Insulin and nutrients activate hepatic p70 S6 kinase (S6K1) to regulate protein synthesis. Paradoxically, activation of S6K1 also leads to the development of insulin resistance. In this study, we investigated the effect of TRB3, which acts as an endogenous inhibitor of Akt, on S6K1 activity in vitro and in vivo. In cultured cells, overexpression of TRB3 completely inhibited insulin-stimulated S6K1 activation by mammalian target of rapamycin, whereas knockdown of endogenous TRB3 increased both basal and insulin-stimulated activity. In C57BL/6 mice, adenoviral overexpression of TRB3 inhibited insulin-stimulated activation of hepatic S6K1. In contrast, overexpression of TRB3 did not inhibit nutrient-stimulated S6K1 activity. We also investigated the effect of starvation, feeding, or insulin treatment on TRB3 levels and S6K1 activity in the liver of C57BL/6 and db/db mice. Both insulin and feeding activated S6K1 in db/db mice, but only insulin activates in the C57BL/6 strain. TRB3 levels were 3.5-fold higher in db/db mice than C57BL/6 mice and were unresponsive to feeding or insulin, whereas both treatments reduced TRB3 in C57BL/6 mice. Akt was activated by insulin alone in the C57BL/6 strain and but not in db/db mice. Both insulin and feeding activated mammalian target of rapamycin similarly in these mice; however, feeding was unable to activate the downstream target S6K1 in C57BL/6 mice. These results suggest that the nutrient excess in the hyperphagic, hyperinsulinemic db/db mouse primes the hepatocyte to respond to nutrients resulting in elevated S6K1 activity. The combination of elevated TRB3 and constitutive S6K1 activity results in decreased insulin signaling via the IRS-1/phosphatidylinositol 3-kinase/Akt pathway.

The p70 ribosomal protein S6 kinase 1 (S6K1) participates in a variety of intracellular signaling events, including mRNA translation, gene transcription, and cell cycle control (1–3). Insulin, and several nutrients such as amino acids, are known to increase the phosphorylation and activation of S6K1 (4). A negative feedback loop involving S6K1 phosphorylation of IRS1 serves to limit insulin signaling (5, 6). Thus, the absence of S6K1 results in an enhancement of insulin sensitivity and a reduction of body fat in mice (6). Conversely, S6K1 activity is elevated in several tissues of animal models of obesity, and/or type 2 diabetes, and correlates with increased insulin resistance (6).

Both insulin and amino acid induction of S6K1 activity are mediated by mTOR, an evolutionarily conserved serine/threonine kinase that is the mammalian target of rapamycin (7, 8). mTOR is found in a complex (mTORC1) with raptor and mLST8 and is activated by the small GTPase Rheb (9, 10). The tuberous sclerosis proteins TSC1 and TSC2 maintain Rheb in the inactive GDP-bound state because of the Rheb-GAP enzyme activity of TSC2. Activation of mTOR by insulin is associated with phosphorylation of TSC2, dissociation of the Rheb-TSC1-TSC2 complex, and relief of Rheb repression (11–13). One of the key enzymes responsible for phosphorylation of TSC2 by insulin is the serine/threonine kinase Akt (14, 15), which is activated in a class I phosphoinositide 3-kinase (PI 3-kinase)-dependent manner (16, 17). In contrast, nutrients such as amino acids activate the mTOR-S6K1 pathway independently of the insulin pathway, via the class III PI 3-kinase hVps34 (18, 19). Unlike insulin, amino acids cause GTP loading on Rheb in a TSC2-independent manner suggesting a parallel pathway leading to mTOR activation. This nutrient-regulated pathway is essential for Akt/TSC2/mTOR signaling, however, because inhibition of hVps34 or its product PI(3)P prevents the insulin stimulation of S6K1 (19).

Recently, Du et al. (21) identified TRB3, a mammalian homolog of Drosophila tribbles, also called NIPK (neuronal cell death-inducible protein kinase) (20), as an endogenous inhibitor of Akt. TRB3 binds to unphosphorylated Akt and prevents its activation. Expression of TRB3 mRNA is induced in the liver by fasting and is elevated in db/db mice. Adenoviral expression of TRB3 impairs insulin-mediated glycogen synthesis and prevents inhibition of gluconeogenesis, thus increasing blood glucose levels (21, 22). These observations have led to the suggestion that TRB3 elevation contributes to the development of insulin resistance. In support of this notion, knockdown of TRB3 improves glucose tolerance in C57BL/6 mice, and TRB3 overexpression reverses the insulin-sensitive phenotype of PGC1-deficient mice (22). However, TRB3-mediated inhibition of Akt may also inhibit insulin-induced S6K1 activation.
Effect of TRB3 on Hepatic p70 S6 Kinase Activity

**FIGURE 1. Effect of overexpression of TRB3 on insulin-stimulated S6K1 activity in vitro.** A, TRB3 inhibits insulin stimulation of S6K1 activity. Primary mouse hepatocytes were infected with the m.o.i. (pfu/cell) of adenovirus indicated 24 h prior to treatment with (+) or without (−) insulin (100 nM) for 15 min. S6K1 activity was measured in vitro on cell extracts. B, TRB3 inhibits insulin-stimulated phosphorylation of Akt, TSC2/tuberin, mTOR, S6K1, S6 ribosomal protein, and 4E-BP1 but not of ERK1/2. Primary mouse hepatocytes were infected with increasing doses of adenovirus and stimulated with insulin, as described above. Cells were harvested, and the total cell lysates were analyzed by immunoblotting using the antibodies indicated. C, TRB3 inhibits the Akt-mediated phosphorylation of S6K1. Primary mouse hepatocytes were infected with an m.o.i. of 20 were stimulated with or without pervanadate (PV, 100 μM) for 15 min. Phosphorylation of Thr-389 and Thr-421/Ser-424 of S6K1 was assayed by immunoblotting. Data in A are expressed as mean ± S.D. for five samples in each group. Data in B and C are representative of three independent experiments. *, p < 0.05; **, p < 0.01, when compared with noninfected or GFP-infected, insulin-treated cells.

and protein synthesis. Inhibition of S6K1 is associated with insulin sensitization because of negative feedback on IRS1 (6), so TRB3 might mitigate insulin resistance.

In this study, we investigated the role of TRB3 in nutrient- and insulin-induced activation of S6K1, both in vitro and in vivo. We find that TRB3 is a potent inhibitor of insulin-stimulated, but not nutrient-stimulated, S6K1 activation. TRB3 protein levels are constitutively elevated in db/db mice and are not responsive to nutrients or insulin. Consequently, Akt signaling is severely compromised, but surprisingly, insulin activation of S6K1 is normal in db/db mice despite the elevated TRB3.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP was purchased from Amersham Biosciences. Phosphospecific rabbit polyclonal antibodies against p-Akt (Thr-380 and Ser-473), p-TSC2/tuberin (Thr-1462), p-mTOR (Ser-2448), p-S6K1 (Thr-389), p-S6K1 (Thr-421/Ser-424), p-S6 ribosomal protein (Ser-235/236), p-4E-BP1 (Ser-65), and p-GSK3α/β (Ser-21/9) and antibodies against Akt, mTOR, S6K1, 4E-BP1, GSK3, and p44/42 mitogen-activated protein kinase (ERK1/ERK2) were from Cell Signaling (Beverly, MA). Antibodies against TRB3-(1–145) were purchased from Calbiochem. Analytical grade porcine insulin was from Sigma.

**Plasmids**—Mammalian expression plasmids were used to express the following proteins: TRB3 (Dr. M. Montminy, Salk Institute for Biological Studies, La Jolla, CA); FLAG-tagged 4E-BP-1 (FLAG-4E-BP1) (Dr. K. L. Guan, University of Michigan Medical School, Ann Arbor, MI); and HA-tagged S6K1 (HA-S6K1) (Dr. J. Blenis, Harvard Medical School, Boston).

**Preparation of Recombinant Adenovirus**—The recombinant adenoviruses encoding TRB3, or green fluorescent protein (GFP), were generated using the AdEasy system, as described previously (23). The recombinant adenovirus was amplified in HEK293 cells, and the 50% tissue culture infectious dose (TCID50) was determined as pfu/ml (24).

**Cell Culture and Recombinant Adenovirus Infection**—HepG2 cells and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Mouse hepatocytes were isolated from the livers of male ddY mice (25–35 g, 8–10 weeks) using collagenase perfusion (25) and were plated onto culture dishes. Prior to experimen-
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FIGURE 2. Effect of siRNA against TRB3 on insulin-stimulated S6K1 activity in vitro. A, TRB3 knockdown increases basal and insulin-stimulated S6K1 activity. HepG2 cells were transfected with the siRNAs indicated 24 h prior to treatment without (−) or with (+) insulin (100 nM) for 15 min. S6K1 activity was measured in vitro on cell extracts. Data are expressed as mean ± S.D. for five samples in each group. B, TRB3 knockdown enhances insulin-stimulated phosphorylation of S6 and 4E-BP1. HepG2 cells were transfected with the siRNAs indicated and were stimulated with insulin, as described above. Cells were harvested, and total cell lysates were obtained and then analyzed with immunoblotting, using the antibodies indicated. Blots are representative of three independent experiments. C and D, quantification of phosphorylation of S6 and 4E-BP1. Data are expressed as mean ± S.D. from three independent experiments. *, p < 0.05; **, p < 0.01, when compared with GFP RNAi-transfected control cells.

Stimulation of Epitope-tagged S6K1 or 4E-BP1 by Nutrients in HepG2 Cells—HepG2 cells were transiently co-transfected with plasmids encoding HA-S6K1 and FLAG-4E-BP1, and then nutrient stimulation was conducted 48 h following transfection. Cell extracts were immunoprecipitated using 2 μg of anti-HA tag antibody (Sigma) or anti-FLAG tag antibody (Sigma) and protein A-Sepharose beads (Amersham Biosciences). The precipitated proteins were eluted from the beads with SDS sample buffer and analyzed by immunoblotting, using the antibodies indicated.

Immunoblotting—The antibodies used for immunoblotting were as indicated, and signals were detected using horseradish peroxidase-mediated chemiluminescence (Amer sham Biosciences), as described previously (24).

Assay of S6K1 Activity—S6K1 activity was measured using the p70S6 kinase assay kit following the manufacturer’s protocol (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, cells were homogenized using ultrasonication in lysis buffer (10 nM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 50 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), and the endogenous S6K1 in the supernatant was immunoprecipitated using 2 μg of anti-S6K1 antibody and protein A-Sepharose. The immunoprecipitates were resuspended in dilution buffer (20 mM MOPS at pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). The kinase reaction was performed for each precipitated sample in an assay mixture containing 50 μM substrate peptide (AKRRRLSSLR) and 0.2 μCi of [γ-32P] ATP. Following incubation for 10 min at 30 °C, aliquots were applied onto P81 phosphocellulose squares and washed, and radioactivity was measured using a scintillation counter.

Animal Experiments—In vivo experiments were conducted using 8–9-week-old male C57BL/6 and db/db mice. The mice were housed under controlled light/dark (12/12 h) and temperature conditions. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Subjects Committee of the University of California, San Diego. Mice were fed normal rodent chow (type MF, Oriental Yeast, Tokyo, Japan) and tap water ad libitum.

Exogenous overexpression of TRB3 or GFP was induced in the livers of C57BL/6 mice via the injection of 5 × 10^8 pfu/ml of virus into the tail vein as described (27). Mice were euthanized 7 days following virus injection. During the course of the experiments, mice were fasted for 24 h with free access to water, and the fasting blood glucose was monitored. Mice were refed for the following 24 h, and blood glucose was again determined. This fasting-feeding protocol was repeated for 7 days. All mice were sacrificed for blood and liver collection at the end of the experiments. Blood samples were collected from the
tail vein. Plasma was obtained by centrifugation of collected blood and assayed for insulin. Blood glucose and plasma insulin levels were determined as described previously (28). Some of the mice were subjected to insulin injection (0.75 units/kg body weight, 10 min) prior to sacrifice. For glucose tolerance tests, mice fasted for 24 h were injected intraperitoneally with 2 g of glucose per kg of body weight. For insulin tolerance tests, mice fasted for 24 h were injected intraperitoneally with 0.75 units of insulin per kg of body weight. Blood glucose level was measured in tail vein blood collected at the designated times.

**Statistical Analysis**—Data are expressed as means ± S.D. and were analyzed using analysis of variance and Bonferroni multiple comparison tests. *p < 0.05 was accepted as statistically significant.

**RESULTS**

**TRB3 Inhibits Insulin-induced Activation of S6K1 Both in Vitro and in Vivo**—TRB3 is an endogenous inhibitor of Akt (21). Therefore, enhanced expression of TRB3 is expected to inhibit insulin-induced activation of S6K1 through the inhibition of Akt. Adenoviral overexpression of TRB3 in primary mouse hepatocytes inhibited insulin-stimulated S6K1 activity in a dose-dependent manner (Fig. 1A). TRB3 overexpression inhibited phosphorylation of Akt (Thr-308 and Ser-473), TSC2/tuberin (Thr-1462), and mTOR (Ser-2448), all of which are located upstream of S6K1 activation in the insulin signaling pathway, and also inhibited phosphorylation of the S6 ribosomal protein (Ser-235/236), a substrate of S6K1 (Fig. 1B). It should be noted that stimulation of endogenous S6K activity as measured by S6 phosphorylation is greater than measurement of S6K activity in vivo. We ascribe this difference to the high basal activity associated with the in vitro kinase assay. Additionally, overexpression of TRB3 inhibited the insulin-induced phosphorylation of another mTOR target, the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which regulates the initiation of 5′-capped mRNA translation (29). Phosphorylation of the major mTOR-regulated site (Ser-65) is dramatically inhibited (30) (Fig. 1B). In contrast, TRB3 had little effect on insulin-induced phosphorylation of extracellular signal-regulated protein kinase 1/2 (Fig. 1B).

Similar results are seen when cells are stimulated with sodium pervanadate (21). The level of S6K1 phosphorylation at Thr-389, a site targeted by mTOR (31), was elevated by pervanadate treatment (Fig. 1C). No phosphorylation of S6K1 on Thr-421/Ser-424 was observed (Fig. 1C). These are the rapamycin-insensitive phosphorylation sites in mouse hepatocytes (31). Adenoviral overexpression of TRB3 greatly inhibited the phosphorylation of Thr-389 in response to pervanadate (Fig. 1C), in agreement with the insulin results.

We examined the effect of RNA interference (RNAi) of TRB3 in HepG2 cells that are known to express endogenous TRB3 (21). Knockdown of TRB3 enhanced basal S6K1 activity 1.2-fold (*p < 0.01), and enhanced insulin-stimulated S6K1 activity 1.6-fold (*p < 0.05) (Fig. 2A). Transfection with TRB3 RNAi caused a 70% reduction in endogenous TRB3 protein levels in HepG2 cells (Fig. 2B). We also examined the effects of TRB3 RNAi on the insulin-stimulated phosphorylation of the S6 ribosomal protein and 4E-BP1. Knockdown of TRB3 resulted in no effect on basal S6 and 4E-BP1 phosphorylation but increased the insulin-stimulated phosphorylation of S6 ribosomal protein and 4E-BP1 2.5-fold (Fig. 2, B and C). Thus, endogenous TRB3 provides tonic inhibition of insulin-stimulated S6K1 activity in hepatocytes.

To investigate the effect of TRB3 on insulin stimulation of S6K1 in vivo, we infected adult male C57BL/6 mice with adenoviruses encoding TRB3 or GFP as control. TRB3-infected mice were treated with or without insulin (0.75 units/kg body weight) for 10 min, and the hepatic S6K1 activity was measured. As shown in Fig. 3A, adenoviral overexpression of TRB3 completely suppressed the insulin-induced hepatic S6K1 activation in mice. TRB3 also suppressed the insulin-stimulated phospho-

**TABLE 1**

Metabolic parameters in adenoviral infected mice

Data are expressed as mean ± S.D. (n = 6), *p < 0.05 compared with control GFP infected mice.

| Virus | Weight | Food intake | Insulin | Glucose |
|-------|--------|-------------|---------|---------|
|       | Fed | Fasted | Fed | Fasted | Fed | Fasted | Fed | Fasted |
| GFP  | TRB3 | TRB3 | TRB3 | TRB3 | TRB3 | TRB3 | TRB3 | TRB3 |
| g/day | 17.8 ± 0.56 | 17.4 ± 0.84 | 2.56 ± 0.23 | 2.68 ± 0.26 | 1.6 ± 0.45 | 1.6 ± 0.23 | 0.4 ± 0.15 | 0.6 ± 0.23 |
| 108 ± 12.2 | 110 ± 10.8 | 7.2 ± 8.3 | 7.3 ± 7.3 | 81 ± 7.5 | 85 ± 8.3 |
| Day 3 | 18.0 ± 0.67 | 18.6 ± 0.65 | 2.68 ± 0.42 | 2.77 ± 0.38 | 1.5 ± 0.29 | 1.8 ± 0.43 | 0.5 ± 0.19 | 0.5 ± 0.23 |
| 110 ± 10.6 | 133 ± 11.4 | 7.3 ± 7.3 | 7.5 ± 7.8 | 74 ± 9.2 | 74 ± 9.8 |
| Day 5 | 18.8 ± 0.45 | 18.9 ± 0.71 | 2.79 ± 0.24 | 2.59 ± 0.43 | 1.4 ± 0.32 | 2.1 ± 0.54 | 0.4 ± 0.13 | 0.6 ± 0.22 |
| 105 ± 11.6 | 150 ± 12.6 | 78 ± 7.2 | 82 ± 7.3 | 78 ± 7.2 | 82 ± 7.3 |
| Day 7 | 19.5 ± 0.86 | 19.7 ± 0.99 | 2.76 ± 0.38 | 2.69 ± 0.56 | 1.6 ± 0.21 | 2.3 ± 0.83 | 0.5 ± 0.13 | 0.6 ± 0.24 |
| 114 ± 10.4 | 155 ± 13.3 | 81 ± 7.5 | 85 ± 8.3 |
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FIGURE 4. Effect of TRB3 overexpression on intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test in vivo. A, glucose tolerance test of mice infected with control GFP or TRB3 adenovirus. Mice were injected intraperitoneally with glucose (2 g/kg), and blood glucose levels were monitored at the intervals indicated. B, insulin tolerance test of mice infected with control GFP or TRB3 adenovirus. Mice were injected intraperitoneally with insulin (0.75 units/kg), and blood glucose levels were monitored at the intervals indicated.

To determine the physiological effects of exogenous TRB3 overexpression, we examined body weight, food intake, and metabolic parameters in these animals. Control GFP-infected mice and TRB3-infected mice had similar increases in body weight over 7 days, although food consumption did not change (Table 1). TRB3 expression had no effect on either insulin or blood glucose levels in the fasted state. Plasma insulin concentrations in the fed state were modestly increased in TRB3-infected mice (2.3 ng/ml) versus GFP-infected mice (1.6 ng/ml) on day 7 following viral infection. Blood glucose levels were also increased in the fed state (155 versus 114 mg/dl, n = 6). As shown in Fig. 4, A and B, overexpression of TRB3 elevated the blood glucose excursion during a glucose tolerance test and impaired the glucose lowering effect of insulin during an insulin tolerance test as has been published previously (21). Given the results from both in vitro and in vivo studies, we conclude that TRB3 inhibits insulin signaling via the Akt/TSC2/mTOR/S6K1 pathway in the liver and creates a state of insulin resistance.

TRB3 Does Not Inhibit Nutrient-stimulated Activation of S6K1 in Vitro—Nutrient signaling to mTOR has been shown to be independent of the insulin-regulated Akt/TSC2 pathway. Therefore, we investigated whether overexpression of TRB3 affects nutrient-induced activation of S6K1. HepG2 cells infected with or without the TRB3-expressing adenovirus were stimulated with either the amino acid mixture (two times the normal culture levels) or DMEM culture medium. Both the amino acid mixture and the DMEM stimulated S6K1 activity 1.5-fold (p < 0.01; Fig. 5A). The addition of insulin together with the nutrients showed an additive effect (2.3-fold, p < 0.01). Rapamycin, an inhibitor of mTOR, strongly inhibited both the insulin and nutrient-stimulated S6K1 activity (Fig. 5A), confirming a central role for mTOR in the regulation of S6K1 activation. Wortmannin also inhibited the insulin-stimulated S6K1 activity, consistent with signaling through the class I PI 3-kinases but had no effect on nutrient-stimulated S6K1 (Fig. 5A). TRB3 overexpression blocked insulin-stimulated S6K1 as shown in vivo earlier but did not have a significant effect on the nutrient stimulation of S6K1. To determine whether the upstream kinase mTOR was activated normally, we measured the phosphorylation state of two mTOR targets S6K1 and 4E-BP1. In these experiments, we transfected HepG2 cells with tagged S6K1 and 4E-BP1 to show the effect of nutrient stimulation more clearly. The nutrient-induced phosphorylation of the exogenously expressed S6K1 and 4E-BP1 was weakly, but significantly, inhibited by overexpression of TRB3 (Fig. 5B). As shown in Fig. 5, C and D, S6K1 phosphorylation on Thr-389 was inhibited 28 ± 6.6%, and 4E-BP1 phosphorylation on Thr-65 was inhibited 15 ± 4.5%.

We also examined the effect of RNAi knockdown of TRB3 on nutrient-stimulated activation of S6K1 in HepG2 cells. Although knockdown of TRB3 enhanced basal S6K1 activity, there was no enhancement in the nutrient stimulation (Fig. 6A). This was further verified by measuring the phosphorylation of the endogenous substrate S6. Knockdown of TRB3 had no effect on the nutrient-stimulated phosphorylation of S6 (Fig. 6, B and C). There was no effect on the phosphorylation of 4E-BP1 either indicating that mTOR activation was unaffected (Fig. 6D). These results suggest that nutrient stimulation of S6K1 is mediated by mTOR but is only partially blocked by TRB3 inhibition of Akt.

Feeding Increases Hepatic S6K1 Activity in db/db Mice without Phosphorylation of Akt—We also investigated the effect of starvation, feeding, or insulin treatment on TRB3 levels and S6K1 activities in the liver in vivo in C57BL/6 and db/db mice. The body weights, food intake, and fasting insulin and glucose levels for the animals used in this study are shown in Table 2. By 6 weeks of age, the db/db mice are hyperinsulinemic and hyperglycemic.

In C57BL/6 control mice, feeding or insulin treatment reduced TRB3 levels 50% (Fig. 7A). In contrast, TRB3 protein levels in db/db mice are constitutively elevated (3.5-fold) and do not respond to feeding or insulin treatment. Fasting S6K
activity was no different between the two strains consistent with the lack of effect of adenoviral TRB3 overexpression on hepatic S6K1 activity in the basal state. Insulin stimulation enhanced S6K1 activity significantly in C57BL/6 mice but feeding did not (Fig. 7B). Both feeding and insulin induced a significant increase in S6K1 activity in db/db mice. The S6K1 activity correlated closely with phosphorylation of the endogenous S6 protein, although the magnitude of the effect is much greater for the endogenous substrate. Insulin increased phosphorylation of S6 on Ser-235/236 in both C57BL/6 and db/db mice, but feeding only increased phosphorylation in db/db mice (Fig. 7C). Phosphorylation of 4E-BP1 followed the same pattern (Fig. 7D) as S6 phosphorylation, although a small increase in phosphorylation was evident in the fed state in C57BL/6 mice. The upstream kinases mTOR and TSC2 are phosphorylated on Ser-2448.
and Thr-1462 by both feeding and insulin treatment in both strains of mice, although the level of phosphorylation tended to be lower in db/db mice (Fig. 7, E and F). In agreement with previous literature, insulin but not feeding stimulated phosphorylation of Akt in C57BL/6 mice (Fig. 7, G and H). The db/db mice are profoundly insulin-resistant, and no insulin stimulation of Akt is observed. To verify that Akt activity was decreased, we measured the phosphorylation of the endogenous substrate glycogen synthase kinase-3 (GSK3). Phosphorylation of Ser-9/21 of GSK3α/β paralleled Akt phosphorylation (Fig. 7I). In contrast, ERK phosphorylation did not change with feeding or insulin treatment in either the C57BL/6 or db/db strains (Fig. 7J).

**DISCUSSION**

In this study, overexpression of TRB3 completely inhibited the insulin-induced activation of Akt, mTOR, and S6K1. TRB3 functions upstream of Akt and controls not only glycogen synthase kinase 3 (GSK3), as described previously (21), but also S6K1 and 4E-BP1. The effect of TRB3 overexpression was also verified in vivo in the livers of C57BL/6 mice. We observed that overexpression of TRB3 in the liver did not alter fasting insulin and glucose levels but increased both insulin and glucose levels in the fed states. Glucose and insulin tolerance tests confirmed that the mice are insulin-resistant. Our results are consistent with the accepted mechanism for insulin signaling to S6K1 via IRS-1, PI 3-kinase, PDK1, Akt, TSC2, and mTOR. Insulin activation of S6K1 provides an important negative feedback loop dephosphorylating IRS-1 on Ser-307/632/635 and uncoupling PI 3-kinase activity (5, 6). Elevated mTOR and S6K1 activity have been observed in rodent models of insulin resistance and obesity leading to the suggestion that exaggerated negative feedback might play a causative role in insulin resistance (6). Conversely, S6K1-deficient mice are more insulin-sensitive and are protected from diet-induced obesity and insulin resistance (6).

Since mTOR, S6K1, and 4E-BP1 are central to the nutrient regulation of protein synthesis (32), we investigated the effect of TRB3 on nutrient signaling. Overexpression of TRB3 did not inhibit nutrient-stimulated S6K1 activity, and deletion of TRB3 by siRNA had no effect on either S6K or mTOR activity, suggesting that the Akt/TSC2 pathway is a minor contributor to nutri-

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**TABLE 2**

**Metabolic parameters in C57BL/6 and db/db mice**

Data are expressed as mean ± S.D. (n = 6), *p < 0.05 compared with control C57BL/6 mice.

| Virus | Weight | Food intake | Insulin | Glucose level |
|-------|--------|-------------|---------|--------------|
|       | C57BL/6 | db/db       | C57BL/6 | db/db       | C57BL/6 | db/db       |
|       | g      | g/day       | ng/ml   | mg/ml        |
| 4 weeks | 14.3 ± 0.76 | 24.4 ± 0.79 | 2.67 ± 0.48 | 5.58 ± 0.33 | 1.3 ± 0.36 | 1.9 ± 0.34 | 101 ± 11.9 | 151 ± 12.1 |
| 5 weeks | 15.4 ± 0.66 | 29.6 ± 0.85 | 2.60 ± 0.65 | 6.34 ± 0.68 | 1.4 ± 0.39 | 2.3 ± 0.35 | 109 ± 11.8 | 173 ± 11.4 |
| 6 weeks | 16.8 ± 0.89 | 32.0 ± 1.12 | 2.67 ± 0.86 | 8.50 ± 0.75 | 1.4 § 0.23 | 2.5 ± 0.52 | 107 ± 10.1 | 233 ± 22.5 |
| 7 weeks | 17.7 ± 0.74 | 35.9 ± 0.91 | 2.47 ± 0.54 | 8.99 ± 1.03 | 1.3 ± 0.23 | 3.1 ± 0.62 | 107 ± 10.7 | 355 ± 27.6 |
| 8 weeks | 18.5 ± 0.96 | 39.2 ± 1.19 | 2.99 ± 0.78 | 8.84 ± 1.36 | 1.5 ± 0.19 | 4.0 ± 0.54 | 107 ± 11.6 | 396 ± 33.7 |

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**FIGURE 6. Effect of siRNA against TRB3 on nutrient-stimulated S6K1 activity in vitro.** A, TRB3 knockdown increases basal and nutrient-stimulated S6K1 activity. HepG2 cells were transfected with the siRNAs indicated 24 h prior to treatment without (−) or with (+) DMEM for 30 min. S6K1 activity was measured *in vitro* on cell extracts. Data are expressed as mean ± S.D. for five samples in each group. *, p < 0.05 compared with control C57BL/6 mice. B, Immunoblot; phospho-S6 (pSer235/236) and S6, phospho-4E-BP1 (pSer65), and 4E-BP1 were detected. C and D, Quantification of phosphorylation of S6 and 4E-BP1. Data are expressed as mean ± S.D. from three independent experiments. *, p < 0.05, when compared with GFP RNAi transfected control cells.
ent signaling. This agrees with previous reports that amino acids phosphorylate mTOR on Ser-2448 and increase mTOR kinase activity in both adipocytes and hepatocytes in the absence of measurable Akt activation (18, 33). It is also consistent with our observed lack of activation of Akt but marked phosphorylation of mTOR and TSC2 in liver with feeding in C57BL/6 (31).

The weak effect with overexpression of TRB3 is similar to effects seen with TSC2. Overexpression of TSC1 or -2 impairs, but does not eliminate, the ability of amino acids to activate S6K1, and loss of TSC1 or -2 elevates basal S6K1 activity and makes it more resistant to inhibition by amino acid depletion (12, 34). These manipulations alter GTP loading on Rheb, the small GTPase upstream of mTOR. Overexpression of TSC2 also inhibits mTOR phosphorylation and activity, whereas depletion of TSC2 increases GTP loading on Rheb activating mTOR. This activation can be mimicked by overexpression of Rheb suggesting that the balance of GDP-GTP on Rheb is critical for regulation of mTOR. However, the ability of amino acids to stimulate S6K1 activity in TSC2−/− cells indicates that amino acids, and perhaps other nutrients, do not signal via the TRB3/Akt/TSC2 pathway.

The missing piece of the puzzle is the upstream signal that connects nutrient status to mTOR activation. Two recent papers have shown that the class III PI 3-kinase hVps34 is the missing nutrient-regulated kinase upstream of mTOR and S6K1 (19, 35). This kinase phosphorylates phosphatidylinositol to phosphatidylinositol 3-phosphate and controls endosomal trafficking. hVps34 activity is increased by amino acid supplementation and inhibited by either amino acid or glucose starvation. It is a target for AMP-activated protein kinase inhibition as activity is decreased by treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside to activate AMP-activated protein kinase or oligomycin to deplete ATP (35). hVps34 activity and its lipid product PI(3)P are also required for insulin stimulation of mTOR as microinjection of inhibitory antibodies, expression of a PI(3)P-binding protein, or siRNA knockdown of hVps34 block insulin activation of S6K1, but insulin does not activate hVps34 (35). The downstream targets of hVps34 in this sig-
naling pathway are not known. PI(3)P might provide a signal to downstream kinases in a manner analogous to the phosphatidylinositol 1,4,5-trisphosphate activation of PDK1 and Akt. Alternatively, the known role for hVps34 in regulating endosomal trafficking through interactions with Rab5 and -7 might indicate that internalization of a plasma membrane protein is required for the propagation of the signal from the insulin receptor to mTOR (36–38). The receptor proximal components of the signaling cascade are PI(3)P-independent as activation of Akt and phosphorylation of TSC2 are not altered by inhibition of hVps34, and GTP-loading of Rheb is unchanged. Interestingly, Rheb is localized to endosomal membranes, the site of hVps34 action, via a carboxyl-terminal CVSM motif (39). Farnesylation of Rheb and membrane localization are essential for GTP loading but not for downstream signaling (40). mTOR has been localized to the endoplasmic reticulum/Golgi or nucleus in different cells, and shuttling of mTOR between these compartments and the cytoplasm is important for activation of S6K1, which is predominantly cytoplasmic (41, 42). These findings suggest a model (Fig. 8) whereby insulin activates Akt in the plasma membrane leading to phosphorylation of TSC2 and activation of Rheb. Rheb must translocate to the nucleus or endoplasmic reticulum via the endosomal pathway to activate mTOR, which is then exported from the nucleus to the cytoplasm to activate S6K1. Amino acid activation of hVps34 is a crucial regulator of endosomal trafficking allowing activation of mTOR by GTP-bound Rheb. This provides a mechanistic checkpoint preventing aberrant activation of protein translation in the absence of a suitable supply of amino acids or nutrients. Two recent studies support this model, as overexpression of active GTP-bound Rheb causes the appearance of large late endosomal vacuoles, and mTOR contains a composite nuclear import/export sequence that is required for activation of S6K (43, 44).

How is the normal regulation of insulin signaling perturbed in the db/db mouse? The observed high levels of hepatic TRB3 expression in db/db mice potentially contribute to insulin resistance through inhibition of Akt (21). The mTORC2 complex containing mTOR and rictor has been suggested to be the Akt Ser-473 kinase (45, 46). As mTOR is active in db/db mice, the lack of Akt activation could be due to a direct effect of TRB3 to block PDK-1 mediated phosphorylation of Thr-308 as well as mTORC2-mediated phosphorylation of Ser-473. Indeed in a recent human study, Prudente et al. (47) demonstrated that TRB3 is associated with insulin resistance. TRB3 has been proposed to be a nutrient sensor as TRB3 levels increase with glucose or amino acid starvation in PC-3 cells (48). The increase is dependent on PI 3-kinase signaling via p110β. In C57BL/6 mice, we observe an increase in TRB3 protein in liver with starvation that is reversed by feeding or insulin treatment, in agreement with the findings in PC-3 cells, but TRB3 levels in the liver of db/db mice are constantly elevated despite the nutrient excess and hyperinsulinemia. It is possible that the nutrient excess state in these mice is associated with increased p110β activity mimicking a state of perpetual starvation, perhaps because of the lack of leptin signaling.

Other sources of insulin resistance are also possible in these animals. There is striking difference in the mTOR/S6K axis between C57BL/6 and db/db mice. Activation of S6K1 is greater in fed db/db mice than C57BL/6 mice. This elevated S6K1 activity may contribute to insulin resistance through phosphorylation of IRS-1. S6K can also phosphorylate mTOR on serine 2448 setting up a positive feedback loop whereby S6K activation maintains mTOR in an active state (49). Feeding does not greatly activate S6K in the C57BL/6 strain, so it is unlikely to create a positive feedback loop, whereas the strong S6K activation in the db/db background may be sufficient to initiate the feedback, which would exacerbate insulin resistance through the negative effect on IRS-1. The results also suggest that the initial phosphorylation of mTOR on serine 2448 may not be mediated by S6K as we observe phosphorylation on this site (and also on TSC2).
Effect of TRB3 on Hepatic p70 S6 Kinase Activity

FIGURE 8. Possible model integrating insulin and nutrient signaling pathways that regulate mTOR and S6K1.

without measurable S6K activity in fed C57BL/6 mice. Sustained activation of mTOR, on the other hand, may be downstream of S6K as has been published.

What causes the elevated S6K1 activity in the fed db/db mouse? The db/db mice are also hyperinsulinemic, as well as hyperphagic. The combination of these two factors may be necessary for constitutive activation of S6K1 and inhibition of Akt. If hyperinsulinemia is needed for S6K1 activation in the fed state, then it is likely via an Akt-independent pathway in these animals as no Akt activation is observed. Selective insulin resistance has been reported previously. Insulin-resistant states in animals and humans are associated with increases in farnesylation and signaling of p21 Ras, another related GTPase (50). So Rheb farnesylation and signaling might be increased in the db/db mice. Interestingly, mTOR is active in all fed or insulin-treated animals but cannot activate S6K1 in the C57BL/6 background in the absence of exogenous insulin. This suggests that mTOR activation is not sufficient for S6K1 phosphorylation. A similar effect was seen in TSC2−/− MEFs where mTOR is phosphorylated on Ser-2448 because of constitutive activation of Rheb, but S6K1 is inactive. The defect in mTOR signaling may be specific for S6K1 as another substrate 4E-BP1 is partially phosphorylated by feeding in the C57BL/6 strain. Together the results suggest that insulin provides another signal that allows active mTOR to phosphorylate S6K1, possibly by promoting nuclear export or localization of mTOR to discrete substrates. Again, this signal is likely Akt-independent as activation of S6K1 is observed in db/db mice in the absence of Akt activation. Further studies will be needed to test the model of nutrient regulation of mTOR and to define the additional pathways used to facilitate activation of S6K1.

In conclusion, our observations indicate a novel role for TRB3 in the integration of signals from insulin and nutrients to mTOR-S6K1. These data support the proposition that TRB3 in the integration of signals from insulin and nutrients to mTOR-S6K1. These data support the proposition that TRB3 helps to modulate the mTOR-S6K1 axis, an additional pathway used to facilitate activation of S6K1.

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