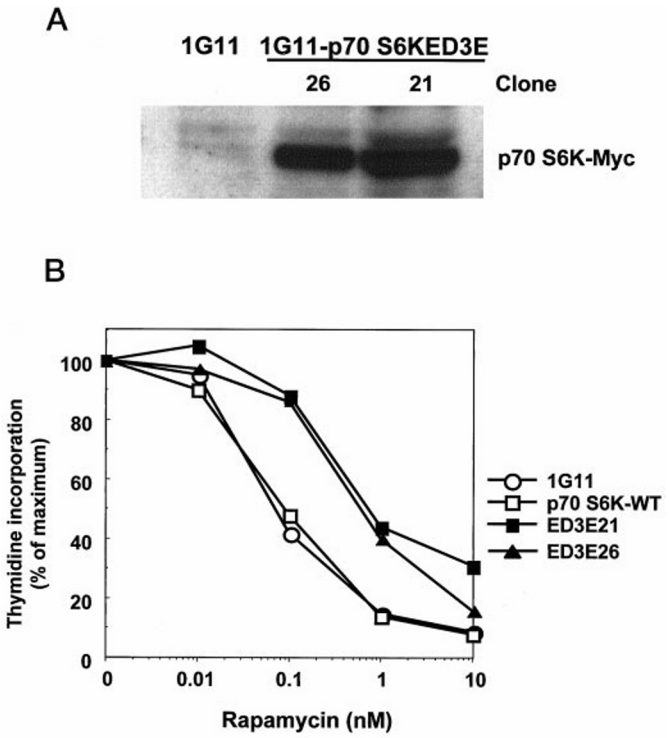

FIG. 9. **Overexpression of a p70 S6K rapamycin-resistant form (p70 S6KE$_{389}$D$_3$E) attenuates inhibition of DNA synthesis by rapamycin.** *A*, 1G11 parental cells or overexpressing 1G11-p70 S6KE$_{389}$D$_3$E cells (two different cell clones, 21 and 26) were lysed, and overexpression of p70 S6KE$_{389}$D$_3$E-Myc was immunodetected with a monoclonal antibody against Myc. A representative Western blot is shown. *B*, quiescent parental 1G11 cells or different clones overexpressing wild type p70 S6K (p70 S6KWT) or p70 S6KE$_{389}$D$_3$E (clones ED3E 21 and 26) were stimulated with 20% FCS in presence of the indicated concentrations of rapamycin and DNA synthesis measured. Results are expressed as a percentage of maximal incorporation in presence of FCS alone for each cell clone. A representative experiment is shown.

synthesis of one of the essential members of the Cdk-cyclin complexes of the G_1 phase, cyclin D1, precluding the phosphorylation of Rb product and therefore the entry into S phase. Moreover, it is interesting to note that treatment with rapamycin discriminates between growth factor-regulated gene products, inhibiting cyclin D1 and p21 synthesis but not the expression of MAPK phosphatase 1, an early gene product. These results can be explained by the presence of an oligopyrimidine tract in the 5′-untranslated region of the cyclin D1 mRNA. However, other mRNA determinants could be involved, since about 30% of newly synthesized proteins are inhibited by rapamycin (19, 44). Rapamycin's target is the immunophilin FK506-binding protein, which binds to the kinase FRAP/mTOR. FRAP/mTOR is a major upstream component of the p70/p85 S6K activation and plays a direct role in the stimulation of protein synthesis. Thus, by phosphorylation FRAP/mTOR inactivates the translation inhibitor eIF4E BP1/PHAS-1 (33–35). Although we cannot discard the possibility of a direct effect of FRAP/mTOR on protein synthesis and proliferation of endothelial cells, we demonstrate in this study that overexpression of a rapamycin-resistant form of p70 S6K partially overcomes the effect of rapamycin. This result reinforces the notion that activation of p70 S6K by itself is an important step for endothelial cell cycle progression. Finally, we cannot exclude other effects of the stimulation of p70 S6K important for the mitogenic cascade in endothelial cells, but it seems that this is not due to the persistence of high levels of the Cdk-cyclin inhibitor p27 after growth factor stimulation. The role of p27 in the anti-proliferative effect of rapamycin is controversial (32, 45–47). In endothelial 1G11 cells, we have not found any effect of rapamycin on p27 regulation. It is plausible that, due to the decrease in cyclin D1 levels, the residual amounts of p27 are sufficient to explain the cell cycle arrest in presence of rapamycin.

In conclusion, at least two pharmacologically separate growth-signaling cascades are operating in vascular endothelial cells. These cascades lead to activation of critical protein

kinases that cooperate at the level of gene induction (p42/p44 MAPK) and protein synthesis (p70 S6K). The extreme sensitivity of vascular endothelial cells to immunosuppressive drugs such as rapamycin as shown here could have great potential in therapeutic intervention.

Acknowledgments—We thank G. Thomas for the generous gift of pBabe-p70S6K wild type and $E_{389}D_3E$, A. Vecchi for 1G11 cells, L. Claesson-Welch for LIBE cells, V. Vouret-Craviari and D. Grall for the preparation of HUVEC cells, D. E. Richard for editorial support, Y. Fantei for technical assistance, and all laboratory members for support.

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