The major function of mammalian target of rapamycin (mTOR) is the control of cell growth. Insulin and amino acids regulate the mTOR pathway, and both are needed to promote its maximal activation. To further understand mTOR regulation by insulin and amino acids, we have studied the enzyme in primary cultures of hepatocytes. We show that insulin increases mTOR phosphorylation on Ser2448, a consensus phosphorylation site for protein kinase B (PKB). Ser2448 phosphorylation is also increased by amino acids, although they do not activate PKB. Furthermore, insulin and amino acids have an additive effect, indicating that they act through distinct pathways. We also show that phosphorylation of Ser2448 does not seem to modulate in vitro phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) by mTOR. However, stimulation of hepatocytes with insulin and amino acids leads to an increase in mTOR kinase activity. Rapamycin has no effect on insulin-, glucagon-, and 8-(4-chlorophenylthio)adenosine-cAMP-induced amino acid transport. Surprisingly, glucagon and 8-(4-chlorophenylthio)adenosine-cAMP, which do not activate PKB, stimulate the phosphorylation on Ser2448 of mTOR. However, glucagon inhibits amino acid- and insulin-induced activation of ribosomal S6 protein kinase 1 and phosphorylation of the translational repressor eukaryotic initiation factor 4E-binding protein 1 by mTOR. Our results demonstrate that glucagon, which is not able to activate but rather inhibits the mTOR pathways, stimulates the phosphorylation of mTOR on Ser2448. This finding suggests that phosphorylation of this site might not be sufficient for mTOR kinase activity but is likely to be involved in other functions.

It is now established that cells respond to amino acid availability by inducing an intracellular pathway involving the mammalian target of rapamycin (mTOR). First identified by antiproliferative activity by inducing an intracellular pathway involving the mammalian target of rapamycin (mTOR). First identified by

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1 The abbreviations used are: mTOR, mammalian target of rapamycin; PKB, protein kinase B; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K1, ribosomal protein S6 kinase 1; CPT, 8-(4-chlorophenylthio)adenosine; MEM, minimum essential medium; BSA, bovine serum albumin; GST, glutathione S-transferase; AIB, 2-aminoisobutyric acid; FKB12, FK506-binding protein 12.
concerning the regulation of mTOR kinase activity by these agents. Similarly, the role of mTOR in insulin action (except the regulation of protein synthesis) is not well defined, especially in the hepatocytes. Therefore, we have studied mTOR kinase in the physiological model of primary cultures of rat hepatocytes. In these cells, we have determined mTOR phosphorylation and kinase activity in the presence of insulin and/or amino acids. We also have investigated the role of mTOR in amino acids transport in response to insulin, as well as in response to an insulin antagonist, i.e. glucagon. Although our results show that system A amino acids transport is regulated by a rapamycin-insensitive pathway, we demonstrate for the first time that glucagon stimulates mTOR phosphorylation on Ser2448. Moreover, we show a concomitant decrease in insulin- and amino acid-induced phosphorylation of S6K1 on Thr389 and of 4E-BP1 on Ser64 and Thr36/45 in the presence of glucagon, indicating that the hormone has an inhibitory effect on proteins downstream of the mTOR pathway. Our study points to a multi-factorial regulation of mTOR phosphorylation on Ser2448 in hepatocytes by insulin, amino acids, and glucagon.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase type IV, 8-(4-chlorophenylthio)adenosine 3':5'- cyclic monophosphate (CPT-cAMP) were purchased from Sigma; cell culture solutions and supplements, reagents for SDS-PAGE and protein A-Sepharose were from Invitrogen. Amino acids mixture (50×) in MEM was from Invitrogen, and amino acid concentrations in a 1× mixture were as follows: Arg, 0.6 mM; Cys, 0.2 mM; Gln, 2 mM; His, 0.2 mM; Ile, 0.4 mM; Leu, 0.4 mM; Lys, 0.4 mM; Met, 0.1 mM; Phe, 0.2 mM; Thr, 0.47 mM; Trp, 0.05 mM; Tyr, 0.2 mM; and Val, 0.4 mM. L-Glutamine (2 mM) is added to amino acids mixture before use. Fetal Bovine Serum (Hyclone) was provided by Pierce. Insulin (recombinant human) and glucagon were from the American Type Culture Collection, and okadaic acid was from BIOMOL Research Laboratories, Inc. Rapamycin and FK-506 were from Calbiochem (La Jolla, CA), and 2-amino-[1-14C]isobutyric acid, and [3H]inulin were purchased from Amersham Biosciences. mTOR Antibodies—mTab2 is an anti-peptide antibody directed against residues 1272-1290 of mTOR (18), phosphospecific antibodies to Ser2448 of mTOR (anti-Ser(P)2448) are directed against mTOR residues as described previously (21). The antibody directed against a C-terminal peptide of 4E-BP1 and the antibody directed against a C-terminal peptide of 4E-BP1 phosphorylated in Thr36/45 and Ser64 were obtained as described previously (20). The antibody directed against a C-terminal peptide of 4E-BP1 and the antibody directed against a C-terminal peptide of 4E-BP1 phosphorylated in Thr36/45 and Ser64 were obtained as described previously (20). The antibody directed against a C-terminal peptide of 4E-BP1 and the antibody directed against a C-terminal peptide of 4E-BP1 phosphorylated in Thr36/45 and Ser64 were obtained as described previously (20).

**Isolation and Primary Culture of Hepatocytes**—Hepatocytes were isolated from adult male Wistar rats (150-175 g, from Elevage Janvier, Le Genest St. Isle, France) by collagenase dissociation of the liver as described previously (21). freshly isolated hepatocytes were incubated in KRb buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 24 mM NaHCO3, 1.3 mM CaCl2, 1.2 mM KH2PO4, pH 7.5) supplemented with 1% (w/v) BSA and were gassed with a mixture of 5% CO2 and 95% O2. The cells were plated at a final concentration of 106 cells/6-well dish and 5 × 105 cells/10-cm-diameter dish in Waymouth medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) and maintained at 37 °C, 5% CO2. After 4 h the medium was replaced with serum-free Waymouth medium containing 0.2% (w/v) BSA, penicillin, streptomycin, and gentamycin (250 μg/ml) for 17 h. The cells were then stimulated in different conditions, as described below. When the cells were used to study the effects of amino acids, the cells were washed twice with KRb buffer and incubated for 150 min in KRb buffer with 0.2% (w/v) BSA containing gentamycin prior to cell stimulation.

**Stimulation and Lysis of Primary Cultured Rat Hepatocytes**—Depending on the experiments, the cells were stimulated with the following reagents: insulin (10−7 M), glucagon (10−7 M), or CPT-cAMP (10 μM), in the presence or absence of a 1× MEM amino acid solution plus l-glutamine. For stimulation, the cells were washed twice with ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.7 mM KH2PO4, pH 7.4), scraped from dishes, and homogenized at 4 °C in 250 μl (for one 6-well dish) or 1 ml (for one 10-cm diameter dish) of buffer A (50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 mM potassium phosphate, 0.2% (v/v) Tween 20, 1 mM sodium orthovanadate, 1 mM 1,4-dithiothreitol, and 50 mM β-glycerophosphate, pH 7.4) containing the protease inhibitors 5 μg/ml leupeptin, 150 μg/ml aprotinin, and 250 μg/ml 4-2-aminomethyl)benzene-sulfonfylfluoride. Homogenization was performed at 4 °C with an Elvehjem Potter (30 s at 2000 min−1). Before use, the homogenates were centrifuged at 16,000 × g for 15 min at 4 °C, and the supernatants were used for the experiments described below.

**Affinity Purification of mTOR**—Recombinant GST-FKBPI2 was expressed in bacteria and purified as described previously (1, 22). To purify mTOR, GST-FKBPI2 (100 μg) was incubated with 30 μl of Glutathione-Sepharose in buffer A containing 0.5% (w/v) BSA. After 90 min at 4 °C, the resin was washed three times with buffer A and incubated (final volume, 1 ml) for 90 min at 4 °C with cell homogenate. The primary cultures of rat hepatocytes were lysed in lysis buffer containing 1 mM ATP, 1 mM Na-HEPES, 50 mM β-glycerophosphate, pH 7.4). The beads were resuspended in 20 μl of buffer C, and the kinase reaction was initiated by adding 30 μl of buffer C supplemented with 0.1 mM γ-32P-ATP (5000 C/mmol), 10 mM MnCl2, and 40 μg/ml of His14E-BP1. After 1 h at room temperature, the reaction solution (containing [His14E-BP]4E-BP1) was separated from the beads (containing immunopurified mTOR) before adding Laemmli sample buffer.

**Electrophoretic Analyses and Western Blotting**—The samples were subjected to SDS-PAGE by using the method of Laemmli. For experiments in which mTOR was purified using GST-FKBPI2, the proteins were transferred to polyvinylidene difluoride (Millipore; Millipore), and Western blotting was performed using affinity purified anti-Ser(P)2448 antibodies (0.5 μg/ml). After an overnight incubation at 4 °C, the membranes were washed with wash buffer (140 mM NaCl, 0.5% (v/v) Tween 20, and 10 mM Tris-HCl, pH 7.4). Incubation with the secondary anti-rabbit antibody peroxidase-conjugated (from Jackson Laboratories, Inc., Copenhagen, Denmark) was then performed for 1 h at room temperature, and the membranes were washed again with wash buffer. Antibody binding was detected by enhanced chemiluminescence. To assess total amount of affinity-purified mTOR, the membranes were stripped (for 30 min at 50°C) and reprobed with mTab2, the binding of which is not affected by phosphorylation of Ser2448.

**System A Amino acid Transport**—The effect of rapamycin on hormone-stimulated system A amino acid transport was measured in primary cultures of rat hepatocytes. In the presence of l-glutamic acid (AIB), a nonmetabolizable analogue of alanine, as described previously (23). Briefly, the cells were incubated for 16 h with 50 mM rapamycin or MeSO (0.05%) (v/v) in Waymouth medium containing 0.2% (w/v) BSA. The cells were washed twice with phosphate-buffered...
saline, incubated in KRB buffer/0.2% BSA, and stimulated for 3 h with insulin (10^{-7} M), glucagon (10^{-7} M), or CPT-cAMP (10 μs), in the presence of freshly added rapamycin or Me2SO. Transport assays were performed at the end of the incubation period by adding [14C]AIB (0.1 mM, 57 mCi/mmol) for 30 min at 37°C, 5% CO2. Medium containing nonincorporated radioactivity was removed, and the cells were washed twice with phosphate-buffered saline. The uptake data have been corrected for extracellular trapping (assessed by [3H]inulin) and expressed per mg of cell protein.

**RESULTS**

**Effects of Insulin and Amino Acids on Ser2448 Phosphorylation and on Kinase Activity of mTOR**—To determine whether insulin and amino acids have any effect on the phosphorylation of Ser2448, primary cultures of rat hepatocytes were stimulated for 30 min in the presence of insulin or amino acids, or both. The cells were lysed, and incubation of cell lysates with GST-FKB12 beads was performed in the presence of rapamycin to purify mTOR. As controls, incubations were also performed with Me2SO (solvent for rapamycin) or FK506 (26). The samples were then subjected to SDS-PAGE and analyzed by immunoblotting with the anti-Ser(P)2448 antibodies followed by mTab2 (Fig 1A, upper and lower panels, respectively). The pull-down with FKB12-rapamycin complex reveals one molecular species corresponding to mTOR. As expected, no mTOR protein was purified when FKB12 alone or the FKB12-FK506 complex was used for the pull-down assay (Fig. 1A). In the control cells, basal phosphorylation of Ser2448 was seen. Insulin treatment increased this phosphorylation as well as amino acids. Moreover, the combination of insulin and amino acids induced a greater phosphorylation of Ser2448 than insulin or amino acids alone. As shown on the immunoblot with mTab2, approximately the same amount of mTOR protein was purified in each condition of stimulation. Quantification of independent experiments revealed that amino acids and insulin induce Ser2448 phosphorylation by about 5- and 3-fold, respectively, and that their effects are significantly additive (almost 8-fold increase when both are present; Fig. 1B).

Next, we measured in vitro kinase activity of mTOR after being phosphorylated by insulin and/or amino acids in primary cultures of hepatocytes. To this aim, mTOR was immunoprecipitated from cell lysates using the anti-Ser(P)2448 antibodies, and an in vitro kinase assay was performed as described under “Experimental Procedures.” Fig. 2A shows a representative autoradiogram of 32P-labeled [His]4E-BP1, as well as the amount of phosphorylated substrate relative to that obtained in control cells. mTOR kinase activity is stimulated 3- and 5.6-fold in the presence of insulin or amino acids, respectively. The increase reaches 12-fold in the presence of both insulin and amino acids. Moreover, the activity is tightly correlated to the amount of immunoprecipitated phospho-mTOR (Fig. 2B). This result suggests that the increased activity observed when mTOR is phosphorylated corresponds to a greater amount of immunoprecipitated mTOR protein. Thus, we tried to determine mTOR kinase activity after immunoprecipitation of total
mTOR protein. Hepatocytes were stimulated or not in the presence of insulin and amino acids for 30 min, mTOR was immunoprecipitated using mTab2, and the same experiment as the one described in Fig. 2A was performed. Compared with control cells, mTOR kinase activity was significantly increased by 2.2-fold after insulin and amino acid treatment (Fig. 2C). This effect was observed without any change in the amount of mTOR protein in mTab2 immunoprecipitates (not shown). This result indicates that insulin and amino acids have a stimulatory effect on mTOR kinase activity.

**Effect of Rapamycin on Amino Acid Transport Stimulated by Insulin and Glucagon**—Because amino acids are involved in the activation of the mTOR pathway to promote protein synthesis, notably in response to insulin, we investigated whether mTOR pathway could have any effect on the regulation of amino acid transport into the cell. We were interested in the system A amino acid transport because it is increased in response to insulin. Primary cultures of rat hepatocytes were treated with rapamycin for 16 h and then stimulated with insulin or glucagon for 3 h. Indeed, generally speaking, glucagon antagonizes insulin action, but both hormones are able to stimulate the system A amino acid transport in hepatocytes (21). As shown in Fig. 3A, insulin induces a 3-fold increase in AIB uptake, and rapamycin does not significantly modify this effect. Glucagon stimulates the amino acid transport to the same extent as does insulin, even in the presence of rapamycin. Because glucagon increases cAMP production in hepatocytes, we also looked at the effect of CPT-cAMP, a nonmetabolizable analogue of cAMP. AIB influx is increased by 4-fold in the presence of CPT-cAMP, and rapamycin has no further effect (Fig. 3A). Thus, system A amino acid transport does not seem to be regulated by the mTOR pathway in hepatocytes.

We investigated in the same experiment the phosphorylation of mTOR on Ser2448 in the absence of rapamycin (Fig. 3B). After 3 h, insulin has little, if any, effect on mTOR phosphorylation on this site, compared with what was observed after 30 min (Fig. 1). Surprisingly, it seems that phosphorylation of Ser2448 occurs when cells are treated with glucagon or CPT-cAMP (Fig. 3B).
Characterization of mTOR Phosphorylation on Ser^{2448} by Glucagon—We further investigated the effect of glucagon on Ser^{2448} phosphorylation. First, we incubated hepatocytes with glucagon for increased periods of time and analyzed Ser^{2448} phosphorylation after affinity purification of mTOR using FKBP12 and rapamycin (Fig. 4A). Phosphorylation of Ser^{2448} increases in the presence of glucagon after 10 min and reaches a maximum within 30 min (6-fold increase compared with the control condition). Phosphorylation persists until 1 h and then slowly decreases up to 3 h. Thus, like insulin and amino acids, glucagon increases mTOR phosphorylation on Ser^{2448} in primary cultures of hepatocytes.

To compare the effect of glucagon on Ser^{2448} phosphorylation with that of insulin, we also monitored the phosphorylation of this site of mTOR after insulin stimulation between 10 min and 3 h. After cell lysis, mTOR was affinity-purified using GST-FKBP12 and rapamycin, and its phosphorylation was analyzed after SDS-PAGE and immunoblotting using the anti-Ser(P)^{2448} antibodies (Fig. 4B). Insulin maximally stimulates phosphorylation of Ser^{2448} after 10 min, and its effect slowly decreases up to 60 min to reach a steady-state level just above the level of phosphorylation seen in the control condition. In conclusion, in primary cultures of hepatocytes insulin transiently increases mTOR phosphorylation on Ser^{2448}, whereas glucagon has a more sustained effect.

In our initial experiment, the effect on mTOR phosphorylation was studied using a high concentration of glucagon (10^{-7} M). Thus, we determined the phosphorylation of Ser^{2448} within a range of glucagon concentrations, varying from 0.1 nM to 1 uM (Fig. 5). The length of stimulation was 30 min, because this length of incubation appears to lead to the maximal glucagon effect (Fig. 4A). mTOR Phosphorylation on Ser^{2448} was detectable with a concentration as low as 0.1 nM (1.7-fold increase compared with the control condition). Then it gradually augments with increased doses of glucagon to reach a maximum at 100 nM (about 8-fold increase). The EC_{50} is around 1 nM, indicating that phosphorylation of Ser^{2448} by glucagon occurs at physiological hormone concentrations.

Next, we investigated the effect of amino acids on glucagon-induced Ser^{2448} phosphorylation. To do so, hepatocytes were incubated for 10 min with glucagon or CPT-cAMP in the presence or in the absence of 1X amino acids. Fig. 6A shows representative immunoblots of anti-Ser(P)^{2448} antibodies (upper panel) and mTab2 (lower panel). Compared with control conditions, glucagon and CPT-cAMP stimulate Ser^{2448} phosphorylation, and their effect is similar to the one obtained with amino acids. Moreover, the presence of amino acids in the incubation medium does not seem to modify the glucagon- or the CPT-cAMP-induced phosphorylation of Ser^{2448}. Indeed, quantification of the phosphorylated mTOR in the different conditions (Fig. 6B) shows no additive effect of amino acids, contrary to what was observed in the case of insulin (Fig. 1).

Effects of Insulin, Amino Acids, and Glucagon on PKB Activation and on Downstream Targets of mTOR—We were interested in studying the effects of amino acids and glucagon on proteins lying upstream (PKB) or downstream (S6K1 and 4E-BP1) of mTOR. To this aim, hepatocytes were incubated with glucagon in the presence or in the absence of amino acids and lysed, and immunoblots using different antibodies were performed (Fig. 7). Concerning PKB, we looked at the phosphorylation state of Ser^{473} and Thr^{389} because both sites are necessary for its activation (27). As a positive control for PKB activation, the cells were also exposed to insulin. Insulin indeed phosphorylates Ser^{473} and Thr^{389} of PKB (Fig. 7A). Amino acids alone have no effect, and they do not modulate insulin-induced PKB phosphorylation. Regardless of the presence of amino acids, glucagon is not able to induce PKB phosphorylation. In the case of S6K1, we analyzed the phosphorylation state of the protein on Thr^{389}. Phosphorylation of this site is necessary for its activation, and moreover Thr^{389} is a rapamycin-sensitive site (28). Compared with control
cytes were incubated for increased periods of time with glucagon (10^{-11} M). Pharmacological inhibitors of protein phosphatases 1 and 2A were used in their physiological concentrations to test whether glucagon could inhibit S6K1 phosphorylation by activating protein phosphatases. We previously showed that Ser^{64} phosphorylation is required for the generation of the γ species (20). As seen with the phosphospecific antibodies, glucagon inhibits Ser^{64} phosphorylation (Fig. 7C, top panel). This is reflected by the predominance of the α species after incubation with glucagon, whereas the γ species is more abundant in the presence of insulin and/or amino acids (Fig. 7C, middle panel). In conclusion, amino acids and glucagon do not activate PKB in hepatocytes. However, the amino acids lead to phosphorylation of S6K1 and 4E-BP1, whereas glucagon inhibits phosphorylation of Thr^{389}, i.e. full activation of S6K1. Glucagon does not induce any phosphorylation of Thr^{389}. Interestingly, glucagon markedly inhibits S6K1 phosphorylation induced by insulin, amino acids, or the combination of both. Immunoblot with anti-S6K1 antibodies (Fig. 7B, lower panel) shows that the phosphorylation of Thr^{389} is found only in the molecular species with the lowest electrophoretic mobility, indicating that the phosphorylated site is present exclusively in the fully activated form of S6K1. Glucagon appears to inhibit phosphorylation of 4E-BP1 on Ser^{64}, because the γ species of 4E-BP1 decreases or totally disappears after insulin and/or amino acids stimulation (Fig. 7C, middle panel). The pattern of 4E-BP1 species also reflects glucagon-induced inhibition of 4E-BP1 phosphorylation. We previously showed that Ser^{64} phosphorylation is required for the generation of the γ species (20). As seen with the phosphospecific antibodies, glucagon inhibits Ser^{64} phosphorylation (Fig. 7C, top panel). This is reflected by the predominance of the α species after incubation with glucagon, whereas the γ species is more abundant in the presence of insulin and/or amino acids (Fig. 7C, middle panel). In conclusion, amino acids and glucagon do not activate PKB in hepatocytes. However, the amino acids lead to phosphorylation of S6K1 and 4E-BP1, whereas glucagon inhibits their phosphorylation.

The Inhibitory Effect of Glucagon on S6K1 Activation Does Not Appear to Be Mediated by a Protein Phosphatase Type 1/Protein Phosphatase Type 2A-dependent Pathway—To test whether glucagon could inhibit S6K1 phosphorylation by activating protein phosphatases, we used okadaic acid, an inhibitor of protein phosphatases 1 and 2A (29). In the absence of any stimulation, okadaic acid slightly increases S6K1 phosphorylation on Thr^{389} (Fig. 8), and it potentiates the positive effect of insulin and amino acids on this site. As shown in Fig. 7, glucagon prevents phosphorylation of Thr^{389}, and this inhibition persists, even in the presence of okadaic acid (Fig. 8). This result indicates that protein phosphatases 1 and 2A do not appear to play an important role in glucagon-induced inhibition of S6K1 phosphorylation.

**DISCUSSION**

Insulin and amino acids are known activators of the mTOR pathway. To study their mode of action on mTOR, we have looked at the phosphorylation of serine residue 2448 of mTOR. Indeed, this PKB consensus phosphorylation site has been proposed to play a positive role in mTOR kinase activity (10). We used primary cultures of rat hepatocytes, because mTOR has been poorly studied in this insulin-sensitive cellular model. First, we show that insulin alone induces the phosphorylation of Ser^{2448}. This result correlates with those found in other cell types (13, 14, 19). A mixture of amino acids at physiological concentrations is also able to increase Ser^{2448} phosphorylation. Moreover, we find that insulin and amino acids have an additive effect on Ser^{2448} phosphorylation, which implies that they use distinct signaling pathways. In the case of insulin we can assume that the phosphatidylinositol 3-kinase/PKB pathway is involved, because insulin activates PKB in hepatocytes (Fig. 7A). However, PKB is not activated by amino acids. Thus, their positive effect on Ser^{2448} phosphorylation could be explained by the inhibition of a phosphatase and/or the activation of a "PKB-like" kinase that would recognize Ser^{2448} and phosphorylate it.

The use of rapamycin was without effect on amino acid transport stimulated by different agents (Fig. 3A). This indicates that this cellular response is independent of mTOR or is.

**FIG. 4.** Time-course of glucagon- and insulin-induced phosphorylation of mTOR on Ser^{2448}. A, primary cultures of rat hepatocytes were incubated for increased periods of time with glucagon (10^{-7} M). mTOR was affinity-purified using rapamycin and GST-FBP12. The samples were subjected to SDS-PAGE, and an immunoblot was prepared with the anti-Ser^{2448} antibodies. Phosphorylated mTOR was quantitated, and the values were corrected for total mTOR, which was determined from mTab2 blots. The results are expressed as percentages of the control values and are the means ± S.E. from three independent experiments, each performed in duplicate. B, same experiment as the one described in A, except that hepatocytes were stimulated for increased periods of time with insulin (10^{-7} M). The results are expressed as percentages of the control values and are the means ± S.E. from two independent experiments, each performed in duplicate.

**FIG. 5.** Dose-response of glucagon-induced phosphorylation of mTOR on Ser^{2448}. Primary cultures of rat hepatocytes were stimulated for 30 min with increased concentrations of glucagon. mTOR was affinity-purified using rapamycin and GST-FBP12. The samples were subjected to SDS-PAGE and immunoblots were prepared with the anti-Ser^{2448} antibodies. Phosphorylated mTOR was quantitated, and the values were corrected for total mTOR, which was determined from mTab2 immunoblots. The results are expressed as percentages of the control values and are the means ± S.E. from three independent experiments, each performed in duplicate.

conditions, insulin and amino acids slightly increase Thr^{389} phosphorylation. (Fig. 7B, upper panel). However, the presence of both insulin and amino acids is needed to obtain a maximal.

mTOR Regulation by Glucagon

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mTOR-dependent but insensitive to rapamycin. Indeed, the complex formed between rapamycin and its intracellular receptor, the FKBP12 protein, binds mTOR but does not inhibit directly its catalytic activity (30, 31). It has been demonstrated by McMahon et al. (32) that the phosphorylation sites in 4E-BP1 and p70S6K do not have the same degree of sensitivity toward rapamycin inhibition. Moreover, Edinger et al. (33) have recently shown in FL5.12 lymphocytic cells that the amino acid transporter trafficking was altered by a kinase-inactive mutant of mTOR but not by rapamycin. Therefore, it would be of interest to see whether such a mutant could impair amino acid transport in our cellular model, before reaching a final conclusion about the role of mTOR in this effect. Nevertheless, our investigation on the potential role of mTOR in the stimulation of amino acid transport allowed us to demonstrate that glucagon stimulates Ser^2448 phosphorylation of mTOR. This unexpected effect of glucagon occurs for hormone concentrations as low as 1 nM. Glucagon is more potent than insulin in inducing Ser^2448 phosphorylation, because the magnitude of the effect is greater and lasts longer. Contrary to what is seen with insulin, the effect of glucagon is not further increased by the presence of amino acids. This finding can be explained either by the fact that the effect of glucagon by itself is maximal or that glucagon and amino acids activate the same pathway to promote Ser^2448 phosphorylation. Like amino acids, glucagon does not activate PKB, and the kinase(s) involved in in vivo mTOR phosphorylation remains to be identified.

We investigated in vitro mTOR kinase activity after immunoprecipitation of phosphorylated mTOR from primary cultures of hepatocytes. Our results do not argue for a positive role of this site. Indeed, we show that phosphorylation of Ser^2448 in mTOR does not seem to significantly modulate or that glucagon and amino acids activate the same pathway to promote Ser^2448 phosphorylation. Like amino acids, glucagon does not activate PKB, and the kinase(s) involved in in vivo mTOR phosphorylation remains to be identified. We investigated in vitro mTOR kinase activity after immunoprecipitation of phosphorylated mTOR from primary cultures of hepatocytes. Our results do not argue for a positive role of this site. Indeed, we show that phosphorylation of Ser^2448 in mTOR does not seem to significantly modulate or that glucagon and amino acids activate the same pathway to promote Ser^2448 phosphorylation. Like amino acids, glucagon does not activate PKB, and the kinase(s) involved in in vivo mTOR phosphorylation remains to be identified.

We investigated in vitro mTOR kinase activity after immunoprecipitation of phosphorylated mTOR from primary cultures of hepatocytes. Our results do not argue for a positive role of this site. Indeed, we show that phosphorylation of Ser^2448 in mTOR does not seem to significantly modulate or that glucagon and amino acids activate the same pathway to promote Ser^2448 phosphorylation. Like amino acids, glucagon does not activate PKB, and the kinase(s) involved in in vivo mTOR phosphorylation remains to be identified.
Fig. 7. Effects of insulin, amino acids and glucagon on PKB S6K1 and 4E-BP1 phosphorylation in primary cultures of rat hepatocytes. Hepatocytes were stimulated as follows: insulin (10^{-7} M), MEM amino acids (1× final concentration), glucagon (10^{-7} M), or the different combinations of the three agents. A, cell extracts were subjected to SDS-PAGE and immunoblotted with phosphospecific PKB antibodies (anti-Ser(P)473 and anti-Thr(P)308) or anti-PKB antibodies. B, cell extracts were subjected to SDS-PAGE and immunoblotted with phosphospecific S6K1 antibodies (anti-Thr(P)389) or anti-S6K1 antibodies. C, to detect 4E-BP1 phosphorylation, the protein was immunoprecipitated from 700 µg of cell lysates, using 3 µg of 4E-BP1 antibody. The samples were immunoblotted with phosphospecific antibodies to Thr(P)^36/45 and Ser(P)^64 of 4E-BP1 (top and middle panel, respectively). To detect the relative amounts of the α, β, and γ forms of 4E-BP1 in each condition of cell stimulation, the cell extracts were immunoblotted with 4E-BP1 antibody (bottom panel).
ate 4E-BP1 in vitro. Moreover, the regulation of the kinase activity seems to be more complicated than anticipated by the recent identification of raptor (regulated associated protein of mTOR). Indeed, the interaction of raptor with mTOR and its substrates is necessary for effective mTOR-catalyzed phosphorylation (34). Regardless of the role of Ser2448 phosphorylation, we have shown in this study that insulin and amino acids significantly increase mTOR kinase activity. This might be due to an increase either in its intrinsic catalytic activity or in its association with raptor. However, the effect of insulin and amino acids could be independent of mTOR and because of a regulation of proteins lying between PKB and mTOR, such as the TSC1-TSC2 complex and the small GTP-binding protein Rheb (4). For example, it has been shown that PKB phosphorylates TSC2, modulating its activity (35–37). Therefore, the activation of S6K1 by insulin and amino acids is due, in part, to increased mTOR activation.

Scott and Lawrence (38) have shown, in 3T3-L1 adipocytes, that increasing cAMP levels attenuates the effects of insulin on 4E-BP1 and p70S6K by inhibiting the phosphorylation at the C terminus of mTOR and its activation. Because we show that glucagon and CPT-cAMP both increase mTOR phosphorylation, we were interested in looking at the effect of increased cAMP levels on downstream effectors of mTOR in hepatocytes. We show that in the presence of glucagon, S6K1 and 4E-BP1 are no longer phosphorylated by insulin and amino acids, at least on the rapamycin-sensitive sites Thr389 for S6K1, Ser424 and Thr3645 for 4E-BP1. Therefore, it seems that glucagon has contradictory effects on the mTOR pathway. Indeed, although it enhances mTOR phosphorylation, it reduces S6K1 activation and inhibits the phosphorylation of the translational repressor eBP1.

We tried to test different hypotheses to understand the molecular mechanism leading to inhibition of the mTOR/S6K1 pathway by glucagon. Our experiments with okadaic acid lead us to suggest that the glucagon-mediated pathway does not involve protein phosphatases 1 and 2A. It has been proposed in Jurkat cells that mTOR inhibits protein phosphatase type 2A, preventing 4E-BP1 and S6K1 dephosphorylation (39). However, our study clearly shows that okadaic acid, which mainly inhibits protein phosphatase type 2A, markedly increases the effect of insulin and amino acids on S6K1 phosphorylation at Thr389 site. It should be stressed that okadaic acid by itself has a weak effect on Thr389 phosphorylation and does not prevent the reduced phosphorylation of this site upon glucagon stimulation. Therefore our result also indicates that in hepatocytes the mTOR pathway does not primarily inhibit the phosphatase 2A to promote p70S6K phosphorylation, at least on the rapamycin-sensitive site Thr389. Another possibility is that glucagon could act on mTOR directly. We have not yet determined the effect of glucagon on in vitro mTOR kinase activity. However, in addition to increasing Ser2448 phosphorylation, glucagon could phosphorylate other sites in the enzyme, leading to an impairment of mTOR kinase activity. In this regard, glucagon and cAMP could also stimulate the phosphorylation of Thr2446 leading to mTOR inhibition, as recently proposed by Cheng et al. (40). Indeed, the authors show a differential phosphorylation of Ser2448 and Thr2446 leading to positive and negative control of mTOR, respectively. However, if, as stated, Ser2448 phosphorylation inhibits Thr2446, one might expect that glucagon and cAMP prevent Thr2446 phosphorylation, because we show that both agents lead to strong Ser2448 phosphorylation. Finally, in co-immunoprecipitation experiments performed between mTOR and raptor, we did not find modifications of the interaction in the presence of glucagon (not shown). This indicates that the hormone does not appear to act by blocking the access of mTOR to its substrates.

In conclusion, we show here in rat hepatocytes that insulin and amino acids positively regulate the mTOR pathway and that they increase mTOR kinase activity. They also stimulate mTOR phosphorylation on Ser2448, but this site does not seem to be involved in mTOR activation. We demonstrate, for the first time to the best of our knowledge, that glucagon increases mTOR Ser2448 phosphorylation. This is unexpected because glucagon inhibits the mTOR pathways, arguing against a positive role of Ser2448 in mTOR activation. We favor the idea that phosphorylation of this site, rather than playing a role in the activation of the enzyme, could be involved in the association of a protein that might be important for the intracellular localization and/or stability of mTOR. However, we cannot exclude the possibility that phosphorylation on Ser2448 affects the phosphorylation of mTOR substrates, which remain to be identified. Finally, our findings suggest that insulin and glucagon have an antagonistic effect on S6K1 and 4E-BP1, which might be important in the control of cell growth by regulating differently S6K1- and eukaryotic initiation factor 4E-dependent mRNA translation regulated by 4E-BP1.

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