Rapamycin-insensitive Regulation of 4E-BP1 in Regenerating Rat Liver*

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In cultured cells, growth factor-induced phosphorylation of two translation modulators, p70 S6 kinase and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), is blocked by nanomolar concentrations of the immunosuppressant rapamycin. Rapamycin also attenuates liver regeneration after partial hepatectomy, but it is not known if this growth-suppressive effect is due to dephosphorylation of p70 S6 kinase and/or 4E-BP1. We found that partial hepatectomy induced a transient increase in liver p70 S6 kinase activity and 4E-BP1 phosphorylation as compared with sham-operated rats. The amount of p70 S6 kinase protein in regenerating liver did not increase, but active kinase from partially hepatectomized animals was highly phosphorylated. Phosphorylated 4E-BP1 from regenerating liver was unable to form an inhibitory complex with initiation factor 4E. Rapamycin blocked the activation of p70 S6 kinase in response to partial hepatectomy in a dose-dependent manner, but 4E-BP1 phosphorylation was not inhibited. By contrast, functional phosphorylation of 4E-BP1 induced by injection of cycloheximide or growth factors was partially reversed by the drug. The mammalian target of rapamycin (mTOR) has been proposed to directly phosphorylate 4E-BP1. Western blot analysis using phospho-specific antibodies showed that phosphorylation of Thr-36/45 and Ser-64 increased in response to partial hepatectomy in a rapamycin-resistant manner. Thus, rapamycin inhibits p70 S6 kinase activation and liver regeneration, but not functional phosphorylation of 4E-BP1, in response to partial hepatectomy. These results indicate that the effect of rapamycin on 4E-BP1 function in vivo can be significantly different from its effect in cultured cells.

Most of the cells in the adult mammalian liver are in a quiescent state, but they are induced to re-enter the cell cycle by chemical or surgical treatments that remove or destroy a portion of the liver. A commonly used method for inducing liver regeneration in the rat is by performing a partial hepatectomy in which the two main lobes of the liver are excised, resulting in 68% tissue loss (1). After partial hepatectomy, the remaining liver cells rapidly undergo one or two proliferative cycles so that the mass of the liver remnant nearly doubles after 2 days and approaches the original liver weight by 7 days. The kinetics of this proliferative response have been well documented (reviewed in Ref. 2). DNA synthesis in parenchymal cells (hepatocytes) begins about 12 h after partial hepatectomy and reaches a peak by 20–24 h, followed by a gradual decline. Mitosis follows 6–8 h later. Nonparenchymal cells lag 24 h behind hepatocytes in initiating DNA synthesis and mitosis. DNA synthesis and restoration of the cell deficit in the liver remnant are mostly complete by 72 h.

Partial hepatectomy also leads to an increase in hepatic protein synthesis that is essential for regeneration (3, 4). Superimposed on a general increase in the rate of protein synthesis are marked increases in translation of specific mRNAs (5). Experiments using a variety of model systems have shown that increased translation of these growth-regulated mRNAs is exerted mainly at the level of initiation. In translation initiation, the 43 S preinitiation complex binds to the m7GpppN (where N is any nucleotide) cap on the 5' end of the mRNA, the complex translocates to the initiation codon, and then the 60 S ribosomal subunit is added to form an active 80 S ribosome (reviewed in Refs. 6 and 7). In general, binding of the 43 S preinitiation complex to mRNA is the rate-limiting step in translation initiation. This step is mediated by eukaryotic initiation factor (eIF)4F. One subunit of the eIF4F complex, eIF4E, binds directly to the m7GpppN cap on the mRNA, and a second subunit, eIF4G, interacts with the 43 S preinitiation complex. Activation of translation initiation results in increased loading of ribosomes onto growth-regulated mRNAs.

After partial hepatectomy, a variety of growth factors and hormones carried in the bloodstream activate signaling pathways that stimulate proliferation of the remaining liver cells (reviewed in Refs. 2 and 8). Experiments done mainly in cultured cells have shown that some of these signaling pathways activate protein synthesis by inducing the phosphorylation of various components of the translational machinery (6, 7, 9, 10). For example, many growth factors promote the phosphorylation of the S6 protein in 40 S ribosomal subunits (9). The major kinase that phosphorylates S6 is the 47,000 S6 kinase (p70 S6 kinase) (9, 11). p70 S6 kinase is activated by phosphorylation of the enzyme at multiple serine and threonine residues (12, 13). Phosphorylation of the 40 S ribosomal subunit by p70 S6 kinase is thought to selectively increase translation of a class of growth-regulated mRNAs characterized by the presence of an m7GpppN cap.

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§ The abbreviations used are: eIF, eukaryotic initiation factor; p70 S6 kinase, M Delegate = 70,000 ribosomal protein S6 kinase; 4E-BP1, eIF4E-binding protein 1; mTOR, mammalian target of rapamycin; PH, rats subjected to partial hepatectomy; SHAM, rats subjected to SHAM operation; RAPA, rats treated with rapamycin and partial hepatectomy; Me2SO, rats treated with dimethyl sulfoxide and partial hepatectomy; CONT, unhandled control rats; HA, hemagglutinin; GST, glutathione S-transferase.

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ence of a polypyrimidine tract adjacent to the m5GpppN cap (5′-terminal oligopyrimidine mRNAs) (14, 15). A second mechanism that mediates growth factor-induced activation of protein synthesis is phosphorylation of the translation repressor eIF4E-binding protein 1 (4E-BP1) (10). In quiescent cells, hypophosphorylated forms of 4E-BP1 bind tightly to eIF4E on the mRNA cap, thus excluding eIF4G from the eIF4F complex. Treatment of cells with growth factors leads to phosphorylation of 4E-BP1 on multiple sites and its dissociation from eIF4E, thereby allowing assembly of a functional eIF4F complex (10, 16). Translation of mRNAs with extensive secondary structure at the 5′ end is thought to be particularly sensitive to regulation by 4E-BP1.

Treatment of cultured cells with a variety of stimuli leads to the simultaneous phosphorylation of both p70 S6 kinase and 4E-BP1. In addition, growth factor-induced activation of p70 S6 kinase and disruption of the 4E-BP1-eIF4E complex in vitro is blocked by nanomolar concentrations of the immunosuppressant rapamycin (17–20). The drug exerts this effect by inhibiting the phosphorylation of specific functionally important sites in both proteins (13, 21). Rapamycin, when bound to its intracellular receptor FKBP12, inhibits the function of the mammalian target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidylinositol 3-kinase (22, 23). Although it is well accepted that mTOR activity is required for p70 S6 kinase activation and 4E-BP1 phosphorylation, the mechanism of action of mTOR is controversial. On one hand, it has been proposed that mTOR is a growth factor-activated kinase (24, 25) that directly phosphorylates the two proteins at functionally important sites (26–28). Alternatively, it has been suggested that mTOR inhibits a phosphatase that can dephosphorylate p70 S6 kinase and 4E-BP1 (29; see discussion in Ref. 13).

Francaviglia et al. (29) showed earlier that injection of rats with rapamycin before partial hepatectomy strongly inhibited DNA synthesis and mitosis in the liver remnant. Since rapamycin is currently being used in liver transplant patients (30), it will be important to determine whether the drug exerts a similar growth inhibitory effect in humans and, if so, to elucidate the inhibitory mechanism. Results from studies in cultured cells would suggest that the growth inhibitory effect of rapamycin in rat liver is due to a reduction in p70 S6 kinase activity and/or 4E-BP1 phosphorylation. However, no in vivo studies have been done to test this hypothesis. In this study, we asked whether rapamycin targets p70 S6 kinase and 4E-BP1 to inhibit liver regeneration. We first tested if p70 S6 kinase activity and functional phosphorylation of 4E-BP1 are altered after partial hepatectomy. Then we determined whether p70 S6 kinase activity and 4E-BP1 phosphorylation are reduced in rapamycin-treated rats. Our data indicate that rapamycin, at a dose that significantly attenuates liver regeneration, selectively inhibits p70 S6 kinase activation but not phosphorylation of 4E-BP1 at sites that regulate binding to eIF4E. These results suggest that the in vivo effect of rapamycin on 4E-BP1 function might differ significantly from that observed in cell culture models, which may have important therapeutic implications for this clinically useful drug.

EXPERIMENTAL PROCEDURES

Animals and Surgical Procedures—Male Harlan Sprague-Dawley rats weighing ~250 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The rats were housed in a quiet room with a 12/12-h light/dark cycle. At specific times postoperatively, animals were weighed and sacrificed by decapitation, and the livers were removed. Livers were weighed, rinsed in cold phosphate-buffered saline, and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C. Then we measured [32P] incorporation into liver and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotonin and leupeptin). Homogenates were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were stored at −80 °C.
plate. The fragment was digested with EcoRI and BamHI and ligated into pGEX-5X-3 (Amersham Pharmacia Biotech). Bacteria were transformed with the expression construct, and the GST-4E-BP1 fusion protein was purified on glutathione-Sepharose.

Analysis of 4E-BP1 Mutants—Cos7 cells were seeded at 5 × 10^3 cells/6-cm dish in growth medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum). The next day the cells were transfected with 3 μg of DNA in 3 ml of Opti-MEM plus 7.5 μl of LipofectAMINE (Life Technologies, Inc.). After 5 h, the transfection solutions were replaced with growth medium, and the cells were allowed to grow for 2 days. After treatment with insulin or LY294002, the cells were rinsed twice with growth medium, and the cells were allowed to grow for 2 days.

RESULTS

Rapamycin Inhibits Liver Regeneration—Francavilla et al. (29) show that injection of rats with 0.1–1 mg/kg rapamycin for 4 days before partial hepatectomy inhibited DNA synthesis and mitosis measured 24–26 h after surgery. A different treatment protocol was used here to examine the effect of rapamycin on the weight of regenerating liver over a longer time course. Rats were injected with vehicle (MeSO group) or with 0.4 mg of rapamycin/kg of body weight (RAPA group) 2 h before surgery and given each day after partial hepatectomy. Rats in the PH group were subjected to partial hepatectomy only. Livers were removed and weighed 2 days and 4 days after surgery. As expected, the liver remnant of PH rats nearly doubled in size after 2 days and grew to about 80% of the original liver weight by 4 days post-partial hepatectomy (Fig. 1). Liver regeneration was suppressed at 2 days in the MeSO control group but was equivalent to the PH animals after 4 days. By contrast, liver weight in RAPA animals was significantly lower than in the MeSO controls at both 2 days and 4 days (Fig. 1). Average liver weight in the RAPA group was less than 60% of that in the MeSO animals 4 days after partial hepatectomy. Inhibition of liver regeneration by rapamycin is probably not due to the immunosuppressive action of the drug because treatment of partially hepatectomized rats with two other immunosuppressants, cyclosporin A and FK506, augments liver regeneration (35). Thus, liver regeneration after partial hepatectomy is strongly inhibited by rapamycin treatment of rats using this experimental protocol.

Partial Hepatectomy Activates p70 S6 Kinase—The inhibitory effect of rapamycin seen in Fig. 1 suggests that p70 S6 kinase and/or 4E-BP1 might play a role in liver regeneration. Gressner and Wool (36) reported nearly three decades ago that the phosphate content of ribosomal protein S6 increases significantly within 24 h after partial hepatectomy. Two subsequent studies showed that total S6 kinase activity is elevated in extracts of regenerating rat liver (37, 38), but reagents were not available at that time to specifically assay p70 S6 kinase. Here we used specific immunocomplex kinase assays to determine whether p70 S6 kinase is activated by partial hepatectomy. Fig. 2 shows that p70 S6 kinase was activated almost 50-fold in the liver of rats 24 h after partial hepatectomy as compared with unhandled rats in the CONT group. Kinase activity in PH rats dropped sharply between 24 and 48 h but was still slightly elevated 96 h after surgery. By contrast, p70 S6 kinase activity in sham-operated animals was not higher than CONT levels at the 24-h time point (Fig. 2). Kinase activity in the SHAM group increased ~3-fold between 24 and 48 h to a level equivalent to that measured in PH animals. Western blot analysis showed that the amount of p70 S6 kinase protein in SHAM and PH livers at the 24-h time point was similar, but active enzyme by PH animals exhibited an upward mobility shift on SDS-polyacrylamide gels that indicates increased phosphorylation of the enzyme (see Fig. 4B).

Partial Hepatectomy Induces Functional Phosphorylation of 4E-BP1—We next tested whether partial hepatectomy induces hyperphosphorylation of 4E-BP1 by using gel mobility shift assays. 4E-BP1 migrates in SDS-polyacrylamide gels as three bands (α, β, and γ). The α band is the least phosphorylated form, and the β and γ bands are more highly phosphorylated species. 4E-BP1 in the liver of CONT rats appeared on Western
Rapamycin Inhibits p70 S6 Kinase Activation in Regenerating Liver—Having established that partial hepatectomy induces both p70 S6 kinase activation and 4E-BP1 phosphorylation, we tested if rapamycin attenuates either of these responses. Animals were injected with increasing dosages of rapamycin 2 h before partial hepatectomy, and p70 S6 kinase activity was measured in liver extracts 24 h after surgery. As shown in Fig. 4A, rapamycin strongly inhibited the activation of p70 S6 kinase in the liver of PH rats at every dose examined. The lowest dose tested (0.1 mg/kg) decreased p70 S6 kinase activity ∼80%, whereas 0.4 mg/kg rapamycin/kg body weight reduced the amount of kinase activity to the basal level seen in sham-operated animals (Fig. 4A). Western blot analysis showed that the amount of p70 S6 kinase protein in liver extracts was not decreased by rapamycin treatment, but the drug prevented the appearance of phosphorylated species of the protein (Fig. 4B). In a second experiment, rats were injected with vehicle or with 0.4 mg/kg rapamycin before surgery and on each day after partial hepatectomy, according to the protocol described in Fig. 1. p70 S6 kinase activity remained much lower in the RAPA group than in the DMSO animals 2 and 4 days after surgery (Fig. 4C). Thus, treatment of rats with rapamycin prevents the phosphorylation and activation of p70 S6 kinase in response to partial hepatectomy. It should be noted that p70 S6 kinase activity in MeSO animals was higher than in PH animals 2 and 4 days post-partial hepatectomy (compare Figs. 2 and 4C). Daily handling and injection of vehicle into the MeSO rats may induce a stress response that causes p70 S6 kinase to remain active for a longer time.

Phosphorylation of 4E-BP1 in Regenerating Liver Is Rapamycin-resistant—The in vivo effect of rapamycin on 4E-BP1 function was examined in the same liver extracts that were used to analyze p70 S6 kinase activity. m7GTP-Sepharose binding assays detected a large amount of 4E-BP1 bound to eIF4E in liver extracts of sham-operated rats, and phosphorylation of 4E-BP1 in response to partial hepatectomy disrupted the 4E-BP1-eIF4E complex (Fig. 5A, upper panel). However, to our surprise, 4E-BP1 did not coprecipitate with eIF4E in liver extracts of rats treated with increasing dosages of rapamycin before partial hepatectomy (Fig. 5A, middle panel). The Western blot used for these assays was stripped and reprobed with an antibody to eIF4E to confirm that the amount of eIF4E pulled down by m7GTP-Sepharose was not altered by partial hepatectomy or rapamycin treatment (Fig. 5A, middle panel). These results suggested that rapamycin does not inhibit the phosphorylation of 4E-BP1 in response to partial hepatectomy. To fur-
FIG. 5. Effect of rapamycin on 4E-BP1 phosphorylation. A, rats were injected intraperitoneally with increasing dosages of rapamycin 2 h before partial hepatectomy. Livers were harvested 24 h after surgery, and 4E-BP1 binding to m7GTP-Sepharose was measured in extracts (upper panel). Equal amounts of liver extract protein were also subjected to immunoblotting, and the various phosphorylated forms of 4E-BP1 were detected (lower panel). Results from individual animals are shown. B, rats undergoing partial hepatectomy were treated with rapamycin or vehicle as described under "Experimental Procedures." Liver extracts were prepared at the indicated times after surgery, and 4E-BP1 binding to m7GTP-Sepharose was measured in extracts (upper panel). Equal amounts of protein were also subjected to immunoblotting, and the various phosphorylated forms of 4E-BP1 were detected (lower panel). Data from individual animals in each group are shown. The numerical values represent the fraction of 4E-BP1 bound to m7GTP-Sepharose (obtained by dividing the integrated density of bands in the upper panel by that of bands in the lower panel). DMSO, Me2SO.

m7GTP-Sepharose binding assays were also performed on liver extracts from rats treated for 2 or 4 days with vehicle or rapamycin after partial hepatectomy. At both time points, there was less 4E-BP1 bound to the resin in drug-treated animals than in the controls (Fig. 5B, upper panel). This result could be obtained if (a) 4E-BP1 is more highly phosphorylated at sites that regulate binding in RAPA rats than in Me2SO animals or (b) the total amount of 4E-BP1 in RAPA animals is much less than in Me2SO rats. Quantitation of total 4E-BP1 protein by densitometric scanning of Western blots (Fig. 5B, lower panel) showed that RAPA livers contained on average 45% and 25% less 4E-BP1 than Me2SO livers at 2 and 4 days, respectively. To correct for the lower level of 4E-BP1 in drug-treated animals, the amount of 4E-BP1 bound to m7GTP-Sepharose was divided by the total amount of 4E-BP1 for each animal. These values (shown above the upper panel in Fig. 5B) confirm that the fraction of 4E-BP1 bound to m7GTP-Sepharose is significantly less in RAPA rats than in the Me2SO controls. Quantitation of individual bands in the lower panel of Fig. 5B indicated that the highly phosphorylated γ species still represented a slightly higher percentage of the total 4E-BP1 in 4E-BP1 was represented a slightly higher percentage of the total 4E-BP1 in the 4-day RAPA rats than in the Me2SO controls. Thus, rapamycin treatment of rats inhibits the activation of p70 S6 kinase but seems to slightly augment the functional phosphorylation of 4E-BP1 in response to partial hepatectomy. Furthermore, liver regeneration after partial hepatectomy is inhibited by rapamycin at a concentration that completely inhibits p70 S6 kinase activation but not 4E-BP1 phosphorylation.

Rapamycin Sensitivity of 4E-BP1 Phosphorylation Induced by Cycloheximide or Growth Factors—The above result was unexpected, as growth factor-induced phosphorylation of 4E-BP1 has been shown to be inhibited by rapamycin in a variety of cultured cells. One possible explanation for our data is that higher concentrations of rapamycin might be required to inhibit 4E-BP1 phosphorylation in vivo. Alternatively, metabolism of rapamycin in the rat might cause production of a substance that selectively inhibits p70 S6 kinase (39). If such were the case, one might expect that 4E-BP1 phosphorylation in rat liver would be rapamycin-resistant regardless of the stimulus used. To test this hypothesis, rats were injected with vehicle or with 0.4 mg/kg rapamycin, and 24 h later were treated with cycloheximide or growth factors to induce 4E-BP1 phosphorylation. Western blot analysis showed that exposure of vehicle-treated rats to cycloheximide, insulin, or epidermal growth factor caused increased phosphorylation of 4E-BP1 (Fig. 6A), and m7GTP-Sepharose binding assays showed a corresponding increase in the amount of 4E-BP1 bound to eIF4E (Fig. 6B). More important, the phosphorylation of 4E-BP1 in control and agonist-treated rats was reduced in the presence of rapamycin (Fig. 6A), and increased formation of the 4E-BP1-eIF4E complex was also observed (Fig. 6B). Of the three agonists tested, insulin was the most potent in disrupting the binding between 4E-BP1 and eIF4E, and it was also the least sensitive to rapamycin (Fig. 6B). p70 S6 kinase activity measured in the same extracts was increased by these agonists and strongly inhibited in each case by rapamycin treatment (data not shown). Together, our results demonstrate that 4E-BP1 phosphorylation...
induced in vivo by cycloheximide or growth factor treatment is relatively rapamycin-sensitive, whereas the pathway that mediates 4E-BP1 phosphorylation in response to partial hepatectomy is rapamycin-resistant.

Characterization of 4E-BP1 Phospho-specific Antibodies—
4E-BP1 is phosphorylated on five Ser/Thr-Pro sites in cells (21), and mTOR has been reported to preferentially phosphorylate two of these sites, Thr-36 and Thr-45, in vitro (27, 40–42). Paradoxically, phosphorylation of Thr-36 and Thr-45 is relatively resistant to rapamycin, whereas phosphorylation of two other sites, Ser-64 and Thr-69, is rapamycin-sensitive (21, 43).

A model has been proposed by Sonenberg and coworkers (41) proposing that phosphorylation of 4E-BP1 on Thr-36 and Thr-45 by mTOR is required for subsequent phosphorylation of the other sites by growth factor-activated kinases. To further elucidate the mechanisms that control 4E-BP1 binding to eIF4E in this in vivo model system, we decided to examine individual phosphorylation sites in 4E-BP1 using phospho-specific antibodies.

The specificity of these antibodies was first tested using HA-tagged 4E-BP1 mutants in which Thr-36, Thr-45, Ser-64, or Thr-69 were changed to Ala (see “Experimental Procedures.”) COS7 cells expressing wild-type or mutant proteins were either stimulated with insulin or treated with LY294002, suggesting either that the antibody is not phospho-specific or that incubation of cells with LY294002 does not reduce the phosphorylation of Thr-69.
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Procedures" and the legend of Fig. 4

and with or without cd2c, a kinase that phosphorylates Ser/Thr-Pro sites. Western blot

other possibilities, purified recombi-

sites after partial hepatectomy, but none of these phosphoryl-

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eIF4E complex (Fig. 3). The kinetics of these two responses were

The inhibitory effect of rapamycin on liver regeneration sug-

Effect of Rapamycin on the Phosphorylation of Individual Sites in Rat Liver 4E-BP1—The results in Fig. 7 indicated that the phospho-specific antibodies could be used to examine the phosphorylation of rat liver 4E-BP1. A low level of phosphorylation was detected in Thr-36/Thr-45 in unhandled CONT rats (Fig. 8, upper panel). The amount of phosphate was relatively unchanged after sham operation, but a substantial increase was detected 24 h after partial hepatectomy. Rapamycin treatment increased the phosphorylation of Thr-36/45 in response to partial hepatectomy. The bottom panel in Fig. 8 shows that the total amount of 4E-BP1 in the PH samples with or without rapamycin is the same, so signals on the phospho-specific antibody blots for these samples can be directly compared with each other. Similarly, phosphorylation of Ser-64 was strongly increased in PH rats relative to CONT animals, and rapamycin slightly increased this response (Fig. 8, second panel). This stimulatory effect of rapamycin on 4E-BP1 phosphorylation may be indirect, for example, via the release of humoral factors from other tissues, since these experiments are conducted in vivo. Nonetheless, increased phosphorylation of 4E-BP1 in response to such indirect mechanisms is still not inhibited by rapamycin. It should be noted that one of the SHAM animals exhibited elevated phosphorylation of 4E-BP1 that was most evident on the phospho-Ser-64 blot. Finally, Thr-69 appeared to be constitutively phosphorylated in CONT rats, and there was little change in the amount of phosphate after partial hepatectomy or rapamycin treatment (Fig. 8, third panel). These results indicate that 4E-BP1 is phosphorylated at the expected sites after partial hepatectomy, but none of these phosphorylation events is inhibited by rapamycin treatment.

FIG. 8. Effect of rapamycin on 4E-BP1 phosphorylation at individual sites. Rats were treated as described under “Experimental Procedures” and the legend of Fig. 4A. Rapamycin was used at 0.4 mg/kg. Equal amounts of liver extract protein were subjected to immuno-

To distinguish between these possibilities, purified recombi-

gnant GST-4E-BP1 was incubated in vitro with or without cd2c, a kinase that phosphorylates Ser/Thr-Pro sites. Western blot analysis showed that the phospho-Thr-69 antibody reacted only with phosphorylated 4E-BP1 protein (Fig. 7C).

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DISCUSSION

The results presented here demonstrate that treatment of rats with rapamycin at a dose that impairs the regenerative response after partial hepatectomy selectively inhibits p70 S6 kinase activation but not the functional phosphorylation of 4E-BP1. Other investigators such as Lawrence and Abraham (45) and Denton and co-workers (46) have previously reported that under conditions in which p70 S6 kinase activity is abol-

- insensitive pathways are involved (reviewed in Refs. 45 and 46). However, these investigators also found that insulin-in-

duced 4E-BP1-eIF4E complex dissociation was blocked by rapa-

To distinguish between these possibilities, purified recombi-

gnant GST-4E-BP1 was incubated in vitro with or without cd2c, a kinase that phosphorylates Ser/Thr-Pro sites. Western blot analysis showed that the phospho-Thr-69 antibody reacted only with phosphorylated 4E-BP1 protein (Fig. 7C).

the phospho-specific antibodies could be used to examine the phosphorylation of rat liver 4E-BP1. A low level of phosphorylation was detected in Thr-36/Thr-45 in unhandled CONT rats (Fig. 8, upper panel). The amount of phosphate was relatively unchanged after sham operation, but a substantial increase was detected 24 h after partial hepatectomy. Rapamycin treatment increased the phosphorylation of Thr-36/45 in response to partial hepatectomy. The bottom panel in Fig. 8 shows that the total amount of 4E-BP1 in the PH samples with or without rapamycin is the same, so signals on the phospho-specific antibody blots for these samples can be directly compared with each other. Similarly, phosphorylation of Ser-64 was strongly increased in PH rats relative to CONT animals, and rapamycin slightly increased this response (Fig. 8, second panel). This stimulatory effect of rapamycin on 4E-BP1 phosphorylation may be indirect, for example, via the release of humoral factors from other tissues, since these experiments are conducted in vivo. Nonetheless, increased phosphorylation of 4E-BP1 in response to such indirect mechanisms is still not inhibited by rapamycin. It should be noted that one of the SHAM animals exhibited elevated phosphorylation of 4E-BP1 that was most evident on the phospho-Ser-64 blot. Finally, Thr-69 appeared to be constitutively phosphorylated in CONT rats, and there was little change in the amount of phosphate after partial hepatectomy or rapamycin treatment (Fig. 8, third panel). These results indicate that 4E-BP1 is phosphorylated at the expected sites after partial hepatectomy, but none of these phosphorylation events is inhibited by rapamycin treatment.

DISCUSSION

The results presented here demonstrate that treatment of rats with rapamycin at a dose that impairs the regenerative response after partial hepatectomy selectively inhibits p70 S6 kinase activation but not the functional phosphorylation of 4E-BP1. Other investigators such as Lawrence and Abraham (45) and Denton and co-workers (46) have previously reported that under conditions in which p70 S6 kinase activity is abolished by rapamycin, phosphorylation of 4E-BP1 was only partly inhibited, suggesting that both rapamycin-sensitive and
inactivation of the enzyme. However, in some reports mTOR has been shown to exhibit a significant amount of kinase activity in the presence of the rapamycin-FKBPL2 complex in vitro (24, 27, 50). In addition, a truncation mutant of p70 S6 kinase is phosphorylated on Thr-389 in rapamycin-treated cells, suggesting that the drug does not inhibit the activity of the kinase that modifies this putative mTOR site (51). These results suggest that mTOR might regulate p70 S6 kinase activity indirectly by inhibiting a protein phosphatase that inactivates the enzyme.

This conclusion is further supported by genetic studies in yeast suggesting that Tap42 and type 2A-related protein phosphatases are components of a TOR signaling pathway that controls translation initiation. The TOR proteins in yeast appear to phosphorylate Tap42 and promote its association with the phosphatases in a rapamycin-sensitive manner (52, 53). A mammalian protein related to Tap42, a4, has also been shown to associate with phosphatase 2A and related enzymes, but there is disagreement about whether the binding is disrupted in the presence of rapamycin (54–56). We showed earlier that the major phosphatase activity in cell extracts that inactivates p70 S6 kinase is protein phosphatase 2A (57). The same phosphatase was recently demonstrated to exhibit increased catalytic activity when isolated from rapamycin-treated cells (58). Together, these results suggest a model in which mTOR-mediated phosphorylation of a4 results in binding to and inhibition of phosphatase 2A, thus preventing the dephosphorylation of p70 S6 kinase. Rapamycin would relieve the inhibitory effect of mTOR on the phosphatase, leading to dephosphorylation and inactivation of p70 S6 kinase. It should be noted that control of protein phosphorylation directly by mTOR kinase activity or indirectly by mTOR inhibition of a phosphatase is not necessarily mutually exclusive. Additional experiments will be required to determine whether rapamycin utilizes either of these two mechanisms to inhibit p70 S6 kinase activity in the liver of partially hepatectomized rats.

In contrast to its potent effect on p70 S6 kinase, rapamycin did not inhibit the functional phosphorylation of 4E-BP1 in regenerating liver (Fig. 5). This result was unexpected, as growth factor-induced disruption of the 4E-BP1-eIF4E complex has been shown to be blocked by rapamycin in a variety of cell types in vitro. Expression of a rapamycin-resistant mutant of mTOR confers rapamycin resistance to 4E-BP1 phosphorylation (26), indicating that mTOR regulates 4E-BP1 in cultured cells. mTOR is thought to control 4E-BP1 by directly phosphorylating the protein, but there is disagreement about which sites are involved. Brunn et al. (49) reported that immunoprecipitated mTOR can phosphorylate recombinant 4E-BP1 at Thr-36, Thr-45, Ser-64, Thr-69, and Ser-82, but it appears that Thr-36 and Thr-45 are preferentially modified by mTOR in vivo. In intact cells, phosphorylation of Ser-64 and Thr-69 is more sensitive to rapamycin than that of Thr-36 and Thr-45 (21, 43). We found that partial hepatectomy induced the phosphorylation of Thr-36/45 and Ser-64 and that Thr-69 was already phosphorylated in CONT animals (Fig. 8). However, in contrast to the situation in cultured cells, none of these sites exhibited rapamycin sensitivity (Fig. 8). One possible explanation for these results is that mTOR kinase activity in regenerating liver is not inhibited by rapamycin, so mTOR is able to fully phosphorylate 4E-BP1 in the presence of the drug. However, in light of the potent effect of rapamycin on p70 S6 kinase, it is equally likely that a kinase other than mTOR phosphorylates 4E-BP1 in regenerating liver. Ongoing experiments are being conducted to examine these two possibilities.

In contrast to the results in regenerating liver, phosphorylation of 4E-BP1 in the liver of rats injected with cycloheximide or growth factors did display rapamycin sensitivity (Fig. 6). Rapamycin has also been shown to inhibit the phosphorylation of 4E-BP1 induced by acute exposure to high concentrations of amino acids in a perfused rat liver model system (59). These findings indicate that an mTOR-dependent pathway that can control 4E-BP1 phosphorylation exists in the liver. One explanation for the lack of rapamycin sensitivity in regenerating liver is that partial heptatectomy might activate mTOR much more potently than these acute agonist treatments, so an inhibitory effect of rapamycin on mTOR kinase activity might only be detected using these agonists. A second reason why this rapamycin-sensitive pathway is not manifest after partial heptatectomy may be related to the proliferative state of the tissue. The remaining liver cells in partially heptatectomized animals are rapidly proliferating, whereas cells in an intact liver exposed for a short time to growth factors are in a quiescent state.

We speculate that mTOR may be the dominant factor controlling the functional phosphorylation of 4E-BP1 in the quiescent liver, but a distinct rapamycin-insensitive 4E-BP1 kinase that takes over the function of mTOR is induced in proliferating liver. Because the five phosphorylation sites in 4E-BP1 are found in Ser/Thr-Pro motifs, we expect that this kinase is a proline-directed enzyme, such as those in the mitogen-activated protein kinase or cdc2 families. Indeed, the Erk2 isoform of mitogen-activated protein kinase has been shown to phosphorylate all five Ser/Thr-Pro sites in vitro (21), and an Erk2-dependent pathway has been proposed to regulate 4E-BP1 phosphorylation in vascular smooth muscle cells (60). However, using Western blots probed with phospho-specific antibodies, we found no evidence that the Erk1, Erk2, Jnk, or p38 mitogen-activated protein kinases are activated by partial heptatectomy at any of the times investigated (data not shown). In this study, we showed that cdc2 can phosphorylate 4E-BP1 at Thr-69 in vitro (Fig. 7), and others have shown that cyclin-dependent kinases are activated in regenerating liver (61). Further studies are needed to identify relevant 4E-BP1 kinases in this in vivo model system.

Results from our study indicate that rapamycin shows significant anti-proliferative effects in vivo that might be mediated by selective inhibition of p70 S6 kinase. However, other rapamycin-sensitive enzymes such as S6 kinase 2 (11) might be responsible for the rapamycin effect in vivo. The specific contribution of p70 S6 kinase to liver regeneration could possibly be tested in rapamycin-treated transgenic mice that express a rapamycin-resistant mutant of p70 S6 kinase (51) in the liver. Inhibition of global protein synthesis by injection of puromycin blocks DNA synthesis in regenerating rat liver (3). However, p70 S6 kinase does not control global protein synthesis but rather is thought to regulate translation of a subset of growth-regulated mRNAs (5′-terminal oligopyrimidine mRNAs) including those that encode ribosomal proteins (13). Indeed, studies in perfused liver have shown that treatment with rapamycin exerts a relatively small effect on general protein synthesis (59). The anti-proliferative effect of the drug observed in this liver regeneration model is presumably due to a selective inhibitory action on the synthesis of ribosomal proteins and/or other proteins encoded by 5′-terminal oligopyrimidine mRNAs. The importance of ribosomal protein production during liver regeneration was highlighted in a recent report in which synthesis of 40 S ribosomal subunits was blocked by conditionally knocking out the ribosomal protein S6 gene in mice (62). Liver cells in the knockout mice were able to grow larger in response to refeeding but were unable to proliferate after partial heptatectomy. This selective effect on proliferation suggests that rapamycin and related compounds might be useful therapeutic agents in the treatment of cancer. Indeed, CCI-
779, a rapamycin analog, shows antitumor activity in mice and is currently undergoing phase I clinical trials in humans (63). A better understanding of how rapamycin inhibits cell proliferation in vivo will aid in the design of more specific and potent drugs to treat patients with malignancies and after organ transplantation.

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