Activation of Mammalian Target of Rapamycin (mTOR) by Insulin Is Associated with Stimulation of 4EBP1 Binding to Dimeric mTOR Complex 1*

Received for publication, April 13, 2006, and in revised form, May 30, 2006. Published, JBC Papers in Press, June 23, 2006, DOI 10.1074/jbc.M603566200

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Insulin stimulates protein synthesis by promoting phosphorylation of the eIF4E-binding protein, 4EBP1. This effect is rapamycin-sensitive and mediated by mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a signaling complex containing mTOR, raptor, and mLST8. Here we demonstrate that insulin produces a stable increase in the kinase activity of mTORC1 in 3T3-L1 adipocytes. The response was associated with a marked increase in 4EBP1 binding to raptor in mTORC1, and it was abolished by disrupting the TOR signaling motif in 4EBP1. The stimulatory effects of insulin on both 4EBP1 kinase activity and binding occurred rapidly and at physiological concentrations of insulin, and both effects required an intact mTORC1. Results of experiments involving size exclusion chromatography and coimmunoprecipitation of epitope-tagged subunits provide evidence that the major insulin-responsive form is dimeric mTORC1, a structure containing two heterotrimeric units of mTOR, raptor, and mLST8.

As the major anabolic hormone in mammals, insulin stimulates protein synthesis in a wide variety of cell types. This response is mediated in part by mTOR, a phosphatidylinositol 3-kinase-related protein kinase that controls the phosphorylation of multiple factors involved in the control of cell growth and proliferation (1, 2). mTOR functions in two signaling complexes, mTORC1 and mTORC2 (1, 3). Both complexes contain mTOR and mLST8 (also known as GβL), a protein homologous to β subunits of heterotrimeric G proteins (4, 5). One defining feature of the complexes is the third subunit, either raptor in mTORC1 (5–7) or rictor (also known as mAVO3) in mTORC2 (8, 9).

4EBP1 (also known as PHAS-I) is an important target of mTOR signaling. 4EBP1 binds eIF4E, the mRNA cap-binding protein, and it represses cap-dependent translation by competitively blocking the binding of eIF4G to eIF4E (2, 10). Activating mTOR with insulin stimulates the phosphorylation of 4EBP1 in four sites (11, 12), including Thr-36 and Thr-45, the two sites preferred by mTOR in vitro (13, 14), causing 4EBP1 to dissociate from eIF4E. This allows eIF4E to engage eIF4F, a scaffolding protein that binds eIF3 and eIF4A (10, 15). eIF3 is a complex initiation factor that binds the small ribosomal subunit and several key initiation factors, and eIF4A is a helicase that unwinds mRNA to facilitate binding and/or scanning by the 40S ribosomal subunit (10, 15). Thus, the phosphorylation of 4EBP1 leads to the recruitment of the small ribosomal subunit and important initiation factors to the 5′-end of the message to begin the processes of scanning and selection of the start codon.

The finding that the effects of insulin and insulin-like growth factor 1 on 4EBP1 were attenuated by rapamycin provided the first evidence that mTOR controlled 4EBP1 (16, 17). Because rapamycin inhibits mTORC1 but not mTORC2 (8, 9), the sensitivity to rapamycin also implicates mTORC1. The functions of the mTORC1 subunits are not fully understood. mLST8, which consists almost entirely of seven WD40 repeats, binds near the catalytic domain of mTOR and is required for the full activity of the mTOR kinase (4). Raptor possesses a unique NH2-terminal region followed by three HEAT motifs and seven WD40 repeats that are believed to mediate protein-protein interactions (7). Raptor binds the mTOR substrates, 4EBP1 and S6K1, and it has been suggested that raptor might function to present substrates to mTOR for phosphorylation (6). 4EBP1 can be readily phosphorylated in vitro by mTORC1 (6) but not by mTORC2, which lacks raptor (9). The substrate interactions with raptor are mediated by TOR signaling (TOS) motifs (18–22). In 4EBP1 this motif is formed by the COOH-terminal five amino acids (FEMDI) (21). Disrupting the TOS motif by a Phe → Ala point mutation markedly decreases phosphorylation of the protein in response to activation by mTOR signaling in cells (21) and by mTORC1 in vitro (20, 22).

Incubating cells with insulin (23), serum (13), or certain growth factors (24, 25) has been reported to increase the protein kinase activity of mTOR. However, such changes in mTOR activity have not been detected in other studies, and the conclusion that insulin produces a stable increase in the kinase activity of mTOR is controversial. Previous studies of insulin action on mTOR activity in vitro have not discriminated...
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between the two mTOR signaling complexes, and in some cases the conditions used to extract mTOR would have disrupted mTORC1. Because mTORC1 mediates the effects of insulin on the phosphorylation of 4EBP1 in cells, we conducted experiments to measure mTORC1 activity and the interaction of mTORC1 with 4EBP1.

EXPERIMENTAL PROCEDURES

Adipocyte Culture and Extract Preparation—3T3-L1 fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum. Fibroblasts were converted to adipocytes by using differentiation medium as described previously (17). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 10–12 days after adding the differentiation medium. For experiments, the culture medium was replaced with a solution containing 145 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl2, 1.4 mM MgSO4, 25 mM NaHCO3, 5 mM glucose, 5 mg/ml bovine serum albumin, 0.2 mM sodium phosphate, and 10 mM HEPES, pH 7.4. The cells were incubated at 37 °C without or with a maximally effective concentration of insulin (0.6 μU/ml) and/or other additions. To terminate the incubation the adipocytes were rinsed once with chilled phosphate-buffered saline (145 mM NaCl, 5.4 mM KCl, and 10 mM sodium phosphate, pH 7.4) and then homogenized (0.8 ml of buffer/10-cm-diameter dish) in a glass tissue grinder with a Teflon pestle driven at 1,000 rpm. Homogenization Buffer was composed of Buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 0.5 μM microcystin. Buffer A contained 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20 (unless otherwise indicated), 10 mM sodium phosphate, and 50 mM β-glycerophosphate, pH 7.4. Homogenates were centrifuged at 12,000 × g for 10 min, and the supernatants were retained for analyses.

Antibodies—Antibodies to the COOH-terminal region of 4EBP1 (26) and the phosphospecific antibodies to the Thr-36 and Thr-45 sites (12) have been described previously (12, 26). The 4EBP1 antibodies bind wild type 4EBP1 and F113A equally well (22), and the phosphospecific antibodies bind 4EBP1 phosphorylated in either Thr-36 or Thr-45 (12) as the amino acid sequences surrounding these sites are almost identical. The mTOR antibodies, mTAb1 and mTAb2, were described previously (27). Antibodies (designated N-Rap Ab) to the region in raptor (amino acids 36–53) originally targeted by Kim et al. (7) were generated as described previously (22). mTAb2 and N-Rap Ab were used to detect mTOR and raptor, respectively, by immunoblotting. mLST8 antibodies were described previously (28) as were phosphospecific antibodies to the Ser-2448 site in mTOR (29).

In pilot experiments we attempted to immunoprecipitate mTORC1 by using N-Rap Ab. Although raptor was readily detected in immunoprecipitates obtained with this antibody, neither mTOR nor mLST8 were found (results not presented). Thus, N-Rap Ab either promoted dissociation of mTOR and raptor or was unable to bind raptor associated with mTOR. To generate raptor antibodies that could be used to immunoprecipitate mTORC1, a peptide having an NH2-terminal Cys followed by 12 amino acids (YISVYSEKVRV) corresponding to the COOH-terminal region of raptor was coupled to keyhole limpet hemocyanin, and the conjugate was used to immunize rabbits. The resulting raptor antibodies (C-Rap Ab) were purified using a column containing an affinity resin prepared by coupling the peptide to Sulfolink beads (Pierce). Rictor antibodies were generated in a similar manner except that a peptide (CRHSPTDAEGQLKEDRE) based on amino acids 263–278 in mouse rictor was used.

Monoclonal antibody 12CA5, which recognizes the HA epitope tag, was purified from hybridoma culture medium. Phosphospecific antibodies to the Ser-473 site in Akt2, the Thr-389 site in S6K1, and the activating sites in the ERK1 and ERK2 isoforms of mitogen-activated protein kinase were from Cell Signaling Technology Inc.

Purification of Recombinant Proteins—His-tagged forms of wild type 4EBP1 and a 4EBP1 protein having Ala at position 113 (F113A) were expressed in bacteria and purified as described previously (22). To assess purity and to confirm protein concentrations, samples were subjected to SDS-PAGE and then stained with Coomassie Blue (22). Complexes of eIF4E bound to mTOR and raptor (results not shown). Adipocyte extract samples (800 μl) were incubated with C-Rap Ab (2 μg) bound to protein A-agarose beads (15 μl) or with 12CA5 (2 μg) bound to protein G-agarose beads (15 μl) at 4 °C for 12 h with constant mixing. As a control for specificity, rabbit or mouse nonimmune IgG was substituted for the C-Rap Ab or 12CA5, respectively. The beads were then washed once with 1 ml of Buffer A, once with 1 ml of Buffer A plus 0.5 mM NaCl, and then twice with 1 ml of Buffer A.

Expression of HA-raptor in 3T3-L1 Adipocytes by Adenoviral Mediated Gene Transfer—Virus for expressing HA-raptor was prepared using the system developed by He et al. (32). Briefly cDNA encoding HA-tagged raptor was excised with KpnI and NotI from the pBluescript construct described previously (22) and inserted between the KpnI and NotI sites in the shuttle vector, pAdTrack. The resulting plasmid was cotransformed into human embryonic kidney 293 cells to generate virus, which was amplified and then purified by CsCl gradient centrifugation to create a high titer viral stock. 3T3-L1 adipocytes were infected essentially as described by Kasuga and co-workers (33). The efficiency of infection judged by expression of green fluorescent protein, which is also encoded by the HA-raptor virus, was ~50%. Virus encoding β-galactosidase was used as a control.

Expression Constructs for mTOR, Raptor, and mLST8—Most of the expression constructs used have been described previously (14,
22, 28). To generate a construct for expressing Myc-raptor, cDNA encoding raptor was excised from an HA-raptor-pcDNA3 vector (22) by using EcoRI and inserted into the EcoRI site in pcMVTag2B (Stratagene). The construct for expressing untagged mLST8 was prepared by excising mLST8 cDNA from the HA-mLST8 vector (28) and then inserting the mLST8 fragment into pcDNA3. To generate vector for expressing FLAG-mTOR, a fragment corresponding to bp 1–632 of the mTOR coding region was amplified by PCR with AU1-mTOR pcDNA3 template and the following primers: 5′-GGCCAGATCCACCATGGGCGAGGCCCTG-3′ and 5′-GGCAGAGCTCTAGAGCCACAGCTCTTTACGAGATG-3′. The fragment was digested with BamHI and SalI, and the product was inserted between the BamHI and SalI sites in pCMVTag2A (Stratagene) to generate NT-pCMVTag2A. To complete construction of the FLAG-mTOR expression construct (mTOR-pCMVTag2A), EcoRI and XbaI sites in the mTOR coding region in NT-pCMVTag2A and XbaI sites in the NT-pCMVTag2A construct. The region of the FLAG-mTOR cDNA generated by PCR was sequenced and found to be free of errors.

**Immune Complex Assay of mTORC1 Activity**—Immune complex beads were rinsed with 1 ml of Buffer B (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM microcystin LR, 10 mM HEPES, and 50 mM β-glycerophosphate, pH 7.4) and suspended in 60 μl of Buffer B. After removing a sample for immunoblotting mTOR and raptor, the kinase reactions were initiated by adding to 20 μl of the suspension 5 μl of Buffer A supplemented with 0.5 mM [γ−32P]ATP (PerkinElmer Life Sciences, 1,000 mCi/mmol), 50 mM MnCl2, and 1 μg of the Histagged form of either wild type 4EBP1 or F113A. Unless otherwise stated, reactions were terminated after 30 min at 30 °C by adding SDS sample buffer. The relative amounts of 32P incorporated into the 4EBP1 proteins were determined by phosphorimaging following SDS-PAGE. Measurements under these conditions reflect the initial rate of phosphorylation as less than 5% of the available substrates were phosphorylated, and the reactions proceed linearly for 60 min (see later, Fig. 2b).

**4EBP1 Binding to C-Rap Ab Immune Complexes**—Immune complexes from 400 μl of extract (7.5 μl of beads) were suspended in Buffer A (500 μl) containing 50 ng of the His-tagged form of either 4EBP1 or F113A. After incubating at 21 °C for 1 h with constant mixing, the beads were washed four times with 1 ml of Buffer A, twice with Buffer A plus 0.5 mM NaCl, and then once with a solution containing 1 mM EDTA, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The relative amounts of the 4EBP1 proteins retained by the beads were determined by immunoblotting.

**Binding of Raptor to 4EBP1 Affinity Resins**—His-tagged 4EBP1 or F113A proteins (1 mg) were coupled to CNBr-activated Sepharose (86 mg) in 0.5 mM NaCl and 0.1 mM NaHCO3 (pH 8.3). After 12 h at 4 °C, the resin was washed exhaustively as directed by the supplier (Amersham Biosciences). The beads were then suspended in 0.5 ml of buffer (0.5 mM NaCl and 50 mM Tris-HCl, pH 7.4) and stored at 4 °C prior to use. For binding, an aliquot (15 μl) of the beads was added to 800 μl of extract. After incubating at 4 °C for 12 h with constant mixing, the beads were washed as described above for 4EBP1 binding to C-Rap Ab immune complexes. The relative amounts of raptor and mTOR retained by the beads were determined by immunoblotting.

**Electrophoretic Analyses**—SDS-PAGE and immunoblotting were conducted as described previously (12). Binding of primary antibodies was monitored by using the appropriate alkaline phosphatase-conjugated secondary antibodies, which were detected by using CDP-Star reagent (PerkinElmer Life Sciences). Relative signal intensities of bands in immunoblots were determined by scanning laser densitometry of x-ray films or by using a Fujifilm LAS 3000 LCD camera system.

**Gel Filtration**—Adipocytes were incubated without or with insulin, rinsed three times with Buffer A (minus detergents and dithiothreitol) that had been chilled on ice, and scraped from the dishes (six 10-cm-diameter dishes per treatment). To enhance resolution by the column, the volume of extract was minimized by homogenizing cells in 500 μl of Homogenization Buffer. The homogenates were centrifuged at 12,000 × g for 10 min. The supernatants were retained and passed through a 0.45-μm filter. Extract samples (350 μl) were applied to a Superose 6 HR 10/30 column (Amersham Biosciences) that had been equilibrated in Buffer A. The flow rate was maintained at 0.2 ml/min, and 1-ml fractions were collected.

**Other Materials**—Recombinant human insulin (Novolin R) was from Novo Nordisk. Rapamycin, LY294002, and U0126 were from Calbiochem-Novabiochem. Epidermal growth factor was from Upstate Biologicals. Fibroblast growth factor-1 was provided by Dr. David Ornitz (Washington University). Farnesylthiosalicylic acid was provided by Dr. Wayne Bardin (Thyreo, New York, NY). TWEEN 20 was from Fischer. CHAPS was from Roche Applied Science. Caffeine, insulin-like growth factor 1, Nonidet P-40, Triton X-100, and wortmannin were from Sigma.

**RESULTS**

**Stable Activation of mTORC1**—mTORC1 was immunoprecipitated from extracts of 3T3-L1 adipocytes by using an antibody (C-Rap Ab) to the COOH-terminal region of raptor (Fig. 1A). Raptor, mTOR, and mLST8 were detected in the immune complexes, confirming that intact mTORC1 was recovered (Fig. 1B). To measure mTORC1 activity, immune complexes were incubated with [γ−32P]ATP and 4EBP1. Treating adipocytes with insulin increased 4EBP1 phosphorylation by mTORC1 (Fig. 1B). No activity was detected in complexes isolated without nonimmune IgG. The 3-fold increase in mTORC1 activity produced by insulin (Fig. 1B) is comparable to the increase in phosphorylation of endogenous 4EBP1 when 32P-labeled 3T3-L1 adipocytes are incubated with the hormone (17). mTORC1 isolated with C-Rap Ab was not able to phosphorylate F113A (Fig. 1C), a 4EBP1 protein having a point mutation that disrupts the TOS motif (22).

In the present experiments, 3T3-L1 adipocytes were incubated in buffer lacking amino acids to isolate the effects due to insulin from those mediated by amino acids, which also activate mTOR signaling (34). Activating mTOR signaling with insulin has been shown previously to increase 4EBP1 phosphorylation in 3T3-L1 adipocytes incubated in buffer without added amino acids (11, 17). The incubation buffer was supplemented with...
albumin to bind fatty acids released from the adipocytes. To be sure that the albumin was not replacing a requirement for amino acids, control experiments were conducted in buffer lacking albumin. Removing the albumin did not attenuate the effects of insulin on mTOR activity. Thus, the insulin response is not occurring secondarily to an increase in amino acid transport.

To confirm findings with the C-Rap Ab, HA-raptor was overexpressed in 3T3-L1 adipocytes by using an adenovirus vector. HA antibodies were then used to isolate mTORC1 (Fig. 1D). HA-raptor, mTOR, and mLST8 were immunoprecipitated, indicating that the epitope-tagged raptor was incorporated into mTORC1 (Fig. 1E). Insulin did not change the amount of mTOR or mLST8 that coimmunoprecipitated with HA-raptor, but it clearly increased the 4EBP1 kinase activity (Fig. 1E). mTORC1 subunits and kinase activity were not detected in immune complexes isolated with HA antibodies from cells infected with a control adenovirus.

The effects of insulin on mTORC2 were investigated by isolating this complex by using antibodies to rictor (Fig. 2A). Insulin decreased the electrophoretic mobility of rictor, suggesting that phosphorylation of rictor was increased by the hormone. Less mTOR was recovered with the rictor antibodies than with the C-Rap Ab, consistent with the interpretation that mTORC2 is less abundant than mTORC1 in 3T3-L1 adipocytes. Little if any 32P was introduced into 4EBP1 by mTORC2 in the immune complexes isolated with the rictor antibodies. Even after correcting for the amount of mTOR catalytic subunit present, phosphorylation of 4EBP1 by mTORC2 was negligible compared with that by mTORC1 (Fig. 2B). The incorporation of 32P into 4EBP1 by C-Rap Ab immune complexes increased linearly for at least 1 h (Fig. 2B). Thus, 32P incorporation measured after 30 min, as in Fig. 1B, reflects the initial rate of phosphorylation.

To investigate further the 4EBP1 kinase activity in C-Rap Ab complexes and to confirm that the phosphorylation detected was mediated by mTOR, the complexes were incubated with additions that have been shown previously to inhibit mTOR. Incubating C-Rap immune complexes with either rapamycin (not shown) or FKBP12 alone was without effect on the phosphorylation of 4EBP1 (Fig. 2C); however, the combination of rapamycin plus FKBP12 attenuated phosphorylation of 4EBP1 (Fig. 2C). The inhibition of mTOR by rapamycin is unusual in that to inhibit, rapamycin must first bind to an intracellular receptor, FKBP12 (35). When complexed with FKBP12, rapamycin binds with high affinity to a domain, designated the FRB, which is located upstream of the kinase domain in mTOR. Thus, the requirement for both rapamycin and FKBP12 to suppress 4EBP1 kinase activity supports the conclusion that the insulin-stimulated increase in activity is due to mTORC1.

Rapamycin is not an active site inhibitor of mTOR, and as noted in other studies (13, 14), FKBP12-rapamycin did not fully inhibit the mTOR kinase. In contrast, LY294002, which is
believed to bind in the active site of the kinase, essentially abolished activity (Fig. 2C). The kinase activity in the C-Rap immunoprecipitates was also inhibited by two other inhibitors of mTOR, caffeine (36) and farnesylthiosalicylate (28) (Fig. 2C). These results provide additional evidence that the kinase activity in C-Rap Ab immunoprecipitates was due to mTORC1.

It has been proposed that to promote dissociation of eIF4E from 4EBP1, mTOR must phosphorylate the 4EBP1-eIF4E complex. Therefore, we compared the phosphorylation of free 4EBP1 to that of a complex of recombinant 4EBP1-eIF4E that had been purified by using mGTP affinity chromatography. The recombinant proteins were stained with Coomassie Blue to ensure that equal amounts of 4EBP1 were added as substrate (Fig. 2D). The phosphorylation of both the free and the eIF4E-bound forms of 4EBP1 by mTORC1 was increased in response to insulin. Indeed there was virtually no difference in phosphorylation of the two forms (Fig. 2D). The 4EBP isoform, 4EBP2, also has a TOS motif, and its phosphorylation in cells is inhibited by rapamycin (30). Recombinant 4EBP2 was phosphorylated by mTORC1 (Fig. 2D) but somewhat more slowly than 4EBP1.

Enhancement of TOS Motif-dependent Binding of 4EBP1 to mTORC1 by Insulin—Several potential mechanisms may be envisioned through which insulin could activate mTOR. For example, the hormone might act to increase the intrinsic activity of the mTOR kinase. Alternatively kinase activity of mTORC1 could be enhanced as a result of an increase in substrate binding to the complex. To investigate the latter possibility, C-Rap Ab immune complexes were incubated with purified 4EBP1 (Fig. 3A). 4EBP1 that bound to raptor was detected by immunoblotting after washing complexes to remove the unbound protein (Fig. 3B). Insulin increased 4EBP1 binding by ~5-fold (Fig. 3C). Little if any endogenous 4EBP1 was found in the C-Rap Ab immune complexes (Fig. 3B). Thus, the results were not complicated by differences in the occupancy of binding sites in raptor with endogenous 4EBP1. Phosphorylation of 4EBP1 has been shown to inhibit binding of 4EBP1 to raptor (6). Therefore, it seems likely that the failure to recover endogenous 4EBP1 in C-Rap immune complexes is due to the fact that even in control cells 4EBP1 is phosphorylated to some extent (11).

Disrupting the TOS motif dramatically decreased binding of 4EBP1 to the immune complexes (Fig. 3B), and binding of F113A to mTORC1 was not increased by insulin (Fig. 3C). Coomassie Blue staining confirmed that the same amounts of the two recombinant proteins were added to the reaction (for example, see Fig. 1C). Moreover the 4EBP1 antibodies used have been shown to recognize 4EBP1 and F113A equally well (22). Hence the failure to detect F113A binding to the C-Rap Ab immune complexes was due to the effect of the mutation on inhibiting the interaction with raptor and not to inactivity of the 4EBP1 antibody to recognize F113A.

As another approach to investigate binding, recombinant 4EBP1 proteins were coupled to agarose beads, which were then used as an affinity resin to bind mTORC1 (Fig. 3D). Adipocyte extracts were incubated with the beads, which were washed before preparing mTOR and raptor immunoblots (Fig. 3E). Insulin markedly increased the amounts of both mTOR and raptor retained by the beads. Binding of raptor was inhibited by supplementing extracts with 4EBP1 but not by adding F113A (Fig. 3F). Moreover neither mTOR nor raptor was retained by F113A beads (Fig. 3E), indicating that the recovery of mTORC1 required a functional TOS motif. Because equal amounts of 4EBP1 and F113A were coupled to the beads, we were confident that the failure of the F113A beads to capture raptor was not due to the absence of the mutant protein on the beads. Nevertheless we conducted experiments to confirm independently that the F113A protein was present and that the integrity of the protein had not been destroyed by the coupling process. Because the TOS motif does not participate in binding of 4EBP1 to eIF4E (10), functionality of the F113A beads could

**FIGURE 2.** Time course of phosphorylation, effects of inhibitors, and phosphorylation of 4EBP-eIF4E complexes by mTORC1. A3T3-L1 adipocytes were incubated without or with insulin for 30 min before extracts were prepared. A, mTORC1 and mTORC2 were immunoprecipitated by using antibodies to raptor (C-Rap Ab) and rictor, respectively. The relative amounts of mTOR, rictor, and rictor associated with the two mTOR complexes were determined by immunoblotting. The shift in mobility of rictor caused by insulin is indicative of covalent modification of the protein. B, mTORC1 and mTORC2 from insulin-treated cells were incubated with [γ-32P]ATP and 4EBP1 for either 30 or 60 min. The kinase reactions were stopped by adding SDS sample buffer, and aliquots were subjected to SDS-PAGE. The relative amounts of 32P incorporated into 4EBP1 were determined by phosphorimaging. The results presented were corrected for the recovery of mTOR, which was estimated from immunoblots, and are mean values (±S.E.) from three experiments. C, washed C-Rap Ab immune complexes were incubated for 30 min in kinase reaction mixtures containing no additions (None) or the following: 10 μM LY294002, 10 μM GST-FKBPD (FKBP12), 10 μM GST-FKBPD plus 10 μM rapamycin (Rapa), 1 mM caffeine, or 20 μM farnesylthiosalicylate (FTS). A phosphorimage of 32P-labeled 4EBP1 is presented. D, phosphorylation of free and eIF4E-bound forms of recombinant 4EBP1 or 4EBP2 by C-Rap immune complexes or by complexes recovered with nonimmune (NI) IgG was measured after Coomassie Blue staining to normalize the 4EBP concentrations in the different preparations. IP, immunoprecipitate; BP-4E, 4EBP1-eIF4E; BP2-4E, 4EBP2-eIF4E.
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**FIGURE 3.** Activation of mTORC1 by insulin is associated with increased 4EBP1 binding to raptor. 3T3-L1 adipocytes were incubated without and with insulin. A, mTORC1 was immunoprecipitated using C-Rap Ab bound to protein A-agarose and incubated with recombinant 4EBP1 proteins. B, immunoblots of mTOR, raptor, and 4EBP1 proteins bound after washing immune complexes that had been incubated without or with 4EBP1 (wild type (WT)) or F113A. C, relative amounts of 4EBP1 proteins bound, expressed as a percent maximum (means ± S.E. from three experiments) are presented. D, mTORC1 was recovered from adipocyte extracts by using 4EBP1 coupled to agarose beads. E, immunoblots of mTOR, raptor, and elF4E bound to 4EBP1 beads or to F113A beads. F, extracts from insulin-treated cells were supplemented with recombinant 4EBP1 or F113A before incubation with 4EBP1 beads. The amounts of raptor and elF4E bound were determined by immunoblotting. CON, control; INS, insulin; PA, protein A; BP1, 4EBP1.

**FIGURE 4.** Time courses of responses to insulin. 3T3-L1 adipocytes were incubated for increasing times with insulin. A, mTORC1 was isolated from extracts by using C-Rap Ab. Recovery of mTOR and raptor in the C-Rap Ab complexes was monitored by immunoblotting. Kinase activity was assessed by phosphorimaging after incubating samples of the complexes with [γ-32P]ATP. Other samples were incubated with 4EBP1 before the beads were washed, and the amount of 4EBP1 bound was determined by immunoblotting. Extract samples were also subjected to SDS-PAGE, and immunoblots were prepared with phosphospecific antibodies to sites in Akt, mTOR, and S6K1. B, mTORC1 kinase activity and 4EBP1 binding expressed as percentages of the respective maximum responses (means ± S.E. of three experiments). C, 4EBP1 and S6K1 phosphorylation in cells determined from immunoblots with the phospho-Thr-36/45 (P-T36/45) antibodies and the phospho-Thr-389 (P-T389) antibodies, respectively. IP, immunoprecipitate; P, phospho-

be judged by their ability to bind to elF4E. Relatively little elF4E from control cells was retained. However, elF4E was readily detected on both 4EBP1 beads and F113A beads that had been incubated with extracts from insulin-treated cells (Fig. 3E). The increase in response to insulin is due to the effect of the hormone on promoting dissociation of the endogenous 4EBP1-elF4E complex, an action that increases the amount of elF4E available to bind the resin (37). Thus, both 4EBP1 and F113A bound to the beads were competent to bind elF4E.

**Time Course and Concentration Dependence of the Insulin Responses and Effects of Growth Factors and Inhibitors**—With a maximally effective concentration of insulin, increases in both binding and activity were clearly evident after only 2 min (Fig. 4A), and the maximum effects on both were observed after 20 min of incubation with insulin (Fig. 4B). To compare the time course of mTORC1 activation with the phosphorylation of known effectors of insulin, immunoblots were prepared with phosphospecific antibodies to sites in Akt, mTOR, S6K1, and 4EBP1 (Fig. 4A). Insulin markedly increased the phosphorylation of Ser-473 in Akt. This response reached a maximum after only 2 min as did the phosphorylation of mTOR in Ser-2448, which is phosphorylated in response to an increase in Akt activity. Insulin also markedly increased the phosphorylation of sites in 4EBP1 and S6K1. The time courses of the stimulatory effects of insulin on 4EBP1 kinase activity and raptor binding to 4EBP1 were very similar (Fig. 4B).

The phosphorylation of 4EBP1 and S6K1 (Fig. 4C) in response to insulin treatment reached a maximum slightly before mTOR kinase activity reached a maximum. This does not mean that the activation of mTORC1 observed in Fig. 4B is too slow to account for phosphorylation of S6K1 and 4EBP1 because the time required to reach a new level of phosphorylation following activation of a protein kinase in a cell depends on the rate of dephosphorylation the protein.

Significant effects of insulin on both binding and activity were observed at a concentration of 6 nM (Fig. 5A), which is in the physiological range of insulin concentrations. Insulin-like growth factor 1 was as equally effective as insulin in increasing mTORC1 binding to 4EBP1 (Fig. 5B), mTORC1 kinase activity (Fig. 5C), and the activation of Akt and S6K1 (Fig. 5D). In contrast, epidermal growth factor, which was at least as effective as insulin in activating mitogen-activated protein kinase, as assessed by the phosphorylation of ERK1/2.
and endogenous FKBP12 bind mTOR persist following homogenization of the cells and immunoprecipitation of mTORC1.

Stimulation of Kinase Activity and Substrate Binding by Insulin Require Intact mTORC1—To determine whether the effects of insulin on 4EBP1 binding to raptor were retained after disrupting mTORC1, extracts were supplemented with Triton X-100 or Nonidet P-40, which dissociate mTOR and raptor
treatment did reduce mTORC1 kinase activity (Fig. 5E) but too low to inhibit mTOR directly (38). Inhibiting activation of mitogen-activated protein kinase with U0126 (Fig. 4B), although this was not surprising in view of previous studies showing that nonionic detergents abolish the 4EBP1 kinase of mTOR (6). Both insulin-stimulated kinase activity (Fig. 6B) and the hormonal effect on 4EBP1 binding (Fig. 6A) were preserved in the presence of Tween 20 and CHAPS, which do not cause dissociation of mTORC1.

Dimeric mTORC1 Is Insulin-responsive—To define better the nature of the insulin-responsive mTOR signaling complex, extract proteins were size-fractionated by using a Superose 6 HR 10/30 column (Fig. 7). Immunoblotting fractions from the column with antibodies to mTOR (Fig. 7A) revealed two major peaks, centered at fractions 9 and 13 (Fig. 5D), did not increase 4EBP1 binding to mTORC1 (Fig. 5B) or the kinase activity of the complex (Fig. 5C).

To investigate further the pathways leading to the stimulatory effects of insulin on mTORC1 activity and 4EBP1 binding, adipocytes were incubated with inhibitors of several signaling pathways. The effects of insulin on both activity and binding were abolished by treating adipocytes with wortmannin at a concentration (100 nM) sufficient to block Akt activation (Fig. 5, A and B) were abolished by treating adipocytes with wortmannin at a

FIGURE 5. Relative effects of insulin, growth factors, and inhibitors on mTORC1. A, 3T3-L1 adipocytes were incubated for 30 min with the following concentrations of insulin: 0, 0.06, 6, and 600 nM. 4EBP1 binding and kinase activity were assessed in C-Rap immune complexes. The results represent the respective maximum changes due to insulin and are mean values + S.E. of three experiments. B–D, adipocytes were incubated for 15 min without additions (None) or with insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), or fibroblast growth factor (FGF) at a concentration of 5 nM. Extracts were incubated with 4EBP1 beads. After washing the beads to remove unbound proteins, samples were subjected to SDS-PAGE, and immunoblots of mTOR, raptor, and elf4E were prepared. C, mTORC1 was immunoprecipitated using C-Rap Ab. The amounts of mTOR and raptor recovered were determined by immunoblotting. mTORC1 activity was measured using 4EBP1 as substrate. A phosphorimage of the 32P-labeled 4EBP1 product and an immunoblot prepared using phosphospecific antibodies to sites in Akt, mTOR, S6K1, and ERK1/2. Antibodies are shown.

Activation of Dimeric mTORC1

FIGURE 6. Stimulation of 4EBP1 kinase activity by insulin. A, 3T3-L1 adipocytes were incubated without additions (None) or with one of the following: 20 nM rapamycin (Rapa), 100 nM wortmannin (Wm), or 10 μM U0126. After 30 min, insulin was added as indicated, and the incubations were continued for 30 min before extracts were prepared. mTORC1 was immunoprecipitated by using C-Rap Ab. E, mTOR kinase activity and 4EBP1 binding to mTORC1 were measured. F, to confirm the effectiveness of the inhibitors, samples of extracts were subjected to SDS-PAGE, and immunoblots were prepared using phosphospecific antibodies to sites in Akt, mTOR, S6K1, and ERK1/2. IP, immunoprecipitate; P, phospho-.
11 was not changed by insulin, but the amount of raptor in the high M<sub>r</sub> peak was increased somewhat by the hormone. The amount of raptor in the low M<sub>r</sub> peak was not changed by insulin, and the amount of mLST8 was not significantly altered by the hormone in either the high or the low M<sub>r</sub> peaks (Fig. 7, E and F).

Insulin increased the 4EBP1 kinase activity measured in C-Rap immune complexes isolated from both peaks of mTOR, although the bulk of the kinase activity was found in the low M<sub>r</sub> peak (Fig. 7I). Insulin also increased the amount of both mTOR and raptor retained by 4EBP1 beads in both the high and low M<sub>r</sub> peaks (Fig. 7, G and H). In contrast, insulin was without effect on the amount of raptor captured from the peak at fraction 17 (Fig. 7H). Because no mTOR was detected in this peak, the lack of effect of insulin on raptor binding to 4EBP1 in this fraction provides additional evidence that insulin action on 4EBP1 binding to raptor depends on the association of raptor and mTOR.

The predicted M<sub>r</sub> of an mTOR-M<sub>r</sub>/H<sub>11015</sub>289,000-raptor-M<sub>r</sub>/H<sub>11015</sub>149,000-mLST8-M<sub>r</sub>/H<sub>11015</sub>36,000 heterotrimer is M<sub>r</sub>/H<sub>11011</sub>474,000, which is considerably lower than that of the species in the low M<sub>r</sub> peak. Assuming an equal stoichiometry of subunits, a complex containing two such heterotrimers has a predicted M<sub>r</sub> closest to the complexes in the low M<sub>r</sub> peak. However, because
forms of mTOR and raptor together with the estimated sizes of the complexes from the experiments in Fig. 7 are consistent with the view that the low $M_r$ peak contains dimeric mTORC1 (two mTOR-raptor-mLST8 heterotrimers).

The estimated size of epitope-tagged raptor not associated with mTOR ($M_r \approx 3,500,000$) is similar to that ($M_r \approx 3,000,000$) predicted of a raptor dimer. The finding that Myc-raptor coimmunoprecipitated with HA-raptor (Fig. 8F) confirms that raptor dimers existed in this peak, although we cannot exclude the possibility that other smaller proteins are also associated with the raptor dimers. The proteins from the high $M_r$ peak immunoprecipitated less efficiently than those from the low $M_r$ peak (Fig. 8, E and F). This complicated the analyses, although we were able to detect signals indicative of coimmunoprecipitation. Thus, it seems likely that multimers of the mTORC1 subunits are present in the high $M_r$ peak, although additional studies will be needed to define the nature of these complexes.

**DISCUSSION**

Two key findings of the present study are that insulin promotes a stable increase in the kinase activity of dimeric mTORC1 and that the increase in kinase activity is associated with a marked increase in the binding of substrate to raptor. The stimulatory effects of insulin on mTOR activity and binding occurred rapidly and at physiological concentrations of the hormone. Both effects of insulin were dependent upon the TOS motif in the substrate, 4EBP1, and it was essential to preserve intact mTORC1 to detect the effect of insulin on kinase activity and 4EBP1 binding.

Our studies provide the first evidence of dimeric mTORC1, although there is recent evidence of complexes containing multiple TOR proteins in yeast and flies. Zhang et al. (39) used a biochemical approach, as well as a genetic strategy involving intragenic complementation, to demonstrate that *Drosophila melanogaster* cells contain functional complexes harboring multiple TOR proteins. Wullschleger et al. (40) presented evidence of a dimeric TORC2 in *Saccharomyces cerevisiae*. These investigators also showed that mTOR proteins harboring different epitope tags could be coimmunoprecipitated, indicating that complexes containing multiple mTOR proteins exist in mammalian cells; however, they did not determine the number of mTOR proteins found in the coimmunoprecipitating complexes or whether the epitope-tagged proteins were in mTORC1 or mTORC2 (40). Although the present experiments did not directly address which subunits mediate dimerization of mTORC1, the results would be consistent with a model similar to that proposed by Wullschleger et al. (40) in which the structure is maintained by interactions between the HEAT and FAT domains of a pair of TOR proteins. However, our results suggest that interactions between raptor proteins might also contribute to the stability of the higher order complex as raptor dimers not associated with mTOR were detected. In addition to dimeric mTORC1, an even larger complex was evident from the high $M_r$ peak, which eluted in the position of the $M_r$ 2,000,000 marker. The nature of this larger complex is poorly defined, and it may represent mTORC1 associated with other proteins, such as the eIF3 complex, which was shown recently to bind mTOR (41, 42).
Activation of Dimeric mTORC1

We have found that kinase-dead mTOR (43) as well as Δrd mTOR, a protein rendered constitutively active by deletion of a putative regulatory domain (24), associate with raptor and elute in the low Mₙ peak, indicating that formation of the dimeric mTORC1 does not depend on mTOR activity. Insulin did not significantly change the amount of dimeric mTORC1, indicating that the hormonal control is not due to gross alterations in the multimeric state of mTOR. The mechanism through which insulin activates mTORC1 is still undefined, and at this point, any of the subunits in the complex must be considered as candidates for mediating the insulin response.

There have been significant advances in defining the upstream elements in the mTOR signaling pathway. The stimulation of 4EBP1 phosphorylation by insulin depends on activation of Akt (23, 24, 44). The rapidity of the phosphorylation of Ser-473 in response to insulin would be consistent with positioning Akt upstream of mTOR (Fig. 4). Insulin also rapidly increased phosphorylation of mTOR in Ser-2448, which is phosphorylated in response to Akt activation (2). Recent evidence indicates that this site is phosphorylated by S6K1 (45, 46), which is downstream of mTOR. However, rapamycin abolished S6K1 activation by insulin, but it did not did not abolish Ser-2448 phosphorylation in either primary (23) or 3T3-L1 adipocytes (Fig. 5F). Consequently S6K1 cannot be the only kinase phosphorylating Ser-2448 in adipocytes. In any event, it is unlikely that phosphorylation of Ser-2448 is the primary signal for activating mTORC1 as Sekulic et al. (24) found that mTOR harboring an Ala-2448 mutation, which ablates the Ser-2448 phosphorylation site, supported the activation of mTOR signaling by insulin in human embryonic kidney 293 cells.

Another link between Akt and mTOR involves the GTP-binding protein Rheb and the tuberous sclerosis proteins TSC1 and TSC2 (1, 2). These two proteins form a complex that suppresses mTOR activity by functioning as a GTPase-activating protein to decrease the active, GTP-bound form of Rheb. TSC2 is phosphorylated by Akt, which has been proposed to inhibit the GTPase-activating function of TSC2 (47, 48), although this point remains hypothetical. GST-Rheb-GTP binding to HA-mTOR in cells was associated with an increase in mTOR activity measured in vitro with S6K1-(355–525) (49), a fragment lacking the TOS motif necessary for binding to raptor. Moreover, Rheb-GTP did not increase the binding of raptor to 4EBP1 (49). Therefore, it would be premature to conclude that Rheb mediates the TOS-dependent effects of insulin on kinase activity and 4EBP1 binding to mTORC1 described in the present study.

Although the mechanism is unclear, it is reasonable to suspect that the effect of insulin on increasing binding of 4EBP1 to mTORC1 contributes to the increase in mTOR activity. The paradigm in which kinase activity is increased as a result of increased substrate binding to an associated noncatalytic protein represents an unusual mechanism for protein kinase activation. For such a kinase system to operate efficiently, phosphorylation of the substrate must decrease its affinity for raptor. Otherwise the turnover number of the enzyme complex would be much too low. Therefore, the finding of Hara et al. (6) that phosphorylation of 4EBP1 abolishes binding to raptor fulfills an important requirement for raptor as a substrate-binding subunit.

The enhanced binding to 4EBP1 may serve a function not directly related to increasing the kinase activity of mTORC1. For example, the interaction with the 4EBP1-eIF4E complex might localize mTORC1 at the 5′-end of mRNAs to which the 4EBP1-eIF4E complex is bound. In this connection, the recent findings that insulin increases the association of both mTORC1 and eIF4G with eIF3 (41, 42) are intriguing. These interactions (41, 42) and the increase in binding of mTORC1 to 4EBP1 observed in the present study might serve to place eIF4G in a favorable position to bind to eIF4E after 4EBP1 has been phosphorylated by mTOR.

A much more speculative hypothesis is that mTORC1 and 4EBP1 might actually replace eIF4G in an alternative initiation complex. By binding to both eIF3 and 4EBP1-eIF4E, mTORC1 would be expected to recruit the small ribosomal subunit (bound to eIF3) to the 5′-end of the message. Whether such a complex would be competent to initiate scanning is of course hypothetical, but most of the essential initiation factors would be expected to be bound to either eIF3 or the 40S ribosomal subunit. This complex would lack the helicase, eIF4A, if eIF4G was not bound to eIF3. eIF4A is essential for translation of messages having secondary structure in the 5′-untranslated region (10); however, messages with unstructured 5′-untranslated regions can be translated without eIF4A. An interesting implication is that by recruiting mTORC1 and eIF3 to the 5′-end of certain mRNAs, 4EBP1 might facilitate translation instead of acting as a translational repressor. Such a role would help to understand why 4EBP1, like eIF4G (50), actually increases binding of eIF4E to the mRNA cap (51). Future studies are needed to investigate the role of mTORC1 in the control of translation initiation and to identify the modifications in the complex that lead to increased 4EBP1 binding and kinase activity.

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