Rapamycin Dissociates p70S6K Activation from DNA Synthesis Stimulated by Bombesin and Insulin in Swiss 3T3 Cells*

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Dominic J. Withers†, Thomas Seufferlein, David Mann, Bibian Garcia, Nicholas Jones, and Enrique Rozengurt‡

From the Imperial Cancer Research Fund, P. O. Box 123, 44 Lincoln’s Inn Fields, London, WC2A 3PX, United Kingdom

Phosphorylation and subsequent activation of p70 S6 kinase (S6K) are events that are highly conserved in the cellular response to mitogens. The neuropetide bombesin, which is a potent mitogen for Swiss 3T3 cells, stimulated a time- and dose-dependent activation of p70S6K as determined by gel mobility shift and immune complex kinase assays. This effect was inhibited by the immunosuppressant rapamycin at 10 nM, which also completely abolished bombesin-stimulated DNA synthesis at identical concentrations. In striking contrast, the combination of bombesin and insulin in synergy stimulated maximum DNA synthesis in these cells despite persistent inhibition of p70S6K by rapamycin throughout G1. These results indicate that activation of p70S6K is not required for the transition of quiescent cells to the S phase of the cell cycle. The inhibitory effects of rapamycin on bombesin-stimulated cell cycle progression did not involve accumulation of the cyclin-dependent kinase inhibitor p27kip1 but a striking inhibition of the expression of cyclin D1. This effect was circumvented by bombesin with insulin, suggesting that a rapamycin-insensitive pathway stimulated by this combination leads to cyclin D1 expression. Thus, these findings, dissociating the mitogenic effects of bombesin in synergy with insulin from activation of p70S6K, support the hypothesis that this kinase is a component of one of the parallel pathways that can lead to DNA synthesis rather than an obligatory point of convergence in mitogenic signaling.

The understanding of the mechanisms that control cell proliferation requires the identification of the early molecular events that govern the transition of quiescent cells into the S phase of the cell cycle. In this context, phosphorylation and subsequent activation of the serine/threonine kinase p70 S6 kinase (S6K), a highly conserved element in the cellular response to mitogenic stimuli, are attracting intense interest (reviewed in Ref. 1). This kinase was originally identified as the enzyme that phosphorylates the S6 protein of the 40S ribosomal subunit in vivo, an event that may be significant in the regulation of the rate of protein synthesis and in the translation of specific mRNA species (reviewed in Ref. 2). Recent evidence suggests that activation of p70S6K may regulate a wider array of cellular processes involved in the mitogenic response (1, 3).

The immunosuppressant rapamycin, which is a highly potent inhibitor of p70S6K phosphorylation and activation by all known stimuli (4, 5), has emerged as a useful tool with which to define the cellular function of p70S6K (1, 2). Rapamycin inhibits p70S6K indirectly, forming a complex with FK-506-binding protein, which in turn interacts with RAFT/mTOR, a lipid kinase that is a putative upstream regulator of p70S6K (1). Studies with rapamycin have shown that it blocks resting lymphocytes and other cell types from entering the cell cycle, inhibiting the activation of p70S6K and regulating the expression of proteins involved in progression through the restriction point of the cell cycle (4–10). In contrast, rapamycin delayed but did not prevent entry into S phase in fibroblasts (4) and T lymphocytes (11) and failed to inhibit proliferation of a variety of cells that had entered the cell cycle (12–14). However, other studies using serum-stimulated fibroblasts microinjected with antibodies against p70S6K suggested that this enzyme plays an essential role throughout G1 (15). Therefore, from the evidence presented to date using different cell types, it is not clear whether activation of the rapamycin-sensitive p70S6K pathway represents an obligatory signal in the mitogenic response.

Quiescent Swiss 3T3 cells can be stimulated to reactivate DNA synthesis by multiple growth factors that utilize a number of different signaling mechanisms (16). In particular, the neuropetide bombesin induces stimulation of DNA synthesis in these cells both alone and in synergy with insulin via distinct and parallel signal transduction pathways (17, 18). Here we examined the contribution of p70S6K to entry into S phase stimulated by bombesin either alone or in synergistic combination with insulin. Our results demonstrate that rapamycin prevents p70S6K phosphorylation/activation and abolishes initiation of DNA synthesis in bombesin-stimulated Swiss 3T3 cells. In contrast, the combination of bombesin and insulin circumvents the rapamycin block, stimulating the transition into S phase in the absence of p70S6K activation in the same cells. Thus, depending on the combination of growth factors used, the activation of p70S6K is not obligatory for the mitogenic response.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experimental purposes, cells were plated out either in 35-mm Nunc Petri dishes at 105 cells/dish or in 100-mm dishes at 6 × 105 cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent.

DNA Synthesis Measurements—Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing [3H]thymidine (1 μCi/ml, 1 μM) and various additions as described in the figure legends. After 40 h of incubation at 37 °C, cultures were washed twice with PBS.
and washed three times in PBS. Cells (10^6) in a volume of 200 μl were lysed at 4°C for 20 min in 1 ml of a solution containing 50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 40 mM β-mercaptoethanol, and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing 10% fetal bovine serum, centrifuged at 1000 × g for 5 min, and resuspended and washed three times in PBS. Cells (10^6) in a volume of 200 μl were stained by adding 800 μl of a solution containing propidium iodide (50 μg/ml), sodium citrate (1 mg/ml), and Triton X-100 (0.1%). The stained chromosomal DNA was kept on ice for 15 min and analyzed on a FACStar 4 (Becton Dickinson).

Western Blotting and p70S6K Mobility Shift Assay—Confluent, quiescent Swiss 3T3 cells were washed twice with DMEM, treated with various factors in DMEM as indicated in figure legends and then lysed in SDS-polyacrylamide gel electrophoresis sample buffer. Expression of cyclins D1 and E, Rb, and p27kip1 was determined by Western blotting using specific antisera, with immunoreactive bands being visualized with either horseradish peroxidase-conjugated anti-rabbit IgG and subsequent ECL detection. Mobility shift assays were performed using a rabbit polyclonal antibody that recognized both of α and β isoforms of p70S6K (4), with immunoreactive bands being visualized with either ECL or iodinated protein A.

p70S6K and p90rsk Immune Complex Kinase Assays—Quiescent cells were treated with various factors as described in the figure legends and lysed at 4°C for 20 min in 1 ml of a solution containing 50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 40 mM β-glyceroophosphate, 50 mM NaF, 1 mM NaVO₄, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.6 (lysis buffer). Immunoprecipitations were performed using either a rabbit polyclonal anti-p70S6K antibody (directed against a synthetic peptide corresponding to amino acids 287–305 of human p70S6K) or a polyclonal antibody for 2 h with protein A-agarose added for the last hour in each case. The immune complexes were washed three times in lysis buffer and once with p70S6K buffer (20 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol and 10 mM β-glycerophosphate) or p90rsk buffer (15 mM Tris-HCL, 15 mM MgCl₂). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing the appropriate kinase buffer with 0.2 mM labeling buffer (BRL pRNA Labeling System), 20 μM 32P-ATP, 5 μCi/ml [γ-^32P]ATP, 2 μM cAMP-dependent protein kinase inhibitor peptide, and 100 nM microcystin LR. Incubations were performed under linear assay conditions at 30°C for 20 min and, following centrifugation for 10 s, terminated by spotting 25 μl of the supernatant onto Whatman P81 chromatography paper. Filters 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.

Materials—Bombesin, insulin, and cAMP-dependent protein kinase inhibitor peptide were obtained from Sigma. Rapamycin was obtained from Calbiochem-Novabiochem (U. K. Ltd), Nottingham, U. K. Protein A-agarose and the BrdUrd labeling and detection kit were obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). Anti-p70S6K affinity-purified rabbit polyclonal antibody used for Western blotting, the anti-p27kip1, and anti-cyclin E antibodies were obtained from Santa Cruz Biotechnology Ltd., U.S. The N-terminally directed anti-p70S6K rabbit polyclonal antibody used for the immune complex kinase assays was obtained from Upstate Biotechnology Inc., U. S. A. The anti-cyclin A antibody was obtained from Boehringer. The anti-cyclin D1 antibody was a kind gift from Dr. R. Peter, Imperial Cancer Research Fund. ECL reagent, [3H]thymidine, [32P]labeled protein A, and [γ-32P]ATP were obtained from Amersham Corp., U. K. All other reagents used were of the purest grade available.

RESULTS
Bombesin Stimulates a Time- and Dose-dependent Phosphorylation and Activation of p70S6K Which Is Specifically Inhibited by Rapamycin—To examine the effect of bombesin on p70S6K phosphorylation and activation, cell lysates from quiescent Swiss mouse 3T3 cells treated with 10 nM bombesin for various times were analyzed by Western blotting using a specific polyclonal antibody. The enzyme exists in two isoforms, a p70 (αII) cytosolic form and a p85 (αI) nuclear form, derived from the same gene by alternative splicing (19). Therefore, we utilized an antibody that recognizes both isoforms to investigate the activation of both enzymes (4). Activation was determined by the appearance of slower migrating forms in SDS-polyacrylamide gel electrophoresis as a result of phosphorylation on threonines 229 and 389 and serine 404 (20). As shown in Fig. 1A, bombesin stimulated a mobility shift of both isoforms of p70S6K that was detectable after 5 min of stimulation and persisted beyond 180 min. To verify that the phosphorylation-associated mobility shift was accompanied by activation of p70S6K, direct measurements of enzyme activity were performed utilizing immune complex kinase assays. As shown in Fig. 1B, bombesin stimulated a persistent activation of p70S6K that displayed the same kinetics as shown in the mobility shift assay. Additionally, bombesin stimulated a dose-dependent activation of both isoforms of p70S6K as shown in the mobility shift assay, with a maximum effect occurring at 10 nM bombesin (Fig. 1B, inset). In further experiments, bombesin-stimulated activation of p70S6K at both 15 and 60 min was blocked by the bisindolylmaleamide GF 109203X which inhibits the protein kinase C isoforms found in Swiss 3T3 cells (21) (results not shown). In contrast, depletion of intracellular Ca2+ stores with thapsigargin (22) did not attenuate the activation of p70S6K by bombesin (results not shown). Thus, these findings suggest that bombesin stimulates p70S6K activation via mechanisms that are protein kinase C-dependent but does not involve the mobilization of intracellular Ca2+ stores.

Treatment of quiescent Swiss 3T3 cells with the immunosuppressant rapamycin inhibited the p70S6K mobility shift induced by the subsequent addition of bombesin in a dose-dependent manner (Fig. 1C). A marked decrease in the shift was seen at a dose of 0.3 nM rapamycin, and a complete abolition of p70S6K phosphorylation was achieved at 1 nM rapamycin. This compound also completely abolished p70S6K activation by bombesin, as measured in the immune complex kinase assay (Fig. 1D).

To confirm the specificity of the inhibition of p70S6K by rapamycin, immune complex kinase assays for bombesin-stimulated p90rsk activation were performed in parallel cultures in the presence or absence of 10 nM rapamycin. As shown in Fig. 1E, rapamycin did not inhibit bombesin-activation of p90rsk, the kinase most closely related to p70S6K but which is phosphorylated and activated by p42- and p44MAPK (23) and therefore lies on a distinct signal transduction pathway. Thus, the results shown in Fig. 1 demonstrate that bombesin stimulates a persistent activation of both isoforms of p70S6K in Swiss 3T3 cells and that this effect is specifically inhibited by rapamycin.

Rapamycin Dissociates p70S6K Activation from DNA Synthesis Stimulated by Bombesin and Insulin—We next examined the effect of rapamycin on bombesin-stimulated DNA synthesis either alone or in the presence of insulin. Quiescent Swiss 3T3 cells treated with increasing concentrations of rapamycin were stimulated with either bombesin or bombesin with insulin. Cumulative [3H]thymidine incorporation was measured after 40 h of incubation. As shown in Fig. 2, top, rapamycin caused a marked dose-dependent inhibition of bombesin-induced
**FIG. 1.** Bombesin stimulation of p70<sup>S6K</sup> in Swiss 3T3 cells: selective inhibition by rapamycin. A, quiescent Swiss 3T3 cells were washed twice with DMEM and were incubated in DMEM with 10 nM bombesin for various times as indicated. Cells were then lysed in SDS-polyacrylamide gel electrophoresis sample buffer, and mobility shift assays were performed using a polyclonal antibody that recognized αI and αII isoforms of p70<sup>S6K</sup> in Western blotting. B, quiescent Swiss 3T3 cells were treated with 10 nM bombesin for various times as indicated and lysed, and p70<sup>S6K</sup> immune complex kinase assays performed as described under “Experimental Procedures.” Results are the means of duplicates, are expressed as the percentage of maximum of bombesin-stimulated activation, (8,000–10,000 cpm/1.5 × 10<sup>6</sup> cells at 15 min), and are representative of three independent experiments. Inset, quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for 15 min in DMEM with various concentrations of bombesin (Bom) as indicated, lysed in SDS sample buffer, and analyzed by Western blotting. C, quiescent Swiss 3T3 cells washed in DMEM were preincubated for 15 min in DMEM with rapamycin (Rap) at the concentrations indicated and then treated with 10 nM bombesin for 15 min, lysed in SDS sample buffer, and analyzed by Western blotting. D, quiescent Swiss 3T3 cells were treated for 15 min with 10 nM bombesin or vehicle (−) with (+) or without (−) a 15-min preincubation with 10 nM rapamycin and lysed, and p70<sup>S6K</sup> immune complex kinase assays were performed as described under “Experimental Procedures.” E, quiescent Swiss 3T3 cells were treated with 10 nM bombesin or vehicle (−) for 5 min with (+) or without (−) a 15-min preincubation with 10 nM rapamycin (Rap) and lysed, and p70<sup>S6K</sup> immune complex kinase assays were performed as described under “Experimental Procedures.” In D and E, results are the means of duplicates, are expressed as the percentage of bombesin-stimulated activation (10,000–15,000 cpm/1.5 × 10<sup>6</sup> cells at 15 min) and are representative of three independent experiments. The specific activity of [γ-<sup>32</sup>P]ATP used in the experiments shown in B, D, and E was 900–1,200 cpm/μmol.

[^3H]thymidine incorporation. This effect was maximal at 0.3 nM of rapamycin and thus displayed a similar dose dependence as bombesin-stimulated p70<sup>S6K</sup> activation. However, in striking contrast, the stimulation of [^3H]thymidine incorporation induced by the synergistic combination of bombesin and insulin was unaffected by rapamycin at doses up to 10 nM in parallel cultures.
Figure 3. Effect of rapamycin on DNA synthesis stimulated by bombesin or bombesin and insulin. A, confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing BrdUrd and either 10 nM bombesin (Bom) or 10 nM serum and 0.5 μg/ml insulin (Bom + Ins) in the presence or absence of 10 nM rapamycin (Rap). After 40 h, BrdUrd incorporation into cell nuclei was determined by using a specific anti-BrdUrd monoclonal antibody detected by a fluorescein-conjugated secondary antibody. Labeled nuclei were visualized by fluorescence microscopy. Typical fields are presented. B, BrdUrd incorporation was performed as above for the same factors together with vehicle-treated cells (control untreated cultures). Results are presented as the percentage of labeled nuclei and are the means ± S.E. of three distinct fields from two separate experiments. C, confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing either 10 nM bombesin or 10 nM serum with 0.5 μg/ml insulin in the presence or absence of 10 nM rapamycin. After 40 h, all the cultures were subjected to FACS analysis as described under “Experimental Procedures.” The graphs represent the number of cells (y axis) as a function of the amount of DNA (fluorescence intensity) on the x axis and are typical of two independent experiments. Control untreated cultures (−) and cultures treated with 0.5 μg/ml insulin (Ins) are also presented for comparison.
FIG. 4. Rapamycin inhibits phosphorylation and activation of p70S6K throughout G1. A and B, quiescent Swiss 3T3 cells were preincubated for 15 min with (+) or without (−) 10 nM rapamycin (Rap) and then treated for either 15 or 60 min with 0.5 μg/ml insulin (Ins), 10 nM bombesin (Bom) or 10 nM bombesin with 0.5 μg/ml insulin (Bom + Ins). Cells were lysed, and p70S6K immunocomplex kinase assays were performed as described under "Experimental Procedures." Results are the means of duplicates, are expressed as the percentage of insulin-stimulated activation (8,000–10,000 cpm/1.5 × 10^5 cells at 15 min) and are representative of three independent experiments. The specific activity of [γ-32P]ATP used was 900–1,200 cpm/pmol. C, quiescent Swiss 3T3 cells were preincubated for 15 min with (+) or without (−) 10 nM rapamycin and then treated with 0.5 μg/ml insulin (Ins), 10 nM bombesin or 10 nM bombesin with 0.5 μg/ml insulin (B + I) for the times indicated. Cells were lysed, and mobility shift assays were performed using a polyclonal antibody that recognized αI and αII isoforms of p70S6K in Western blotting.

TABLE I
Effect of rapamycin and PD 098059 on DNA synthesis stimulated by bombesin alone or in combination with insulin

| Addition | Rapamycin + PD | [%H]Thymidine incorporation (% of maximum) |
|----------|---------------|------------------------------------------|
| 1 nM bombesin | − | 28 ± 5 | 89 ± 4 |
| 1 nM bombesin | + | 1 ± 0.3 | 25 ± 2 |
| 10 nM bombesin | − | 49 ± 11 | 100 |
| 10 nM bombesin | + | 2 ± 0.3 | 66 ± 4 |

These results suggest that the inhibitory effect of rapamycin on bombesin-stimulated DNA synthesis is mediated by inhibition of cyclin expression rather than by inhibition of the elimination of p27kip1. In cells stimulated with bombesin and insulin, cyclin D1 and E expression was largely unaffected by rapamycin as was Rb hyperphosphorylation and DNA synthesis.

DISCUSSION

The transition of quiescent cells to the S phase of the cell cycle can be induced by multiple parallel signaling pathways that act in a combinatorial and synergistic fashion (16, 28, 29). The redundancy in signaling pathways prior to the R point implies that some early molecular events are required for the stimulation of DNA synthesis by certain stimuli but that they are not necessarily obligatory for the action of all mitogens (24, 30–34). For example, activation of protein kinase C and MAP kinase and an elevation of intracellular calcium are among the early mitogenic signals elicited by bombesin in Swiss 3T3 cells (18, 24). However, distinct mitogens do not recruit all of these pathways; therefore, none of these signaling mechanisms represent obligatory events in the stimulation of DNA synthesis in these pathways (24, 31). In this context, it was not clear whether activation of p70S6K, a highly conserved molecular event in the response of many cell types to mitogens that is maintained throughout G1, is obligatory for the transition to the S phase of the cell cycle (see Refs. 4–14).

Bombesin is the only growth-promoting neuropeptide that induces stimulation of DNA synthesis in Swiss 3T3 cells in the absence of other factors (16). The mitogenic activity of bombesin is, however, potentiated by insulin (16). Interestingly, the combination of bombesin and insulin induces DNA synthesis via signaling pathways that are different from those utilized by bombesin alone. In this study, we utilized Swiss 3T3 cells stimulated by bombesin alone or bombesin in combination with insulin to evaluate the contribution of p70S6K to the reinitiation of the cell cycle.

Our results demonstrate that bombesin stimulates a robust and persistent activation of p70S6K as determined by mobility shift and immune complex kinase assays, and this effect was dependent on the activation of protein kinase C but not the mobilization of intracellular Ca2+. The immunosuppressant rapamycin completely and specifically prevented bombesin-stimulated p70S6K activation. This agent also caused a marked inhibition of bombesin-induced [%H]thymidine incorporation with a dose dependence that paralleled its effects on bombesin-
stimulated p70S6K activation. These results demonstrate that a sustained activation of p70S6K is necessary for bombesin-stimulated DNA synthesis in Swiss 3T3 cells. However, in the same cells, insulin stimulated p70S6K activation to a similar level and with kinetics similar to that seen with bombesin. In contrast to bombesin, insulin alone does not lead to DNA synthesis in Swiss 3T3 cells. Therefore, these results suggest that activation of p70S6K is necessary but not sufficient for bombesin stimulation of DNA synthesis.

The results obtained with cells stimulated with a combination of bombesin and insulin provide a novel insight into the role of p70S6K in the mitogenic response and give striking support to the hypothesis that envisages the existence of alternative pathways leading to DNA synthesis. We have demonstrated, using three distinct techniques, that the synergistic combination of bombesin with insulin is able to stimulate DNA synthesis in Swiss 3T3 cells and progression through the cell cycle in these cells despite the presence of concentrations of rapamycin that strongly inhibited p70S6K phosphorylation and activation throughout G1. Thus, our results demonstrate that stimulation of p70S6K is required for the mitogenic effects of bombesin alone, but they dissociate p70S6K activation from DNA synthesis stimulated by bombesin and insulin.

Furthermore, we have demonstrated that the growth-inhibitory effect of rapamycin on bombesin-stimulated DNA synthesis involves a novel mechanism acting at the level of the cell cycle machinery. Rapamycin has previously been reported to inhibit growth factor-induced down-regulation of the cyclin-dependent kinase inhibitor p27kip1 (26, 27). This accumulation of p27kip1 results in inhibition of G1 to S phase transition and cell cycle arrest. However, the inhibitory effect of rapamycin on bombesin-stimulated DNA synthesis in Swiss 3T3 cells is independent of p27kip1 down-regulation but is associated with a reduction in the expression of cyclins D1 and E and with inhibition of Rb hyperphosphorylation. Interestingly, rapamycin blocks proliferation of T lymphocytes from the recently described p27kip1 knockout mouse (35). This finding also indicates that the growth-inhibitory effects of rapamycin are independent of its inhibition of growth factor-induced down-regulation of p27kip1. Furthermore, we show that the rapamycin-insensitive pathway(s) stimulated by bombesin in combination with insulin can override the block to cyclin expression and results in Rb hyperphosphorylation and in progression through the cell cycle.

Our results have another interesting implication. It is not known whether the effects of rapamycin on p70S6K phosphorylation/activation and p27kip1 levels are mediated by a linear or bifurcated pathway. The finding that rapamycin inhibits bombesin-stimulated p70S6K activation and DNA synthesis but does not lead to p27kip1 accumulation suggests that in Swiss 3T3 cells these events lie on distinct pathways. In contrast, our results demonstrate that bombesin-stimulated cyclin D1 and E expression is rapamycin sensitive. Thus, regulation of cyclin D1 and E expression may involve RAFT/mTOR-dependent pathways including the activation of p70S6K.

Therefore, from the results presented in this study, we conclude that p70S6K is a component of one of the early alternative pathways that can lead to DNA synthesis rather than an obligatory point of convergence in the action of all mitogens. Additionally, the striking inhibitory effect of rapamycin on cell proliferation may involve effects that are independent of expression of the cyclin-dependent kinase inhibitor p27kip1 but involve regulation of the expression of other components of the cell cycle machinery.

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