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Advances in Brief

Regulation of Cell Growth and Cyclin D1 Expression by the Constitutively Active FRAP-p70^{s6K} Pathway in Human Pancreatic Cancer Cells[1]

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Abstract

The FRAP-p70^{s6K} signaling pathway was found to be constitutively phosphorylated/active in MiaPaCa-2 and Panc-1 human pancreatic cancer cells and a pancreatic cancer tissue sample as judged by the retarded electrophoretic mobility of the two major FRAP downstream targets, p70^{s6K} and 4E-BP1. Treatment of cells with rapamycin, a selective FRAP inhibitor, inhibited basal p70^{s6K} kinase activity and induced dephosphorylation of p70^{s6K} and 4E-BP1. Moreover, rapamycin inhibited DNA synthesis as well as anchorage-dependent and -independent proliferation in MiaPaCa-2 and Panc-1 cells. Finally, rapamycin strikingly inhibited cyclin D1 expression in pancreatic cancer cells. Thus, inhibitors of the constitutively active FRAP-p70^{s6K} pathway may provide a novel therapeutic approach for pancreatic cancer.

Introduction

Pancreatic cancer constitutes the fourth leading cause of cancer death in Western countries for both sexes and has a dismal prognosis (1). The growth of pancreatic cancer is driven by multiple factors such as activating mutations in the small GTPase Ki-*ras* and various growth factors acting in an autocrine or paracrine manner (2). However, the downstream targets of these proteins and the arising signal transduction pathways involved in autocrine/paracrine human pancreatic cancer cell growth are poorly understood. Defining these pathways could give rise to novel therapeutic approaches that are urgently needed.

The serine/threonine kinase p70^{s6K} is a highly conserved element in a wide array of cellular processes including the mitogenic response to growth factors (3). This enzyme is activated *in vivo* by phosphorylation mediated in part by a phosphatidyl kinase-related kinase, FRAP[3] or mTOR (mammalian target of rapamycin; Ref. 4). The immunosuppressant rapamycin has emerged as a useful tool to elucidate the cellular function of FRAP and its downstream target, p70^{s6K} (3, 5, 6): rapamycin inhibits FRAP by forming a stable complex with the immunophilin FK506-binding protein, which binds to FRAP. As a result of this interaction, rapamycin induces dephosphorylation of several sites (Thr^{229}, Thr^{389}, and Ser^{404}) on p70^{s6K}, leading to its rapid inactivation (7). Interestingly, rapamycin blocks the proliferation of a variety of cells that have not entered the cell cycle (8, 9). Furthermore, we have recently shown that rapamycin inhibits constitutive p70^{s6K} phosphorylation and cell growth in classical small cell lung cancer cell lines (10). Consequently, there has been considerable interest in the downstream targets of rapamycin and p70^{s6K} that include 5′-terminal oligopyrimidine tract mRNA translation and protein synthesis (11). The translation inhibitor 4E-BP1 is also phosphorylated by the FRAP-p70^{s6K} pathway. This leads to the dissociation of 4E-BP1 from initiation factor eIF-4E, permitting increased protein translation and mitogenesis (12). In contrast, dephosphorylated 4E-BP1 interacts with eIF-4E and thereby inhibits cap structure-dependent protein synthesis and cell growth (13). Interestingly, rapamycin induces dephosphorylation of 4E-BP1, leading to inactivation of eIF-4E (6, 14). In contrast to previous studies, more recent reports suggest that 4E-BP1 is not a direct downstream target of p70^{s6K} but is regulated by FRAP in a parallel manner (14). This view is supported by results obtained in p70^{s6K} −/− cells, demonstrating that rapamycin can still prevent phosphorylation of 4E-BP1 and inhibit growth (15). Thus, rapamycin most likely inhibits growth via both p70^{s6K}-dependent and -independent pathways. The role of these rapamycin-sensitive pathways in the growth of pancreatic cancer cells is unknown.

In the present study, we demonstrate that the FRAP-p70^{s6K} pathway is constitutively phosphorylated/active in MiaPaCa-2 and Panc-1 human pancreatic cancer cell lines and a pancreatic cancer tissue sample. Rapamycin induced p70^{s6K} dephosphorylation and inactivation of constitutively active p70^{s6K} in serum-starved MiaPaCa-2 and Panc-1 cells. Rapamycin also inhibited constitutive phosphorylation of the translation inhibitor 4E-BP1 in these cells. Furthermore, proliferation and colony formation of MiaPaCa-2 and Panc-1 human pancreatic cancer cells were markedly reduced in the presence of rapamycin. Finally, rapamycin profoundly inhibited expression of cyclin D1. Thus, our results suggest that the rapamycin-sensitive FRAP-p70^{s6K} pathway could serve as a novel target for therapeutic intervention in pancreatic cancer.

Materials and Methods

Cell Culture. Human pancreatic cancer cell lines MiaPaCa-2 and Panc-1 were purchased from the American Type Culture Collection (Manassas, VA). Stocks were maintained in DMEM supplemented with 10% (v/v) FBS in a humidified atmosphere of 5% CO_2: 95% air at 37°C. The cells were passaged every 3 days.

Tissue Samples. Pancreatic carcinoma tissue samples were obtained from a patient undergoing a surgical operation for pancreatic cancer at the Department of General Surgery, University of Ulm. Informed consent was obtained from the patient before surgery. The tissue was collected after surgical removal, snap-frozen immediately in liquid nitrogen, and stored at −80°C.

Immunoprecipitations and Western Blotting. MiaPaCa-2 and Panc-1 cells were washed twice in serum-free DMEM and incubated in fresh DMEM for an additional 24 h. Cells were then treated with rapamycin as indicated in the figure legends and lysed in 50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 40 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.6; lysis buffer). For immunoprecipitations, lysates were incubated with a polyclonal anti-4E-BP1 antibody for 2 h at 4°C on a rotating wheel with protein A-Sepharose beads added for the second h. Beads were washed twice in lysis buffer and resuspended in 2× SDS-PAGE sample buffer. Proteins were

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[3] The abbreviations used are: FRAP, FK506-binding protein rapamycin-associated protein; FBS, fetal bovine serum; TGF, transforming growth factor; ERK, extracellular

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clonal anti-eIF-4E antibody or a polyclonal anti-4E-BP1 antibody with immunoreactive bands visualized by enhanced chemiluminescence detection. For p70^{s6K}− and 4E-BP1 mobility shift assays and detection of cyclin D1, cyclin E, and p27^{kip1}, cells were treated as indicated in the figure legends and lysed in SDS-PAGE sample buffer, and samples were further analyzed by SDS-PAGE and Western blotting with specific antisera to these proteins essentially as described above. Pancreatic tissues were lysed and dissociated by sonication. The lysates were subsequently boiled in SDS-PAGE sample buffer for 5 min and further analyzed by SDS-PAGE and Western blotting as described above.

p70^{s6K} and ERK2 Immune Complex Kinase Assays. Serum-starved MiaPaCa-2 and Panc-1 cells were incubated with rapamycin, PD 098059, and TGF-α as indicated in the figure legends. Controls received an equivalent amount of solvent. Cells were then lysed at 4°C in 1 ml of lysis buffer. Immunoprecipitations were performed at 4°C using an anti-p70^{s6K} antibody or an anti-ERK2 antibody for 2 h, with protein A-agarose added for the second hour. Immune complexes were washed three times in lysis buffer and once with p70^{s6K} kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 10 mM β-glycerophosphate] or ERK kinase buffer [15 mM $MgCl_2$, 15 mM Tris-HCl (pH 7.4)], respectively. Kinase reactions were performed by resuspending the protein A-Sepharose pellets in 25 μl of kinase assay mixture containing the appropriate kinase buffer with 0.2 mM S6 peptide (RRRLSSLRA) or myelin basic protein, 20 μM ATP, 5 μCi/ml [γ-^{32}P]ATP, 2 μM cyclic AMP-dependent protein kinase inhibitor peptide, and 100 nM microcystin LR. Incubations were performed under linear assay conditions at 30°C for 20 min and terminated by spotting 25 μl of the supernatant onto Whatman p81 chromatography paper. Papers were washed four times for 5 min in 0.5% *o*-phosphoric acid, immersed in acetone, and dried before scintillation counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample.

DNA Synthesis Assays. MiaPaCa-2 and Panc-1 cells were serum-starved for 24 h and then incubated with various additions of rapamycin as described in the figure legends. Control cells received solvent or DMEM/10% FBS to determine maximum DNA synthesis. After 18 h of incubation at 37°C, [^{3}H]thymidine (0.25 μCi/ml; 1 μM) was added to the cultures, and cells were incubated for another 6 h at 37°C. Cells were then washed with PBS and incubated in 5% trichloroacetic acid at 4°C for 30 min, washed with ethanol, and solubilized in 1 ml of 2% Na_2CO_3, 0.1 M NaOH, and 1% SDS. The acid-insoluble radioactivity was determined by Cerenkov counting in 6 ml of Ultima Gold (Packard). For detection of BrdUrd incorporation into cellular DNA, MiaPaCa-2 and Panc-1 cells were serum-starved in fresh DMEM for 24 h. Cells were then incubated with 20 ng/ml rapamycin or solvent for 24 h at 37°C, with 10 μM BrdUrd added for the last 6 h. Cultures were subsequently washed with PBS, fixed in 70% ethanol for 20 min, and incubated with anti-BrdUrd monoclonal antibody, followed by labeling with an antimouse IgG-fluorescein antibody. Cells were examined using a Zeiss Axiophot immunofluorescence microscope. Data are expressed as the percentage of BrdUrd-labeled nuclei.

Growth Assay. Three days after passage, MiaPaCa-2 and Panc-1 cells were washed in serum-free DMEM, trypsinized, and resuspended in DMEM/1% FBS. Cells were plated at a density of 1×10^4 cells in 1 ml of DMEM/1% FBS in the presence or absence of 20 ng/ml rapamycin in duplicate. At the times indicated in the figure legends, the cell number was determined using a cell counting chamber.

Clonogenic Assay. MiaPaCa-2 and Panc-1 cells were washed, trypsinized, and resuspended in DMEM. The cell number was determined using a cell counting chamber. Cells (3×10^4) were mixed with DMEM/1% FBS containing 0.3% agarose in the presence or absence of rapamycin at the concentrations indicated and layered over a solid base of 0.5% agarose in DMEM/1% FBS in the presence or absence of rapamycin at the same concentrations in 33-mm dishes. The cultures were incubated in humidified 5% CO_2: 95% air at 37°C for 14 days and then stained with the vital stain nitroblue tetrazolium. Colonies of $>$120 μm in diameter (20 cells) were counted using a microscope.

Materials. Rapamycin was obtained from Calbiochem-Novabiochem. Antibodies against p70^{s6K}, 4E-BP1, p27^{KIP1}, cyclin D1, and cyclin E were obtained from Santa Cruz Biotechnology. The monoclonal anti-eIF-4E antibody was from Transduction Laboratories. The NH_2-terminally directed anti-

from Upstate Biotechnology, Inc. The polyclonal anti-ERK2 antibody was a kind gift of Dr. Jo van Lint (Katholieke Universiteit, Leuven, Belgium). PD 098059 was obtained from New England Biolabs. Protein A-Sepharose and the BrdUrd labeling and detection kit were from Boehringer Mannheim. Enhanced chemiluminescence reagents, [^{3}H]thymidine, and [γ-^{32}P]ATP, were from Amersham. All other reagents were of the purest grade available.

Results

Rapamycin Inhibits Constitutive Phosphorylation and Activation of p70^{s6k} in Panc-1 and MiaPaCa-2 Cells. Activation of p70^{s6K} by mitogens can be determined by the appearance of slower-migrating forms in SDS-PAGE due to the phosphorylation of p70^{s6K} on Thr^{229}, Thr^{389}, and Ser^{404}, which are not basally phosphorylated in quiescent cells (3). The phosphorylation of these sites can be prevented or reversed by treatment with rapamycin, which specifically inhibits the p70^{s6K} activator FRAP (3, 4). To examine the status of p70^{s6K} phosphorylation in human pancreatic cancer cells, cultures of Panc-1 and MiaPaCa-2 cells were incubated in the absence or presence of 20 ng/ml rapamycin. Cells were lysed, and the lysates were analyzed by immunoblotting. p70^{s6K} exhibited a retarded electrophoretic mobility characteristic of the phosphorylated form of this enzyme in Panc-1 and MiaPaCa-2 cells. In control experiments, there was no retarded electrophoretic mobility of p70^{s6K} in serum-starved mouse or human fibroblasts (data not shown), in accordance with previous results (3). Treatment of cells with rapamycin induced a striking dephosphorylation of p70^{s6K}, as demonstrated by the increase in the electrophoretic mobility (Fig. 1*A, top panels*). The effect of rapamycin on constitutive p70^{s6K} phosphorylation was concentration dependent; maximum effects were achieved at 6 and 20 ng/ml rapamycin in Panc-1 and MiaPaCa-2 cells, respectively (Fig. 1*A*, *bottom panels*). Dephosphorylation of p70^{s6K} by rapamycin in Panc-1 and MiaPaCa-2 cells was first visible after 5 min and reached a maximum 10 min after the addition of rapamycin to the cells (data not shown). Next we performed immune complex kinase assays to directly assess p70^{s6K} activity. Treatment of cells with rapamycin substantially reduced the basal kinase activity of p70^{s6K} by 92% and 99% in serum-starved Panc-1 and MiaPaCa-2 cells, respectively (Fig. 1*B, top panels*). The effect of rapamycin on p70^{s6K} activity was specific because rapamycin did not affect basal and TGF-α-stimulated activation of the mitogen-activated protein kinase ERK2 in both cell lines. In contrast, the mitogen-activated protein/ERK kinase 1 inhibitor PD 098059 markedly inhibited basal and TGF-α-stimulated ERK2 activation (Fig. 1*B, bottom panels*).

Effect of Rapamycin on 4E-BP1 Phosphorylation in Panc-1 and MiaPaCa-2 Cells. It has been suggested that phosphorylation and thus inactivation of the translation inhibitor 4E-BP1 are also mediated by a rapamycin-sensitive pathway (6, 14). Therefore, we examined the phosphorylation status of 4E-BP1 in serum-starved human pancreatic cancer cells in the absence or presence of rapamycin. To determine 4E-BP1 phosphorylation, a Western blot analysis was performed using a polyclonal antibody against 4E-BP1. Three forms of 4E-BP1 were present in serum-starved Panc-1 and MiaPaCa-2 cells, suggesting constitutive phosphorylation of 4E-BP1 in these cells. Treatment with rapamycin leads to a decreased intensity of the upper two bands and to an increased intensity of the fastest migrating lower band (Fig.1*C, left, top* and *bottom panels*). According to previous studies, the lowest band represents the unphosphorylated form of 4E-BP1 (16, 17). We next examined whether the constitutive phosphorylation of 4E-BP1 was sufficient to decrease the interaction of eIF-4E with 4E-BP1. Serum-starved Panc-1 cells were incubated with solvent or 20 ng/ml rapamycin. Cell lysates were immunoprecipitated with a polyclonal anti-4E-BP1 antibody and analyzed further by Western

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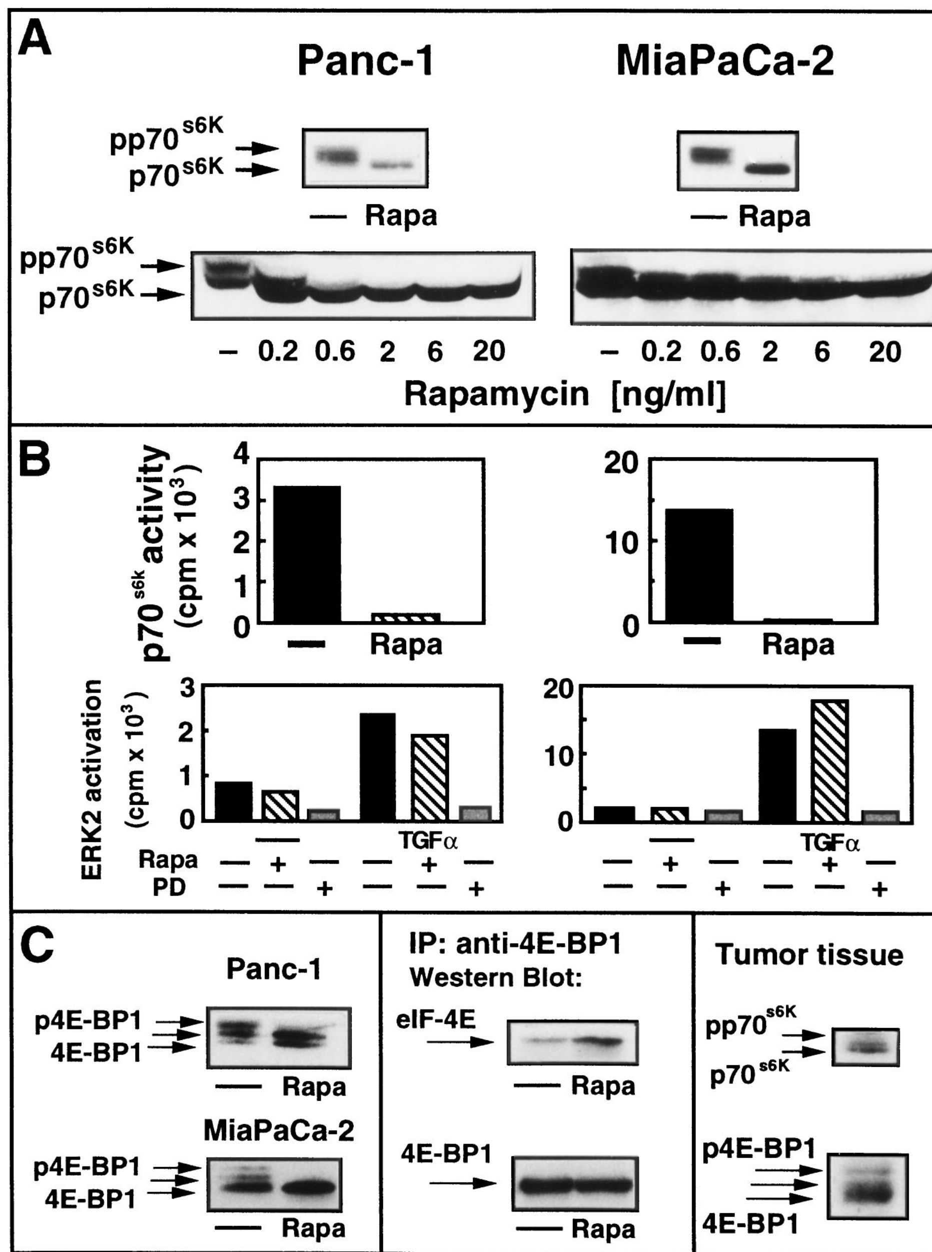

Fig. 1. Constitutive phosphorylation/activation of $p70^{s6K}$ and 4E-BP1 in Panc-1 and MiaPaCa-2 cells. *A*, *top panels*, serum-starved cultures of Panc-1 (*left*) and MiaPaCa-2 cells (*right*) were treated with 20 ng/ml rapamycin (*Rapa*) or received an equivalent amount of solvent (—). $p70^{s6K}$ 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 hypophosphorylated $p70^{s6K}$ (*$p70^{s6K}$*) and the slower-migrating phosphorylated $p70^{s6K}$ (*$pp70^{s6K}$*) are indicated by arrows. *Bottom panels*, serum-starved cultures of Panc-1 (*left*) and MiaPaCa-2 cells (*right*) were treated with various concentrations of rapamycin as indicated for 20 min or received an equivalent amount of solvent (—). $p70^{s6K}$ 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 hypophosphorylated $p70^{s6K}$ (*$p70^{s6K}$*) and the slower-migrating phosphorylated $p70^{s6K}$ (*$pp70^{s6K}$*) are indicated by arrows. *B*, *top panels*, serum-starved cultures of Panc-1 (*left*) and MiaPaCa-2 cells (*right*) were treated with 20 ng/ml rapamycin for 20 min (*Rapa*) or received an equivalent amount of solvent (—). Cells were subsequently lysed, and immune complex kinase assays for $p70^{s6K}$ activity were performed as described in "Materials and Methods." *Bottom panels*, serum-starved cultures of Panc-1 (*left*) and MiaPaCa-2 cells (*right*) were treated with 20 ng/ml rapamycin (*Rapa* +) for 20 min or 20 μM PD 098059 (*PD* +) for 40 min or received an equivalent amount of solvent (—). Cells were then incubated with 50 ng/ml TGF-α for 5 min (*TGF-α* +) and lysed, and immune complex kinase assays for ERK2 activity were performed as described in "Materials and Methods." *C*, *left panels*, serum-starved cultures of Panc-1 (*top*) and MiaPaCa-2 cells (*bottom*) were treated with 20 ng/ml rapamycin (*Rapa*) as indicated for 20 min. Control cells received an equivalent amount of solvent (—). 4E-BP1 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 hypophosphorylated 4E-BP1 (*4E-BP1*) and the slower-migrating phosphorylated forms of 4E-BP1 (*p4E-BP1*) are indicated by *arrows*. *Middle panels*, cells were treated as described above and lysed, and lysates were immunoprecipitated using a specific anti-4E-BP1 antibody as described in "Materials and Methods." Western blotting was performed using an anti-eIF-4E antibody (*top*) or an anti-4E-BP1 antibody (*bottom*) as described in "Materials and Methods." *Right panels*, human pancreatic cancer tissue samples were lysed and subsequently analyzed by SDS-PAGE with an anti-$p70^{s6K}$ (*top*) or an anti-4E-BP1 antibody (*bottom*) as described in "Materials and Methods." The positions of hypophosphorylated and hyperphosphorylated $p70^{s6K}$ (*$p70^{s6K}$* and $pp70^{s6K}$) and 4E-BP1 (4E-BP1 and p4E-BP1) are indicated by arrows.

(*middle, top panel*), a small amount of eIF4E coimmunoprecipitated with 4E-BP1 in nontreated cells. Upon treatment with rapamycin, the amount of eIF-4E that could be detected in 4E-BP1 immunoprecipi-

anti-4E-BP1 antibody, similar amounts of 4E-BP1 protein could be detected in rapamycin-treated and untreated cells (Fig. 1*C, middle, bottom panel*). In addition, using 7-methyl-GTP-Sepharose that spe-

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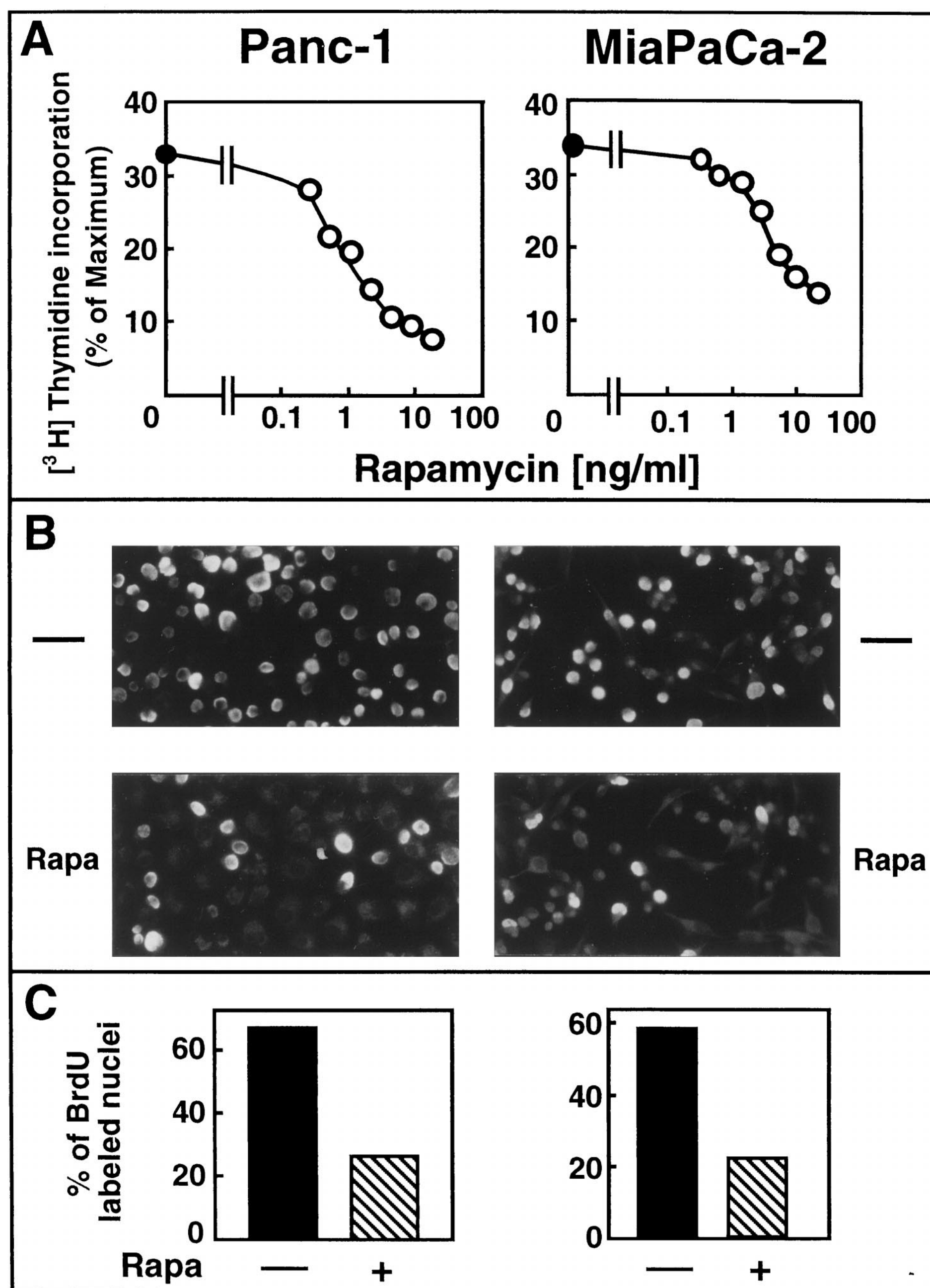

Fig. 2. Rapamycin inhibits DNA synthesis in Panc-1 and MiaPaCa-2 cells. *A*, serum-starved cultures of Panc-1 (*left*) or MiaPaCa-2 cells (*right*) were washed and incubated at 37°C for 24 h in serum-free DMEM containing either 20 ng/ml rapamycin or an equivalent amount of solvent. [^{3}H]Thymidine was added during the last 6 h of the incubation, and [^{3}H]thymidine incorporation was determined as described in "Materials and Methods." Each point represents the mean of three determinations and is representative of at least two independent experiments. *B*, serum-starved cultures of Panc-1 (*left panels*) or MiaPaCa-2 cells (*right panels*) were washed and incubated at 37°C for 24 h in DMEM containing either 20 ng/ml rapamycin (*Rapa; bottom panels*) or an equivalent amount of solvent (—; *top panels*). BrdUrd was added during the last 6 h of the incubation. After 24 h, BrdUrd incorporation into cell nuclei was determined as described in "Materials and Methods." Labeled nuclei were visualized by fluorescence microscopy. Typical fields are presented. *C*, BrdUrd incorporation was performed as described above for rapamycin (*Rapa* +)-treated or vehicle-treated (—) Panc-1 (*left*) or MiaPaCa-2 cells (*right*). Results are presented as the percentage of labeled nuclei and are the means of three distinct fields from two separate experiments.

7-methyl-GTP-Sepharose immunoprecipitates of lysates of Panc-1 cells treated with rapamycin as compared to untreated cells (data not shown). Thus, the level of constitutive phosphorylation of 4E-BP1 in serum-starved pancreatic cancer cells is sufficient to prevent interaction with eIF-4E.

Constitutive p70^{s6K}- and 4E-BP1 phosphorylation was not restricted to pancreatic cancer cells exhibiting activating Ki-*ras* mutations such as MiaPaCa-2 and Panc-1 cells but could also be detected in the human pancreatic cancer cell lines SW 850 and SW 979 that exhibit wild-type Ki-*ras* (data not shown). Thus, constitutive phosphorylation of p70^{s6K} and 4E-BP1 is not due to the activating Ki-*ras* mutation in these cells. Interestingly, constitutive phosphorylation of p70^{s6K} and 4E-BP1 could also be observed in a human pancreatic carcinoma tissue sample (Fig. 1*C, right panels*).

Rapamycin Inhibits DNA Synthesis in Panc-1 and MiaPaCa-2 Cells. We first examined the effect of rapamycin on basal DNA

active FRAP-p70^{s6K} pathway for pancreatic cancer cell growth. Basal DNA synthesis in serum-free DMEM as assessed by [^{3}H]thymidine incorporation was 35% of maximum stimulation in response to 10% FBS in Panc-1 and MiaPaCa-2 cells (Fig. 2*A*). Upon treatment with rapamycin, basal [^{3}H]thymidine incorporation decreased substantially in both cell lines. The effect of rapamycin was concentration dependent, with half-maximal effects at 1 and 3 ng/ml in Panc-1 and MiaPaCa-2 cells, respectively, and maximal effects at 20 ng/ml in both cell lines. At this concentration, basal DNA synthesis was reduced by 77% and 66% in Panc-1 and MiaPaCa-2 cells, respectively (Fig. 2*A*). These results are in good agreement with the data obtained on rapamycin-induced dephosphorylation of p70^{s6K} (Fig. 1*A*). To further substantiate our observations, we applied a distinct technique in which DNA synthesis was determined using an immunofluorescence assay to detect BrdUrd incorporation into cell nuclei. As shown in Fig. 2*B*, BrdUrd incorporation into cell nuclei was markedly

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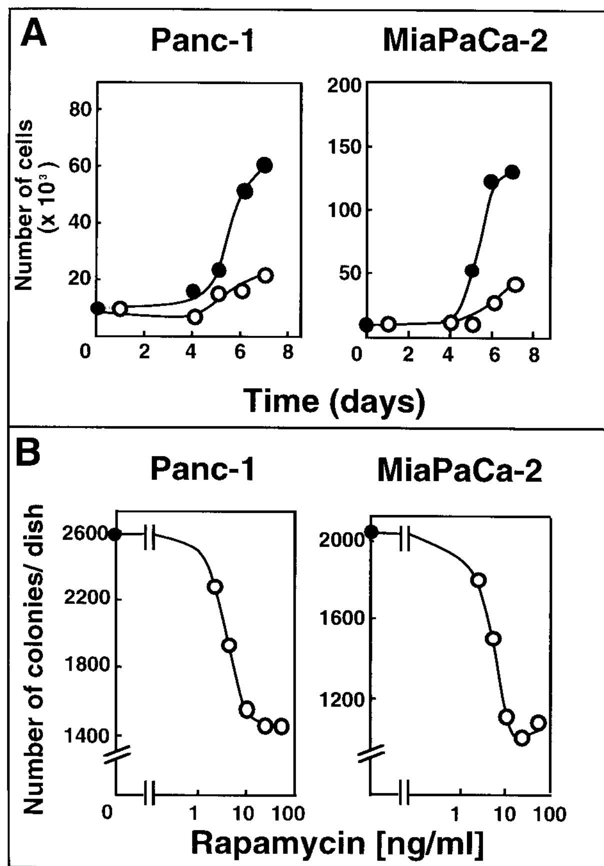

Fig. 3. Rapamycin inhibits anchorage-dependent and -independent proliferation of Panc-1 and MiaPaCa-2 cells. *A*, cultures of Panc-1 (*left*) and MiaPaCa-2 cells (*right*) were incubated at a density of 1×10^4 cells in 1 ml of DMEM containing 1% FBS and 20 ng/ml rapamycin (○) or vehicle (●), and cells were counted at day 0, 1, 4, 5, 6, and 7 as indicated. Each point represents the mean of two determinations and is representative of at least two independent experiments. *B*, single cell suspensions of Panc-1 (*left*) or MiaPaCa-2 cells (*right*) were plated at a density of 3×10^4 cells/dish in agarose medium containing DMEM/1% FBS and various concentrations of rapamycin as indicated. Colonies were counted after 2 weeks of incubation. In all cases, a representative of two independent experiments, each performed in triplicates, is shown.

rapamycin, a maximum 62% reduction in BrdUrd incorporation could be detected in both cell lines (Fig. 2*C*).

Effect of Rapamycin on Anchorage-dependent and -independent Proliferation of Panc-1 and MiaPaCa-2 Cells. Next we examined the effect of rapamycin on actual cellular proliferation. MiaPaCa-2 and Panc-1 cells were incubated in the absence or presence of 20 ng/ml rapamycin, and cell numbers were determined over a period of up to 7 days. In the presence of rapamycin, cell numbers were reduced by a maximum of 66% and 68% at day 7 in Panc-1 and MiaPaCa-2 cells, respectively (Fig. 3*A*). Thus, the constitutively active, rapamycin-sensitive FRAP-p70^{s6K} signaling pathway is likely to participate in sustaining anchorage-dependent growth of human Panc-1 and MiaPaCa-2 cells. In contrast to recent results obtained in rhabdomyosarcoma cells (18), rapamycin did not induce apoptosis in pancreatic cancer cells, as judged by an *in situ* assay to detect DNA fragmentation (data not shown).

Tumors and transformed cells, including human pancreatic cancer cells, are able to grow in an anchorage-independent manner by forming colonies in agarose medium. There is even a positive correlation between the cloning efficiency of tumor cells in soft agar and the histological involvement and invasiveness of the tumor in specimens taken from different carcinomas (19). Consequently, we determined

solid medium in the presence or absence of rapamycin. As shown in Fig. 3*B*, rapamycin markedly inhibited the formation of colonies by MiaPaCa-2 and Panc-1 cells in a concentration-dependent manner. Half-maximal and maximal effects were achieved at 6 and 20 ng/ml rapamycin in both cell lines.

Rapamycin Inhibits Expression of Cyclin D1 but not Cyclin E and p27^{KIP1}. Progression from G_1 to the S phase of the cell cycle is regulated by the expression of cyclins D and E, which modulate the activities of the cyclin-dependent kinases. Rapamycin can block G_1 to S-phase cell cycle progression in a number of cell types by blocking growth factor-stimulated elimination of the cyclin-dependent kinase inhibitor p27^{KIP1} (20, 21). We have recently shown that rapamycin strikingly reduces bombesin-induced expression of cyclins D1, D3, and E in Swiss 3T3 fibroblasts (22). These effects of rapamycin may be a consequence of the inhibition of p70^{s6K} or may be due to a distinct pathway(s) regulated by the FRAP-p70^{s6K} pathway. To further explore the nature of the rapamycin-sensitive and -insensitive mechanisms regulating the basal growth of human pancreatic cancer cells, we determined the expression of cyclin D1, cyclin E, and p27^{KIP1} and correlated these with the activation of p70^{s6K}. As shown in Fig. 4, treatment of cells with rapamycin leads to a sustained dephosphorylation of p70^{s6K} for up to 36 h. Similar results were obtained for 4E-BP1 phosphorylation (data not shown). Rapamycin treatment of MiaPaCa-2 cells at a concentration that inhibited p70^{s6K}– and 4E-BP1 phosphorylation as well as basal cell growth

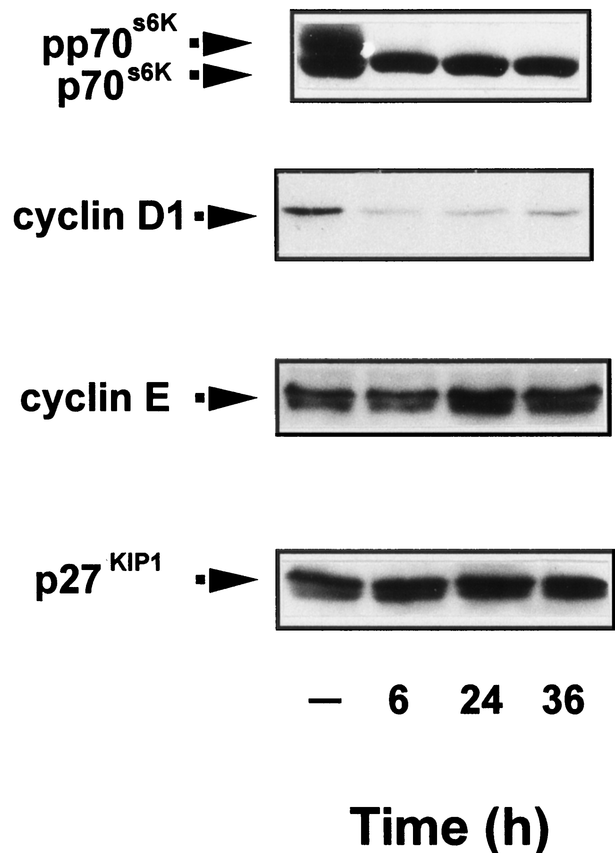

Fig. 4. Inhibition of cyclin D1 expression by rapamycin in MiaPaCa-2 cells. Subconfluent cultures of MiaPaCa-2 cells were treated with 20 ng/ml rapamycin for various times, as indicated. Control cells received an equivalent amount of solvent (—). Cells were lysed and further analyzed by Western blotting with either anti-p70^{s6K} antibody, anti-cyclin D1 antibody, anti-cyclin E antibody, or anti-p27^{KIP1} antibody as indicated by an *arrow*. The results shown in each case are representative of at least three independent experiments. The positions of hypophosphorylated p70^{s6K} (*p70^{s6K}*) and the slower-

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