Interaction of FoxO1 and TSC2 Induces Insulin Resistance through Activation of the Mammalian Target of Rapamycin/p70 S6K Pathway*

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Both TSC2 (tuberin) and forkhead transcription factor FoxO1 are phosphorylated and inhibited by Akt and play important roles in insulin signaling. However, little is known about the relationship between TSC2 and FoxO1. Here we identified TSC2 as a FoxO1-binding protein by using a yeast two-hybrid screening with a murine islet cDNA library. Among FoxOs, only FoxO1 can be associated with TSC2. The physical association between the C terminus of TSC2 (amino acids 1280–1499) and FoxO1 degrades the TSC1-TSC2 complex and inhibits GTPase-activating protein activity of TSC2 toward Rheb. Overexpression of wild type FoxO1 enhances p70 S6K phosphorylation, whereas overexpression of TSC2 can reverse these effects. Knockdown of endogenous FOXO1 in human vascular endothelial cells decreased phosphorylation of p70 S6K. Prolonged knockdown of endogenous FOXO1 in human vascular endothelial cells decreased phosphorylation of Akt and FOXO1 itself even in the presence of serum. These data suggest a novel mechanism by which FoxO1 regulates the insulin signaling pathway through negative regulation of TSC2 function.

Forkhead transcription factors of the FoxO (forkhead box-containing protein, Q subfamily) family are conserved across many species. In Caenorhabditis elegans, DAF-16, the FoxO orthologue, is downstream of the DAF-2/AGE-1/AKT signaling pathway (1, 2). Loss-of-function of daf-2, age-1, or akt causes life span extension in a daf-16-dependent manner (3). In Drosophila, the life span is extended over 50% by ablation of insulin-producing cells or mutations of genes encoding the insulin-like receptor (dInR) or its receptor substrate (chico) (4).

Similarly, dInR phosphorylates and inactivates dFOXO, the Drosophila homologue of DAF-16/FOXO (5). In mammals, InsR/IGF1R-P13K-Akt signaling inhibits transcription by FoxO1, FoxO3a, and FoxO4 (6). These proteins possess a forkhead DNA binding domain consisting of around 110 amino acids and a transactivation domain in the C terminus. FoxOs bind to consensus FoxO-binding sites (GTAAA(C/T)A, T(G/A)TTTAC) in the promoter region of their target genes and activate gene expression (7). It has been reported that FoxOs cause cell cycle arrest through induction of p27, p21, cyclin B, pololo-like kinase, the retinoblastoma family-related protein p130 and cyclin G2, apoptosis through induction of Fas ligand and Bim, DNA repair through GADD45, stress resistance through MnSOD, and regulation of glucose and lipid metabolism through G6pase, apoC-III, and Lgfbp-1 (8). Several FoxO-binding proteins, which include co-activators, transcription factors, signaling molecules, and Sirt1, a NAD-dependent deacetylase, have also been identified (8). These FoxO-binding molecules regulate FoxO-dependent transcription and vice versa. However, there have been few reports that described identification of FoxO-binding proteins by comprehensive strategies.

The PI3K∗-Akt pathway is also important for growth factor stimulation of mammalian target of rapamycin (mTOR) signaling (9). The primary mechanism by which Akt activates mTOR signaling appears to be through direct phosphorylation and inhibition of TSC2 (also known as tuberin). TSC1 (also known as hamartin) and TSC2 were first identified as genes mutated in patients with tuberous sclerosis complex (TSC), an autosomal dominant disease. Affected patients suffer from hamartomas in a wide spectrum of organs. TSC1 and TSC2 physically associate in vivo and form a heterodimeric complex (10–12). TSC2 has been directly linked to cell size regulation by the discovery that mutation in dTsc2 leads to the gigas (large cell) phenotype (13).

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The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; TSC, tuberous sclerosis complex; DMEM, Dulbecco’s modified Eagle’s medium; HUVEC, human vascular endothelial cell; siRNA, small interfering RNA; RNAi, RNA interference; ANOVA, analysis of variance; WT, wild type; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; GAP, GTPase-activating protein; HA, hemagglutinin.
Recent studies revealed that the TSC1-TSC2 complex functions downstream of Akt and upstream of target of rapamycin to restrict cell growth and cell proliferation (14–17). Akt-phosphorylation of TSC2 leads to the functional inactivation of the TSC1-TSC2 complex and results in mTOR activation leading to phosphorylation of two main mTOR substrates, ribosomal p70 S6 kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP1), and elevated mRNA translation (18–20). The TSC2 C-terminal region has homology with the catalytic domain of GTPase-activating proteins (GAPs). An inhibitory target of TSC1-TSC2 has been identified as Ras homologue enriched in brain (Rheb), a small GTPase. GTP-bound Rheb is bound to and activates mTOR (21, 22).

There have been several reports about molecules, which regulate TSC2 function. Energy depletion inhibits mTOR signaling through AMP-activated kinase phosphorylation of TSC2, although it is not known how AMP-activated kinase phosphorylation of TSC2 enhances the ability of TSC1–TSC2 to inhibit downstream signaling to Rheb (23–25). The hypoxia-inducible gene, regulated in development and damage responses (REDD1), is also induced by energy depletion, and this leads to inhibition of mTOR complex 1 signaling to p70 S6K in a TSC2-dependent manner (26). It is important to identify molecules that regulate TSC2 function because these molecules may affect the activity of mTOR/p70 S6K signaling and finally determine the activity of PI3K/Akt pathway through a negative feedback loop (27).

In this study, we identified TSC2 as a novel FoxO1-binding protein by a yeast two-hybrid screening using a murine islet cDNA library. Binding of FoxO1 to TSC2 in cytoplasm inhibits TSC2 function and results in activation mTOR/p70 S6K and inhibition of Akt activity through negative feedback on IRS protein, leading eventually to feedback activation of FoxO1. Here we demonstrate a novel mechanism by which FoxO1 regulates activity of mTOR/p70 S6K signaling pathway and of FoxO1 itself through association with TSC2.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Plasmids**—We purchased anti-FLAG (M2) and anti-tubulin from Sigma; anti-c-Myc (9E10), anti-TSC2 (C-20 and N-19), anti-FOXO1 (H128), anti-FOXO1 (N18), anti-IR52 from Santa Cruz Biotechnology; anti-HA (12CA5) from Roche Applied Science; anti-p70 S6K, anti-phospho-p70 S6K (Thr-P)-389, anti-phospho-FOXO1 (Ser-P)-256, anti-Akt, anti-phospho-Akt (Thr-P)-308 and Ser(P)-473, and anti-phospho-IRS-1 (Ser(P)-307) from Cell Signaling Technology; anti-TSC1 from Zymed Laboratories Inc.; anti-IRS1 from Upstate Biotechnology, Inc.; and anti-GFP from Dr. Michiyuki Matsuda, Osaka University. We used pCAG/FLAG-rTSC2DEE, pMT2/FLAG-p70 S6K (28), pCMV5/cMyc/FOXO1 (29), pCMV5/cMyc/FOXo3a (30), pTB701-FLAG-FOXO4 (31), pCA-EGFP-Rheb (32), and adenoviral vectors encoding wild type or mutant FoxO1 (30). The pCAG/FLAG-rTSC2DEE was constructed by ligation of N-terminal FLAG-tagged full-length cDNA of rTSC2 with pCAG-GS vector (33, 34).

**Construction of Expression Vectors**—For mutagenesis of FoxO1, we performed overlap extension PCR using pCMV5/cMyc/ADA FoxO1 as a template as described previously (35). For construction of pCMV5/cMyc/T24A/S253A/S316A (3A), the following mutagenic primers were used: primer 1, 5′-AGA GCT GCG GCC ATG GAC AAC-3′, corresponding to nucleotide 844–864, and primer 2, 5′-TGT GTC CAT GGC CGC AGC TCT-3′, corresponding to nucleotide 864–884.

**Yeast Two-hybrid Screen**—Amino acids 424–550 of the murine FoxO1 were cloned in-frame into the GAL4 DNA-binding domain plasmid pGBK7 (Clontech). The GAL4 activation domain cDNA library of murine islets was constructed as described previously (36). AH109 yeast strain was used for the library search. The transformation was performed as described in the Clontech Matchmaker two-hybrid system 3 protocol. The transformants were plated on SD/−Ade/−His/−Leu/−Tyr plates in the presence of galactose and then were incubated at 30 °C for 3–4 days. Positive interaction was identified by strong β-galactosidase activity. Individual positive clones were isolated by YEASTMAKER™ yeast plasmid isolation kit (BD Biosciences) and were sequenced by ABI310 automated DNA sequencer and analyzed for homology with sequences in the GenBank™ data base using the BLAST algorithm.

**Cell Culture, Transfection, and Viral Transduction**—HEK293 cells were cultured in DMEM containing 10% fetal calf serum. SV40-transformed hepatocytes used in these studies have been described in previous publication (29). Human vascular endothelial cells (HUVEC) were cultured in HuMedia-E8B (KURABO) supplemented with 2% fetal calf serum, 10 ng/ml human recombinant epidermal growth factor, 1 µg/ml hydrocortisone, 5 ng/ml human recombinant fibroblast growth factor, and 10 µg/ml heparin. Transient transfection was performed using Lipofectamine (Invitrogen) according to manufacturer’s protocol. Adenoviral infection was described in a previous publication (30). We transduced SV40-transformed hepatocytes by incubating them with adenoviral preparations at 10–50 multiplicities of infection for 2 h.

**Cell Isolation and Culture**—Brown adipocytes and their precursor cells were isolated from newborn wild type mice by collagenase digestion as described previously (37). Preadipocytes were immortalized by infection with the retroviral vector pGCDNsamIRES-Puro, encoding SV40T antigen (38) and selected with puromycin (1 µg/ml). Preadipocytes were grown to confluence in culture medium supplemented with 50 nM insulin and 50 nM triiodothyronine (differentiation medium) (day 0). Adipocyte differentiation was induced by treating confluent cells for 24 h in differentiation medium further supplemented with 0.5 mM isobutylmethylxanthine, 0.5 µM dexamethasone, and 0.125 mM indomethacin. After induction, cells were changed back to differentiation medium, which was then changed every day. At day 5, cells were harvested and used for experiments.

**Western Blot Analysis**—We lysed cells in buffer containing 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (Roche Applied Science). After centrifugation to remove insoluble material, each 30 µg of sample was electrophoresed in SDS-PAGE, and Western blotting was performed. For immunoprecipitation, cell lysates were diluted with buffer containing 50
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mm Tris-HCl (pH 7.6), 150 mm NaCl, 0.1% Nonidet P-40, 10% glycerol, 5 mm MgCl₂, and protease inhibitors.

In Vitro Translation and Glutathione S-Transferase Fusion Protein Pulldown Assay—The TSC2 deletions were generated by PCR using specific primers, and they were cloned in-frame into the EcoRI and Sali sites of pGEX-4T-1. These fusion proteins were expressed in 20 μl of 50% slurry beads containing ~2 μg of protein (either GST or alone, or fused to deleted TSC2 mutants), resuspended in 350 μl of binding buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). This was mixed with 10 μl of in vitro translated wild type FoxO1 (Promega TnT reticulocyte lysate system kit). Binding was performed for 6 h at 4 °C. The beads were then washed four times with the binding buffer and resuspended in 2× SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane; the blot was incubated with anti-FOXO1 antibody and developed with an ECL detection system (Amersham Biosciences).

Immunofluorescence—Immunofluorescence using SV40-transformed hepatocytes was performed as described previously (35). After transient transfection with pCAG/FLAG-TSC2 using Lipofectamine (Invitrogen), SV40-transformed hepatocytes were transduced with adenovirus encoding HA-tagged wild type or ADA-FoxO1. HA-tagged FoxO1 was visualized with anti-HA monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG, and TSC2 was visualized with anti-TSC2 polyclonal antibody and rhodamine-conjugated anti-rabbit IgG.

Measurement of GTP- and GDP-bound Rheb—SV40-transformed hepatocytes were cultured in 6-well plates and co-transfected with pCAG/FLAG-rTSC2DEE and pCA-EGFP-Rheb using Lipofectamine 2000 reagent (Invitrogen) and subsequently transduced with an adenovirus encoding WT FoxO1. At 48 h after transfection, the cells were washed once with phosphate-free DMEM (DMEM without sodium phosphate and sodium pyruvate; Invitrogen) and incubated with 1 ml of phosphate-free DMEM for 90 min. Cells were then incubated with 25 μCi of [32P]phosphate/ml (GE healthcare) for 4 h. After the labeling, cells were lysed with prechilled lysis buffer (0.5% Triton X-100, 20 mm Tris (pH 7.5), 150 mm NaCl, 20 mm MgCl₂, 1 mm phenylmethylsulfonlfyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, 600 μl per well of a 6-well plate). To avoid lysing the nuclei, the cells were incubated with lysis buffer for just 30 s with gentle shaking. The lysates were then centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant (500 μl) was transferred to a fresh tube. Sixteen microliters of NaCl (500 mm) was added to 160 μl of supernatant to inhibit GAP activity in the lysates. To immunoprecipitate pCA-EGFP-Rheb, anti-green fluorescent protein and protein A-agarose (GE Healthcare) were added to the supernatant and incubated for 3 h at 4 °C. The beads were washed with lysis buffer twice and with wash buffer (20 mm Tris (pH 7.5), 150 mm NaCl, 20 mm MgCl₂) one time at 4 °C. The Rheb-bound nucleotides were eluted with 15 μl of elution buffer (20 mm Tris (pH 7.5), 20 mm EDTA, 2% SDS) at 68 °C for 10 min. Ten microliters of eluted nucleotides was then applied onto polyethyleneimine-cellulose plates. Before applying sample, the plate was soaked in methanol and dried with a hair dryer. The bottom portion of the plate was immersed in methanol again, and the plate was placed in a sealed chromatography chamber that was filled with 0.75 m KH₂PO₄ (pH 3.4) to a depth of 1 cm. The chamber was closed, and the solvent was allowed to ascend to the top of the plate. The plate was then removed and air-dried. GTP and GDP resolved by thin layer chromatography were visualized and quantified by a BAS-5000 (Fuji Film).

Design and Transfection of siRNAs—We used BLOCK iT RNAi Designer (Invitrogen) to identify target siRNAs and used Stealth RNAi (Invitrogen). The FOXO1-specific sequence was 5′-AACUGCAGAUGUCUGCGAGCAUGU-3′. HUVECs were transfected with Stealth RNAi using Lipofectamine 2000 according to the manufacturer’s instruction (Invitrogen). At 48 h after transfection, cells were harvested and used for Western blotting.

Statistics—We calculated descriptive statistics and ANOVA followed by Fisher’s test using the Statview software (SAS Institute Inc.).

RESULTS

Identification of TSC2 as a FoxO1-binding Protein—To identify proteins that bind to FoxO1, we performed a yeast two-hybrid screen using GAL4-FoxO1 fragment (amino acids 424–550) as bait and a murine islet cDNA library as prey. About 2 × 10⁶ transformants were tested. The strongest colony was isolated and was found to encode the C-terminal fragment of murine TSC2 (amino acids 1280–1815). To confirm the interaction between TSC2 and FoxO1, we co-transfected HEK293 cells with pCAG/FLAG-rTSC2DEE and pCMV5/cMyc-WT FoxO1, and we immunoprecipitated cell lysates using normal mouse IgG or anti-c-Myc mouse antibody and blotted with anti-FLAG antibody. Reciprocal immunoprecipitation/immunoblotting using anti-FLAG and anti-c-Myc antibodies showed that the exogenously expressed FoxO1 could interact with FLAG-tagged full-length-TSC2 (Fig. 1a).

Furthermore, to investigate whether endogenous FoxO1 is physically associated with TSC2 or not, cell lysates from brown adipocytes immortalized by SV40-T antigen were immunoprecipitated with anti-FOXO1 (Fig. 1b, lane 2) or anti-TSC2 (Fig. 1b, lane 3) and followed by an immunoblotting with antibodies against TSC2 (Fig. 1b, lanes 1–3, top panel) or FOXO1 (Fig. 1b, lanes 4–6, top panel). In this cell line, both FoxO1 and TSC2 are expressed abundantly. Our results showed that endogenous FoxO1 was associated with endogenous TSC2 (Fig. 1b). These results suggest that FoxO1 interacts physically with TSC2 in vivo.

TSC2 Binds to Only FoxO1 among FoxO Family Members—FoxOs consist of FoxO1, FoxO3a, and FoxO4. These molecules have highly conserved motifs, which include forhead DNA binding domain, three Akt phosphorylation sites, and several acetylation sites. Therefore, it is interesting to investigate whether TSC2 is associated with other FoxOs or not. To examine whether all three FoxOs can interact with TSC2 equally, we transfected SV40-transformed hepatocytes with cMyc-FoxO1, -FoxO3a, or FLAG-FOXO4 and immunoprecipitated lysates with antibody against TSC2 or normal rabbit serum and immunoblotted with antibody against c-Myc or FLAG. This co-immunoprecipitation study demonstrated that only FoxO1 could
interact with endogenous TSC2 (Fig. 2, lane 3, top panel). These data suggest that FoxO1 interacts with TSC2 among FoxOs specifically.

Identification of FoxO1-binding Site in TSC2 Protein—To examine whether FoxO1 interacts with TSC2 directly, we constructed several glutathione S-transferase (GST) fusion TSC2 fragments. Because the yeast two-hybrid screening identified the C-terminal fragment of TSC2 (amino acids 1280–1815), we constructed several deleted Tsc2 mutants in this region (Fig. 3a). Using in vitro translated wild type FoxO1, pulldown assays with GST-deleted TSC2 fusion proteins were performed and showed that FoxO1 interacted with the C-terminal TSC2 fragment (amino acids 1280–1686) directly. The FoxO1-binding site on TSC2 protein encompasses amino acids 1280–1499 (Fig. 3b, lane 3), located near the GAP domain of TSC2. These data suggest that FoxO1 binds to TSC2 directly.

FoxO1 Co-localizes with TSC2 in Cytoplasm—To examine subcellular localization of the FoxO1/TSC2 interaction, we transfected SV40-transformed hepatocytes with pCAG/FLAG-rTSC2DEE, transduced them with adenovirus encoding with HA-WT or ADA FoxO1 (30), and performed immunofluorescence using anti-TSC2 polyclonal and anti-HA monoclonal antibodies. In this cell line, even in the absence of serum, 70–80% of wild type FoxO1 was located in cytoplasm until 48 h after transduction (data not shown), where it co-localized with TSC2. In contrast, FLAG-TSC2 failed to co-localize with constitutively nuclear HA-tagged ADA FoxO1 (Fig. 4a). These data demonstrate that FoxO1 co-localizes with TSC2 in cytoplasm.
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**FIGURE 4. Co-localization and co-immunoprecipitation of FoxO1 with TSC2 in cytoplasm.**

*Panel a,* FoxO1 is co-localized with TSC2 in cytoplasm and not in nucleus. SV40-transformed hepatocytes were transiently transfected with FLAG-TSC2 followed by transduction with adenovirus encoding a constitutively active HA-ADA FoxO1 (upper panel) or HA wild type FoxO1 (lower panel), and immunofluorescence was performed as described under “Experimental Procedures.” *b,* constitutively active mutant FoxO1 (3A) in which three Akt phosphorylation sites (Thr24, Ser253, and Ser316) were mutated to alanine (T24A/S253A/S316A; 3A FoxO1) and nine residues binds to TSC2 weakly compared with wild type FoxO1. SV40-transformed hepatocytes were co-transfected with c-Myc-WT FoxO1 (lanes 1 and 2) or -3A FoxO1 (lane 3) and FLAG-TSC2 transiently. Cell lysates were immunoprecipitated (IP) with anti-c-Myc mouse monoclonal antibody (lanes 2 and 3) or normal mouse IgG (lane 1) and Western-blotted with anti-FLAG (upper panel), anti-c-Myc (middle panel). The lower panel shows Western blotting with anti-FLAG using cell lysates.

Furthermore, to examine whether FoxO1 interacts with TSC2 in cytoplasm, we used a constitutively active mutant FoxO1 (3A FoxO1) in which all three Akt phosphorylation sites were mutated to alanine (T24A/S253A/S316A; 3A FoxO1) and performed co-immunoprecipitation experiments in the same cell line. Although exogenous WT FoxO1 interacted with TSC2 as well as in HEK293 cells (Fig. 1a), the 3A FoxO1 interacted with TSC2 weakly compared with wild type FoxO1 (Fig. 4b, lanes 2 and 3). These data suggest the possibility that FoxO1 may associate with TSC2 in cytoplasm.

**FoxO1 Inhibits TSC2 and Enhances p70 S6K Phosphorylation**—TSC2 regulates cellular function mainly through its inhibitory effects on mTOR and its targets p70 S6K and 4E-BP1. It is important to elucidate whether binding of FoxO1 to TSC2 affects the activity of the mTOR/p70 S6K pathway. To investigate the effects of the FoxO1/TSC2 interaction on the mTOR pathway, we transfected SV40-transformed hepatocytes with FLAG-p70 S6K followed by transduction with adenovirus encoding HA-WT FoxO1 or -ADA FoxO1, which is localized in the nucleus and active constitutively and is immunoprecipitated with anti-FLAG monoclonal antibody and blotted with anti-phospho-p70 S6K (Thr(P)-389) antibody. After serum deprivation for 24 h, p70 S6K is dephosphorylated, and insulin increases phosphorylation of p70 S6K (Fig. 5a, lanes 1 and 2 and lanes 6 and 7). However, even in the absence of insulin, p70 S6K was phosphorylated in a dose-dependent manner of transduced WT FoxO1 (Fig. 5, a, lanes 3–5, and b, left panel). As described above, in this cell line, even after serum deprivation for 24 h, around 70–80% of transduced wild type FoxO1 is located in the cytoplasm (data not shown) and is phosphorylated (Fig. 5a, lanes 3–5). These data indicate that FoxO1 is constantly phosphorylated in this cell line even in the absence of serum and insulin. In contrast, p70 S6K was dephosphorylated in cells transduced with the ADA-FoxO1 (Fig. 5, a, lanes 8–10, and b, right panel). These data suggest that FoxO1 in cytosol enhances p70 S6K phosphorylation.

To confirm whether enhanced phosphorylation of p70 S6K by FoxO1 is mediated through binding to TSC2, we overexpressed FLAG-TSC2 in SV40-transformed hepatocytes transduced with adenovirus encoding HA-WT FoxO1, and we investigated the effects on phosphorylation of p70 S6K. Overexpression of TSC2 decreased phosphorylation of p70 S6K (Fig. 5c, lanes 1 and 2). Overexpression of WT FoxO1 enhanced phosphorylation of p70 S6K (Fig. 5c, lanes 1 and 3). Even in the presence of WT FoxO1, overexpression of TSC2 decreased phosphorylation of p70 S6K (Fig. 5c, lanes 3 and 4). These data suggest that cytoplasmic FoxO1 enhances phosphorylation of p70 S6K through association with endogenous TSC2. These data also suggest the possibility that cytoplasmic FoxO1 may affect an inhibitory action of TSC2 onto mTOR and activate mTOR.

**FoxO1 Functions Upstream of mTOR for Activation of p70 S6K**—To investigate whether enhanced phosphorylation of p70 S6K by overexpression of WT FoxO1 is mediated through mTOR activation, we treated cells with rapamycin (25 nM) and examined effects on p70 S6K phosphorylation. Overexpression of WT FoxO1 enhanced phosphorylation of p70 S6K (Fig. 6, lanes 2 and 3). In contrast, treatment with rapamycin abolished p70 S6K phosphorylation induced by overexpression of FoxO1 (Fig. 6, lane 4). These data suggest that FoxO1 enhances phosphorylation of p70 S6K through activation of mTOR and mTOR functions downstream of FoxO1 for p70 S6K phosphorylation.

**FoxO1 Reduces the Association between TSC1 and TSC2**—Dimerization of TSC2 with TSC1 is important for functional inhibition on the mTOR/p70 S6K pathway. To elucidate the mechanism of how FoxO1 inhibits TSC2, we transduced HUVEC with adenovirus encoding WT FoxO1 and immunoprecipitated cell lysates with anti-TSC2 (Fig. 7a, lanes 3–5), anti-TSC1 (Fig. 7a, lanes 8–10), or anti-HA antibody (Fig. 7a, lanes 13–15) and immunoblotted with anti-TSC1, anti-TSC2, or anti-HA antibody. Overexpression of WT FoxO1 increased association with TSC2 (Fig. 7a, lanes 3–5, bottom panel). Co-immunoprecipitation of TSC2 and TSC1 was decreased in a dose-dependent manner of transduced FoxO1 (Fig. 7a, lanes 3–5 and lanes 8–10, top panel). TSC1 does not show any physical association with transduced FoxO1 (Fig. 7a, lanes 8–10, bottom panel, and lanes 13–15, bottom panel). Furthermore, immunoblotting with anti-TSC2 antibody (C-20), which recognizes the C terminus of TSC2, detected a short fragment of
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Physical Association of FoxO1 with TSC2 Inhibits GAP Activity toward Rheb—From this study, FoxO1 binds to amino acids 1280–1499 of TSC2, which is near the GAP domain of TSC2. Therefore, it is possible to speculate that binding of FoxO1 may inhibit the GAP activity of TSC2. To investigate whether FoxO1 inhibits the GAP activity of TSC2 toward Rheb, we transfected cells with pCAG/FLAG-rTSC2DEE and pCA-EGFP-Rheb transiently followed by transduction with adenovirus encoding WT FoxO1 in SV40-transformed hepatocytes, and we examined guanylnucleotide binding by EGFP-Rheb. Overexpression of TSC2 decreased %GTP by 33% compared with non-transfected cells (Fig. 8, a, lanes 1 and 2, and b). In contrast, overexpression of both TSC2 and FoxO1 increased %GTP by 30% compared with TSC2-transfected cells (Fig. 8a, lanes 2 and 3, and b). These data suggest that FoxO1 inhibits the GAP activity of TSC2 toward Rheb.

Knockdown of FOXO1 Reduces Phosphorylation of p70 S6K in HUVEC—If endogenous FoxO1 binds to TSC2 and inhibits its function, decreased expression of FoxO1 activates TSC2 and inhibits mTOR-p70 S6K pathway. To investigate whether endogenous FoxO1 inhibits TSC2, we transfected HUVEC with siRNA of FOXO1. In this cell line, both endogenous FOXO1 and TSC2 are expressed abundantly (Fig. 9 and data not shown). Knockdown of FOXO1 in HUVEC decreased FOXO1 protein level by 90% (Fig. 9, a, top panel, and b). Knockdown of FOXO1 inhibited phosphorylation of p70 S6K (Fig. 9, 2nd panel). These data suggest that endogenous FOXO1 inhibits TSC2 and regulates phosphorylation of p70 S6K in vivo.

Prolonged Overexpression of FoxO1 Enhances Phosphorylation of Ser-307 of IRS-1—It has been reported that p70 S6K might be implicated in a negative feedback loop to suppress insulin signaling (27). From this study, we demonstrated that TSC2, which was immunoprecipitated with FoxO1 (Fig. 7a, lanes 14–15, top panel). This short fragment of TSC2 was also detected in SV40-transformed hepatocytes (data not shown). In another set of experiments in HUVEC, WT FoxO1 decreased total amounts of full-length TSC2, which was detected by both C-20 (Fig. 7b, top panel) and N-19, which recognized the N terminus of TSC2 (Fig. 7b, 2nd panels). Furthermore, the amount of short band of TSC2 was increased in a dose-dependent manner of transduced WT FoxO1 (Fig. 7b, top panel). However, we could not detect the fragmented N terminus of TSC2 by immunoblotting with N-19 antibody. These data indicate that WT FoxO1 interacts with TSC2 through its C-terminal domain, fragmented TSC2, and disrupts heterodimeric complex between TSC1 and TSC2.
cytosolic FoxO1 bound to and inhibited TSC2 and enhanced phosphorylation of p70 S6K. We speculate that enhanced phosphorylation of p70 S6K may lead to phosphorylation of serine 307 in IRS-1, which is one of the phosphorylation sites by p70 S6K (39), and finally to decreased phosphorylation of Akt and FoxO1 itself. To investigate whether FoxO1-TSC2 binding affects phosphorylation of serine 307 of IRS1 or not, we transduced SV40-transformed hepatocytes with adenoviruses encoding LacZ or WT FoxO1 and cultured cells in the presence of serum, and we examined phosphorylation of serine 307 of IRS-1 in a time course study. Phosphorylation of p70 S6K in WT FoxO1-transduced cells at 48 and 72 h is increased compared with LacZ-transduced cells because of inhibition of TSC2 by overexpression of FoxO1 from the previous experiments (Fig. 10, 3rd top panel). Amounts of total IRS1 protein level showed no significant differences between LacZ- and WT FoxO1-transduced cells (Fig. 10, lanes 1–6, 2nd top panel). However, phosphorylation of serine 307 of IRS1 in WT FoxO1-transduced cells is increased significantly compared with LacZ-transduced cells at 72 h after transduction (Fig. 10, lanes 3 and 6, top panel, and b). Furthermore, at 72 h after transduction, phosphorylation of both threonine 308 and serine 473 of IRS1 is increased compared with LacZ-transduced cells. Therefore, these results suggest that FoxO1-TSC2 binding affects phosphorylation of serine 307 of IRS1 or not, we transduced SV40-transformed hepatocytes with adenoviruses encoding LacZ or WT FoxO1 and cultured cells in the presence of serum, and we examined phosphorylation of serine 307 of IRS-1 in a time course study. Phosphorylation of p70 S6K in WT FoxO1-transduced cells at 48 and 72 h is increased compared with LacZ-transduced cells because of inhibition of TSC2 by overexpression of FoxO1 from the previous experiments (Fig. 10, 3rd top panel). Amounts of total IRS1 protein level showed no significant differences between LacZ- and WT FoxO1-transduced cells (Fig. 10, lanes 1–6, 2nd top panel). However, phosphorylation of serine 307 of IRS1 in WT FoxO1-transduced cells is increased significantly compared with LacZ-transduced cells at 72 h after transduction (Fig. 10, lanes 3 and 6, top panel, and b). Furthermore, at 72 h after transduction, phosphorylation of both threonine 308 and serine 473 of IRS1 is increased compared with LacZ-transduced cells.
Amino acid sequences in this region of 424–550 of the C terminus of FoxO1 as bait for a yeast two-hybrid screening. Amino acid sequences in this region of FoxO1 was used as bait for a yeast two-hybrid screening. We used a fragment (amino acids 459–463) (44), LL motif (amino acids 459–463), in this study, we identified TSC2 as a novel FoxO1-binding protein. In this study, we identified TSC2 as a novel FoxO1-binding protein. In this study, we identified TSC2 as a novel FoxO1-binding protein. In this study, we identified TSC2 as a novel FoxO1-binding protein. In this study, we identified TSC2 as a novel FoxO1-binding protein. In this study, we identified TSC2 as a novel FoxO1-binding protein. Akts were decreased even in the presence of serum and also phosphorylation of FoxO1 itself was decreased. These data suggest that FoxO1-TSC2 binding leads to enhanced phosphorylation of serine 307 of IRS-1 protein through increased phosphorylation of p70 S6K and finally inhibits phosphorylation of Akt and FoxO1 itself even in the presence of serum.

**DISCUSSION**

In this study, we identified TSC2 as a novel FoxO1-binding protein by a yeast two-hybrid screening using a murine islet cDNA library. FoxOs interact with several kinds of protein and regulate their function and vice versa. For example, FoxO1 binds to the transcriptional co-activator PGC-1α, and PGC-1α potentiates FoxO1-dependent transcription of gluconeogenic genes (40). Acetylation by Cbp/P300 and deacetylation by Sirt1 regulates transcriptional activity of FoxOs (6). Therefore, it is important for understanding the mechanism of how FoxO1 is regulated to identify FoxO1-binding proteins. However, there are few reports about the identification of FoxO1-binding proteins using comprehensive strategies.

We used a murine islet cDNA library for identification of FoxO1-binding proteins because FoxO1 is expressed in pancreatic β-cells abundantly and has been reported already to play an important role for compensatory hypertrophy of β-cells under insulin resistance (41–43). Therefore, we speculated that it might be easy to identify FoxO1-binding proteins by using an islet cDNA library. One of interesting findings in this study is that only FoxO1 binds to TSC2 among FoxOs. We used a fragment (amino acids 424–550) of the C terminus of FoxO1 as bait for a yeast two-hybrid screening. Amino acid sequences in this region of FoxO1, except the LXXLL motif (amino acids 459–463) (44), have low similarity among FoxOs. Therefore, it is reasonable for only FoxO1 to associate with TSC2 physically. These findings suggest the hypothesis that FoxO1 may have specific roles in vivo. Studies using genetically modified mice, such as knock-out and transgenic mice, support this hypothesis. FoxO1-null mice die at embryonic day 10.5 from defects in angiogenesis.
itself was blunted even in the presence of serum. These data suggest a novel mechanism in which FoxO1 in cytosol can regulate Akt/FoxO1 through the TSC2/mTOR/p70 S6K/IRS1 pathway (Fig. 11).

In conclusion, these studies identified TSC2 as a novel interacting partner with FoxO1 and suggested that FoxO1 could negatively regulate TSC2 function. They suggest a novel crosstalk between Akt/FoxO1 and the mTOR p70 S6K pathway and propose the possibility that FoxO1 can induce insulin resistance not only through increased gene expression in the nucleus but also through down-regulation insulin signaling in the cytosol. Therefore, regulation of the association between FoxO1 and TSC2 should be a target of therapy of type 2 diabetes.

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REFERENCES

1. Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997) Nature 389, 994–999
2. Lin, K., Dorman, J. B., Rodan, A., and Kenyon, C. (1997) Science 278, 1319–1322
3. Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001) Nat. Genet. 28, 139–145
4. Stocker, H., and Hafen, E. (2000) Curr. Opin. Genet. Dev. 10, 529–535
5. Puig, O., Marr, M. T., Ruhl, M. L., and Tjian, R. (2003) Genes Dev. 17, 2006–2020
6. Accili, D., and Arden, K. C. (2004) Cell 117, 421–426
7. Furuyama, T., Nakazawa, T., Nakano, I., and Morii, N. (2000) Biochem. J. 349, 629–634
8. Greer, E. L., and Brunet, A. (2005) Oncogene 24, 7410–7425
9. Fingar, D. C., and Blenis, J. (2004) Oncogene 23, 3151–3171
10. The European Chromosome 16 Tuberous Sclerosis Consortium (1993) Cell 75, 1305–1315
11. Hay, N., and Sonenberg, N. (2004) Genes Dev. 18, 1926–1945
12. Sparagana, S. P., and Roach, E. S. (2000) Curr. Opin. Neurobiol. 13, 115–119
13. Ito, N., and Rubin, G. M. (1999) Cell 96, 529–539
14. Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru, B., and Pan, D. (2002) Nat. Cell Biol. 4, 699–704
15. Goncharova, E. A., Goncharov, D. A., Eszterhas, A., Hunter, D. S., Glassberg, M. K., Yeung, R. S., Walker, C. L., Noonan, D., Kwiatkowski, D. J., Chou, M. M., Panettieri, R. A., Jr., and Krymskaya, V. P. (2002) J. Biol. Chem. 277, 30958–30967
16. Kwiatkowski, D. J., Zhang, H., Bandura, J. L., Heiberger, K. M., Glogauer, M., el-Hashemite, N., and Onda, H. (2002) Hum. Mol. Genet. 11, 525–534
17. Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13571–13576
18. Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002) Nat. Cell Biol. 4, 648–657
19. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002) Mol. Cell 10, 151–162
20. Potter, C. J., Pedraza, L. G., and Xu, T. (2002) Nat. Cell Biol. 4, 658–665
21. Manning, B. D., and Cantley, L. C. (2003) Trends Biochem. Sci. 28, 573–576
22. Li, Y., Corradetti, M. N., Inoki, K., and Guan, K. L. (2004) Trends Biochem. Sci. 29, 32–38
23. Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A., and Guan, K. L. (2004) Genes Dev. 18, 1533–1538
24. Inoki, K., Zhu, T., and Guan, K. L. (2003) Cell 115, 577–590
25. Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Komatsa, M., DePinho, R. A., and Cantley, L. C. (2004) Cancer Cell 6, 91–99
26. Sofer, A., Lei, K., Johannissem, C. M., and Ellisen, L. W. (2005) Mol. Cell. Biol. 25, 5834–5845
27. Um, S. H., D’Alessio, D., and Thomas, G. (2006) Cell Metab. 3, 393–402
28. Harra, K., Yonezawa, K., Kozloowski, M. T., Sugimoto, T., Andradi, K., West, Q. P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997) J. Biol. Chem. 272, 26457–26463
29. Nakae, J., Park, B. C., and Accili, D. (1999) J. Biol. Chem. 274, 15982–15985
30. Nakae, J., Kitamura, T., Silver, D., L., and Accili, D. (2001) J. Clin. Investig. 108, 1359–1367
31. Takaichi, H., Konishi, H., Matsuhashi, H., Ono, Y., Shirai, Y., Saito, N., Kitamura, T., Ogawa, W., Kasuga, M., Kikukawa, U., and Nishizuka, Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11836–11841
32. Kamioka, Y., Fujikura, S., Sawa, H., Nagashima, K., Masuda, M., Matsuoka, Y., and Mochizuki, N. (2004) J. Biol. Chem. 279, 40091–40099
33. Tsuji, H., Orimoto, K., Kobayashi, K., and Hino, O. (1996) Cancer Res. 56, 429–433
34. Nishi, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–199
35. Nakae, J., Barr, V., and Accili, D. (2000) EMBO J. 19, 989–996
36. Nishimura, M., Yokoi, N., Miki, T., Horikawa, Y., Yoshioka, H., Takeda, J., Ohara, O., and Seino, S. (2004) DNA Res. 11, 315–323
37. Kleiner, J., Fasshauer, M., Ito, M., Lowell, B. B., Benito, M., and Kahn, C. R. (1999) J. Biol. Chem. 274, 34795–34802
38. Nabekura, T., Otsu, M., Nagasawa, T., Nakauchi, H., and Onodera, M. (2006) Mol. Ther. 13, 301–309
39. Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquín, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J., and Thomas, G. (2004) Nature 431, 200–205
40. Puigserver, P., Rhee, J., Donovan, J., Walkey, C. J., Yoon, J. C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D., and Spiegelman, B. M. (2003) *Nature* **423**, 550–555
41. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., III, Wright, C. V., White, M. F., Arden, K. C., and Accili, D. (2002) *J. Clin. Investig.* **110**, 1839–1847
42. Kitamura, Y. I., Kitamura, T., Kruse, J. P., Raum, J. C., Stein, R., Gu, W., and Accili, D. (2005) *Cell Metab.* **2**, 153–163
43. Okamoto, H., Hribal, M. L., Lin, H. V., Bennett, W. R., Ward, A., and Accili, D. (2006) *J. Clin. Investig.* **116**, 775–782
44. Nakae, J., Cao, Y., Daitoku, H., Fukamizu, A., Ogawa, W., Yano, Y., and Hayashi, Y. (2006) *J. Clin. Investig.* **116**, 2473–2483
45. Hosaka, T., Biggs, W. H., III, Tieu, D., Boyer, A. D., Varki, N. M., Cavenee, W. K., and Arden, K. C. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2975–2980
46. Nakae, J., Biggs, W. H., Kitamura, T., Cavenee, W. K., Wright, C. V., Arden, K. C., and Accili, D. (2002) *Nat. Genet.* **32**, 245–253
47. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W. H., III, Arden, K. C., and Accili, D. (2003) *Cell* **11**, 1457–1466
48. Castrillon, D. H., Miao, L., Kollipara, R., Horner, J. W., and DePinho, R. A. (2003) *Science* **301**, 215–218
49. Potter, C. J., Huang, H., and Xu, T. (2001) *Cell* **105**, 357–368
50. Gao, X., and Pan, D. (2001) *Genes Dev.* **15**, 1383–1392
51. Tapon, N., Iti, N., Dickson, B. J., Treisman, J. E., and Hariharan, I. K. (2001) *Cell* **105**, 345–355
52. Nellist, M., van Slegtenhorst, M. A., Goedbloed, M., van den Ouweland, A. M., Halley, D. J., and van der Sluijs, P. (1999) *J. Biol. Chem.* **274**, 35647–35652
53. Benvenuto, G., Li, S., Brown, S. J., Braverman, R., Vass, W. C., Cheadle, J. P., Halley, D. I., Sampson, J. R., Wienecke, R., and DeClue, J. E. (2000) *Oncogene* **19**, 6306–6316
54. Li, Y., Inoki, K., and Guan, K. L. (2004) *Mol. Cell. Biol.* **24**, 7965–7975
55. Wienecke, R., König, A., and DeClue, J. E. (1995) *J. Biol. Chem.* **270**, 16409–16414
56. Xiao, G. H., Shoiranejad, F., Jin, F., Golemis, E. A., and Yeung, R. S. (1997) *J. Biol. Chem.* **272**, 6097–6100
57. Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003) *Mol. Cell* **11**, 1457–1466
58. Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003) *Curr. Biol.* **13**, 1259–1268
59. Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003) *Genes Dev.* **17**, 1829–1834
60. Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003) *Nat. Cell Biol.* **5**, 578–581
61. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003) *Nat. Cell Biol.* **5**, 559–565
62. Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., and Edgar, B. A. (2003) *Nat. Cell Biol.* **5**, 566–571
63. Harrington, L. S., Findlay, G. M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N. R., Cheng, S., Shepherd, P. R., Gout, I., Downes, C. P., and Lamb, R. F. (2004) *J. Cell Biol.* **166**, 213–223
64. Craparo, A., Freund, R., and Gustafson, T. A. (1997) *J. Biol. Chem.* **272**, 11663–11669