

Mechanisms Involved in the Coordinate Regulation of mTORC1 by Insulin and Amino Acids^{*[5]}

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In this study, we explored the coordinate regulation of mTORC1 by insulin and amino acids. Rat livers were perfused with medium containing various concentrations of insulin and/or amino acids. At fasting (1×) or 2× (2×AA) concentrations of amino acids, insulin maximally stimulated Akt phosphorylation but had no effect on global rates of protein synthesis. In the absence of insulin, 4×AA produced a moderate stimulation of protein synthesis and activation of mTORC1. The combination of 4×AA and insulin produced a maximal stimulation of protein synthesis and activation of mTORC1. These effects were accompanied by decreases in raptor and PRAS40 and an increase in RagC associated with mTOR (mammalian target of rapamycin). The studies were extended to a cell culture model in which mTORC1 activity was repressed by deprivation of leucine and serum, and resupplementation with the amino acid and insulin acted in an additive manner to restore mTORC1 activation. In deprived cells, mTORC1 was activated by expressing either constitutively active (ca) Rheb or a caRagB-caRagC complex, and coexpression of the constructs had an additive effect. Notably, resupplementation with leucine in cells expressing caRheb or with insulin in cells expressing the caRagB-caRagC complex was as effective as resupplementation with both leucine and insulin in non-transfected cells. Moreover, changes in mTORC1 activity correlated directly with altered association of mTOR with RagB/RagC, Rheb, raptor, and PRAS40. Overall, the results suggest that amino acids signal through the Rag complex and insulin through Rheb to achieve coordinate activation of mTORC1.

Global rates of protein synthesis in the liver are repressed after an overnight fast but recover rapidly in response to refeeding (e.g. Ref. 1). The feeding-induced stimulation of protein synthesis is in large part due to modulation of signaling through mTORC1 (mammalian target of rapamycin (mTOR) complex 1), as evidenced by ablation of the response by the selective inhibitor rapamycin (2). Signaling through mTORC1 acutely stimulates protein synthesis through multiple mechanisms, including phosphorylation and activation of several down-

stream proteins involved in the binding of mRNA to the 40 S ribosomal subunit. mTORC1 is activated by hormones, such as insulin, and nutrients, such as amino acids, with the branched-chain amino acid leucine being the most potent in the liver (3). Thus, the stimulation of hepatic protein synthesis in refeed animals could be due to either increased plasma insulin or amino acid concentrations or both. In this regard, a study utilizing a pancreatic/amino acid clamp to precisely maintain insulin and amino acids at specific concentrations showed that at fasting insulin concentrations, increasing amino acids from fasted to fed values led to increased rates of hepatic protein synthesis (4). In contrast, increasing insulin at fasting amino acid concentrations had no effect on protein synthesis. However, when insulin and amino acid concentrations were simultaneously elevated, the magnitude of the increase in protein synthesis was greater than when either alone was raised. Thus, the stimulation of global rates of hepatic protein synthesis in response to refeeding is likely a consequence of increases in plasma concentrations of both insulin and amino acids acting in a coordinate manner to activate mTORC1.

Insulin-induced activation of mTORC1 occurs primarily through the PI3K/Akt signaling pathway (5, 6). Activation of Akt by insulin leads to the phosphorylation of at least two proteins involved in the regulation of mTORC1, PRAS40 (proline-rich Akt substrate of 40 kDa) and TSC2 (tuberous sclerosis complex 2). PRAS40 binds to raptor (regulatory-associated protein of mTOR), a component of mTORC1, and blocks its interaction with substrates such as S6K1 and 4E-BP1, thereby preventing their phosphorylation. Phosphorylation of PRAS40 by Akt results in its dissociation from mTORC1, allowing raptor to recruit S6K1 and 4E-BP1 to the complex for phosphorylation. TSC2, in a complex with TSC1, acts as a GTPase activator protein for Rheb (Ras homolog enriched in brain). Through an incompletely defined mechanism, binding of Rheb-GTP, but not Rheb-GDP, to mTORC1 results in its activation. Phosphorylation of TSC2 by Akt results in inhibition of its GTPase activator activity, leading to increased GTP loading on Rheb and consequently increased mTORC1 activity. Although the mechanism through which amino acids act to stimulate mTORC1 activity is incompletely defined, they are thought to function through a pathway distinct from either TSC2 or Rheb. Instead, recent studies have implicated the heterodimeric Rag GTPases in the amino acid-induced activation of mTORC1 (7, 8). Based on those studies, a model has been proposed (9) in which the Rag GTPases bind to mTORC1 in an amino acid-dependent manner and, via interaction with a complex termed Ragulator, promote its translocation to lysosomal membranes, where

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1 and Tables S1–S4.

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mTORC1 can interact with Rheb-GTP, thereby resulting in its activation.

The purpose of this study was to develop a better understanding of how insulin- and amino acid-induced signaling inputs coordinately regulate mTORC1 signaling and protein synthesis in the liver. The hypothesis being tested was that insulin and amino acids act in a coordinate manner to activate mTORC1 and consequently to stimulate protein synthesis. The results provided herein support the hypothesis and are consistent with a model in which insulin signals primarily through Rheb, but not the Rag GTPases, whereas amino acids signal to mTORC1 through the Rag GTPases, but not Rheb. The findings also suggest that insulin and amino acids activate mTORC1 by altering its interaction with regulatory proteins such as raptor, PRAS40, Rheb, and the Rag GTPases.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitor mixture was purchased from Sigma, and ECL Western blotting detection reagent from Pierce. Horseradish peroxidase-conjugated anti-mTOR, anti-S6K1, anti-phospho-S6K1 (Thr-389), and goat anti-rabbit IgG antibodies were purchased from Bethyl Laboratories. Anti-phospho-mTOR (Ser-1261) antibody was a kind gift from Dr. Diane C. Fingar (Cell and Developmental Biology, University of Michigan). Anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, and all other antibodies were purchased from Cell Signaling Technology. Preparation of anti-eIF4G antibody has been described previously (10). Cell culture medium lacking leucine, histidine, and pyruvate was a custom formulation purchased from Atlanta Biologicals; histidine was added to the medium prior to use.

In Situ Liver Perfusion—Male Sprague-Dawley rats weighing ~125 g were maintained on a 12/12-h light/dark cycle, with food (Harlan Teklad) and water provided *ad libitum*. The experimental protocol used for the studies described herein was reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Livers were perfused *in situ* with a non-circulating medium at a flow rate of 7 ml/min as described previously (11), with the following modifications. Insulin (1.74 mM in 0.155 M NaCl (pH 2.0)) was infused directly into the inflow line at a rate of 0.2 ml/min to produce a final calculated concentration of 1 or 10 nM. The perfusate also contained amino acids at one (1×), two (2×), or four (4×) times the concentrations present in the arterial plasma of a fasted rat (12). The total perfusion time was 20 min. For determination of global rates of protein synthesis, livers were perfused for an additional 15 min with [³H]phenylalanine (1 μCi/ml) and prepared for analysis as described previously (13). Briefly, following excision, a portion of the liver was weighed and homogenized in 7 volumes of buffer consisting of 20 mM HEPES (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate using a Dounce homogenizer. A 250-μl aliquot of homogenate was used to assess the rate of incorporation of [³H]phenylalanine into liver protein, from which the global rate of protein synthesis was calculated as described previously (14) using the specific radioactivity of [³H]phenylalanine in the

perfusate. The elapsed time for incorporation was calculated as the time from the start of perfusion with the radiolabel to the time the tissue was homogenized.

Processing of Liver Samples—For the analysis of protein phosphorylation state, a portion (~0.3 g) of liver was homogenized in 7 volumes of CHAPS lysis buffer (40 mM HEPES, 0.3% CHAPS, 1 mM EDTA, 50 mM NaF, 120 mM NaCl, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM benzamidine, 200 mM sodium vanadate, and 10 μl/ml protease inhibitor mixture) using a Polytron homogenizer. The homogenate was centrifuged at 1000 × *g* for 3 min at 4 °C, and the resulting supernatant was subjected to SDS-PAGE and Western blot analysis as described previously (11). Phosphorylation of S6K1, Akt, 4E-BP1, mTOR, and TSC2 was measured in the supernatants using phospho-specific antibodies as described previously (11).

Cell Culture and Transfections—Cultures of Rat2 fibroblasts were maintained in Dulbecco's modified Eagle's medium lacking sodium pyruvate and containing high glucose (Invitrogen) supplemented with 10% fetal bovine serum (Atlas) and 1% penicillin/streptomycin (Invitrogen). Transfections were performed using FuGENE HD (Roche Applied Science) with an 8:2 ratio of reagent to DNA (μl/μg) according to the manufacturer's instructions. The following plasmids were used in transfections: pRK5 and pCMV5 control plasmids; pRK5-HA-GST-Rheb and pRK5-Myc-Rheb-S16H (kindly provided by Dr. Kun-Liang Guan); pRK5-HA-GST-RagC, pRK5-HA-GST-RagC-S75L, pRK5-HA-GST-RagB, and pRK5-HA-GST-RagB-Q99L (purchased from Addgene; submitted to Addgene by Dr. David M. Sabatini); and pCMV5-HA-caAkt (purchased from Addgene; submitted to Addgene by Dr. Mien-Chie Hung). Constitutively active (ca)² Akt lacks the pleckstrin homology domain (amino acids 4–129) but has an Src myristoylation signal sequence added to the N terminus (called myrAktΔ4–129) and a HA tag added to the C terminus. After 6 h, the transfection reagent was removed, and cells were maintained overnight in fresh medium. Cells were deprived of serum and leucine for 2 h and then treated with 0.76 mM leucine (Sigma) and/or 10 nM insulin (Novolin) for 30 min. Cells were harvested in 1× SDS sample buffer for analysis of cell lysate or CHAPS lysis buffer for immunoprecipitation.

Immunoprecipitations—Immunoprecipitations were performed by incubating 1000 × *g* supernatants of liver homogenates or cell lysates overnight with anti-mTOR polyclonal, anti-raptor monoclonal (Cell Signaling Technology), or anti-eIF4E monoclonal antibody. Homogenate or lysate containing ~1 mg of protein was incubated with 0.5 μg of antibody overnight at 4 °C. The next morning, 200 μg of BioMag goat anti-rabbit or anti-mouse IgG beads (Qiagen), previously blocked with CHAPS lysis buffer containing 1% milk, was added to each sample, and the suspension was rocked at 4 °C for 2 h. Beads were washed twice with 1 ml of ice-cold CHAPS lysis buffer, suspended in 1× SDS sample buffer, and then boiled for 5 min. Supernatants were subjected to Western blot analysis using antibodies to mTOR, raptor, RagC, PRAS40, S6K1, and 4E-BP1,

² The abbreviations used are: ca, constitutively active; AA, amino acids.

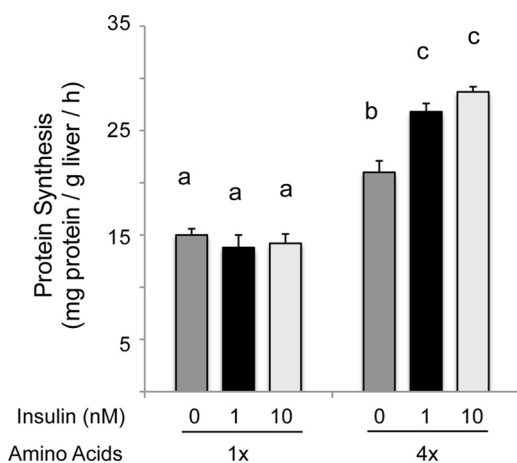


FIGURE 1. Amino acids are necessary for insulin-induced stimulation of protein synthesis in perfused liver. Rat livers were perfused *in situ* with a non-recirculating medium containing either 1×AA or 4×AA in combination with 0, 1, or 10 nM insulin. Rates of hepatic protein synthesis were measured by the incorporation of [³H]phenylalanine into protein as described under "Experimental Procedures." Values are means ± S.E. ($n = 6$). Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different ($p < 0.05$).

and the results were normalized for the amount of the target protein in the immunoprecipitate.

Statistical Analysis—The data are expressed as means ± S.E. One-way analysis of variance and Student's *t* test were used to compare differences among groups. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Insulin and Amino Acids on Liver Protein Synthesis—In this study, the coordinate roles of insulin- and amino acid-induced signaling in the regulation of mTORC1 were investigated using, as a model system, rat livers perfused with amino acids at concentrations occurring in the post-absorptive plasma of fasted rats (referred to as 1×AA) or at 2 (2×AA) or 4 (4×AA) times those concentrations in the presence or absence of 1 or 10 nM insulin. In the absence of insulin, 4×AA were sufficient to significantly stimulate hepatic protein synthesis (Fig. 1). The effect of 4×AA on protein synthesis was further enhanced by either 1 or 10 nM insulin. However, in the presence of 1×AA, insulin had no significant effect on protein synthesis. In combination, 4×AA and 10 nM insulin resulted in nearly a 2-fold increase in global rates of protein synthesis compared with perfusion with 1×AA alone. These findings are in agreement with studies showing that changes in plasma insulin in response to feeding a protein-free diet are not sufficient to stimulate protein synthesis (1), suggesting a requirement for signaling inputs from both dietary protein and insulin.

Regulation of Cell Signaling in Liver by Insulin and Amino Acids—In livers perfused in the absence of insulin, increasing amino acid concentrations from 1×AA to 4×AA led to a significant decrease in the amount of 4E-BP1 bound to eIF4E (Fig. 2A), a significant increase in the phosphorylation of eIF4G at Ser-1108 (Fig. 2B), and an increase in both the proportion of S6K1 in the hyperphosphorylated form (Fig. 2C) and in the phosphorylation of the protein at Thr-389 (Fig. 2D). In contrast, in livers perfused with 1×AA, insulin had no effect on

either 4E-BP1 binding to eIF4E or the phosphorylation of eIF4G, although the phosphorylation of S6K1 was modestly increased. Importantly, a combination of 4×AA and insulin caused a significantly greater change in each end point compared with either input alone, suggesting that amino acids and insulin act in a coordinate manner to activate mTORC1.

A possible explanation for the lack of effect of insulin on dissociation of the 4E-BP1·eIF4E complex or stimulation of eIF4G phosphorylation in livers perfused with 1×AA is that increased amino acid concentrations are required for insulin-induced activation of the PI3K/Akt signaling pathway. To further examine this possibility, changes in Akt phosphorylation at Ser-473 were assessed. As shown in Fig. 2E, insulin-induced Akt phosphorylation was increased to the same extent regardless of perfusate amino acid concentrations. However, amino acids in the absence of insulin had no effect, a result consistent with previous findings in cell culture that amino acids act downstream of Akt and TSC2 to activate mTORC1 (15). To further confirm the finding that insulin acts independently of amino acids to activate Akt, mTOR phosphorylation on Ser-2448, a site directly phosphorylated by Akt, was assessed (16). As shown in Fig. 2F, in the absence of insulin, raising perfusate amino acids from 1× to 4× had no significant effect on the phosphorylation of mTOR at Ser-2448. In contrast, insulin promoted the phosphorylation mTOR at Ser-2448 regardless of amino acid concentrations. Notably, no synergy between insulin and amino acids was evident.

Effect of Insulin and Amino Acids on mTORC1 Assembly in Liver—As discussed further below, current models propose that activation of mTORC1 results in a conformational change leading to destabilization of the complex (17). In this study, the association of mTOR with raptor was evaluated by immunoprecipitating either mTOR or raptor from liver homogenates and assessing the amount of each protein in the immunoprecipitates. As shown in Fig. 3 (A and B), the mTOR-raptor association was weakened in livers perfused with either 4×AA or 1×AA supplemented with 10 nM insulin compared with control livers perfused with 1×AA alone. A further reduction in mTOR-raptor association was seen in livers perfused with a combination of 4×AA and insulin, which resulted in a significantly larger reduction in the amount of raptor in mTOR immunoprecipitates compared with perfusion with either 4×AA or insulin alone (Fig. 3A). Notably, the effect of insulin and amino acids on mTOR-raptor interaction was greater in raptor compared with mTOR immunoprecipitates (compare the *fourth lanes* in Fig. 3, A and B). The difference was likely due to the presence of both mTORC1 and mTORC2, which contains rictor rather than raptor, in the mTOR immunoprecipitates.

A similar outcome was observed for the effects of insulin and amino acids on PRAS40 association with mTOR. The amount of PRAS40 present in mTOR immunoprecipitates was highest in livers perfused with 1×AA alone and was reduced by ~20% when livers were perfused with either 4×AA or 1×AA plus insulin (Fig. 3C). The effects of insulin and amino acids were additive, as livers perfused with 4×AA plus insulin showed an ~40% drop in the association of PRAS40 with mTOR. These findings support previous studies in cells in culture showing

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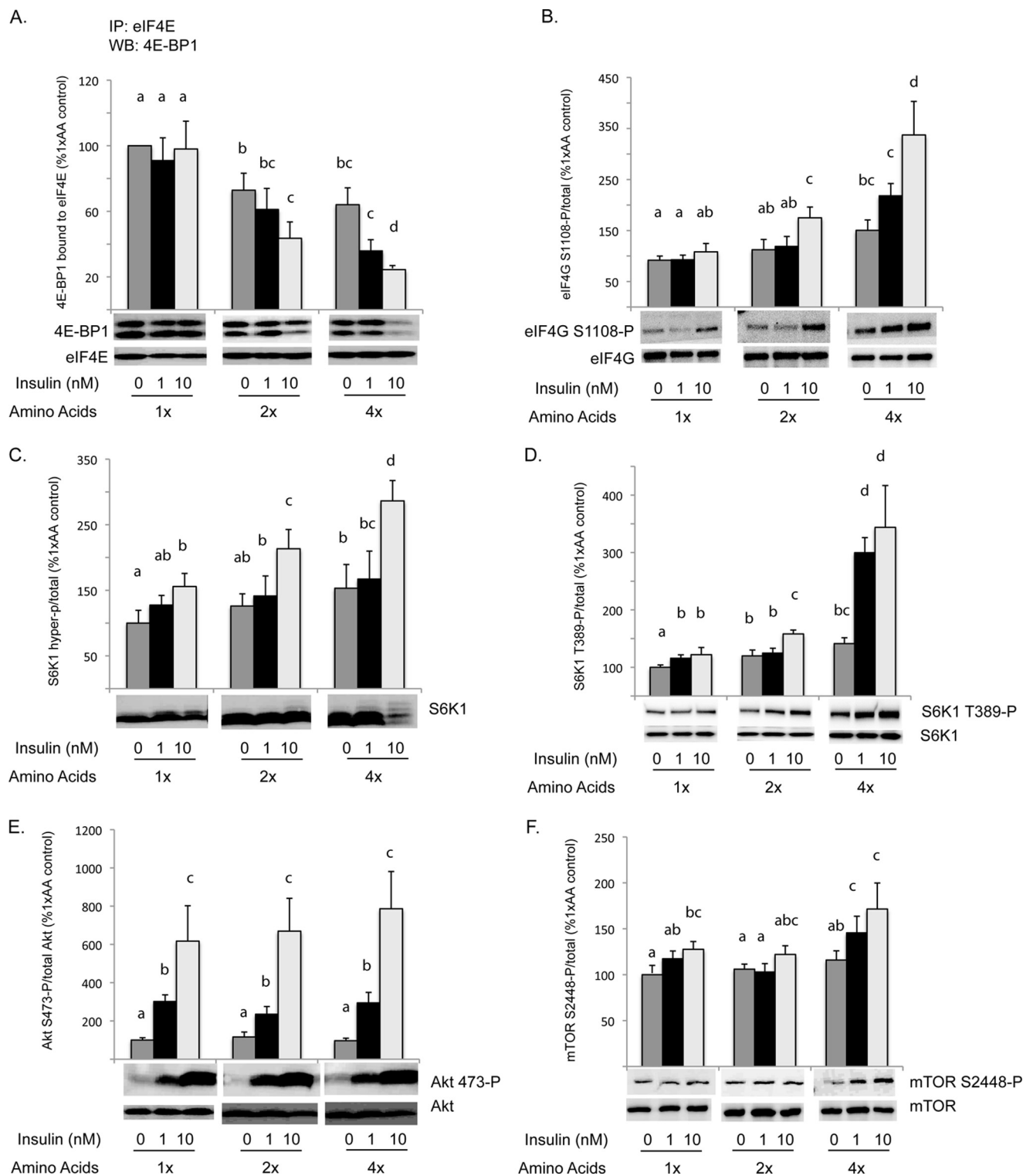


FIGURE 2. Effects of insulin and amino acids on mTORC1 signaling in perfused liver. Rat livers were perfused as described in the legend to Fig. 1. Livers were harvested, and mTORC1 signaling was assessed by Western blot analysis. *A*, the association of eIF4E with 4E-BP1 was examined by immunoprecipitating (IP) eIF4E and measuring the amount of eIF4E and 4E-BP1 present in the immunoprecipitate. *B*, eIF4G phosphorylation at Ser-1108 was analyzed by Western blotting (WB). *C*, S6K1 hyperphosphorylation was assessed by measuring the proportion of the protein present in the β , γ , and δ forms relative to the total amount of S6K1 present in all four forms ($\alpha + \beta + \gamma + \delta$). *D–F*, S6K1 phosphorylation at Thr-389, Akt phosphorylation at Ser-473, and mTOR phosphorylation at S-2448, respectively, were analyzed by Western blotting. *Insets* show representative blots. Values are means \pm S.E. ($n = 6$). Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different ($p < 0.05$).

that PRAS40 disassociates from mTORC1 in response to insulin and nutrients (18, 19) and extend them to a more physiologically relevant model, *i.e.* an intact organ.

In contrast to the finding with raptor and PRAS40, the association of RagC with mTOR was significantly increased when livers were perfused with both insulin and 4 \times AA compared

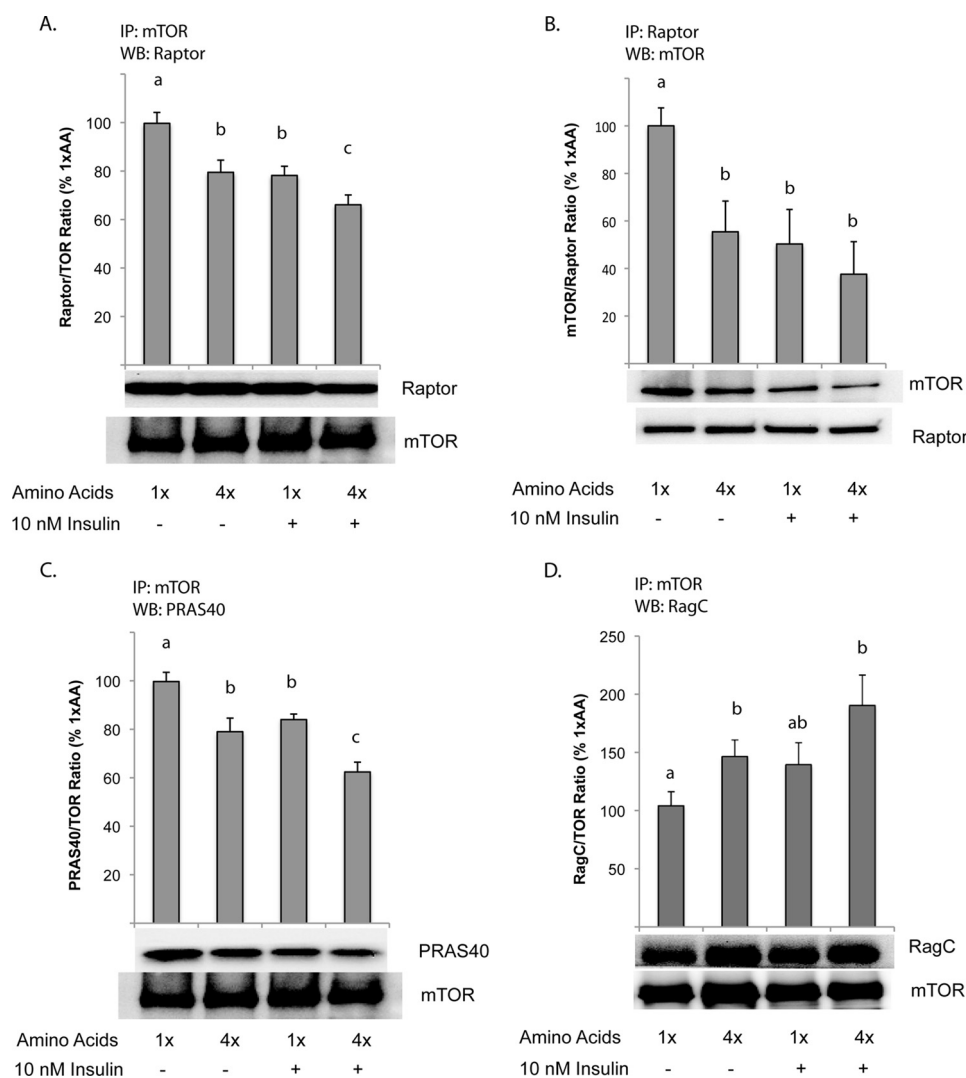


FIGURE 3. Effects of insulin and amino acids on mTOR complex assembly in perfused liver. Rat livers were perfused as described in the legend to Fig. 1. The association of mTOR with raptor (A), PRAS40 (C), and RagC (D) was examined by immunoprecipitating (IP) mTOR from liver homogenates and measuring the amount of each protein in the immunoprecipitates by Western blot (WB) analysis. Similarly, raptor was immunoprecipitated from homogenates, followed by Western blot analysis of the immunoprecipitates for raptor and mTOR (B). Insets show representative blots. Values are means \pm S.E. ($n = 4-8$). Statistical significance is denoted by the presence of different letters above the bars. Bars not sharing a letter are statistically different ($p < 0.05$).

with the 1 \times AA control (Fig. 3D). This finding is not only the first evidence of a mTORC1-Rag interaction in an intact organ but is also the first to demonstrate that the interaction between endogenous mTOR and RagC is influenced by growth factors in addition to nutrients.

Effects of Leucine and Insulin on mTORC1 Signaling and Complex Assembly in Rat2 Cells in Culture—To further evaluate the effects of insulin- and amino acid-induced signaling inputs on mTORC1, Rat2 cells were employed as an experimental model. These cells were chosen as a model system because mTORC1 signaling displays rapid responses to changes in hormones and nutrients, and the effect of amino acids occurs at physiological concentrations. In this study, the cells were deprived of serum and the single amino acid leucine for 2 h prior to the addition of 10 nM insulin and/or 0.76 mM leucine. The addition of leucine to deprived cells led to increased phosphorylation of mTOR at Ser-2448 and S6K1 at Thr-389, but not TSC2 at Thr-1461 or Akt at Ser-473 (Fig. 4A and supplemental Table S1). In contrast, in the absence of leu-

cine, insulin promoted the phosphorylation of mTOR at Ser-2448, TSC2 at Thr-1461, S6K1 at Thr-389, Akt at Ser-473, PRAS40 at Thr-246, and 4E-BP1 at Thr-37/Thr-46 compared with values seen in serum- and leucine-deprived cells. Importantly, combined treatment with leucine and insulin led to an larger increase in the phosphorylation of mTOR at Ser-2448, S6K1 at Thr-389, and 4E-BP1 at Thr-37/Thr-46 compared with either one alone.

To evaluate effects on mTORC1 assembly, mTOR was immunoprecipitated from cells, and the amount of various proteins in the immunoprecipitate was evaluated by Western blot analysis. Deprivation of cells of serum and leucine for 2 h increased the amount of raptor and PRAS40 present in the mTOR immunoprecipitates compared with cells maintained in complete medium (Fig. 4B and supplemental Table S2). Similar to the findings shown earlier for liver homogenates, treatment of cells with insulin reduced the amount of raptor and PRAS40 associated with mTOR. The association was further reduced when cells were treated with leucine in combination with insu-

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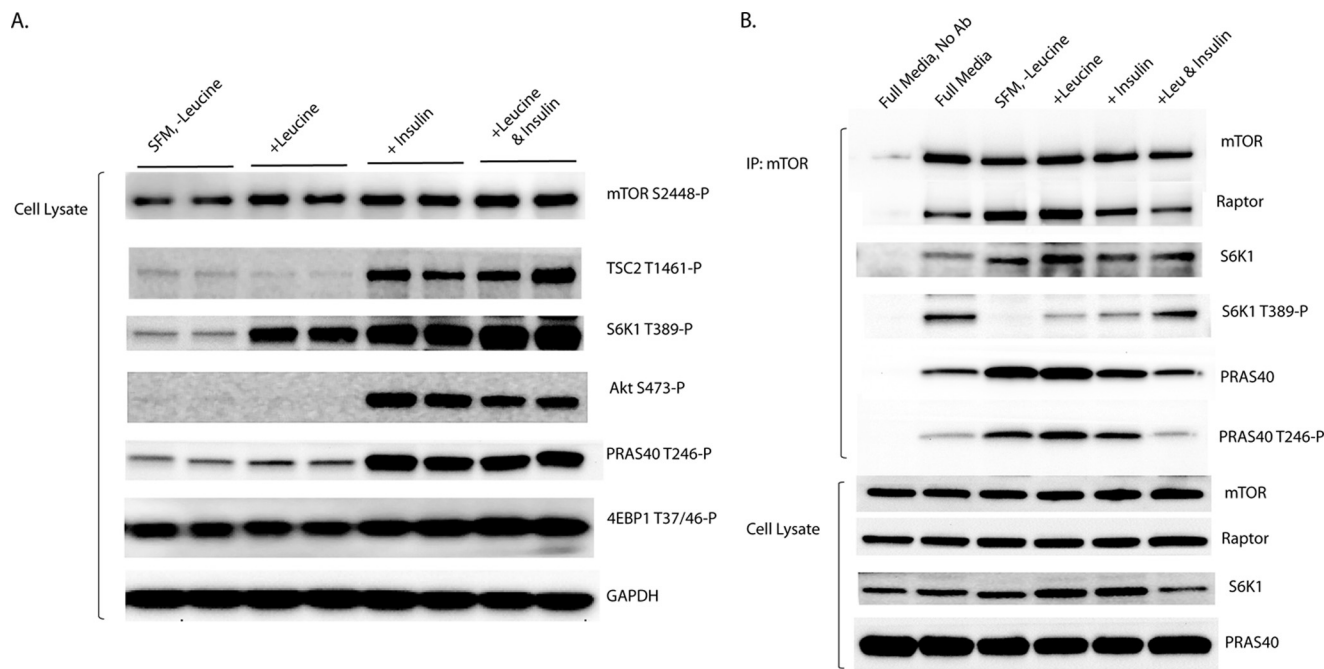


FIGURE 4. Effects of insulin and amino acids on mTORC1 signaling and complex assembly in Rat2 fibroblasts. Cells were deprived of serum and leucine for 2 h prior to the addition of 0.76 mM leucine and/or 10 nM insulin. *A*, the phosphorylation of mTOR at Ser-2448, TSC2 at Ser-1461, S6K1 at Thr-389, Akt at Ser-473, and 4E-BP1 at Thr-37/Thr-46 was assessed by Western blot analysis using antibodies specific for the phosphorylated form of the protein. GAPDH content was used as a control for equal protein loading. Representative blots are shown ($n = 4$). All blots are from the same gel, but the blots for phospho-S6K1 (Thr-389) and GAPDH were stripped and reprobed for the phosphorylation of Akt at Ser-473 and 4E-BP1 at Thr-37/Thr-46 as described under "Experimental Procedures." *SFM*, serum-free medium. *B*, the effects of insulin and leucine on the association of mTOR with raptor, PRAS40, and S6K1 were examined by immunoprecipitating (IP) mTOR from cell homogenates and measuring the amount of each protein in the immunoprecipitates by Western blot analysis. *No Ab*, no anti-mTOR antibody added to the sample. Representative blots are shown ($n = 4$). For quantitation of key measurements, see [supplemental Tables S1 and S2](#).

lin, suggesting that signaling inputs from both are required for optimal assembly of the complex.

Effects of Rag and Rheb Activation on Leucine- and Insulin-stimulated mTORC1 Signaling—The results presented above suggest that insulin and amino acids (leucine) signal through parallel pathways to promote mTORC1 activation. On the basis of recent studies (7, 8), we hypothesized that insulin acts through Akt and Rheb and that amino acids (leucine) act through Rag GTPases to activate mTORC1. To test this theory, cells were transfected with a control plasmid, a plasmid expressing a Rheb variant that preferentially binds GTP (caRheb), and/or a combination of plasmids expressing a variant of RagB that preferentially binds GTP and a variant of RagC that preferentially binds GDP (caRag). The latter two plasmids were chosen based on previous studies (7, 8) showing that mTORC1 is activated when RagB is in the GTP-bound form and RagC is in the GDP-bound form. In serum- and leucine-deprived Rat2 cells, individual expression of caRag or caAkt led to activation of mTORC1, and combined expression of caRag and caAkt led to a further activation compared with either alone (Fig. 5*A*). Leucine readdition to deprived cells transfected with empty vector resulted in mTORC1 activation, and the effect was enhanced by expression of caAkt, but not caRag (Fig. 5*B*). No further increase was observed when caRag and caAkt were both expressed. Conversely, mTORC1 activity was greater in insulin-treated cells expressing caRag compared with cells transfected with empty vector or expressing caAkt (Fig. 5*C*). Combined treatment with insulin and leucine activated mTORC1 in cells transfected with empty vector (compare the *first two lanes*

in Fig. 5*A* with those in Fig. 5*D*), but no additional activation was observed in cells expressing caRag, caAkt, or both (Fig. 5*D*), suggesting that the activation of mTORC1 by combined treatment with insulin and leucine is functionally equivalent to simultaneous expression of caRag and caAkt. Similar results were obtained in cells transfected with a plasmid expressing caRheb instead of caAkt (Fig. 5, *E–H*). Thus, leucine activated mTORC1 in cells expressing caRheb, but not caRag, whereas insulin activated mTORC1 in cells expressing caRag, but not caRheb. Overall, the results are consistent with a model (Fig. 6) in which leucine acts through the Rag GTPases and insulin through Akt and Rheb to activate mTORC1.

Insulin and Leucine Stimulate the Association of Rag and Rheb with mTOR—To evaluate the association of Rag proteins and Rheb with mTORC1, cells were transfected with plasmids expressing HA-tagged wild-type Rheb or a combination of HA-tagged wild-type RagB and RagC. The association of exogenously expressed Rag proteins and Rheb with endogenous mTOR was assessed by Western blot analysis of mTOR immunoprecipitates. The amount of HA-RagB and HA-RagC present in immunoprecipitates from cells deprived of serum and leucine was dramatically reduced compared with cells maintained in complete medium (Fig. 7*A* and [supplemental Table S4](#)). The addition of either leucine or insulin to deprived cells modestly increased mTOR-Rag association. However, when provided in combination, a greater increase in association was observed. The association of exogenously expressed Rheb with endogenous mTOR was also stimulated independently by the addition of either leucine or insulin to deprived cells (Fig. 7*B*). However,

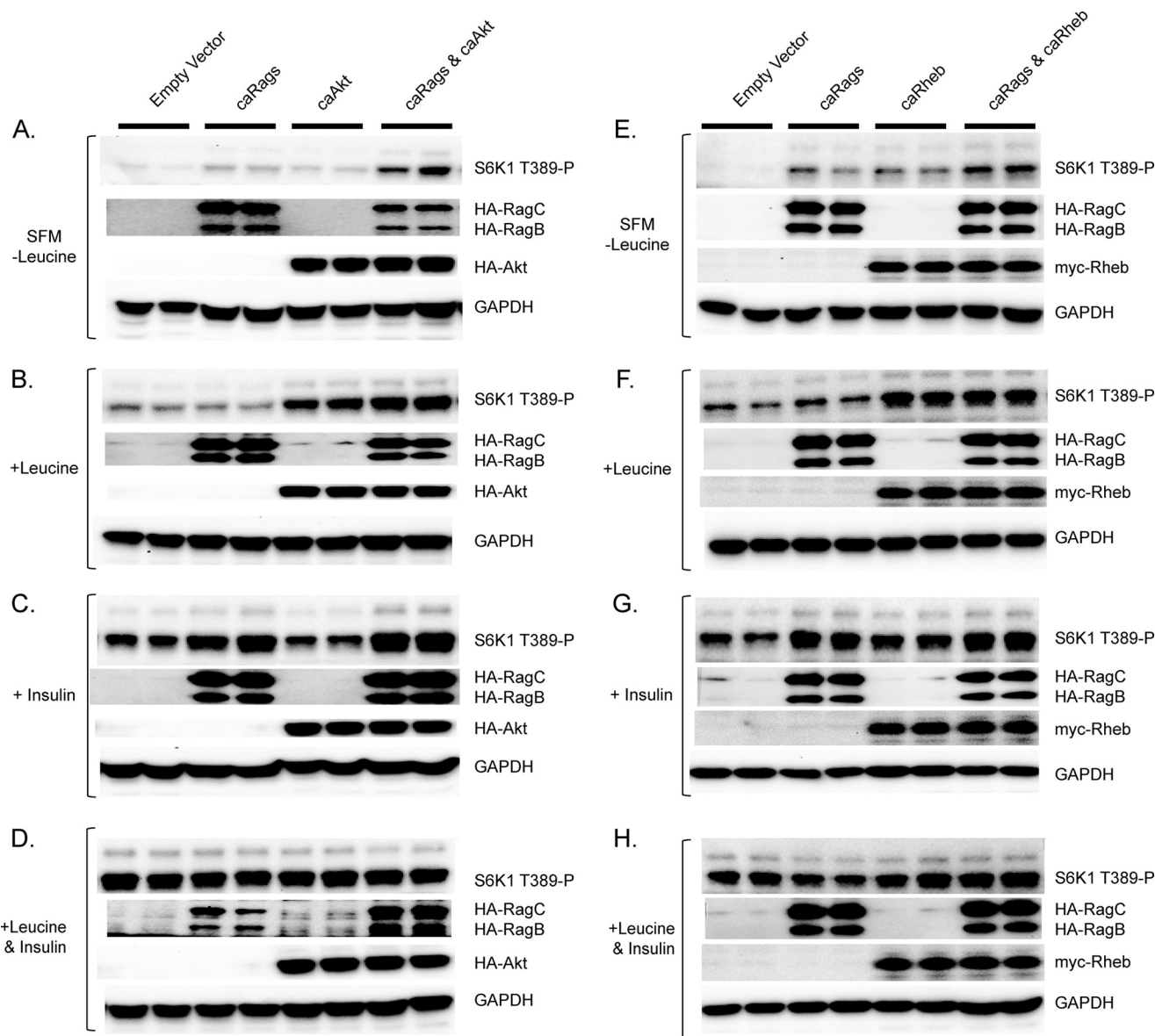


FIGURE 5. Effects of exogenous expression of caRagB/caRagC, caRheb, and/or caAkt on insulin- and leucine-stimulated mTORC1 signaling in Rat2 fibroblasts. *A–D*, cells were transfected with a combination of plasmids expressing caRagB (pRK5-HA-GST-RagB-Q99L), caRagC (pRK5-HA-GST-RagC-S75L), and/or caRheb (pRK5-Myc-Rheb-S16H). *E–H*, cells were transfected with a combination of plasmids expressing caRagB (pRK5-HA-GST-RagB-Q99L), caRagC (pRK5-HA-GST-RagC-S75L), and/or caAkt (pCMV5-HA-caAkt), and mTORC1 activation was assessed by the phosphorylation of S6K1 at Thr-389. The next day, the cells were treated as described in the legend to Fig. 4, and mTORC1 activity was assessed by Western blot analysis of S6K1 phosphorylation at Thr-389 using a phospho-specific antibody. Representative blots are shown ($n = 4$). For quantitation of S6K1 Thr-389 phosphorylation, see [supplemental Table S3](#). *SFM*, serum-free medium.

in contrast to the Rag GTPases, the effect on Rheb did not appear to be additive. The association of endogenous raptor and PRAS40 with mTOR showed a similar pattern of change that was opposite that for either the Rag GTPases or Rheb (Fig. 7, *A* and *B*). Thus, the amount of both proteins was increased in mTOR immunoprecipitates from serum- and leucine-deprived cells compared with cells maintained in complete medium. The addition of either leucine or insulin to deprived cells resulted in reduced recovery of raptor and PRAS40 in immunoprecipitates, and a combination of both inputs had a greater effect compared with either alone.

The readdition of leucine or insulin to deprived cells also led to increased mTOR phosphorylation at Ser-1261. This finding supports previous studies showing that the phosphorylation of

mTOR at Ser-1261 is increased by signaling through TSC/Rheb in an amino acid-dependent manner, which suggests a functional role for site-specific phosphorylation in mTORC1 activation, as Ser-1261 phosphorylation promotes mTORC1-mediated phosphorylation of S6K1 (20).

DISCUSSION

In contrast to the relatively well characterized signaling pathway through which insulin activates mTORC1, the pathway(s) through which amino acids act is less well defined. Several studies (21–23) have suggested a role for the type III PI3K Vps34 (vacuolar protein sorting 34) in the activation of mTORC1 by amino acids in mammalian cells. However, in *Drosophila*, Vps34 does not act upstream of mTORC1 (24), even though, as

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in mammalian cells, it plays a critical role in regulating autophagy. Other studies (25, 26) have suggested a role for MAP4K3 in amino acid-induced activation of mTORC1. However, the mechanism(s) through which either Vps34 or MAP4K3 acts to regulate mTORC1 is as yet undefined.

More recently, the Rag GTPases have been implicated in amino acid signaling to mTORC1. The Rag GTPase family currently consists of four members, RagA, RagB, RagC, and RagD. RagA and RagB were originally identified in a screen for novel G proteins (27), and RagC and RagD were subsequently identified as proteins that interact with RagA (28). Interestingly, the Rag GTPases function as heterodimers consisting of RagA or RagB in a complex with RagC or RagD. In both mammals (7) and *Drosophila* (8), repressing Rag GTPase expression attenuates

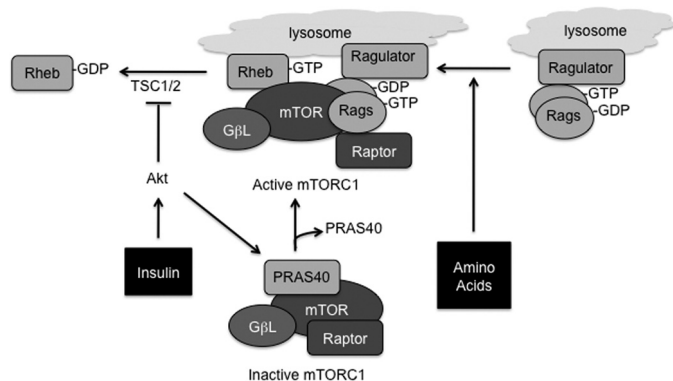
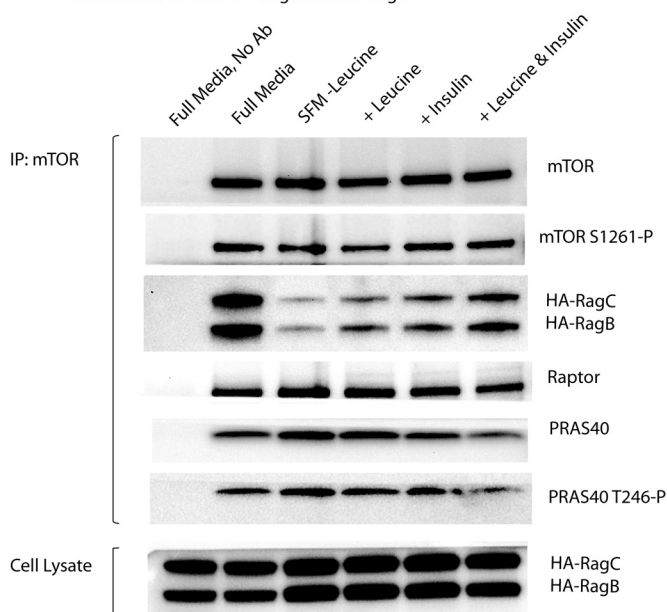


FIGURE 6. Model for insulin and leucine signaling to mTORC1. Maximal activation of mTORC1 occurs in response to inputs from both insulin and leucine. mTORC1 is composed of the serine/threonine protein kinase, mTOR, the regulatory associated protein of TOR, raptor, and the G-protein β -subunit like protein, G β L.

activation of mTORC1 by amino acids, but not insulin, whereas exogenous expression of constitutively active variants of the proteins prevents amino acid deprivation-induced down-regulation of mTORC1 signaling. A more recent study (9) identified a heterotrimeric complex referred to as Ragulator that targets the Rag GTPases to the lysosomal membrane and showed that localization of mTORC1 to the Rag-Ragulator complex at the membrane is necessary for amino acid-induced activation of the kinase. Indeed, constitutive localization of mTORC1 to the lysosomal membrane is sufficient to render mTORC1 activity resistant not only to knockdown of Rag GTPases or components of the Ragulator complex but also to amino acid deprivation. Based on these results, a model was proposed (9) in which amino acids cause mTORC1 to bind to the Rag-Ragulator complex at the lysosomal membrane, and insulin, through repression of TSC2 GTPase-activating protein activity, promotes accumulation of Rheb in the GTP-bound form. Although not experimentally verified, another presumed function of insulin is to promote migration of Rheb-GTP to the same membrane compartment as the mTORC1-Rag-Ragulator complex, allowing it to interact with and thereby activate mTORC1.

In apparent contradiction of the proposed model, in some studies (e.g. Ref. 7), exogenous expression of Rheb renders mTORC1 activity resistant not only to insulin deprivation but also to amino acid deprivation, suggesting that amino acids might signal through both the Rag GTPases and Rheb. In confirmation of the findings of Sancak *et al.* (7), in this study, we found that exogenous expression of Rheb at high levels (~10-fold above endogenous levels) resulted in an apparent insensitivity of mTORC1 to leucine deprivation (supplemental Fig. 1). However, when expressed at lower levels (~2.5-fold above

A. Transfected cDNAs: HA-RagB and HA-RagC



B. Transfected cDNA: HA-Rheb

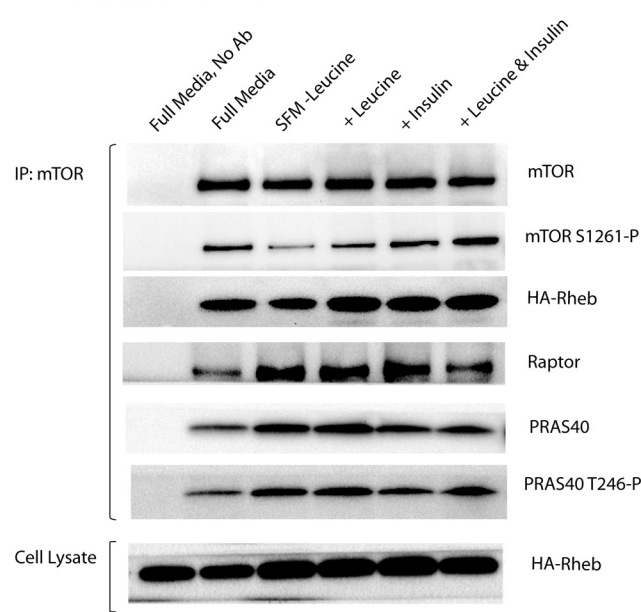


FIGURE 7. Effects of insulin and amino acids on mTORC1 assembly in Rat2 fibroblasts. Cells were transfected with plasmids expressing HA-tagged RagB and HA-tagged RagC (A) or HA-tagged Rheb (B). The next day, the cells were treated as described in the legend to the Fig. 4. mTORC1 complex assembly was assessed by immunoprecipitation (IP) of mTOR from cell lysates, followed by Western blot analysis of immunoprecipitates for raptor, PRAS40, and HA-tagged Rag or Rheb. mTORC1 phosphorylation at Ser-1261 was assessed by Western blot analysis using a phospho-specific antibody. In addition, PRAS40 phosphorylation at Thr-246 was measured by Western blot analysis using a phospho-specific antibody. Representative blots are shown ($n = 4$). For quantitation of HA-Rag and HA-Rheb association, see supplemental Table S4. SFM, serum-free medium.

endogenous levels), leucine deprivation repressed and leucine readdition restored mTORC1 signaling (e.g. Fig. 5). Importantly, even when expressed at lower levels, Rheb prevented down-regulation of mTORC1 activity in response to serum deprivation, and in the absence of leucine, insulin had no effect on mTORC1 signaling in cells expressing low levels of Rheb. Thus, at the level of expression utilized in this study, Rheb functionally substituted for insulin (but not leucine) signaling to mTORC1. A possible explanation for the discrepancy between results obtained when Rheb is expressed at different levels is that when it is expressed at high levels, a portion of Rheb is improperly localized and is able to interact with mTORC1 at a location distinct from the lysosomal membrane. An alternative and not necessarily exclusive explanation is that a portion of exogenously expressed Rheb is localized away from TSC2 and therefore is constitutively associated with GTP, *i.e.* is present in the active form.

Another apparent inconsistency between the model proposed by Sancak *et al.* (7) and the results of this study is that insulin and amino acids/leucine independently promoted the association of mTOR with the Rag GTPases both in the perfused liver and in Rat2 cells in culture. As discussed above for exogenously expressed Rheb, the effect of insulin on the association of mTOR with the Rag GTPases in Rat2 cells may be related to improper localization of a portion of the exogenously expressed proteins, allowing for non-physiological interactions to occur. However, in the liver, the association of endogenous (rather than exogenously expressed) Rheb and mTOR was assessed, and consequently, in the liver, this explanation is inappropriate. An alternative but speculative explanation for the results observed in the liver is that the binding of Rheb-GTP to mTORC1 stabilizes its interaction with the Rag GTPases and vice versa. Studies designed to address this possibility will be a subject of future investigations.

In this study, global rates of protein synthesis were greater in livers perfused with medium containing 4×AA compared with 1×AA. The addition of insulin to the perfusate had no effect in livers perfused with 1×AA but significantly stimulated protein synthesis in livers perfused with 4×AA. In agreement with these results, a previous study using a pancreatic/amino acid clamp (4) showed that increasing plasma amino acid concentrations from fasted to fed values resulted in a significant stimulation of global rates of protein synthesis in livers of neonatal piglets. When amino acids were clamped at fasted values, increasing insulin concentrations had no effect on protein synthesis. However, at fed amino acid levels, insulin significantly increased protein synthesis compared with amino acids alone. In addition, in the previous study (4), the stimulation of protein synthesis engendered by amino acids and insulin was positively correlated with increased signaling through mTORC1, suggesting that its activation underlies the observed changes in protein synthesis.

In this study, global rates of protein synthesis in the liver were inversely correlated with the amount of 4E-BP1 associated with eIF4E. Binding of 4E-BP1 to eIF4E prevents assembly of the eIF4F complex (consisting of eIF4E, eIF4G, and eIF4A) that mediates binding of mRNA to the 40 S ribosomal subunit (29). Thus, the amino acid- and insulin-induced decrease in 4E-BP1

bound to eIF4E would be expected to be accompanied by a corresponding increase in eIF4G bound to eIF4E, leading to increased rates of protein synthesis. Unfortunately, in this study, we were unable to accurately assess the amount of eIF4G in eIF4E immunoprecipitates due to degradation of eIF4G during immunoprecipitation.

Overall, the results this study support a model (Fig. 6) in which insulin and amino acids signal through parallel pathways to regulate mTORC1 activity. Specifically, insulin acts through Rheb and amino acids through the Rag GTPases to activate the kinase. Signaling through either pathway alone has a minor effect on mTORC1 activity, but a combination of both inputs additively increases mTORC1 activity. Moreover, the results are consistent with a model in which insulin and amino acids act independently to promote association of the mTORC1 complex with Rheb and the Rag GTPases, as well as its dissociation from PRAS40.

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