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The TSC1-TSC2 Complex Is Required for Proper Activation of mTOR Complex 2[▽]

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The mammalian target of rapamycin (mTOR) is a protein kinase that forms two functionally distinct complexes important for nutrient and growth factor signaling. Both complexes phosphorylate a hydrophobic motif on downstream protein kinases, which contributes to the activation of these kinases. mTOR complex 1 (mTORC1) phosphorylates S6K1, while mTORC2 phosphorylates Akt. The TSC1-TSC2 complex is a critical negative regulator of mTORC1. However, how mTORC2 is regulated and whether the TSC1-TSC2 complex is involved are unknown. We find that mTORC2 isolated from a variety of cells lacking a functional TSC1-TSC2 complex is impaired in its kinase activity toward Akt. Importantly, the defect in mTORC2 activity in these cells can be separated from effects on mTORC1 signaling and known feedback mechanisms affecting insulin receptor substrate-1 and phosphatidylinositol 3-kinase. Our data also suggest that the TSC1-TSC2 complex positively regulates mTORC2 in a manner independent of its GTPase-activating protein activity toward Rheb. Finally, we find that the TSC1-TSC2 complex can physically associate with mTORC2 but not mTORC1. These data demonstrate that the TSC1-TSC2 complex inhibits mTORC1 and activates mTORC2, which through different mechanisms promotes Akt activation.

The TSC1-TSC2 complex has emerged as a central signal-integrating node within the cell. Mutations in the tumor suppressor genes encoding TSC1 and TSC2 give rise to a multisystemic tumor syndrome called tuberous sclerosis complex (TSC), which is characterized by widespread dysplastic and neoplastic lesions (i.e., hamartomas) (reviewed in reference 7). *TSC2* mutations are also found in sporadic cases of lymphangioleiomyomatosis (LAM), which is characterized by aberrant smooth muscle cell proliferation and cystic destruction in the lung (5). In addition, the TSC1-TSC2 complex lies downstream of numerous oncogenes (e.g., RTKs, phosphatidylinositol 3-kinase [PI3K]–Akt, and Ras) and tumor suppressors (e.g., PTEN, LKB1, and NF1) and, therefore, is predicted to be functionally altered by posttranslational modifications in the majority of human cancers (11, 17).

Within the TSC1-TSC2 complex, TSC1 stabilizes TSC2 (3, 6), while TSC2 acts as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) (10, 18, 39, 44, 45, 55). GTP-bound Rheb potently activates the mammalian target of rapamycin complex 1 (mTORC1) (26, 35), which plays an evolutionarily conserved role in promoting cell growth and proliferation (49). When active, the TSC1-TSC2 complex inhibits mTORC1 by stimulating the conversion of Rheb-GTP to Rheb-GDP. Therefore, the ability of a variety of upstream pathways to affect mTORC1 activity is dependent on modifications that functionally inhibit or activate the TSC1-TSC2 complex.

Target of rapamycin (TOR) proteins are PI3K-related Ser/Thr kinases found in two functionally distinct complexes that are conserved in eukaryotic cells from yeast to humans (49). In mammalian cells, mTORC1 is comprised of mammalian target of rapamycin (mTOR), RAPTOR, and mLST8 (13, 21, 22, 25) and is acutely sensitive to the compound rapamycin. Within mTORC1, RAPTOR contributes to mTOR substrate specificity by binding to substrates via TOR signaling motifs on those proteins (33, 40). The best characterized of these substrates are the ribosomal S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E-binding proteins (4E-BP1 and 4E-BP2). Phosphorylation by mTORC1 leads to S6K activation and 4E-BP inhibition, thereby stimulating cap-dependent translation (15, 27). The second mTOR-containing protein complex, mTORC2, is comprised of mTOR, RICTOR, mSIN1, and mLST8 (8, 19, 25, 36, 50). More recently, the protein PROTOR/PRR5 was also found to be associated with mTORC2 (34, 48). Within this complex, mTOR is resistant to acute rapamycin treatment, but prolonged exposure to rapamycin can block the assembly of mTORC2 components (37). Unlike mTORC1, very little is known regarding the regulation and function of mTORC2. However, both biochemical and genetic evidence have demonstrated that mTORC2 phosphorylates Akt at S473 (12, 19, 38, 43), thereby contributing to the activation of this important cell survival kinase (1). S473 on Akt lies within a hydrophobic motif conserved among AGC (protein kinase A, G, and C) family kinases, including the S6Ks. Interestingly, mTOR within mTORC1 phosphorylates this same motif on the S6Ks (T389 in the 70-kDa isoform of S6K1). While the TSC1-TSC2 complex and Rheb are critical regulators of mTORC1, whether these proteins likewise regulate mTORC2 is not known.

Activation of mTORC1 has been found to negatively impact Akt phosphorylation in response to insulin or IGF1 (reviewed in reference 28). As Akt is an important upstream activator of mTORC1 in response to these growth factors, this serves as a negative feedback loop. The mechanism of this feedback regulation has been attributed to the phosphorylation of serine

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residues on insulin receptor substrate-1 (IRS-1) by mTORC1 and its downstream target S6K1, resulting in decreased IRS-1 protein stability (e.g., see references 14, 41, 42, 46, and 47). Upon ligand binding, the insulin and IGF1 receptors phosphorylate IRS-1 on tyrosine residues, thereby creating binding sites for the p85 regulatory subunit of class I PI3K. IRS-1-bound PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which triggers PDK1 and Akt membrane recruitment and stimulates phosphorylation events on Akt at T308 and S473 through PDK1 and mTORC2, respectively (reviewed in reference 4). Therefore, mTORC1-driven feedback inhibition of IRS-1 leads to an inability of insulin or IGF1 to activate PI3K and, subsequently, Akt. This feedback mechanism is most obvious in cell culture models with defects in the TSC1-TSC2 complex, where mTORC1 and S6K1 are constitutively active, resulting in the hyperphosphorylation and degradation of IRS-1 (14, 41, 42). Although the mechanism is unknown, platelet-derived growth factor receptor β (PDGF-Rβ) has also been found to be downregulated in TSC1- and TSC2-deficient murine embryonic fibroblasts (MEFs) and to contribute to attenuation of PI3K signaling (52, 53). However, whether the pronounced defect in Akt phosphorylation observed in cells lacking the TSC1-TSC2 complex can be attributed to additional mechanisms is unknown.

Here, we report that mTORC2 kinase activity is lost upon disruption of the TSC1-TSC2 complex. Interestingly, this effect can be separated from mTORC1-dependent feedback mechanisms affecting IRS-1 and PI3K. Furthermore, the TSC1-TSC2 complex promotes mTORC2 activity in a manner that is, at least partially, independent of its GAP activity toward Rheb. Surprisingly, we found that the TSC1-TSC2 complex can physically associate with mTORC2 but not mTORC1. Taken together, our data suggest that the TSC1-TSC2 complex inhibits mTORC1 and activates mTORC2, which through different mechanisms promotes Akt phosphorylation and activation. We discuss the potential consequences of this unusual behavior for a tumor suppressor.

MATERIALS AND METHODS

Antibodies and reagents. Unless otherwise stated, all antibodies were obtained from Cell Signaling Technology. Exceptions include antibodies to RICTOR and mSIN1 (Bethyl Laboratories), antibodies to the FLAG epitope and actin (Sigma), antibodies to the hemagglutinin (HA) epitope (Covance), and preimmune rabbit immunoglobulin G (IgG) (Santa Cruz). Antibodies to p85 and Rheb were generous gifts from L. C. Cantley (Beth Israel Deaconess Medical Center, Boston, MA) and R. F. Lamb (Institute of Cancer Research, London, United Kingdom), respectively. Growth factors and kinase inhibitors used were from Sigma (insulin, epidermal growth factor [EGF]), Austral Biologicals (PDGF), or Calbiochem (rapamycin, LY294002).

Cell culture, constructs, and small interfering RNAs (siRNAs). HeLa, HepG2, MCF7, HEK293, and MEF lines were maintained in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose containing 10% fetal bovine serum. 3T3-immmortalized *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ MEFs and the littermate-derived pair of *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs (both p53$^{-/-}$) were provided by D. J. Kwiatkowski (Brigham and Women's Hospital, Boston, MA) and were described previously (23, 53). A retroviral internal ribosome entry site-hygromycin construct encoding a human *TSC2* cDNA was provided by D. J. Kwiatkowski. The isogenic pair of control and reconstituted *Tsc2*$^{-/-}$ MEFs was generated by infection of the 3T3-immortalized MEFs with retroviruses containing empty vector (Tsc2-V) or the above *TSC2* construct (Tsc2-T2), and stable pools or individual clones were selected with hygromycin (100 μg/ml). Retroviral constructs (pBabe-puro based) encoding the wild-type, myristoylated, E545K, or H1047R alleles of p110α were obtained from T. M. Roberts (Dana Farber Cancer Center, Boston, MA) (56) via Addgene, and stable pools of MEFs expressing these constructs were obtained following infection and selection with puromycin (2 μg/ml).

All mammalian cell transfections, including those with siRNAs, were performed using Lipofectamine 2000 (Invitrogen), by following the manufacturer's protocol. Mammalian expression constructs of mTOR, kinase-dead mTOR, Rictor, mSin1, and mLST8 were obtained from D. M. Sabatini (Massachusetts Institute of Technology, Cambridge, MA) via Addgene. The original myc-mSIN1 and myc-mLST8 constructs were subcloned into the pRK7 vector for the experiments described. Transient RNA interference experiments were carried out with SMARTpool siRNAs (Dharmacon). Eighty-nanomolar control (D-00126-14), mouse Raptor (M-058754-00), or mouse Rheb (M-057044-00) siRNAs were transfected into MEFs, and experiments were carried out 48 h after transfection.

Short hairpin RNAs (shRNAs), expressed as miR30 fusions, targeting firefly luciferase or human *TSC2* were in the pMSCV-PM vector and were provided by the laboratory of S. J. Elledge (Harvard Medical School, Boston, MA). The *TSC2*-targeting sequences were as follows: T2a, 5′ AGCCTGCCCTTCCGGAA GGATT; and T2b, 5′ ACCTGTCAGTGAAATAAATAAA. Stable pools expressing these shRNAs were generated by retroviral infection of HeLa or HepG2 cells followed by puromycin selection at 0.5 μg/ml.

Cell lysis and immunoprecipitations. For immunoprecipitations with epitope-tagged proteins, 100-mm dishes containing 6×10^6 HEK293 cells were transfected with Myc- or FLAG-tagged cDNA constructs and grown in full serum for 24 h before lysis. For anti-TSC1 immunoprecipitations, lysates were generated from 80% confluent 100-mm dishes of MCF7 cells, HeLa cells, HEK293 cells, or MEFs. Lysates were prepared in 1-ml mTORC lysis buffer, derived from previous studies on the mTOR complexes (21, 38), containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 10 mM glycerol 2-phosphate, 0.5 mM sodium orthovanadate, 0.3% CHAPS {3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate}, and the Sigma protease inhibitor cocktail. For IRS-1 immunoprecipitations, cell lysates were prepared in NP-40 lysis buffer, as described previously (31). The soluble fraction of the lysates was incubated with the precipitating antibody for 2 h followed by 1-h incubation with protein A/G-agarose (Pierce) or 3 h with anti-myc-agarose or anti-FLAG (M2) affinity gel (Sigma). Immunoprecipitates were washed four times with mTORC lysis buffer before boiling in sodium dodecyl sulfate sample buffer.

mTORC2 kinase assays. Kinase assays on endogenous mTORC2 were performed as described previously (38). Near-confluent 100-mm plates of the given cell line were lysed in 1 ml mTORC lysis buffer, and mTORC2 was immunoprecipitated with 1.5 μg anti-RICTOR antibodies, as described above. Inactive Akt1/PKBα (Upstate Biotechnology) was used as the substrate for these reactions, and phosphorylation was detected by immunoblotting. For mTORC2 assays using kinase-dead Akt as a substrate, a previously described HA-Akt-K179D mutant (31) was isolated from serum-starved HEK293E cells. Lysates of the indicated HeLa cell derivatives (see Fig. 2F) were generated, and endogenous mTORC2 complexes bound to an anti-RICTOR antibody, or control IgG, were precipitated using the protein A/G-agarose with HA-Akt-K179D bound. These ice-cold immune complexes containing mTORC2 and the Akt substrate were then subjected to the mTORC2 kinase assay conditions described elsewhere (38).

RESULTS

The TSC1-TSC2 complex is required for proper Akt phosphorylation. Previous studies have found that *Tsc1*- or *Tsc2*-deficient MEFs are impaired in insulin or PDGF-responsive Akt phosphorylation relative to the levels in wild-type littermate-derived MEFs (14, 42, 53). In order to confirm this defect in Akt activation in an isogenic cell line, we reconstituted a *Tsc2*$^{-/-}$ MEF line (3T3 immortalized) with empty vector (Tsc2-V) or human *TSC2* (Tsc2-T2) and selected stable pools (Fig. 1A). The Tsc2-T2 cells express slightly higher levels of human TSC2 than the levels of mouse TSC2 found in wild-type MEFs, but they express very similar levels of TSC1 (Fig. 1B). As expected, in the Tsc2-V cells, S6K1 phosphorylation at T389, a marker of mTORC1 activity in the cell, was elevated during serum starvation and not further stimulated by growth factors, while human TSC2 completely restored the growth factor dependence to mTORC1 activity in the Tsc2-T2 cells

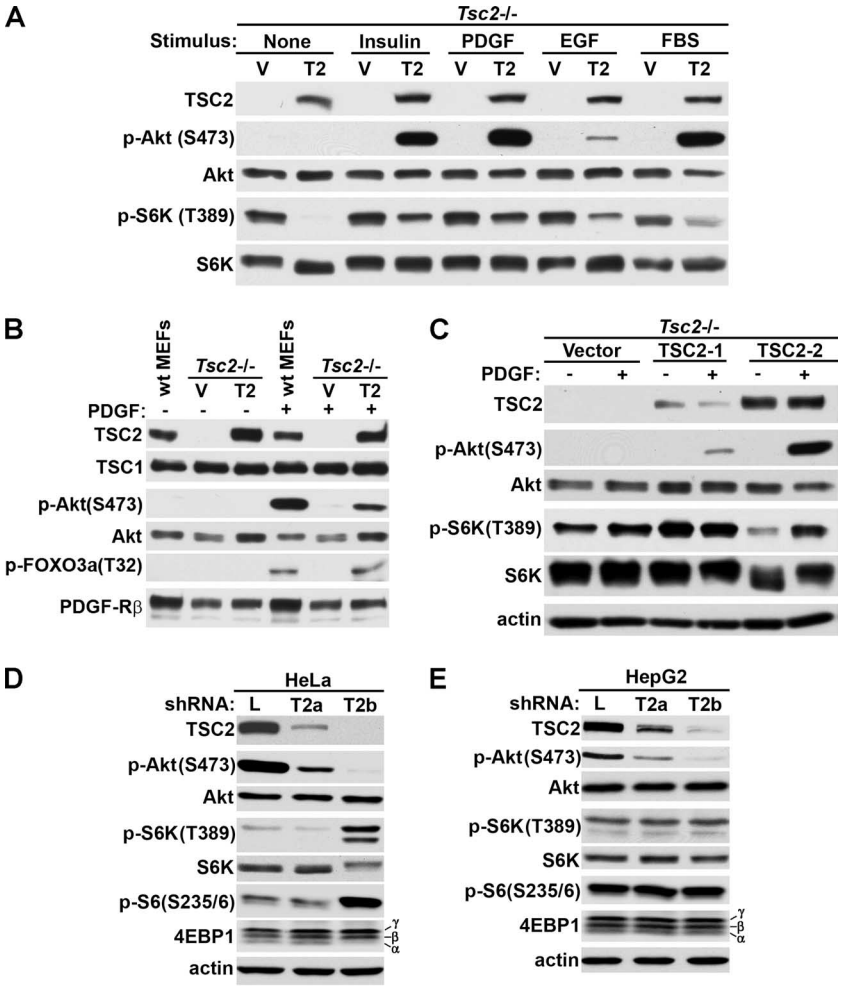

FIG. 1. General attenuation of Akt phosphorylation upon decreased TSC2 expression. (A) Growth factor-stimulated phosphorylation of Akt is impaired in cells lacking the TSC1-TSC2 complex. Pools of isogenic *Tsc2*$^{-/-}$ MEFs (3T3 immortalized) stably expressing either vector alone (V) or human *TSC2* (T2) were stimulated with insulin (100 nM), PDGF (5 ng/ml), EGF (5 ng/ml), or 10% fetal bovine serum (FBS), as indicated, for 30 min prior to lysis. (B) Levels of TSC2 and Akt activity in wild-type (wt) MEFs compared to *Tsc2*$^{-/-}$ MEFs reconstituted with *TSC2*. 3T3-immortalized wild-type MEFs and the pair of vector and TSC2-expressing *Tsc2*$^{-/-}$ MEFs, described for panel A, were serum starved overnight and stimulated with 5 ng/ml PDGF for 30 min prior to lysis. (C) Reconstituted *Tsc2*$^{-/-}$ MEFs expressing low levels of TSC2 display partial restoration of growth factor-dependent Akt phosphorylation before noticeable effects on mTORC1 signaling. Two individual clones of Tsc2$^{-/-}$ MEFs reconstituted with human *TSC2* (TSC2-1 and TSC2-2), or vector control cells, were serum starved overnight and stimulated with PDGF (5 ng/ml) for 30 min prior to lysis. (D, E) Dose-dependent decrease in Akt phosphorylation upon shRNA-mediated knockdown of TSC2. HeLa (D) and HepG2 (E) cells stably expressing shRNAs targeting firefly luciferase (L) or two different shRNAs targeting human *TSC2* (T2a and T2b) were cultured under normal growth conditions (i.e., in full serum) prior to lysis. The phosphorylation status of 4E-BP1 is detected as a shift from the unphosphorylated α form to the phosphorylated β and γ forms.

(Fig. 1A). Consistent with previous findings, the Tsc2-V cells were defective in insulin and PDGF-stimulated Akt phosphorylation. However, these cells were also defective in their response to EGF and full serum for Akt phosphorylation. This attenuation was due to a lack of TSC2, as the Tsc2-T2 cells displayed Akt phosphorylation in response to all of these growth factors. In response to PDGF, Akt phosphorylation in the Tsc2-T2 cells was lower than in wild-type MEFs, and this correlated with lower levels of PDGF-Rβ in these cells (Fig. 1B). However, PDGF-Rβ levels were not significantly different between the Tsc2-V and Tsc2-T2 cells. *TSC2* reconstitution also restored Akt signaling to downstream targets, such as FOXO3a (Fig. 1B). Therefore, disruption of the TSC1-TSC2 complex results in loss of Akt stimulation in response to a variety of growth factors, including those that do not signal through IRS-1. This suggests that either multiple independent feedback mechanisms exist as a result of high mTORC1 activity in the TSC-deficient cells or a more-general mechanism of Akt attenuation occurs when the TSC1-TSC2 complex is disrupted.

In the generation of stable reconstituted MEF lines, we found a surprising dose dependence for the rescue of Akt signaling in the *Tsc2*$^{-/-}$ MEFs. We isolated single-cell-derived clones of Tsc2-T2 cells that expressed lower levels of TSC2 than the original stable pool. Although most of the clones expressed TSC2 at levels sufficiently high to suppress the growth factor-independent phosphorylation of S6K1, some clones expressed low levels of TSC2 that could not suppress

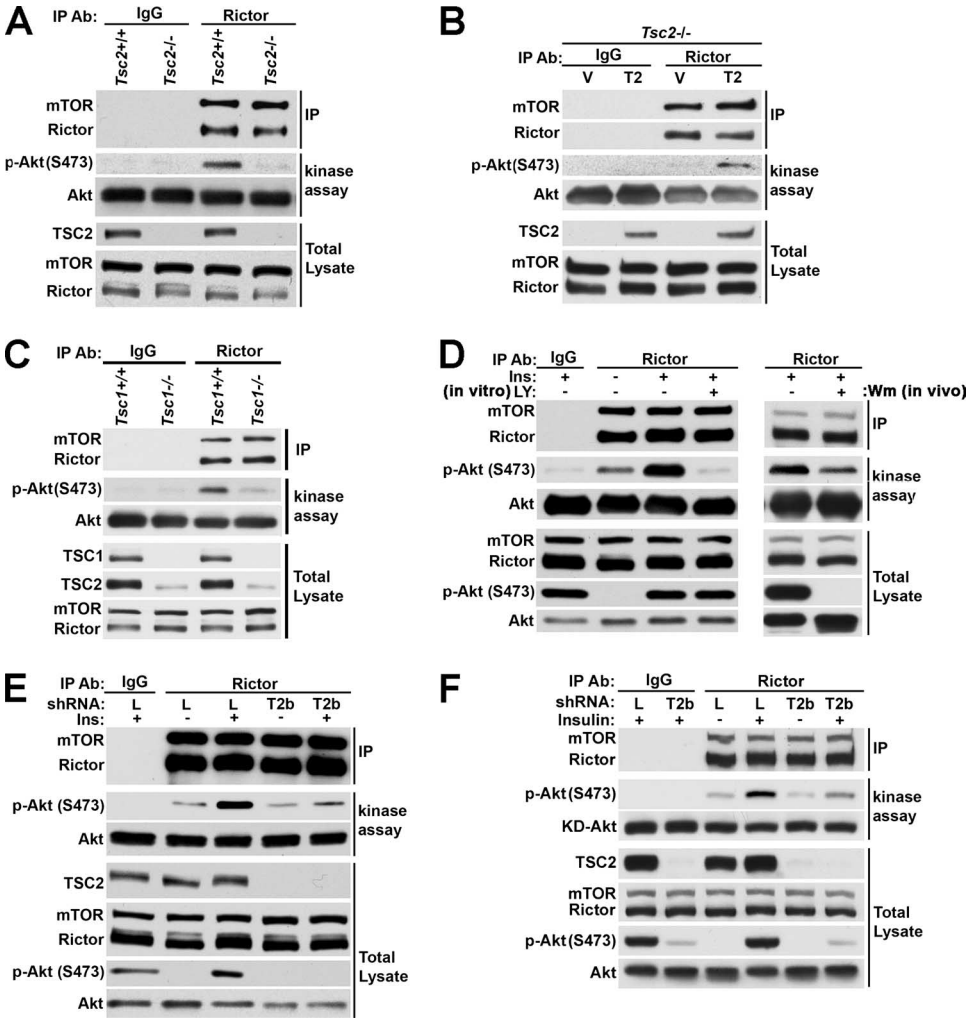

FIG. 2. Loss of mTORC2 kinase activity in cells lacking the TSC1-TSC2 complex. (A) mTORC2 kinase activity is impaired in *Tsc2*$^{-/-}$ MEFs. Littermate-derived *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were grown in full serum before lysis and immunoprecipitation (IP) with control IgG or anti-RICTOR antibodies (Ab). mTORC2 kinase activity was assessed in these immunoprecipitates using inactive Akt1 as a substrate. (B) mTORC2 kinase assays, performed as described for panel A, on *Tsc2*$^{-/-}$ MEFs stably expressing empty vector (V) or human *TSC2* (T2) demonstrate that the impaired mTORC2 activity in *Tsc2*$^{-/-}$ cells is rescued by wild-type *TSC2*. (C) mTORC2 kinase activity is impaired in *Tsc1*$^{-/-}$ MEFs. Littermate-derived *Tsc1*$^{+/+}$ and *Tsc1*$^{-/-}$ MEFs were grown in full serum, and mTORC2 kinase activity was assayed as described for panel A. (D) Insulin can stimulate mTORC2 kinase activity. HeLa cells were serum starved overnight and stimulated with insulin (Ins; 100 nM) for 15 min before lysis. mTORC2 kinase assays were performed as described for panel A. Where indicated, LY294002 (LY; 15 μM) was added to the kinase reaction. In the right two lanes, HeLa cells were treated as above but, where indicated, were pretreated for 30 min with wortmannin (Wm; 100 nM) prior to insulin stimulation. (E) Knockdown of TSC2 impairs insulin-stimulated mTORC2 kinase activity. HeLa cells stably expressing shRNAs targeting luciferase (L) or *TSC2* (T2b) were treated as described for panel D, and mTORC2 activity was assayed as described for panel A. (F) The differences between mTORC2 kinase activity in cells lacking or expressing TSC2 can also be detected using a kinase-dead (KD) Akt as a substrate. Cells were treated as described for panel D, and mTORC2 kinase activity in RICTOR immunoprecipitates was assayed using an HA-Akt-K179D substrate.

this aberrant mTORC1 signaling. Interestingly, the stimulation of Akt phosphorylation in these clones correlated with the level of TSC2 expression rather than detectable effects on mTORC1 signaling (e.g., Fig. 1C, clone TSC2-1). In reciprocal experiments, we used two distinct retroviral shRNA constructs targeting human *TSC2* (T2a and T2b) to stably knock down TSC2 expression in HeLa and HepG2 cells. Importantly, the degree of knockdown of TSC2 protein levels varies for these two shRNAs, with T2b being significantly more effective than T2a (Fig. 1D and E), allowing us to further assess whether TSC2 protein levels differentially affect mTORC1 and mTORC2 signaling events. Under full-serum growth conditions, HeLa cells expressing either a control shRNA targeting firefly luciferase or the T2a shRNA exhibit low levels of mTORC1 activity, as indicated by phosphorylation of S6K1, S6, and 4E-BP1, whereas the T2b-expressing line showed a significant increase in mTORC1 signaling (Fig. 1D). Despite a lack of detectable changes in mTORC1 signaling, the T2a line showed a substantial decrease in Akt phosphorylation relative to controls, and the T2b line showed a near-complete loss of Akt phosphorylation. In HepG2 cells, mTORC1 signaling was basally elevated under these growth conditions and was not

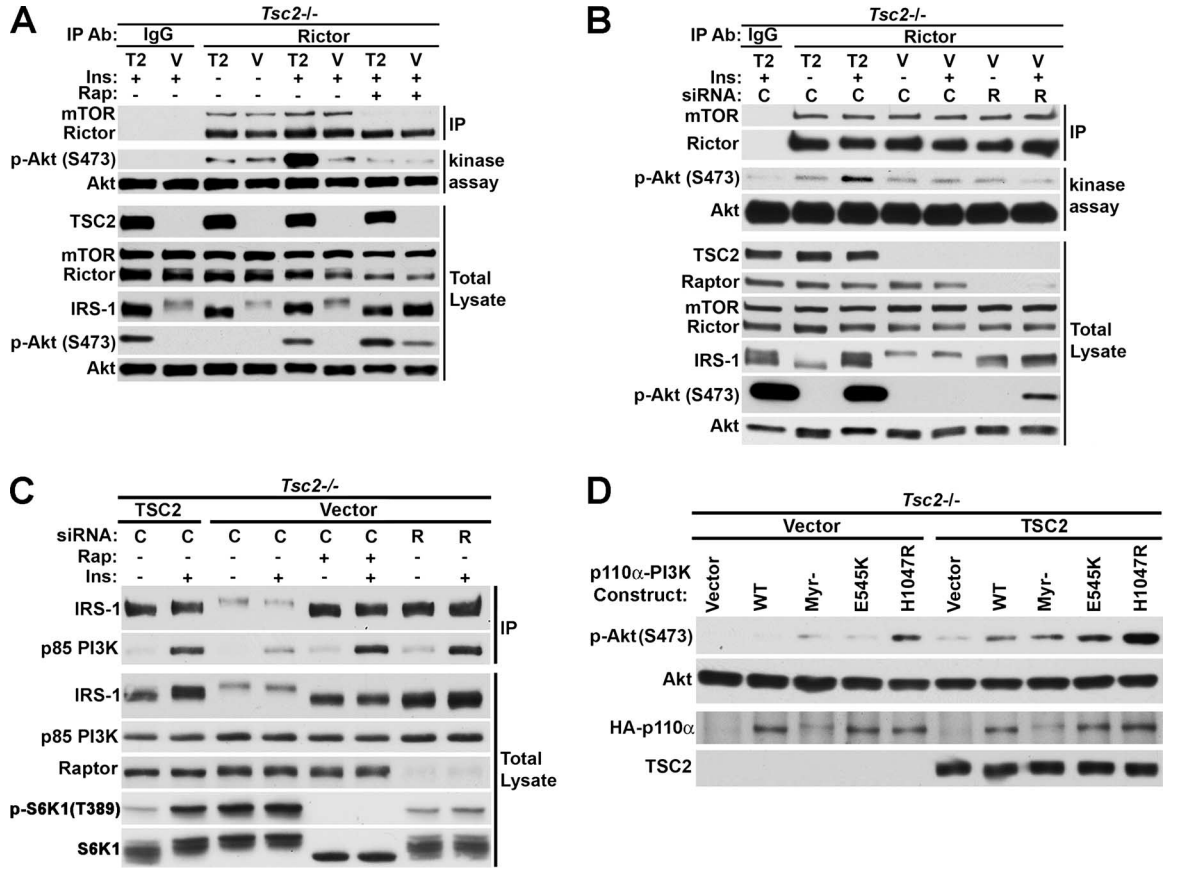

FIG. 3. Impaired mTORC2 activity in cells lacking the TSC1-TSC2 complex can be separated from mTORC1-dependent feedback mechanisms affecting IRS-1 and PI3K. (A) Prolonged rapamycin treatment does not restore mTORC2 kinase activity to $Tsc2^{-/-}$ MEFs. $Tsc2^{-/-}$ MEFs stably expressing empty vector (V) or human *TSC2* (T2) were serum starved overnight and stimulated with 100 nM insulin (Ins) for 15 min before lysis. Where indicated, cells were treated with rapamycin (Rap) during the serum starvation. Immunoprecipitation (IP) was performed with control IgG or anti-RICTOR antibodies (Ab), and mTORC2 kinase activity was assessed in these immunoprecipitates using inactive Akt1 as a substrate. (B) Inhibition of mTORC1 activity with siRNAs to RAPTOR does not restore mTORC2 kinase activity to $Tsc2^{-/-}$ MEFs. The cells described for panel A were transfected with either control (C) or RAPTOR (R) siRNAs, and insulin-stimulated mTORC2 kinase activity was assessed, as described for panel A. (C) Inhibition of mTORC1 activity with prolonged rapamycin treatment or siRNAs to RAPTOR restores insulin-responsive recruitment of the PI3K regulatory subunit p85 to IRS-1 in *Tsc2* null cells. $Tsc2^{-/-}$ MEFs stably expressing empty vector or human *TSC2* were serum starved overnight and stimulated with insulin as described for panel A. Where indicated, cells were treated with rapamycin as described for panel A or were transfected with control or RAPTOR siRNAs as described for panel B. The binding of endogenous p85 to IRS-1 was then examined by immunoprecipitating endogenous IRS-1. (D) Akt phosphorylation stimulated by activated alleles of the PI3K catalytic subunit p110α is attenuated in *Tsc2* null cells. HA-tagged wild-type (WT), myristoylated (Myr-), or oncogenic (E545K and H1047R) alleles of p110α were stably expressed in the cells described for panel A. Akt phosphorylation was then assessed in these cells following overnight serum starvation. The levels of expression of the different p110α alleles were determined in anti-HA immunoprecipitates from these cells.

further increased by either shRNA (Fig. 1E). However, as in the HeLa cells, Akt phosphorylation decreased as the levels of TSC2 decreased with the two shRNAs. This direct correlation between TSC2 levels and Akt phosphorylation suggests the intriguing possibility that the absence of TSC2 affects Akt phosphorylation through a mechanism that is, at least in part, independent of its effects on mTORC1 activity.

Loss of the TSC1-TSC2 complex impairs mTORC2 kinase activity. It is worth noting that phosphorylation of Akt on both T308 and S473 was affected by the levels of TSC2 in the above experiments. Although phosphorylation of T308 and S473 can occur independent of one another in mouse knockouts of PDK1 or components of mTORC2 (12, 32), reductions in mTORC2 components result in the loss of both T308 and S473 phosphorylation of Akt (e.g., see references 16, 38, and 50). Therefore, the complexity of Akt regulation within cells led us to directly assay mTORC2 kinase activity. Endogenous mTORC2 was isolated from littermate-derived $Tsc2^{+/+}$ or $Tsc2^{-/-}$ MEF lysates by immunoprecipitating RICTOR, and its kinase activity was assayed using an exogenous Akt1 substrate. Importantly, there was no difference in the amounts of mTOR associated with RICTOR in the $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs. However, mTORC2 kinase activity was greatly impaired in the $Tsc2^{-/-}$ cells (Fig. 2A), and this was restored upon *TSC2* reconstitution (Fig. 2B). $Tsc1^{-/-}$ MEFs also have substantially lower mTORC2 activity than do littermate-derived wild-type MEFs, with no significant differences in the levels of mTOR associated with RICTOR (Fig. 2C). Therefore, loss of TSC1 or TSC2 expression impairs mTORC2 kinase activity.

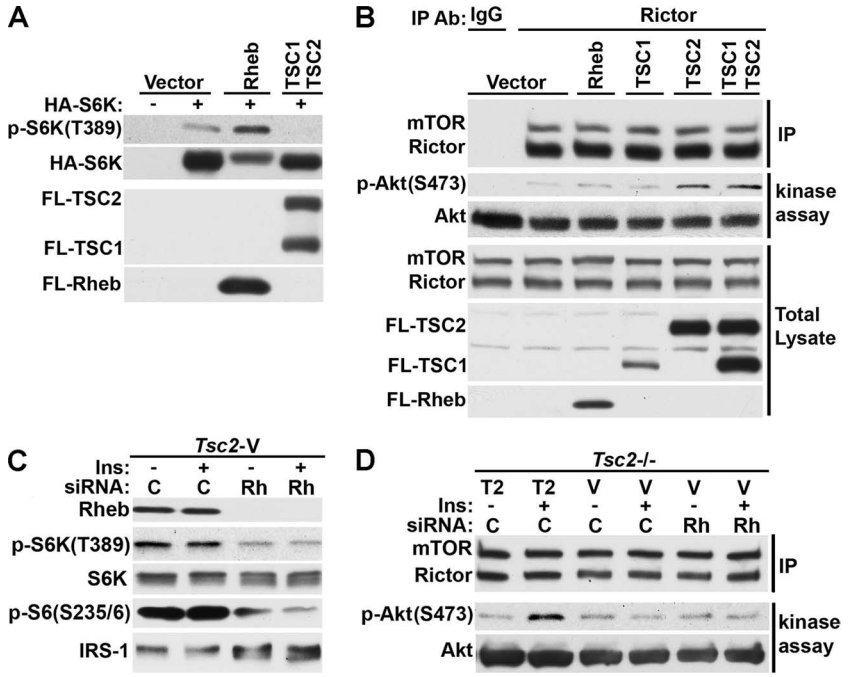

FIG. 4. Increased TSC1-TSC2 expression activates mTORC2 kinase activity, while neither Rheb overexpression nor knockdown affects mTORC2. (A) TSC1-TSC2 overexpression decreases and Rheb overexpression increases mTORC1 signaling. HA-S6K1 was coexpressed with empty vector, FLAG-Rheb, or both FLAG-TSC1 and FLAG-TSC2 in HEK293 cells grown in full serum, and the level of S6K1 phosphorylation was detected. (B) TSC1-TSC2 overexpression increases mTORC2 kinase activity, while Rheb overexpression has no effect on mTORC2. Empty vector, FLAG-Rheb, FLAG-TSC1, FLAG-TSC2, or both FLAG-TSC1 and FLAG-TSC2 were expressed in HEK293 cells grown in full serum. Endogenous mTORC2 kinase activity was assayed in these cells following immunoprecipitation (IP) with anti-RICTOR antibodies (Ab) or preimmune IgG as a control. (C) siRNA-mediated knockdown of Rheb decreases mTORC1 signaling in *Tsc2* null cells. *Tsc2*$^{-/-}$ MEFs (Tsc2-V) were transfected with either control (C) or Rheb (Rh) siRNAs, and the effect on S6K and S6 phosphorylation was determined. Ins, insulin. (D) siRNA-mediated knockdown of Rheb has no effect on mTORC2 kinase activity in *Tsc2* null cells. The kinase activity of endogenous mTORC2 was assayed in anti-RICTOR immunoprecipitates from the *Tsc2*$^{-/-}$ (Tsc2-V) cell lysates shown in panel C or from those reconstituted with human *TSC2* (T2). The effects of control (C) and Rheb (Rh) siRNAs on insulin-stimulated (100 nM, 15 min) mTORC2 activity were assessed.

In order to further explore the regulation of mTORC2, we tested whether mTORC2 kinase activity can be stimulated by insulin. In HeLa cells, the kinase activity of mTORC2 in RICTOR immunoprecipitates was low during serum starvation and was markedly increased when cells were stimulated with insulin (Fig. 2D). This kinase activity was mTOR dependent, since addition of the PI3K and PI3K-related Ser/Thr kinase inhibitor LY294002 to the kinase reaction blocked Akt-S473 phosphorylation. This result is consistent with previous studies demonstrating that mTORC2 kinase activity is increased by insulin (8, 50). We also found that treating cells with the pan-PI3K inhibitor wortmannin prior to insulin stimulation could partially block mTORC2 activation, suggesting that mTORC2 might be regulated by a PI3K isoform (Fig. 2D, two right-hand lanes). In order to determine whether the stimulation of mTORC2 kinase activity by insulin requires the TSC1-TSC2 complex, we compared HeLa cells with stable knockdowns of TSC2 (HeLa-T2b) to control lines targeting luciferase (HeLa-L). The insulin-stimulated increase in mTORC2 kinase activity seen in HeLa-L cells was significantly blunted in the HeLa-T2b cells (Fig. 2E). A similar result was seen in *Tsc2* null MEFs (Fig. 3A), and insulin-stimulated mTORC2 activity was restored in cells reconstituted with human *TSC2*. Finally, to rule out that Akt phosphorylation in these mTORC2 assays is due to autophosphorylation, we used a kinase-dead version of Akt1 (K179D) as a substrate. As in the previous assays, the kinase activity of immunoprecipitated mTORC2 against this substrate was stimulated by insulin and attenuated in cells with reduced levels of TSC2 (Fig. 2F). Therefore, an intact TSC1-TSC2 complex is required for proper mTORC2 activation under both full-serum and insulin-stimulated conditions.

The TSC1-TSC2 complex affects mTORC2 kinase activity independent of its effects on mTORC1. The above data suggest that the TSC1-TSC2 complex might positively influence mTORC2 activity in a manner independent of its inhibitory effects on mTORC1. However, the fact that mTORC2 activity is insulin responsive and partially sensitive to wortmannin raises the question of whether its activity might be subjected to feedback regulation by the mTORC1-dependent inhibition of IRS-1 and PI3K, shown previously to occur in TSC-deficient cells (14, 42). Prolonged exposure (>12 h) of *Tsc2*$^{-/-}$ cells to the mTORC1 inhibitor rapamycin can block feedback mechanisms affecting IRS-1 (14, 42, 54). However, there was no detectable insulin-stimulated mTORC2 activity in RICTOR immunoprecipitates from either *Tsc2* null (Tsc2-V) or reconstituted (Tsc2-T2) cells following overnight rapamycin treatment. Consistent with previous observations that prolonged rapamycin treatment can disrupt mTORC2 (37), there was a significant loss of mTOR associated with RICTOR following rapamycin treatment. This effect on mTORC2 assembly pre-

cludes the use of rapamycin to test whether elevated mTORC1 signaling negatively influences mTORC2 activity in these cells. As an alternative, we used siRNAs to knock down the mTORC1-specific component RAPTOR in *Tsc2* null cells (Tsc2-V). The RAPTOR siRNAs greatly reduced RAPTOR protein levels in these cells but did not alter the level of mTOR coimmunoprecipitating with RICTOR, thereby allowing a comparison of mTORC2 activities in the presence and absence of mTORC1 (Fig. 3B). However, RAPTOR knockdown did not restore insulin-stimulated mTORC2 activity in the *Tsc2*-deficient cells. Interestingly, despite a lack of detectable mTORC2 kinase activity, Akt phosphorylation was partially responsive to insulin in the cell lysates of *Tsc2* null cells following mTORC1 inhibition with rapamycin (Fig. 3A) or RAPTOR siRNAs (Fig. 3B). This is reflective of the fact that both of these treatments efficiently blocked the known feedback mechanisms affecting IRS-1 in these cells. This is illustrated by a restoration of IRS-1 protein levels and loss of hyperphosphorylation, as detected by an increase in electrophoretic mobility. Furthermore, both overnight rapamycin and RAPTOR siRNAs reestablished wild-type levels of recruitment of the PI3K regulatory subunit (p85) to IRS-1 in *Tsc2* null cells in response to insulin (Fig. 3C). These data demonstrate that the attenuation of mTORC2 kinase activity upon disruption of the TSC1-TSC2 complex can be separated from the accompanying mTORC1-driven feedback mechanisms.

To further address whether a defect in growth factor stimulation of PI3K is the sole cause of Akt attenuation in *Tsc2* null cells, we generated *Tsc2* null (Tsc2-V) and reconstituted (Tsc2-T2) lines stably expressing a variety of activated alleles of the p110α catalytic subunit of PI3K. We tested the ability of these alleles and wild-type p110α to stimulate growth factor-independent phosphorylation of Akt in the presence (Tsc2-T2) or absence (Tsc2-V) of the TSC1-TSC2 complex. Strikingly, the wild-type, myristoylated, E545K, and H1047R alleles of p110α all stimulated Akt phosphorylation to a greater extent in the TSC2-expressing cells (Fig. 3D). These data further demonstrate that the loss of Akt phosphorylation observed in the absence of the *TSC* genes is not due exclusively to defects in PI3K activation caused by feedback or other unknown mechanisms.

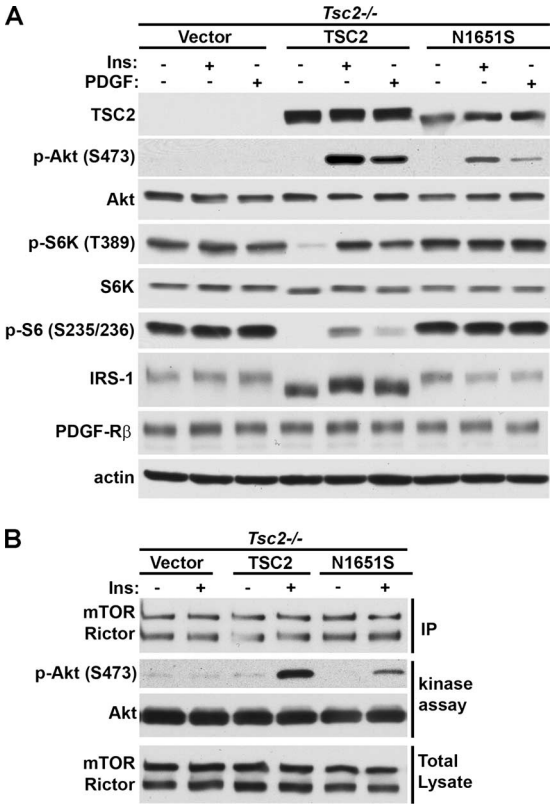

FIG. 5. The TSC1-TSC2 complex promotes mTORC2 activity in a manner that is, at least partially, independent of its GAP activity. (A) The reconstitution of $Tsc2^{-/-}$ cells with a GAP-dead mutant of TSC2 partially restores growth factor-stimulated Akt phosphorylation, while failing to suppress constitutive mTORC1 signaling. Stable pools of $Tsc2^{-/-}$ MEFs expressing empty vector, wild-type TSC2, or a TSC2 mutant with impaired GAP activity (N1651S) were serum starved overnight and then stimulated for 15 min with insulin (Ins; 100 nM) or PDGF (5 ng/ml), as indicated. (B) A GAP-dead mutant of TSC2 can restore mTORC2 kinase activity to $Tsc2^{-/-}$ MEFs. The cells described for panel A were serum starved overnight and stimulated for 15 min with insulin (100 nM) before mTORC2 kinase activity was assayed in anti-RICTOR immunoprecipitates (IP).

The ability of the TSC1-TSC2 complex to promote mTORC2 activity can be separated from its GAP activity toward Rheb. Since loss of the TSC1-TSC2 complex leads to inactivation of mTORC2, we tested whether the reciprocal was true by overexpressing TSC1, TSC2, or their downstream target Rheb in HEK293 cells. Rheb overexpression potently activated mTORC1 signaling, while increased expression of the TSC1-TSC2 complex inhibited mTORC1 signaling (as detected by S6K1-T389 phosphorylation) (Fig. 4A). Consistent with previous studies (51), Rheb overexpression did not significantly decrease or increase mTORC2 kinase activity relative to vector controls (Fig. 4B). However, overexpression of the TSC1-TSC2 complex strongly increased mTORC2 kinase activity in RICTOR immunoprecipitates. These data suggest that the TSC1-TSC2 complex activates mTORC2 kinase activity in a manner independent of Rheb and its effects on mTORC1. To further test this possibility, we used siRNAs to knock down Rheb expression in *Tsc2* null (Tsc2-V) cells, which led to a corresponding decrease in the constitutive mTORC1 signaling in these cells (Fig. 4C). However, Rheb knockdown had no effect on mTORC2 kinase activity, which remained unresponsive to insulin in the Tsc2-V cells relative to the reconstituted Tsc2-T2 cells (Fig. 4D). Therefore, while increasing or decreasing Rheb levels profoundly affects mTORC1 signaling, Rheb does not appear to affect mTORC2 activity.

The above data suggest that the TSC1-TSC2 complex positively influences mTORC2 activity independent of its GAP activity toward Rheb. To critically test this possibility, we reconstituted $Tsc2^{-/-}$ MEFs with a GAP domain mutant of TSC2 (N1651S), which has been previously shown to lack Rheb-GAP activity (45), and examined mTORC1 and mTORC2 signaling. Unlike wild-type TSC2, the N1651S mutant failed to suppress the constitutive mTORC1 signaling, resembling vector control cells for growth factor-independent phosphorylation of S6K1 and S6 (Fig. 5A). Furthermore, this mutant failed to block the feedback inhibition of IRS-1, as indicated by the presence of the slower-migrating hyperphosphorylated form and the lower abundance of IRS-1 in the

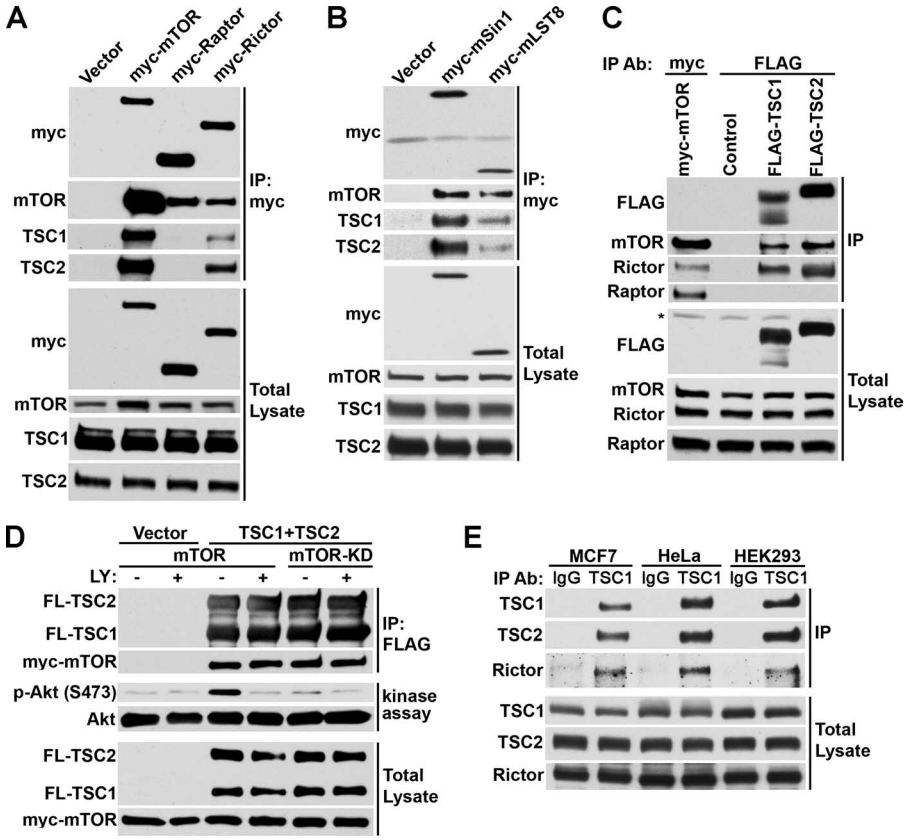

FIG. 6. The TSC1-TSC2 complex associates with mTORC2 but not mTORC1. (A, B) Endogenous TSC1 and TSC2 coimmunoprecipitate with mTORC2 components. HEK293 cells were transfected with empty vector, myc-mTOR, myc-RAPTOR, and myc-RICTOR (A) or myc-mSIN1 and myc-mLST8 (B). The myc-tagged proteins were immunoprecipitated (IP), and coprecipitation of endogenous TSC1 and TSC2 was determined by immunoblotting. (C) Endogenous mTOR and RICTOR coimmunoprecipitates with TSC1 and TSC2. The same cells were transfected with myc-mTOR, empty vector, FLAG-TSC1, or FLAG-TSC2 and were subjected to immunoprecipitation with anti-myc or anti-FLAG antibodies (Ab), as indicated. The immunoprecipitates were immunoblotted for the presence of endogenous mTOR, RICTOR, and RAPTOR. *, cross-reacting band. (D) The TSC1-TSC2 complex immunoprecipitates an mTOR-containing complex that phosphorylates Akt. HEK293 cells (grown in full serum) expressing FLAG-TSC1 and FLAG-TSC2 with myc-tagged mTOR or kinase-dead mTOR (mTOR-KD) were lysed, and FLAG immunoprecipitates were used in mTORC2 kinase assays with inactive Akt1 as the substrate. Where indicated, LY294002 (LY; 15 μM) was added during the kinase reaction to inhibit mTOR kinase activity. (E) Endogenous RICTOR coimmunoprecipitates with endogenous TSC1. Lysates of MCF7, HeLa, and HEK293 cells were incubated with control IgG or anti-TSC1 antibodies. Immunoprecipitates were then probed for TSC1, TSC2, and RICTOR.

vector and N1651S cells relative to levels in the wild-type TSC2-expressing cells. However, the N1651S mutant partially restored growth factor-stimulated Akt phosphorylation to the Tsc2-deficient cells (Fig. 5A), and this was also reflected by a partial rescue of mTORC2 kinase activity in these cells (Fig. 5B). These data further demonstrate that the TSC1-TSC2 complex promotes mTORC2 activity in a manner that is, at least in part, independent of its inhibitory effects on Rheb and mTORC1.

The TSC1-TSC2 complex binds to mTORC2 but not mTORC1. Prior to knowledge of the existence of two distinct mTOR complexes and the discovery of Rheb as the functional link between the TSC1-TSC2 complex and mTORC1, overexpression studies in *Drosophila* S2 cells found that the TSC1-TSC2 complex could associate with *Drosophila* TOR (9). As our data above suggest that the TSC1-TSC2 complex regulates mTORC2 through a distinct mechanism from mTORC1, we revisited this previously described physical interaction with a focus on distinguishing between the two mTOR complexes. We confirmed that exogenously expressed mTOR could immunoprecipitate endogenous TSC1 and TSC2 (Fig. 6A). Immunoprecipitates of exogenously expressed RAPTOR and RICTOR contain similar amounts of endogenous mTOR, but interestingly, TSC1 and TSC2 were only found associated with the mTORC2 component RICTOR. Endogenous TSC1 and TSC2 also coimmunoprecipitated with mSIN1, which is an mTORC2-specific component, and mLST8, which is found in both complexes (Fig. 6B). Reciprocally, endogenous mTOR and RICTOR, but not RAPTOR, could coimmunoprecipitate with exogenously expressed TSC1 and TSC2 (Fig. 6C). In a control experiment, both RAPTOR and RICTOR were found associated with exogenous mTOR in a parallel immunoprecipitation. In order to determine if mTORC2 associated with the TSC1-TSC2 complex maintains its kinase activity, we coexpressed FLAG-tagged TSC1 and TSC2 with wild-type or kinase-dead mTOR and performed mTORC2 kinase assays on anti-FLAG immunoprecipitates. The TSC1-TSC2 complex associated similarly with both wild-type and kinase-dead mTOR,

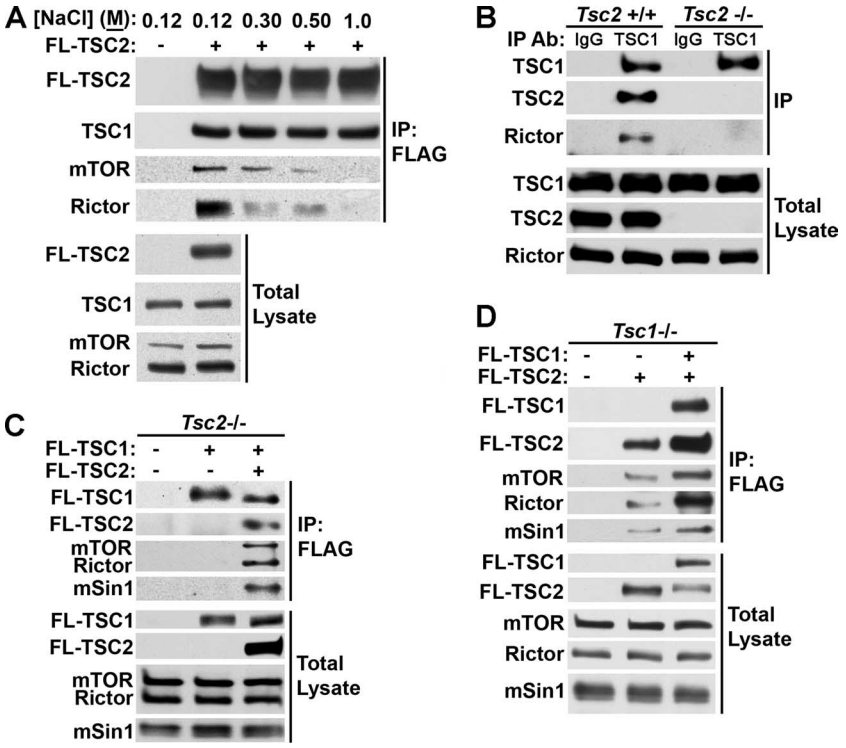

FIG. 7. The interaction between the TSC1-TSC2 complex and mTORC2 is salt sensitive and mediated by TSC2. (A) The interaction between the TSC1-TSC2 complex and mTORC2 is disrupted by high salt concentrations. HEK293 cells were transfected with empty vector (−) or FLAG-TSC2, and the anti-FLAG immunoprecipitates (IP) were divided into four equal fractions. Each fraction was then subjected to four washes with buffer containing the indicated concentrations of NaCl. Binding of endogenous TSC1, mTOR, and RICTOR was then assessed by immunoblotting. (B) The interaction between RICTOR and the TSC1-TSC2 complex requires TSC2. Lysates of littermate-derived $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs were incubated with either an anti-TSC1 antibody (Ab) or control IgG, and immunoprecipitates were probed for endogenous TSC1, TSC2, and RICTOR. (C) mTORC2 components do not associate with TSC1 in the absence of TSC2. Empty vector (−), FLAG-TSC1, or both FLAG-TSC1 and FLAG-TSC2 were coexpressed in $Tsc2^{-/-}$ MEFs with components of mTORC2, and the anti-FLAG immunoprecipitates were immunoblotted for components of mTORC2. (D) mTORC2 components can associate with TSC2 in the absence of TSC1. Empty vector (−), FLAG-TSC2, or both FLAG-TSC2 and FLAG-TSC1 were coexpressed in $Tsc1^{-/-}$ MEFs with components of mTORC2. The transfected cells were pretreated with MG-132 (7.5 μM) for 4 h prior to cell lysis to allow TSC2 expression in the absence of TSC1, and the anti-FLAG immunoprecipitates were immunoblotted for components of mTORC2.

but mTORC2 activity was only detected when wild-type mTOR was bound (Fig. 6D). As a further control for the specificity of this reaction for mTOR kinase activity, the LY294002 compound was added to the kinase reactions and found to inhibit the in vitro phosphorylation of Akt. Finally, in order to confirm this interaction with endogenous levels of the TSC1-TSC2 complex and mTORC2, we used anti-TSC1 antibodies to immunoprecipitate the TSC1-TSC2 complex from human cell lines derived from different tissues, including MCF7 (breast), HeLa (cervix), and HEK293 (kidney). In all cases, both TSC2 and RICTOR (Fig. 6E), but not RAPTOR (not shown), coimmunoprecipitated with the anti-TSC1 antibody but not a rabbit IgG control. A specific interaction between the endogenous complexes was also seen in wild-type MEFs (Fig. 7B). Therefore, the TSC1-TSC2 complex can physically associate with mTORC2 but not mTORC1.

While our data above demonstrate a clear physical interaction between the TSC1-TSC2 complex and mTORC2, we do not believe that these two complexes are part of a tightly associated larger complex. This is illustrated by the fact that we could dissociate mTOR and RICTOR from the TSC1-TSC2 complex by washing immunoprecipitates with increasing concentrations of salt. While the association of TSC1 and TSC2 is unaffected even at 1 M NaCl, mTOR and RICTOR can be removed at significantly lower concentrations (Fig. 7A). We conclude that the TSC1-TSC2 complex and mTORC2 are physically distinct complexes and associate via weaker, salt-sensitive interactions that are transient in nature.

In order to determine the relative importance of TSC1 and TSC2 in the binding of mTORC2, we examined these interactions in $Tsc1^{-/-}$ and $Tsc2^{-/-}$ MEFs. Unlike in wild-type cells ($Tsc2^{+/+}$), anti-TSC1 antibodies failed to immunoprecipitate RICTOR from $Tsc2^{-/-}$ cells (Fig. 7B), suggesting a requirement for TSC2. To further test the role of TSC2 in this interaction, we expressed TSC1 or both TSC1 and TSC2 in the $Tsc2^{-/-}$ MEFs together with the components of mTORC2. Indeed, mTOR, RICTOR, and mSIN1 coimmunoprecipitated with the TSC1-TSC2 complex but not with TSC1 alone (Fig. 7C). "Free" TSC2 not associated with TSC1 is susceptible to proteasomal degradation (3, 6), making it difficult to express significant levels of TSC2 in $Tsc1^{-/-}$ cells. Therefore, in order to determine whether TSC2 was both necessary and sufficient to bind to mTORC2, we expressed TSC2 with or without TSC1 in $Tsc1^{-/-}$ MEFs and treated the cells with the proteasome

inhibitor MG-132, which allowed TSC2 expression. TSC2 alone was sufficient to immunoprecipitate mTOR, RICTOR, and mSIN1 in the absence of TSC1 (Fig. 7D), demonstrating that, within the TSC1-TSC2 complex, TSC2 is essential for the association with mTORC2.

DISCUSSION

This study demonstrates that, in addition to previously characterized mTORC1-dependent feedback mechanisms, loss of the TSC1-TSC2 complex leads to a general reduction in Akt phosphorylation due to defects in mTORC2 activation. Relative to mTORC1, little to nothing is currently known regarding regulation of mTORC2. Using Akt as an in vitro substrate to assay its kinase activity, mTORC2 was found in this and previous studies to be stimulated by serum (38) and insulin (8, 50). Our data demonstrate that an intact TSC1-TSC2 complex is required for this growth factor-stimulated mTORC2 activity, without obvious effects on mTORC2 levels. The molecular mechanism of TSC1-TSC2-mediated activation of mTORC2 is currently unknown, but we have demonstrated that this regulation can be separated from its Rheb GAP activity and subsequent inhibition of mTORC1. However, since a GAP-dead mutant of TSC2, which fails to suppress mTORC1 signaling, only partially restores mTORC2 activity to *Tsc2* null cells, it remains possible that mTORC2 is also somewhat sensitive to mTORC1-dependent feedback mechanisms. Such a mechanism could affect a putative input from PI3K to mTORC2, as we found that wortmannin could partially inhibit mTORC2 activation by insulin in wild-type cells and that constitutively active versions of PI3K could stimulate Akt phosphorylation in *Tsc2* null cells, albeit much less efficiently than in wild-type cells. However, our data strongly suggest the existence of mTORC1-independent mechanisms of mTORC2 regulation by the TSC1-TSC2 complex. This mode of regulation is likely related to the physical association between the TSC1-TSC2 complex and mTORC2 found in this study. However, to date, we have yet to find a stimulus or pharmacological inhibitor that increases or decreases this interaction, suggesting a more-complex regulatory mechanism.

Through the differential regulation of mTORC1 and mTORC2, the TSC1-TSC2 complex promotes Akt activation (Fig. 8A). Feedback inhibition stemming from constitutive mTORC1 signaling combined with loss of mTORC2 activity results in a strong attenuation of Akt signaling in cells lacking a functional TSC1-TSC2 complex (Fig. 8B). As TSC1 and TSC2 are tumor suppressors (7) and Akt is an oncogene activated in many cancers (2), this role for the TSC1-TSC2 complex in ensuring proper activation of Akt is somewhat puzzling. However, TSC1 and TSC2 are unusual tumor suppressors. There are many oncogenes and tumor suppressors comprising the pathways upstream of the TSC1-TSC2 complex, and genetic lesions affecting these pathways lead to a myriad of cancer predisposition syndromes and sporadic malignancies (11, 17). Despite these facts, TSC1 and TSC2 mutations have not been found in sporadic cancers, and TSC is a benign tumor syndrome with a low risk of malignancy. Given the importance of Akt activation in tumor progression, an inability to activate Akt is a likely explanation for the benign nature of the TSC disease and the lack of evidence for loss of the TSC genes in cancer. In support of this notion, we have detected defects in Akt activation and signaling to its downstream target FOXO1 within benign tumors of a TSC mouse model (30). In addition, benign renal angiomyolipomas, which occur frequently in TSC and LAM patients, display loss of TSC2 expression and a corresponding loss of Akt phosphorylation (20). Therefore, while mTORC1 activation is likely to drive tumor formation in TSC patients, loss of mTORC2 activation might limit the progression of these tumors. In light of our findings that a TSC patient-derived missense mutation of TSC2 that lacks GAP activity could partially restore mTORC2 activity to $Tsc2^{-/-}$ MEFs, without blocking the constitutive mTORC1 signaling, it will be interesting to revisit genotype-phenotype correlations in the TSC disease. Intriguingly, in a study of human breast cancers, it was found that high levels of TSC2 expression correlated with increased tumor invasiveness and poor prognosis (24). Taken together with our current study, these findings suggest that in some cellular contexts the TSC1-TSC2 complex

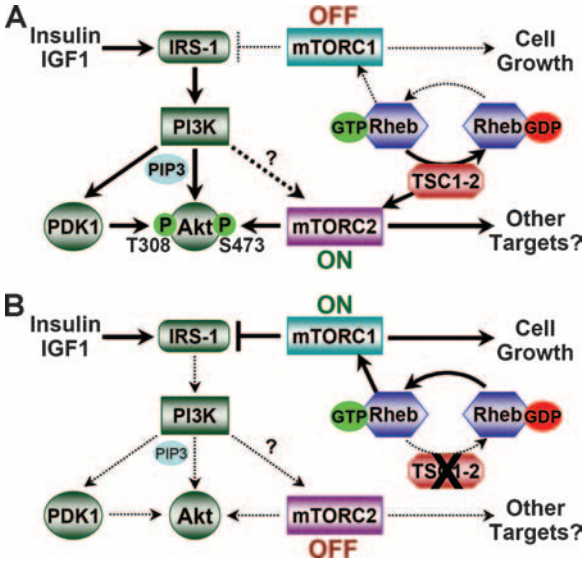

FIG. 8. The TSC1-TSC2 complex promotes Akt activation through differential regulation of the two mTOR complexes. (A) The TSC1-TSC2 complex inhibits mTORC1 through its Rheb-GAP activity, while it activates mTORC2 through a mechanism independent of this activity. When the TSC1-TSC2 complex is active, the resulting decrease in Rheb-GTP levels inactivates mTORC1 and blocks its cell growth-promoting activity and mechanisms of IRS-1 inhibition. In response to insulin/IGF1 receptor activation, IRS-1 binds PI3K and recruits it to the plasma membrane, where it produces lipid second messengers, such as PIP_3. PIP_3 recruits PDK1 and Akt to the membrane, resulting in phosphorylation of Akt at T308. As shown in this study, the TSC1-TSC2 complex is required for activation of mTORC2, which phosphorylates Akt on S473, leading to its full activation. However, whether mTORC2 activity itself is also regulated by PI3K is currently unknown. (B) In cells lacking the TSC1-TSC2 complex, mechanisms activating Akt are impaired through differential effects on mTORC1 and mTORC2. Upon loss of the TSC1-TSC2 complex, elevated levels of Rheb-GTP strongly activate mTORC1 and its growth-promoting functions. This enhanced mTORC1 activity also triggers feedback mechanisms inhibiting IRS-1 and its signaling through PI3K to Akt. In addition, loss of the TSC1-TSC2 complex impairs the proper activation of mTORC2, leading to a further decrease in Akt phosphorylation and activation.

might act in an oncogenic manner through activation of mTORC2 and Akt.

There is still much we do not know regarding mTORC2 regulation and function. While we are using Akt phosphorylation as the only reliable and direct readout of mTORC2 activity currently available, the regulation of mTORC2 by the TSC1-TSC2 complex might ultimately be more critical for control of some other cellular process. Knowledge of other functions of mTORC2 might also reveal a role for its activation in the tumor suppressor activity of the TSC1-TSC2 complex. While TSC is a benign tumor syndrome, the sheer number and location of the tumors lead to severe clinical manifestations. mTORC1 inhibitors are now in clinical trials for the treatment of TSC and LAM. However, it seems likely that the loss of mTORC2 activation in the tumors of these patients will offer additional therapeutic opportunities. As Akt signaling promotes cell survival through many downstream targets (29), the loss of mTORC2-mediated activation of Akt in cells lacking a functional TSC1-TSC2 complex should present an Achilles' heel that sensitizes the cells within TSC and LAM lesions to some apoptotic stimuli. We must now consider both aberrant mTORC1 activation and mTORC2 inactivation when considering the molecular pathogenesis and treatment options for these diseases.

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