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The rapid activation of protein synthesis by growth hormone requires signaling through mTOR

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[1]*Institute of Food Nutrition and Human Health, Massey University, and Metabolism and Microbial Genomics Section, AgResearch Limited, Palmerston North, New Zealand; and* [2]*Department of Biochemistry and Molecular Biology, Life Sciences Centre, University of British Columbia, Vancouver, British Columbia, Canada*

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Hayashi AA, Proud CG. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. *Am J Physiol Endocrinol Metab* 292: E1647–E1655, 2007. First published February 6, 2007; doi:10.1152/ajpendo.00674.2006.—An important function of growth hormone (GH) is to promote cell and tissue growth, and a key component of these effects is the stimulation of protein synthesis. In this study, we demonstrate that, in H4IIE hepatoma cells, GH acutely activated protein synthesis through signaling via the mammalian target of rapamycin (mTOR) and specifically through the rapamycin-sensitive mTOR complex 1 (mTORC1). GH treatment enhanced the phosphorylation of two targets of mTOR signaling, 4E-BP1 and ribosomal protein S6. Phosphorylation of S6 and 4E-BP1 was maximal at 30–45 min and 10–20 min after GH stimulation, respectively. Both proteins modulate components of the translational machinery. The GH-induced phosphorylation of 4E-BP1 led to its dissociation from eIF4E and increased binding of eIF4E to eIF4G to form (active) eIF4F complexes. The ability of GH to stimulate the phosphorylation of S6 and 4E-BP1 was blocked by rapamycin. GH also led to the dephosphorylation of a third translational component linked to mTORC1, the elongation factor eEF2. Its regulation followed complex biphasic kinetics, both phases of which required mTOR signaling. GH rapidly activated both the MAP kinase (ERK) and PI 3-kinase pathways. Signaling through PI 3-kinase alone was, however, sufficient to activate the downstream mTORC1 pathway. Consistent with this, GH increased the phosphorylation of TSC2, an upstream regulator of mTORC1, at sites that are targets for Akt/PKB. Finally, the activation of overall protein synthesis by GH in H4IIE cells was essentially completely inhibited by wortmannin or rapamycin. These results demonstrate for the first time that mTORC1 plays a major role in the rapid activation of protein synthesis by GH.

mammalian target of rapamycin; protein synthesis; eukaryotic initiation factor 4E-binding protein-1; eukaryotic elongation factor-2

GROWTH HORMONE (GH) IS SECRETED into the circulation by the anterior pituitary and subsequently binds to membrane receptors in target tissues to stimulate tissue growth (40) and elicit changes in protein, carbohydrate, and fat metabolism (17).

Although it plays a key role in controlling growth, details of the mechanism by which GH stimulates protein synthesis remain largely unknown. The rate at which protein synthesis occurs depends in the short term upon the efficiency of the translation process. In the longer term (hours to days) its capacity can also be increased. The protein synthesis process is divided into three main stages: initiation, elongation, and termination. Each stage requires a number of translation factors controlled through alterations in their phosphorylation status. Several upstream signals, including hormones, growth factors, and nutrients, activate protein synthesis. In a number of cases, it has been shown (56) that this activation is at least partially inhibited by rapamycin, implying that signaling through the mammalian target of rapamycin (mTOR) is required for this. mTOR forms two types of complexes, mTORC1 and mTORC2, of which mTORC1 is sensitive to rapamycin (58). Sensitivity to rapamycin thus implies a role specifically for mTORC1.

mTOR controls at least three types of components of the translational machinery (56). The ribosomal protein S6 kinases (S6Ks) are activated by insulin and other agents and phosphorylate S6 (S6), a component of the 40S ribosomal subunit complex (6). However, despite extensive study over many years, the function of S6 phosphorylation remains obscure (41). A second example of an mTOR target is typified by eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1), which binds to and inhibits the mRNA cap-binding protein eIF4E, thereby blocking the formation of active initiation factor complexes containing the scaffold protein eIF4G (23). Activation of mTOR signaling leads to the phosphorylation of 4E-BP1 and to its release from eIF4E, which is thus free to associate with eIF4G. Third, mTOR controls the activity of elongation factor-2 (eEF2) kinase such that activation of mTOR leads to inhibition of eEF2 kinase and the dephosphorylation and activation of eEF2 (11). Thus, mTOR controls both the initiation and elongation steps of translation. Their regulation is sensitive to rapamycin, which inhibits certain functions of mTOR that are mediated through mTORC1 (58).

There is now considerable evidence (35, 58) that mTORC1 plays an important role in the control of cell size. However, it is not fully clear how cell size is regulated downstream of mTORC1. One candidate for this regulation is S6K. Knockout studies of this kinase in *Drosophila* and of the S6 kinase 1 gene in mice (36, 44) gave rise to a phenotype characterized by small animal size. Other data (21) suggest a role for another mTORC1 target, 4E-BP1, in regulating cell size.

Recent studies (9, 37) have implied that the mTORC1 pathway may play an important role in skeletal muscle hypertrophy. Agents such as insulin activate mTORC1 through signaling via phosphatidylinositide (PI) 3-kinase and protein kinase B (PKB, also termed Akt) (32, 33). PKB phosphorylates and apparently inactivates the tuberous sclerosis complex (TSC). This consists of two proteins, TSC1 and TSC2. TSC2 acts as the GTPase activator protein for a small G protein,

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Rheb, which is a positive regulator of mTORC1. PKB directly phosphorylates TSC2. An alternative pathway by which mTORC1 can be activated involves signaling via the extracellular ligand-regulated kinase (ERK) pathway. This again appears to be mediated through phosphorylation of TSC2, but it is unclear whether this is catalyzed directly by ERK (30) or by its downstream effector $p90^{RSK}$ (38, 39).

The binding of GH to the GH receptor increases the affinity of Janus kinase 2 (JAK2) for the GH receptor and activates JAK2 (5). The phosphorylation of tyrosines within JAK2 and the GH receptor form high-affinity binding sites for a variety of signaling proteins containing Src homology 2 and other phosphotyrosine-binding domains. Recruitment of these signaling molecules to GH receptor/JAK2 complexes activates signaling pathways, including the STAT (26), the MAP kinase (ERK) transduction pathways (48), and events that involve insulin receptor substrate (IRS) proteins (16, 18, 45).

Although there is ample evidence that GH activates protein synthesis (14, 19, 22), and it is clear that mTOR is important for the regulation of protein synthesis (56, 58), there are no studies relating the effects of GH to signaling through the mTOR pathway. In particular, it has not previously been tested whether the activation of protein synthesis by GH involves mTORC1, a key regulator of cell growth (58). Given the roles of both mTOR and GH in promoting growth, such links would be logical and potentially important. The aims of this study were, therefore, to determine whether GH activates mTORC1 signaling, how it does this, and the importance of mTORC1 signaling for the activation of protein synthesis by GH.

Here, we demonstrate that GH activates 4E-BP1 and other targets for mTORC1 signaling in hepatoma cells. Importantly, we show for the first time that the short-term activation of protein synthesis by GH is dependent upon mTORC1 signaling.

MATERIALS AND METHODS

Materials. Recombinant rat GH was purchased from the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program. Cell culture media were purchased from Invitrogen; Immobilon P membranes were from Millipore. L-[^{35}S]methionine and ECL reagents were purchased from Amersham Biosciences (GE Healthcare). PD098059, rapamycin, and wortmannin were from Calbiochem. Other chemicals were purchased from Merck or Sigma unless otherwise noted.

Antibodies. Primary antibodies were as follows: for total S6, phospho-S6 ($Ser^{235/236}$); phospho-4E-BP1 (Ser^{65}), phospho-4E-BP1 ($Thr^{37/46}$); total ERK, phospho-ERK ($Thr^{202/204}$); total Akt/PKB, phospho-Akt/PKB (Ser^{473}); total TSC2, phospho-TSC2 (Ser^{939}); total eIF4G; total eIF4E (Cell Signaling Technology, Beverly, MA). Antisera for total 4E-BP1, total eEF2, and phospho-eEF2 (Thr^{56}) were described previously (8). Horseradish peroxidise-conjugated goat anti-rabbit secondary antibody was obtained from Amersham Biosciences (Piscataway, NJ).

Cell culture and treatment. Rat hepatoma H4IIE cells were grown in 6-cm plates in Dulbecco's modified Eagle's Medium (DMEM) medium supplemented with 20% (vol/vol) horse serum, 5% (vol/vol) fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. At 50% confluence cells were removed from serum and maintained in serum-free medium for 30 h prior to the start of the experiment treatments. In a number of experiments, specific signaling inhibitors were employed. PD098059 was used to inhibit the ERK pathway, which it does by blocking the activation of the upstream kinase MAP kinase kinase (MEK)1 (1). Wortmannin (4) was employed as specific inhibitor of PI 3-kinase, and rapamycin was used to inhibit mTORC1 (43). Cells were pretreated with these agents before exposure to GH using the following concentrations and times, which are based upon earlier work in this laboratory (49, 50, 53): 10 μM PD098059 for 30 min, 100 nM wortmannin for 30 min, and 50 nM rapamycin for 30 min. These concentrations used were chosen because other studies (49, 50, 53) have shown that they completely inhibit the relevant target, although they are unlikely to interfere with other processes.

As described in greater detail in RESULTS, it was first necessary to define appropriate conditions for treatment of hepatoma H4IIE cells with GH and then to explore the time courses over which components of signaling pathways and the translational machinery were regulated. Subsequently, we employed the specific signaling inhibitors to examine the signaling events involved in the control of the translational machinery by GH. The design of later studies was, in many cases, prompted by data from our initial experiments.

Protein extraction from H4IIE hepatoma cells. For Western blotting analysis and protein synthesis assays, cells were lysed in extraction buffer containing 50 mM β-glycerophosphate, pH 7.5, 1 mM EGTA, 1mM EDTA, 1% (vol/vol) Triton X-100, 1 mM Na_3VO_4, 100 nM microcystin-LR, 0.1% (vol/vol) β-mercaptoethanol, protease inhibitors (leupeptin, pepstatin, and antipain, each 1 μg/ml), and phenylmethylsulfonyl fluoride (200 μM). Lysates were centrifuged at 13,000 *g* for 10 min to remove debris. Protein concentrations in the resulting supernatants were determined as described (10). Aliquots of lysate containing equal amounts of protein were used for Western blots and for protein synthesis measurements.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed as described earlier (20), as was the affinity chromatography of eIF4E on m^7GTP-Sepharose (54). Blots were visualized using the enhanced chemiluminescence (ECL) method.

Protein synthesis measurements. Cells were starved of serum for 30 h and preincubated with fresh serum-free DMEM for 1 h prior to the start of the treatments. Cells were then incubated with or without signaling inhibitors for 30 min. Treated cells were then stimulated with GH (500 ng/ml) for an additional 30 min before the addition of [^{35}S]methionine (5 μCi/6-cm plate) for an additional 1 h. Cells were washed three times with cold PBS and were then lysed with extraction buffer. Protein was then spotted on the 3-MM filter paper (Whatman) before precipitation with 5% (wt/vol) TCA and measurement of incorporated radiolabel by scintillation counting.

Reproducibility. All experiments were performed at least three times with similar outcomes. Image J software (available at rsb.info.nih.gov/ij/) was used for quantification where indicated. A summary figure with SE is shown where feasible. For example, the Western blots shown are representative of at least three independent experiments in which similar data were obtained. In the case of Western blots, data from a typical experiment are shown.

RESULTS

GH regulates multiple effectors of mTOR signaling. To study whether GH activates mTOR signaling in H4IIE hepatoma cells, it was first necessary to define conditions under which basal mTOR signaling was low. We therefore starved the cells of serum for a range of times from 6 to 30 h and subjected the cell lysates to analysis by SDS-PAGE and Western blotting for multiple targets for mTOR signaling. As shown in Fig. 1, the phosphorylation of both S6 (at $Ser^{235/236}$) and 4E-BP1 (at Ser^{65}) was decreased as early as 6 h after serum withdrawal and continued to decline up to 30 h. The drop in phosphorylation of Ser^{65} in 4E-BP1 (Fig. 1) is likely the cause of the shift from the most highly phosphorylated γ-species to the faster-migrating β-form, which is not phosphorylated at this site. The increase in the signal for total 4E-BP1 suggests that its expression is enhanced during serum starvation, and

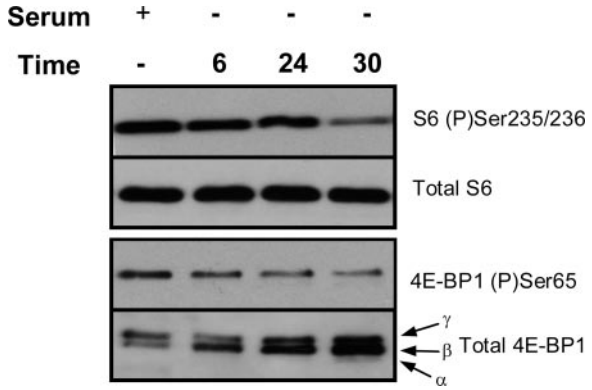

Fig. 1. Effects of serum starvation of H4IIE cells on targets of the mammalian target of rapamycin (mTOR) pathway. H4IIE cells were grown in medium lacking serum for the times indicated (in h) to define the time point at which phosphorylation of eukaryotic factor initiation factor (eIF)4E-binding protein 1 (4E-BP1) (P)Ser^{65} and ribosomal protein S6 (P)Ser$^{235/236}$ was decreased. Cells grown in medium with serum were used as a control. For 4E-BP1, labeled arrows indicate the differentially phosphorylated α-, β-, and γ-species. Total S6 was used as the "loading control".

taking this into account, the fall in the relative level of Ser^{65} phosphorylation is even more pronounced than the change in the signal for the Ser^{65} phosphospecific antibody alone would suggest.

We therefore elected to use cells that had been starved of serum for 30 h in all subsequent experiments. Such cells were treated for various times with GH, and cell lysates were again analyzed for targets of mTOR signaling and for potential upstream regulators of mTOR such as Akt/PKB and ERK. The phosphorylation of S6 was markedly increased by GH, although it rose rather slowly, with only small increases being observed up to ~20 min after GH treatment. S6 phosphorylation then increased substantially, being maximal by 30–45 min and falling again thereafter (Fig. 2*A*). Some minor variation between the kinetics of these responses was seen across the set of experiments that we performed. Therefore, typical data are presented.

GH also enhanced the phosphorylation of a second target for mTOR signaling, 4E-BP1. Its phosphorylation at Ser^{65} was seen by 10 min (Fig. 2*A*), was maximal at 20 min, and fell toward basal levels again by 45 min. The increase in 4E-BP1 phosphorylation is also demonstrated by its mobility shift on SDS-PAGE from mainly running as the partly phosphorylated β-species in untreated cells to running almost entirely as the hyperphosphorylated γ-form after GH treatment (Fig. 2*A*). The γ-form is the only one of the three resolved on SDS-PAGE that is phosphorylated at Ser^{65} and does not bind to eIF4E (54). Therefore, the time courses for the phosphorylation of S6 and 4E-BP1 differ, with the latter changing more rapidly.

The binding of 4E-BP1 to eIF4E prevents eIF4E from binding eIF4G to form complexes that are competent for cap-dependent mRNA translation (24, 31). Thus, by causing the release of 4E-BP1 from eIF4E, the phosphorylation of 4E-BP1 is expected to enhance the binding of eIF4G to eIF4E. To study this, we isolated eIF4E and its binding partners on m^{7}GTP-Sepharose beads and analyzed the bound material by SDS-PAGE and Western blotting. As shown in Fig. 2*B*, GH treatment enhanced the association of eIF4G with eIF4E, as assessed by analysis of material bound to m^{7}GTP-Sepharose (which binds eIF4E and thus also its partner proteins). This effect was blocked by rapamycin, which also decreased the basal level of eIF4G bound to eIF4E (Fig. 2*B*). GH thus promotes formation of initiation factor complexes that are required for the initiation of cap-dependent translation.

We also examined a third translational regulator that is regulated by mTOR, the elongation factor eEF2. Its phosphorylation was also regulated by GH, but in a more complex manner. GH caused a rapid decrease in the phosphorylation of eEF2 at Thr^{56} (Fig. 2*A*), which corresponds to its activation, since phosphorylated eEF2 is inactive, as it cannot bind ribosomes (15). However, by ~20 min, eEF2 phosphorylation had returned to basal levels, but fell again by 30–45 min, before rising once more by 2 h. We have previously observed a rather similar biphasic regulation of eEF2 in cardiomyocytes in response, e.g., to insulin (52). The mechanism that underlies this response to GH is studied in more detail below.

Rapamycin treatment blocked the ability of GH to promote the phosphorylation of S6 (Fig. 2*C*) and of 4E-BP1 (as judged from analysis with phosphospecific antisera and by its mobility on SDS-PAGE; Fig. 2*C*). This indicates that, as anticipated, the GH-induced phosphorylation of S6 and 4E-BP1 requires signaling through mTOR, and in particular, the rapamycin-sensitive type of mTOR complex, mTORC1 (58).

GH activates two pathways that can function upstream of mTOR. mTOR can be controlled by through PI 3-kinase (32, 34) or through ERK signaling (30, 39, 51). It was important to check whether GH activated these pathways in H4IIE hepatoma cells. GH elicited the rapid phosphorylation of Akt/PKB, as evidenced by the increased phosphorylation by 10 min of PKB at Ser^{473}, a COOH-terminal site involved in its activation. Increased phosphorylation was sustained for ≥120 min (Fig. 2*D*). Activation (T-loop phosphorylation) of ERK1/2 was also observed; however, the rise in ERK phosphorylation was slower (only being seen by 15 min) and transient (it returned essentially to basal levels by 120 min; Fig. 2*D*).

It was important to examine whether rapamycin affected potential upstream signaling pathways, e.g., ERK and PKB. Rapamycin treatment had little effect on GH-induced ERK phosphorylation (Fig. 2*C*) and actually enhanced GH-induced PKB phosphorylation (Fig. 2*C*; see also Fig. 3). This likely reflects inhibition by rapamycin of the negative feedback loop whereby S6K1 phosphorylates IRS1 and impairs signaling through it (25, 47).

GH activates mTOR via PI 3-kinase. As reported above, GH activates both PKB and ERK signaling. To study whether either or both of the above pathways mediate the activation of mTOR signaling by GH, we made use of pathway-specific inhibitors. PD098059 is a rather specific inhibitor of ERK signaling that acts at the level of the upstream kinase MEK (1). Wortmannin is an irreversible inhibitor of PI 3-kinase (4) but may also inhibit mTOR at certain concentrations (13).

Treatment of cells with PD098059 completely eliminated the phosphorylation of ERK1/2 (Fig. 3), confirming its efficacy in blocking this pathway, but had no effect upon the activation of the phosphorylation of S6 or 4E-BP1 by GH (Fig. 3), ruling out ERK signaling as a significant component in the control of mTOR by GH. On the other hand, wortmannin (which completely prevented GH-induced phosphorylation of PKB; Fig. 3) clearly inhibited the phosphorylation of S6 and of 4E-BP1 that was induced by GH (Fig. 3).

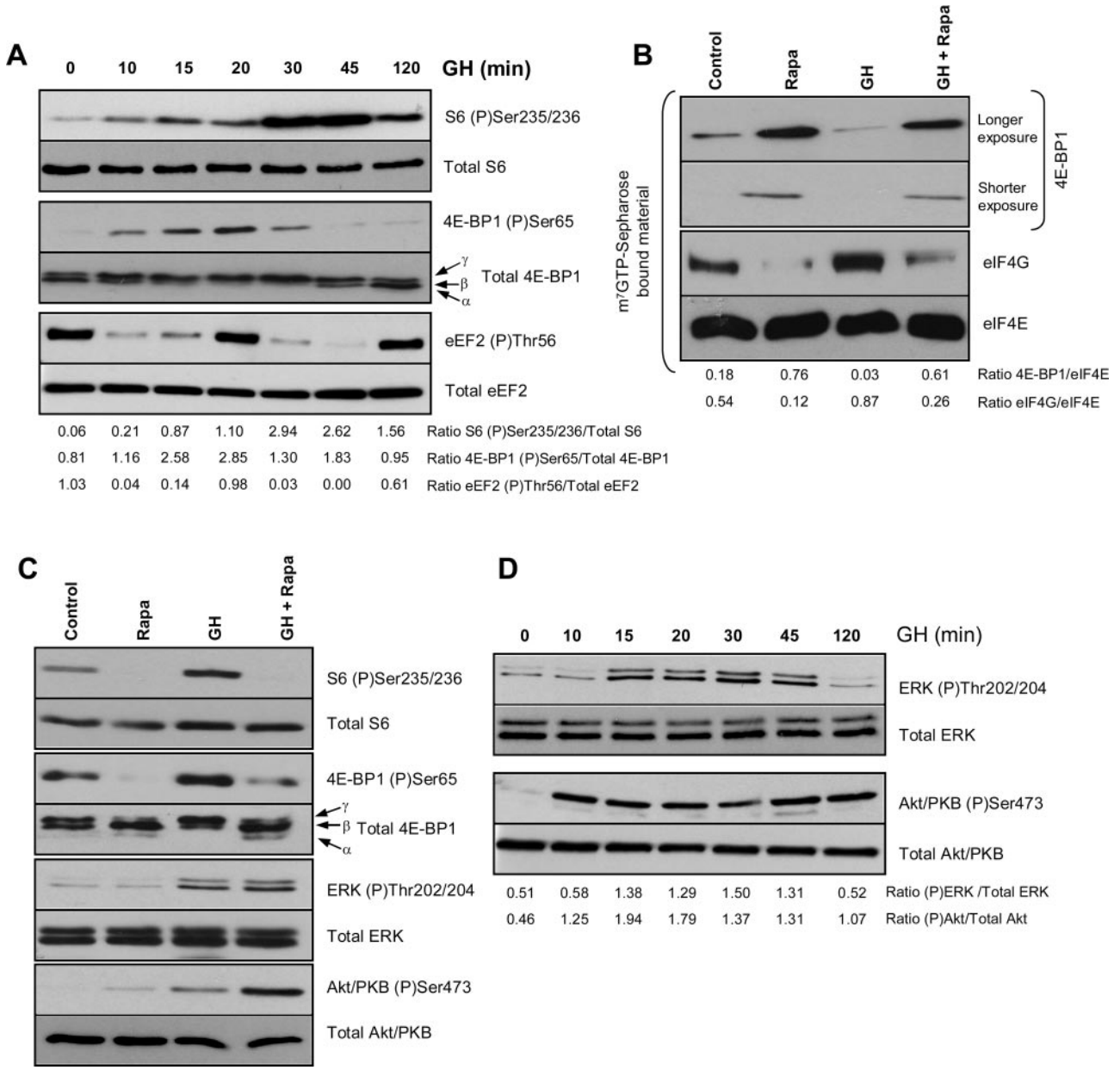

Fig. 2. Growth hormone (GH) stimulates targets of mTOR signaling and potential upstream regulators of mTOR. Cells were serum starved for 30 h and then treated with GH (500 ng/ml) for the times indicated (in min). Clarified whole cell extracts were analyzed by SDS-PAGE and Western blotting using the indicated antisera, including antibodies to assess total levels of each protein to which levels of the phosphospecies should be compared. In all cases, blots are representative of at least 3 independent experiments. The ratio values represent the individual densitometric analysis of the immunoblots shown in the figures. A summary figure with SE is not presented because the precise time courses of some responses varied slightly between experiments. *A*: for 4E-BP1, labeled arrows indicate the differentially phosphorylated α-, β-, and γ-species. Ratios represent the signal observed with the phosphospecific antibody normalized to the total amount of the corresponding protein. (*B* and *C*) Cells were pretreated with or without rapamycin (Rapa) prior to GH treatment (30 min). *B*: cell extracts were then subjected to affinity chromatography on m^7GTP-Sepharose, as described in MATERIALS AND METHODS, and the bound material was analyzed by SDS-PAGE and Western blotting using antisera against eIF4E, 4E-BP1, and eIF4G. Ratios represent the quantitation of 4E-BP1 and eIF4G relative to total eIF4E. *C*: Cell lysates were analyzed by SDS-PAGE and Western blotting using antisera for the indicated proteins. *D*: antisera for the phosphorylated active forms of Akt/PKB and ERK, and for the total amounts of these proteins, were used to assess activation of potential upstream regulators of mTOR. Ratios represent the signal seen with the phosphospecific antibody normalized to the total amount of the corresponding protein. eEF2, eukaryotic elongation factor 2.

This inhibition could reflect a requirement for PI 3-kinase signaling, or it might be a consequence of the ability of wortmannin to inhibit mTOR itself. To distinguish between these possibilities, we examined the effect of a range of concentrations of wortmannin on the phosphorylation of the NH_2-terminal sites in 4E-BP1 whose basal phosphorylation requires amino acid-dependent mTOR signaling, but not PI 3-kinase (53). At all concentrations tested, wortmannin eliminated the GH-induced phosphorylation of PKB, S6, and Ser^{65} in 4E-BP1 (Fig. 4*A*). In contrast, wortmannin had no effect upon the phosphorylation of $Thr^{37/46}$ in 4E-BP1, strongly implying that its ability to impair the GH-induced phosphorylation of (other sites in) 4E-BP1 is a consequence of its ability to block PI 3-kinase rather than a direct effect on mTOR. We

have shown earlier that the phosphorylation of these sites in 4E-BP1 is mediated via mTORC1 (53) but is not sensitive to inhibition of PI 3-kinase by wortmannin (51). At the highest concentration used, wortmannin decreased the phosphorylation of ERK. The reason for this is unknown, but this effect has been noted in other studies (see, e.g., Ref. 57).

Thus, it appears that GH activates mTORC1 in a manner that requires signaling via PI 3-kinase. This likely corresponds to

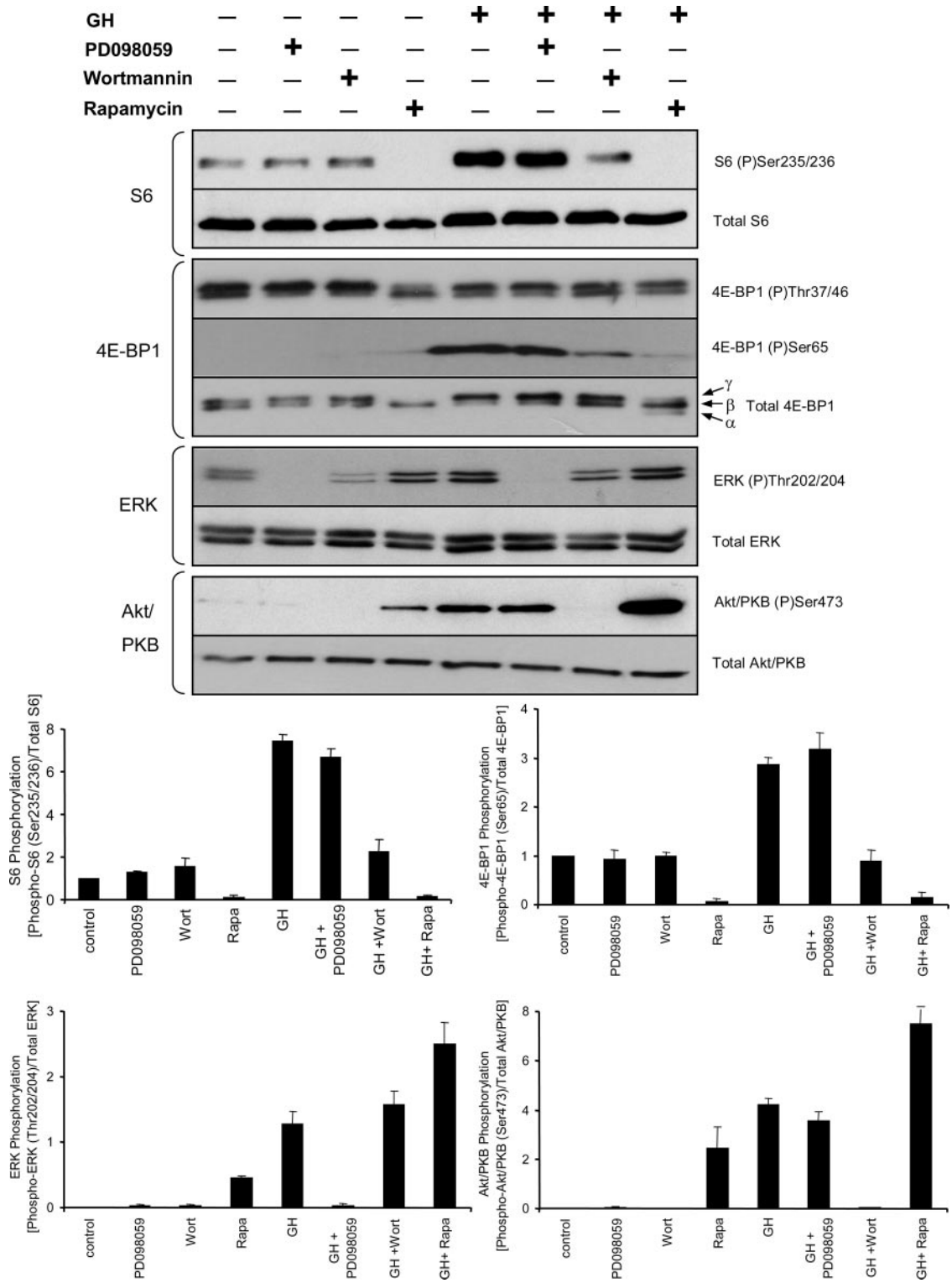

Fig. 3. Blocking phosphatidylinositide (PI) 3-kinase, but not ERK signaling, inhibits the activation of mTOR complex 1 (mTORC1) signaling by GH. After 30-h serum starvation period, cells were treated with 10 μM PD098059, 100 nM wortmannin (wort), or 50 nM rapamycin (rapa), as noted, and then treated with GH for 30 min. Clarified whole cell extracts were analyzed by SDS-PAGE and Western blotting using the indicated antisera. Graphs show the mean ± SE of individual densitometric analyses of several immunoblots for the indicated phospho-antisera normalized to the total amount of the relevant protein.

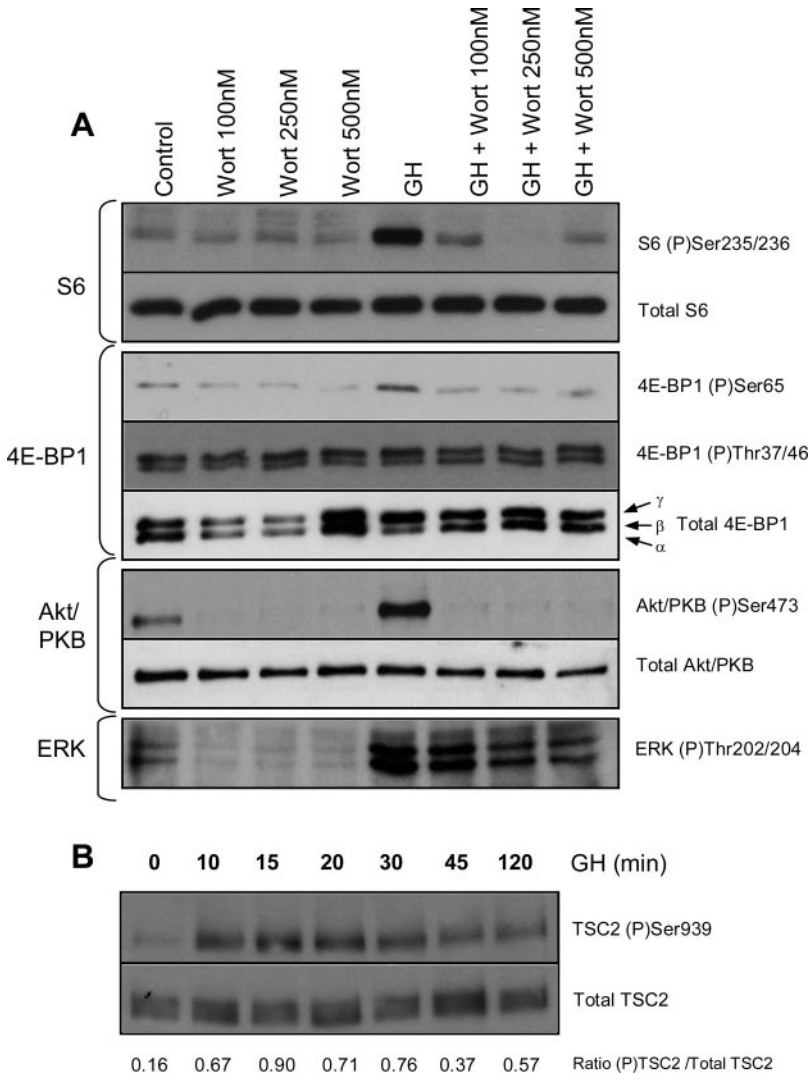

Fig. 4. GH regulates the mTOR pathway through activation of the PI 3-kinase signaling pathway. *A*: H4IIE cells were pretreated with wortmannin (wort) at the indicated concentrations (for 30 min) and then treated with GH for 30 min (or left for an additional 30 min without GH). Clarified whole cell extracts were analyzed by SDS-PAGE and Western blotting using the indicated antisera. For 4E-BP1, labeled arrows indicate the differentially phosphorylated α-, β-, and γ-species. *B*: immunoblot showing the level of tuberous sclerosis complex 2 (TSC2) phosphorylation at Ser^{939} assessed with a specific phosphospecific antibody. Anti-TSC2 is used as a loading control. Ratios represent the signal observed with the phosphospecific antibody normalized to the total amount of the corresponding protein.

the widely-accepted model where phosphorylation of TSC2 (a negative regulator of mTOR) by PKB/Akt inhibits TSC2 function, allowing the activation of mTORC1 (32, 33). One of the sites in TSC2 that is modified by PKB/Akt is Ser^{939} (27, 46), which is not affected by ERK signaling (38, 39). As assessed using a phosphospecific antibody directed against this site, it was clear that GH enhanced the phosphorylation of this site in TSC2 (Fig. 4*B*).

mTOR controls early and late stages of GH-induced eEF2 dephosphorylation. GH caused the dephosphorylation of eEF2 at Thr^{56} in a biphasic manner (Fig. 2*A*). As noted above, the first phase is faster than the changes in the phosphorylation of other targets of mTOR signaling, such as 4E-BP1 and S6. In addition, the control of eEF2 kinase and thus of eEF2 phosphorylation is complex (11, 12), raising the possibility that the first phase of eEF2 phosphorylation might be mediated in a different manner, perhaps independently of mTOR.

To study this, we examined the effect of rapamycin on the early (15 min) and late (45 min) phases of eEF2 dephosphorylation. Rapamycin completely inhibited both, showing that both require mTOR signaling (Fig. 5).

Rapamycin inhibits the activation of protein synthesis by GH. The above data show that, via mTOR, GH quickly regulates components of the translational machinery that are associated with its activation. It was important to investigate whether GH also switched on protein synthesis. Treatment of hepatoma cells with GH led to a rapid increase in the rate of protein synthesis (130%; Fig. 6), as monitored by following the incorporation of radiolabeled [^{35}S]methionine into protein.

Given that a number of steps in translation are subject to control, and not all these events are mediated via mTORC1, it was important to test to what extent activation of protein synthesis by GH required mTOR. To test this, we examined the effect of rapamycin on the GH-induced stimulation of protein synthesis. Rapamycin markedly inhibited the increased rate of incorporation of radiolabel (106%; Fig. 6). In such experiments, it is critical to take into account any effect of the drug on basal rates of protein synthesis: in fact, rapamycin did have a small but reproducible inhibitory effect on this (85%; Fig. 6). However, even when this is taken into account, it is clear that rapamycin significantly inhibits the GH-induced acceleration of protein synthesis (stimulation drops to 106%, $P < 0.01$). In

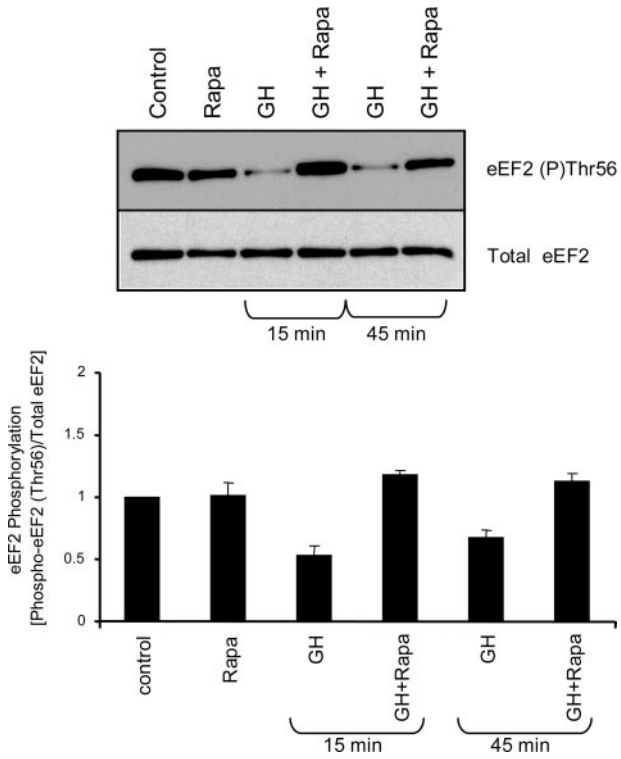

Fig. 5. Both phases of the GH effect on eEF2 phosphorylation are blocked by rapamycin (rapa). Cells were treated with/without rapa for 30 min after serum starvation for 30 h. Cells were preincubated with rapamycin (rapa; 50 nM), and GH was then added for 15 or 45 min. Phosphorylation of eEF2 at Thr^{56} was assessed using the phosphospecific antibody. Total eEF2 levels are shown as a loading control. Graph shows the mean ± SE of individual densitometric analysis of several immunoblots of indicated phospho-antisera normalized to the total amount of the relevant protein.

this experiment, we also checked the effect of the wortmannin on the GH-stimulated protein synthesis. Wortmannin was used at concentrations to specifically inhibit PI 3-kinase. Wortmannin treatment numerically but not statistically reduced the basal rate of protein synthesis (94%; Fig. 6). However, the inhibitory effect of wortmannin on the GH-induced stimulation of protein synthesis was significant (stimulation was decreased to 106%, $P < 0.01$).

DISCUSSION

In this study, we set out to examine how GH rapidly activates protein synthesis. Our data clearly show that GH rapidly stimulates protein synthesis (over the minute to hour timescale) and that this effect requires signaling through mTOR, as judged by the ability of rapamycin to block completely the GH-induced stimulation of protein synthesis. Both GH and mTORC1 play key roles in regulating cell and tissue growth, so this connection between GH and mTORC1 is not surprising. Nonetheless, this is the first time that GH has been shown to activate mTORC1-dependent components that regulate the translational machinery and the first time that GH has been shown to turn on protein synthesis in a mTORC1-dependent manner.

GH had been shown previously to stimulate S6 kinase in 3T3-F442A preadipocytes (2, 3). However, the contribution, if any, of S6 phosphorylation to the short-term activation of protein synthesis remains unclear (41, 42). Here, we show that GH activates two key steps in mRNA translation. These are, first, the formation of eIF4F complexes (the binding of eIF4G to eIF4E) that participate in cap-dependent translation initiation. Second, GH also stimulates the dephosphorylation of eEF2, which is associated with activation of translation elongation. Thus, GH activates both the initiation and elongation stages of translation. The effect of GH on both steps was blocked by rapamycin, consistent with the fact that rapamycin also blocked the overall activation of protein synthesis by GH. Our data also show that GH enhances the phosphorylation of S6, consistent with earlier data that it activates S6 kinase.

As we have noted previously (53), the phosphorylation of $Thr^{37/46}$ in 4E-BP1 is insensitive to rapamycin. Although this is a characteristic of signaling through mTORC2 (58), the phosphorylation of these sites is dependent upon amino acids (53), which regulate mTORC1 (28). This suggests that the phosphorylation of $Thr^{37/46}$ is likely to be a rapamycin-resistant effect of mTORC1, since mTORC2 is not known to be affected by amino acids.

Interestingly, the GH-induced dephosphorylation of eEF2 was clearly biphasic. GH induced a rapid dephosphorylation of eEF2, such that phospho-eEF2 was almost undetectable 10 min after addition of GH. eEF2 phosphorylation then returned almost to control levels by 20 min, and this was followed by second dephosphorylation phase, such that it was essentially completely dephosphorylated by 45 min. eEF2 phosphorylation returned to control levels by 2 h after GH treatment. Importantly, both phases were blocked by rapamycin, indicating that mTORC1 drives both. Thus it appears that, although GH signals through mTORC1 to activate both the initiation (4E-BP1, S6) and the elongation processes of protein synthesis (eEF2), the earliest effects on eEF2 are the most rapid.

There are three known inputs from mTOR into eEF2 kinase: phosphorylation at Ser^{78} (12), which prevents CaM binding; phosphorylation at Ser^{359} (29), which also inhibits maximal eEF2 kinase activity; and phosphorylation at Ser^{366}, which

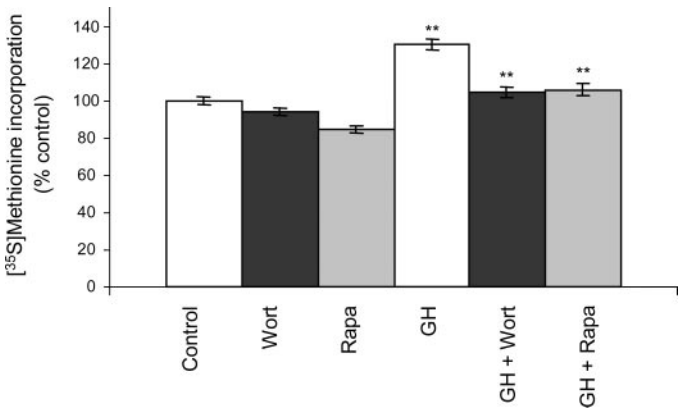

Fig. 6. mTOR is required for the activation of protein synthesis by GH in H4IIE cells. After the 30-h serum starvation, H4IIE cells were treated with signaling inhibitors as indicated for 30 min prior to addition of GH. After 30 min, [^{35}S]methionine was added, and 1 h later, cells were extracted and the incorporation of radiolabel into TCA-insoluble material was assessed as described in MATERIALS AND METHODS. Since the inhibitors slightly affected basal rates of protein synthesis, data for GH plus inhibitors are shown in relation to control cells treated with the inhibitor; i.e., for GH, control was DMSO (control); for GH + wort, control was wort; for GH + rapa, control was rapa. Data are given as means ± SD, $n = 4$. $^{**}P < 0.01$.

makes eEF2 kinase less sensitive to activation by Ca/CaM (55). Since there was very little change in S6 phosphorylation by 10 min, the first phase of eEF2 dephosphorylation is presumably not due to S6 kinase and reflects another input. The S6 kinase site, Ser^{366}, can also be phosphorylated by $p90^{RSK}$, which lies downstream of ERK (55). However, since ERK activation was slower than the change in eEF2 phosphorylation, the first rapid phase of eEF2 regulation is unlikely to be mediated by ERK/$p90^{RSK}$ signaling. In any case, it was blocked by rapamycin, ruling out a significant role for MEK/ERK signaling. The second phase does match the kinetics of S6 kinase activation, as judged by S6 phosphorylation, with both being maximal at 45 min. Unfortunately, our phosphospecific anti-eEF2 kinase antisera are not sensitive enough to allow us to study changes in the phosphorylation of specific sites in the endogenous eEF2 kinase in hepatoma cells.

How does GH activate mTOR? The best-characterized mechanism for the activation of mTOR by hormones involves the phosphorylation of TSC2, a negative regulator of mTOR, by PKB/Akt (33). This is mediated through PI 3-kinase. Our data are consistent with GH employing this mechanism. First, the effects of GH on the phosphorylation of 4E-BP1 and S6 were blocked by the PI 3-kinase inhibitor wortmannin, used at concentrations that do not interfere with mTOR function (50, 53). Second, these effects of GH were not affected by the MEK inhibitor PD098059, which completely inhibited the activation of ERK by GH. Thus, although wortmannin did interfere with the activation of ERK by GH, the fact that PD098059 did not impair the activation of mTORC1 signaling by GH indicates that this "side effect" of wortmannin may be ignored in interpreting the present data. It is not known why wortmannin impairs ERK activation, but this effect has been noted in other studies (see, e.g., Ref. 57). Third, GH induced the phosphorylation of TSC2 at Ser^{939}, a major site of phosphorylation by PKB, but one that is not controlled by MEK/ERK signaling (7, 38, 39). This is completely consistent with the above conclusion that GH signals via PKB/Akt to activate mTOR. Importantly, the change in the phosphorylation of TSC2, an upstream regulator of mTOR, is at least as rapid as the change in eEF2 phosphorylation and faster than the increases in the phosphorylation of S6 and 4E-BP1. Furthermore, the rapidity of activation of PKB (Fig. 2*D*) and of TSC2 phosphorylation (Fig. 4*B*) is consistent with one another.

Our data thus demonstrate that GH acts through PI 3-kinase and probably PKB/Akt to activate mTOR signaling, leading to the rapid activation of eEF2 and formation of eIF4F complexes. The fact that the activation of protein synthesis by GH is completely blocked by rapamycin is consistent with the conclusion that these changes in translation factor function contribute to the activation of protein synthesis by GH. Thus, GH activates targets for rapamycin-sensitive mTORC1 signaling, such as eIF4E and eEF2, and treatment with rapamycin or wortmannin inhibits the ability of GH to stimulate protein synthesis. mTOR also positively controls ribosome biogenesis and the synthesis of several components of the translational machinery, thereby increasing the translational capacity of the cell/tissue. It is thus likely that the longer-term effects of GH on protein synthesis and cell growth also involve GH-activated mTOR signaling.

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