MOLECULAR AND CELLULAR BIOLOGY, Nov. 2009, p. 5657–5670 Vol. 29, No. 21
0270-7306/09/$12.00 doi:10.1128/MCB.00735-09

Characterization of Rictor Phosphorylation Sites Reveals Direct Regulation of mTOR Complex 2 by S6K1▽†

Christian C. Dibble,[1] John M. Asara,[2,3] and Brendan D. Manning[1]*

Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, Massachusetts[1]; Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, Massachusetts[2]; and Department of Medicine, Harvard Medical School, Boston, Massachusetts[3]

Received 7 June 2009/Returned for modification 10 August 2009/Accepted 19 August 2009

The mammalian target of rapamycin (mTOR) functions within two distinct complexes (mTORC1 and mTORC2) to control cell growth, proliferation, survival, and metabolism. While there has been great progress in our understanding of mTORC1 regulation, the signaling mechanisms that regulate mTORC2 have not been defined. In this study, we use liquid chromatography-tandem mass spectrometry analyses to identify 21 phosphorylation sites on the core mTORC2 component Rictor. We find that one site, T1135, undergoes growth factor-responsive phosphorylation that is acutely sensitive to rapamycin and is phosphorylated downstream of mTORC1. We find that Rictor-T1135 is directly phosphorylated by the mTORC1-dependent kinase S6K1. Although this phosphorylation event does not affect mTORC2 integrity or in vitro kinase activity, expression of a phosphorylation site mutant of Rictor (T1135A) in either wild-type or *Rictor* null cells causes an increase in the mTORC2-dependent phosphorylation of Akt on S473. However, Rictor-T1135 phosphorylation does not appear to regulate mTORC2-mediated effects on SGK1 or PKCα. While the precise molecular mechanism affecting Akt is unknown, phosphorylation of T1135 stimulates binding of Rictor to 14-3-3 proteins. We provide evidence that Rictor-T1135 phosphorylation acts in parallel with other mTORC1-dependent feedback mechanisms, such as those affecting IRS-1 signaling to PI3K, to regulate the response of Akt to insulin.

The mammalian target of rapamycin (mTOR) is a protein kinase conserved in all eukaryotes that controls key cellular processes, including growth, proliferation, survival, and metabolism (66). Its activity is dependent on association with regulatory proteins that cooperate to form functional kinase complexes that are directed toward specific downstream substrates. At least two mTOR-containing complexes exist in eukaryotic cells, mTOR complexes 1 and 2 (mTORC1 and -2), which consist of distinct sets of proteins and appear to perform nonoverlapping functions (34). In mammals, both complexes contain mTOR and mLST8, along with core components that are essential for the specific functions of each complex: Raptor (16, 30) in mTORC1 and Rictor (28, 50) and mSIN1 (11, 70) in mTORC2. In addition, these complexes contain other interacting proteins that are not functionally essential to the core complex, including PRAS40 in mTORC1 (10, 41, 49, 58, 64) and Protor/PRR5 in mTORC2 (42, 58, 65). Finally, both complexes have been found to interact with a negative regulator dubbed DEPTOR (44).

While there are likely to be many direct substrates of the mTOR complexes, specific members of the protein kinase A, G, and C (AGC) family are among the best-characterized downstream effectors of mTOR signaling (reviewed in reference 29). These kinases include the mTORC1 substrates S6K1 and S6K2 (5, 32) and the mTORC2 substrates Akt1/2/3, SGK1, and PKCα (8, 12, 22, 26, 52). Many AGC kinases share a requirement for phosphorylation of three conserved regions for their full activation and/or stability: the activation loop within the kinase domain, a linker region termed the turn motif, and a C-terminal hydrophobic motif (reviewed in reference 39). Phosphorylation of the activation loop is essential for kinase activity and is invariably phosphorylated by PDK1 (37). Phosphorylation of the hydrophobic motif, on the other hand, can either create a docking site for PDK1 to facilitate activation loop phosphorylation, as it appears to do in S6K1 and SGK1 (4), or stabilize the active conformation of the kinase, as it does in Akt (69). Phosphorylation of the turn motif in AGC kinases generally occurs as part of protein maturation and is thought to stabilize the kinase (20). The hydrophobic motif on S6K1 (T389) is the best-characterized substrate of mTORC1, while this same motif on Akt (S473), SGK1 (S422), and PKCα (S657) is phosphorylated in an mTORC2-dependent manner (29). mTORC2 is also essential for turn motif phosphorylation of Akt (T450) and PKCα (S638) (8, 26). How mTORC2 controls the phosphorylation of both growth factor-responsive sites, such as the hydrophobic motifs on Akt and SGK1, and constitutive growth factor-independent sites, such as the turn motifs on Akt and PKCα, is currently unknown (reviewed in reference 2).

Due primarily to its acute sensitivity to rapamycin, most studies over the past 15 years have focused on mTORC1, and therefore, much more is known about its upstream regulation than mTORC2's. Many of the upstream signaling pathways (e.g., Akt, Erk, AMPK, and Wnt, etc.) that regulate mTORC1 do so by affecting the phosphorylation state of a protein complex comprised of the tuberous sclerosis complex tumor suppressors TSC1 and TSC2, which acts as a key negative regula-

* Corresponding author. Mailing address: Harvard School of Public Health, Dept. of Genetics and Complex Diseases, 665 Huntington Ave., SPH2-117, Boston, MA 02115. Phone: (617) 432-5614. Fax: (617) 432-5236. E-mail: bmanning@hsph.harvard.edu.

† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 31 August 2009.

tor of mTORC1 (reviewed in references 7 and 24). In addition, mTORC1 activity is very sensitive to the availability of amino acids through a pathway that culminates in the Rag GTPases, which contribute to the activation of mTORC1 (31, 48). In comparison, we know very little regarding the mechanisms regulating mTORC2. While the catalytic activity of mTORC2 is known to be stimulated by growth factors and might be activated downstream of class I phosphoinositide 3-kinase (PI3K) (22, 23, 52), the molecular mechanisms regulating its cellular activities are unknown. Unlike mTORC1, mTORC2 activity is not acutely inhibited by rapamycin, but prolonged exposure to rapamycin (generally 12 h or more) blocks mTORC2 assembly and leads to a greatly diminished pool of mTORC2 in cells (51).

mTORC1 and its downstream target S6K1 have been shown to exert negative regulatory effects on Akt activation (reviewed in reference 18). The scaffolding adaptor proteins IRS-1 and IRS-2 are required for insulin and IGF-1 to activate PI3K, which is essential for subsequent phosphorylation and activation of Akt by PDK1 and mTORC2. The IRS proteins are downregulated by mTORC1 and S6K1 signaling through a combination of (i) direct Ser phosphorylation, which either increases the degradation of these proteins or blocks their interaction with PI3K (see, e.g., references 19, 33, 47, 57, 60, 62, and 63), and (ii) poorly understood transcriptional effects (17, 55). Therefore, under settings of chronically elevated mTORC1 signaling, such as in cells lacking the TSC1-TSC2 complex, IRS protein levels are diminished and both PI3K and Akt are unresponsive to insulin (17, 55). Prolonged rapamycin treatment is typically required to restore IRS levels and insulin-stimulated PI3K activation in this setting. As mTORC1 and S6K1 are activated by insulin through the PI3K-Akt pathway, these effects on the IRS proteins act as a classical negative-feedback loop, which is a regulatory strategy common to all signaling pathways.

Given our current lack of knowledge regarding the signaling mechanisms that regulate mTORC2, in this study we examine the regulation of this complex by posttranslational modification. We focus here on phosphorylation of the core mTORC2 component Rictor and find that Rictor is heavily phosphorylated within a region whose sequence is unique to vertebrate orthologs. Surprisingly, we find that a single site within this region is directly phosphorylated by S6K1 downstream of mTORC1 and that this phosphorylation event exerts a negative regulatory effect on the mTORC2-dependent phosphorylation of Akt-S473 within cells. This finding indicates that mTORC1 signaling can directly affect mTORC2 signaling, suggesting a novel form of cross talk between the two mTOR complexes.

MATERIALS AND METHODS

Cell culture and reagents. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/liter glucose) with 10% fetal bovine serum (Sigma). HEK-293E cells were provided by J. Blenis (Harvard Medical School). The littermate-derived pair of *Rictor*$^{+/+}$ and *Rictor*$^{-/-}$ (both *p53*$^{-/-}$) mouse embryonic fibroblasts (MEFs) were a gift of D. A. Guertin and D. M. Sabatini (Massachusetts Institute of Technology). HeLa cells stably expressing short hairpin RNAs (shRNAs) targeting TSC2 or firefly luciferase were described previously (23). 3T3-L1 preadipocytes and C2C12 myoblasts were differentiated into adipocytes and myotubes, respectively, using standard protocols. Rapamycin and LY294002 were from Calbiochem, and insulin and epidermal growth factor (EGF) were from Sigma.

MS. For initial identification of the Rictor phosphorylation sites, HEK-293E cells were transfected with wild-type myc-Rictor and grown in full serum for 48 h with a change to fresh medium/serum 16 h prior to lysis. For the targeted analysis of four candidate sites, HEK-293E cells were transfected with wild-type myc-Rictor, starved for 16 h, and treated as indicated in the legend to Fig. 2. For all mass spectrometry (MS) experiments, myc immunoprecipitates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was stained with Coomassie blue, and the myc-Rictor band was excised. Samples were subjected to reduction with dithiothreitol, alkylation with iodoacetamide, and in-gel digestion with trypsin or chymotrypsin overnight at pH 8.3, followed by reversed-phase microcapillary/tandem MS (LC/MS/MS). See the supplemental material for details on all qualitative and quantitative MS analyses using both linear ion trap and hybrid orbitrap instruments.

Bioinformatics. Rictor ortholog GenBank accession numbers used to assess the conservation of phosphorylation sites are as follows: *Homo sapiens*, AAS79796; *Mus musculus*, Q6QI06; *Gallus gallus*, translated from XR027072; *Xenopus tropicalis*, C_scaffold_350000006 (JGI *Xenopus tropicalis* genome assembly v4.1); *Danio rerio*, translated from XM_001921872; *Drosophila melanogaster*, NP_573371; *Caenorhabditis elegans*, CAB04220; and *Saccharomyces cerevisiae*, P40061. The NetPhorest software can be accessed at http://netphorest.info/ (36). The sequence for full-length human Rictor was entered along with the identity and position of each phosphorylated residue. Higher NetPhorest scores, referred to as posterior probabilities, suggest higher confidence in a given prediction. The position-specific scoring matrix for S6K was generously provided by B. E. Turk (Yale University). This matrix was used in combination with Scansite (40; http://scansite.mit.edu/) to identify the top 10 serines and threonines in the full-length sequence of human Rictor predicted to be phosphorylated by S6K1.

Constructs and siRNAs. Mammalian expression constructs of myc-Rictor, myc-mTOR, mSIN1.1-myc, and mLST8-myc were obtained from D. M. Sabatini via Addgene (11, 50). mSIN1.1-myc and mLST8-myc were transferred into a pRK7 backbone. Single and double alanine or aspartic acid mutations of myc-Rictor were made using the QuikChange multisite-directed mutagenesis kit from Agilent Technologies according to the manufacturer's instructions. Following mutagenesis, the myc-Rictor coding region was sequenced to check for errors. The rapamycin-resistant S6K1 mutant (S6K1-F5A-389E-R5A) was obtained from J. Blenis (53), glutathione *S*-transferase (GST)–14-3-3 from R. J. Shaw, and Flag–14-3-3 from L. C. Cantley. Transient transfections were performed using Lipofectamine 2000 (Invitrogen), and cells were typically serum starved 32 h posttransfection and harvested 48 h posttransfection. Transfection of small interfering RNAs (siRNAs) into HEK-293E cells was carried out using siRNAMAX Lipofectamine (Invitrogen) in full serum, with a medium change after 24 h. ON-TARGETplus SMARTpool siRNAs (Thermo Scientific, Dharmacon Division) specifically targeting S6K1 or S6K2, and nontargeting control pools of siRNAs, were used at 20 nM, and cells were harvested 48 h posttransfection.

Antibodies and immunoblots. Antibodies to the following proteins were obtained from the indicated sources: myc epitope (9E10), phospho-IRS-1 (S307), and phospho-Rictor (T1135) antibodies from Millipore (Upstate division); Rictor (C-terminal epitope), mSin1, and mLST8 from Bethyl Laboratories; mTOR for immunoprecipitations (FRAP N-19) from Santa Cruz; SGK1 antibody from Stressgen; Flag epitope from Sigma; and hemagglutinin (HA) epitope (16B12) from Covance. All other antibodies were from Cell Signaling Technology.

For most experiments, cells were lysed in an NP-40 lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% NP-40 [Igepal CA-630], 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, and 0.5 mM sodium orthovanadate). In experiments where it was necessary to maintain interactions of mTORC2 components, cells were lysed in a CHAPS {3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate} lysis buffer (0.3% CHAPS with the same ingredients as noted above for NP-40 lysis buffer but without NP-40 and glycerol) (28, 50). In the experiment whose results are shown in Fig. 4D, cells were lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, with the same ingredients as noted above for the NP-40 buffer but without NP-40). Whole-cell lysates were centrifuged at 16,000 × *g* for 15 min at 4°C, and the supernatant was saved. Protein concentrations were normalized using Bradford assays. Where indicated, the program ImageJ (http://rsbweb.nih.gov/ij/) was used to quantify the relative intensities of individual bands from original film exposures of the immunoblots shown in the figure under discussion.

Endogenous proteins or exogenously expressed epitope-tagged proteins were immunoprecipitated from lysates by incubation with the antibodies indicated in relevant figures for 2 to 4 h, followed by a 1- to 2-h incubation with 15 to 20 μl

TABLE 1. Rictor phosphorylation sites identified in LC/MS/MS analyses[a]

Site[b]	Amino acid at position[c]:											Conservation in[d]:							Predicted kinases[e]
	−5	−4	−3	−2	1	0	+1	+2	+3	+4	+5	*Mm*	*Gg*	*Xt*	*Dr*	*Dm*	*Ce*	*Sc*	
S21	R	G	R	N	D	S	G	E	E	N	V	X	–	X	X	–	–	–	CK2, Pim2
S35	L	T	R	E	P	S	D	N	L	R	E	X	–	X	X	–	–	–	TGFβR2, CLK
S265	F	H	Y	R	H	S	P	D	T	A	E	X	–	–	–	X	?	–	p38, MAPKAPK
T1103	I	L	N	S	L	T	L	P	N	K	K	X	X	–	X	–	–	–	DMPK, MSN
T1135	R	I	R	T	L	T	E	P	S	V	D	X	X	X	X	–	–	–	Akt, S6K
S1138	T	L	T	E	P	S	V	D	F	N	H	X	X	?	X	–	–	–	PKC, TGFβR2
S1219	K	I	R	S	Q	S	F	N	T	D	T	X	X	X	X	–	–	–	S6K, RCK
S1235	S	S	M	S	S	S	P	S	R	E	T	X	X	X	X	–	–	–	CDK2/3, p38
T1271	T	S	H	Y	L	T	P	Q	D	N	H	X	X	–	X	–	–	–	CDK2/3, p38
S1274	Y	L	T	P	Q	S	N	H	L	S	L	X	X	–	X	–	–	–	MAPK1/3/7, GSK3
S1302	P	R	R	A	Q	S	L	K	A	P	S	X	X	X	X	–	–	–	PKC, DMPK
S1313	I	A	T	I	K	S	L	A	D	C	N	X	X	–	X	–	–	–	PKD, DMPK
S1346	M	H	P	S	L	S	H	S	E	A	L	X	X	X	X	X	–	–	CK2, NEK
S1353	S	E	A	L	A	S	P	A	K	D	V	X	X	X	X	–	–	–	p38, JNK
T1376	F	E	A	R	L	T	P	S	R	F	M	X	X	X	X	–	–	–	CDK2/3, DMPK
Y1386	M	K	A	L	S	Y	A	S	L	D	K	X	X	X	–	–	–	–	Tec, InsR
S1388	A	L	S	Y	A	S	L	D	K	E	D	X	X	X	X	X	–	–	CK2, PKC
S1396	K	E	D	L	L	S	P	I	N	Q	N	X	X	X	X	X	–	–	p38, CDK2/3
S1571	V	P	S	K	F	S	G	I	S	G	C	X	X	X	X	X	–	–	Aurora B/C, CK1
S1574	K	F	S	G	I	S	G	C	S	D	G	X	X	X	X	X	–	?	CK1, NEK
S1577	G	I	S	G	C	S	D	G	V	S	Q	X	X	X	X	?	–	?	CK2, CK1

[a] See the expanded version of this table as Table S2 in the supplemental material and MS/MS spectra demonstrating phosphorylation of Rictor-T1135 as Fig. S1 in the supplemental material.

[b] Numbering and sequence for full-length human Rictor (GenBank accession no. AAS79796).

[c] Amino acid sequence centered around the identified phosphorylated residue (0).

[d] Rictor orthologs from each species were individually aligned with human Rictor using BLAST to determine the conservation of each phosphorylation site in *M. musculus* (*Mm*), *G. gallus* (*Gg*), *X. tropicalis* (*Xt*), *D. rerio* (*Dr*), *D. melanogaster* (*Dm*), *C. elegans* (*Ce*), and *S. cerevisiae* (*Sc*). See Materials and Methods for GenBank accession numbers. X, conserved; −, not conserved; ?, weak or questionable conservation.

[e] Top two kinases predicted to phosphorylate the given site using the NetPhorest program (36; http://netphorest.info/; see Materials and Methods). It should be emphasized that these are simply predictions. CK1 and -2, casein kinases 1 and 2; TGFβR2, transforming growth factor β receptor 2; CLK, CDC-like kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; PKC, protein kinase C; CDK2/3, cyclin-dependent kinases 2 and 3; GSK3, glycogen synthase kinase 3; PKD, protein kinase D; JNK, c-jun N-terminal kinase; JnsR, insulin receptor.

(1:1 slurry) of protein A-protein G agarose beads (Thermo Scientific, Pierce Division). Antibody concentrations used for immunoprecipitation of endogenous proteins were as follows: Rictor (1 μg), mSin1 (1 μg), and mTOR (5 μg). For GST pulldown assays, 10 μg GST–14-3-3 or GST alone was incubated with myc-Rictor-containing lysates for 2 h, and glutathione beads (Thermo Scientific, Pierce Division) were added for an additional 2 h. Immune complexes and glutathione beads were washed three times with lysis buffer before being boiled in SDS sample buffer.

Kinase assays. For the demonstration of Rictor phosphorylation by S6K1, whole-cell lysates from confluent HEK-293E cells were prepared in NP-40 lysis buffer and all steps prior to the kinase reaction were carried out at 4°C. Transfected myc-Rictor variants were isolated from serum-starved, rapamycin-treated cells by immunoprecipitation. Transfected HA-S6K1 from cells treated as indicated in the legend to Fig. 4 for a representative experiment were bound to HA epitope antibodies precipitated using protein A-protein G agarose beads with the Rictor substrate already bound. Following three washes in lysis buffer, the beads containing both substrate and kinase were washed twice in S6K1 kinase reaction buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4), and kinase reactions were carried out in 15 μl reaction buffer containing 250 μM ATP and 1 mM dithiothreitol at 30°C for 20 min. Reactions were stopped by boiling the mixtures in SDS sample buffer. mTORC2 kinase assays using kinase-dead Akt (HA–Akt-K179D) as a substrate were carried out as previously described (23).

RESULTS

Multisite phosphorylation of Rictor within a region conserved only in vertebrates. One likely mode of mTORC2 regulation that has not been specifically examined is the phosphorylation of its core components. Through multiple microcapillary LC/MS/MS analyses, we found that human Rictor, purified from HEK-293 cells grown in fresh serum, is heavily phosphorylated, primarily on serine and threonine residues. The 21 unique sites identified with high confidence in these analyses are summarized in Table 1 (see Table S1 in the supplemental material for the interpretation of raw LC/MS/MS spectra). Most of the phosphorylated residues identified are conserved across vertebrate orthologs of Rictor, with a few conserved in *Drosophila* (Table 1). Interestingly, the vast majority of these sites are clustered in a region in the C-terminal half of Rictor, the sequence of which is conserved among vertebrates but is poorly conserved in the *D. melanogaster* and *C. elegans* orthologs of Rictor. In fact, the most highly conserved regions (labeled "A" and "B" in Fig. 1) contain relatively few phosphorylation sites. This finding suggests that the phosphorylation of these sites might represent later-evolving mechanisms of mTORC2 regulation. Several phosphorylation sites had been identified previously on the *S. pombe* ortholog Ste20p (21), but none of these sites clearly align with the phosphorylated residues identified on human Rictor. Finally, we scanned existing large phospho-proteome data sets and databases for previously identified Rictor phosphorylation sites. Strikingly, Rictor is phosphorylated on at least 37 sites, when the sites identified in our analyses are included, and the majority of these are found within the same region unique to vertebrate orthologs of Rictor (the 37 sites and their predicted kinases are summarized in Table S2 in the supplemental material).

Rictor is phosphorylated on T1135 in a growth factor-responsive and rapamycin-sensitive manner. To further charac-

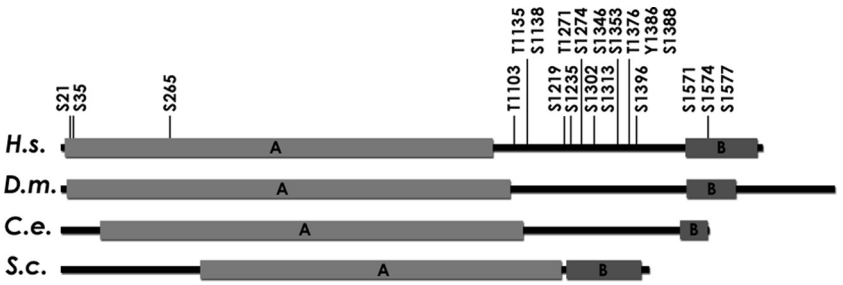

FIG. 1. The phosphorylation sites on Rictor identified by LC/MS/MS are clustered in a region conserved only in vertebrate orthologs. Rictor orthologs from the indicated species are shown schematically. Regions of similarity were determined by individually aligning full-length Rictor orthologs to human Rictor using BLAST. Two major regions of homology exist, the highly conserved region A (amino acids 8 to 1050 in humans) and the less-well-conserved region B (amino acids 1519 to 1694 in humans). The sizes of regions A and B represented in human Rictor correspond to the longest stretch of conservation in any other ortholog. Phosphorylation sites identified in this study are numbered based on full-length human Rictor and are summarized in Table 1. Accession numbers for orthologs are listed in Materials and Methods. *H.s.*, *H. sapiens*; *D.m.*, *D. melanogaster*; *C.e.*, *C. elegans*; *S.c.*, *S. cerevisiae*.

terize these 21 phosphorylation sites, we used a Web-based kinase prediction program (Table 1) (36; NetPhorest; http://netphorest.info/). We were intrigued to find that some of these sites lie within motifs predicted to be phosphorylated by members of the AGC kinase family, a group that includes several kinases dependent on either mTORC1 or mTORC2 for their activation (29). To determine whether Rictor or other core components of mTORC2 (66) are phosphorylated on AGC kinase motifs in response to growth factors, we coexpressed and immunoprecipitated myc-tagged versions of these proteins and immunoblotted with the phospho-Akt substrate antibody. This phosphorylation-specific antibody recognizes a K/R-X-R-X-X-pS/pT motif, where X is any amino acid and pS/pT is phosphorylated Ser or Thr (35, 71). Indeed, Rictor was phosphorylated on a site recognized by this antibody upon stimulation with insulin or EGF (Fig. 2A). However, with the exception of mTOR, which was recognized weakly in response to insulin, the other mTORC2 components were not recognized by this antibody. To confirm that endogenous Rictor is likewise phosphorylated and to assess which signaling pathways might regulate this phosphorylation event, we tested the effects of short pretreatments with inhibitors of candidate pathways, including PI3K (wortmannin), MEK1/2 (UO126), and mTORC1 (rapamycin) (Fig. 2B). The insulin- and EGF-stimulated phosphorylation of Rictor on this motif was very sensitive to PI3K inhibition, while it was resistant to MEK inhibition. Surprisingly, Rictor phosphorylation was also acutely sensitive to rapamycin treatment, suggesting that it is phosphorylated in an mTORC1-dependent manner. Importantly, this short-term rapamycin treatment does not affect mTORC2 stability, which is disrupted during longer treatments with rapamycin (51), as there are no differences in the levels of endogenous mTOR, mSIN1, or mLST8 associated with Rictor following these treatments (Fig. 2B and C). Therefore, the inhibition of Rictor phosphorylation by rapamycin observed here is not a secondary effect of disrupting mTORC2 assembly. In order to confirm that Rictor is phosphorylated in this manner as part of mTORC2, the endogenous Rictor coimmunoprecipitating with mTOR or mSIN1 was immunoblotted with the phospho-Akt substrate antibody. Indeed, the Rictor associated with mTOR and mSIN1 underwent insulin-responsive and rapamycin-sensitive phosphorylation on a motif recognized by this antibody (Fig. 2C). Therefore, Rictor within mTORC2 is phosphorylated on a motif preferentially phosphorylated by AGC kinases in a growth factor-, PI3K-, and mTORC1-dependent manner.

In human Rictor, there are only four sites that match the AGC kinase motif that is most strongly recognized by the phospho-Akt substrate antibody, and our LC/MS/MS analyses identified three of these sites as being phosphorylated (S21, T1135, and S1219) (see the sequences in Table 1). In order to identify the growth factor-stimulated, rapamycin-sensitive site(s), we made phosphorylation site mutations (S/T to A) at these three sites and at a fourth predicted site (S1113). Strikingly, the T1135A mutant alone eliminated the insulin-induced phosphorylation of Rictor detected by the phospho-Akt substrate antibody, while mutation of the other sites had no discernible effect (Fig. 2D). As the T1135A mutant was still weakly recognized by the phospho-Akt substrate antibody, we tested whether this site accounted for all of the rapamycin-sensitive phosphorylation of Rictor. Indeed, in the absence of T1135, rapamycin treatment had no effect on the residual recognition of Rictor by this phospho-motif antibody (Fig. 2E). While these data clearly point to the phosphorylation of T1135 as accounting for the reactivity of the phospho-Akt substrate antibody with Rictor, it remains possible that the other three AGC motifs are also phosphorylated in a growth factor-responsive and/or rapamycin-sensitive manner but are not significantly reactive with this antibody. To address this possibility, we used an LC/MS/MS-based ratio analysis in which the total ion current for targeted phosphorylated and nonphosphorylated peptides is used to quantify the relative levels of phosphorylation at a given site (3, 73). For this experiment, Rictor was purified from cells that were serum starved and either left untreated or stimulated with insulin in the presence or absence of rapamycin (Fig. 2F). This analysis revealed that only the T1135 site is phosphorylated in response to insulin, and it is also the only site sensitive to rapamycin. Although a significant number of counts for S1113-containing peptides were detected, none were phosphorylated. Therefore, T1135 is the primary AGC kinase site on Rictor that is induced by insulin, and this occurs in an mTORC1-dependent manner. The motif surrounding T1135 is highly conserved throughout vertebrates (Fig. 2G) but is not conserved in lower metazoans.

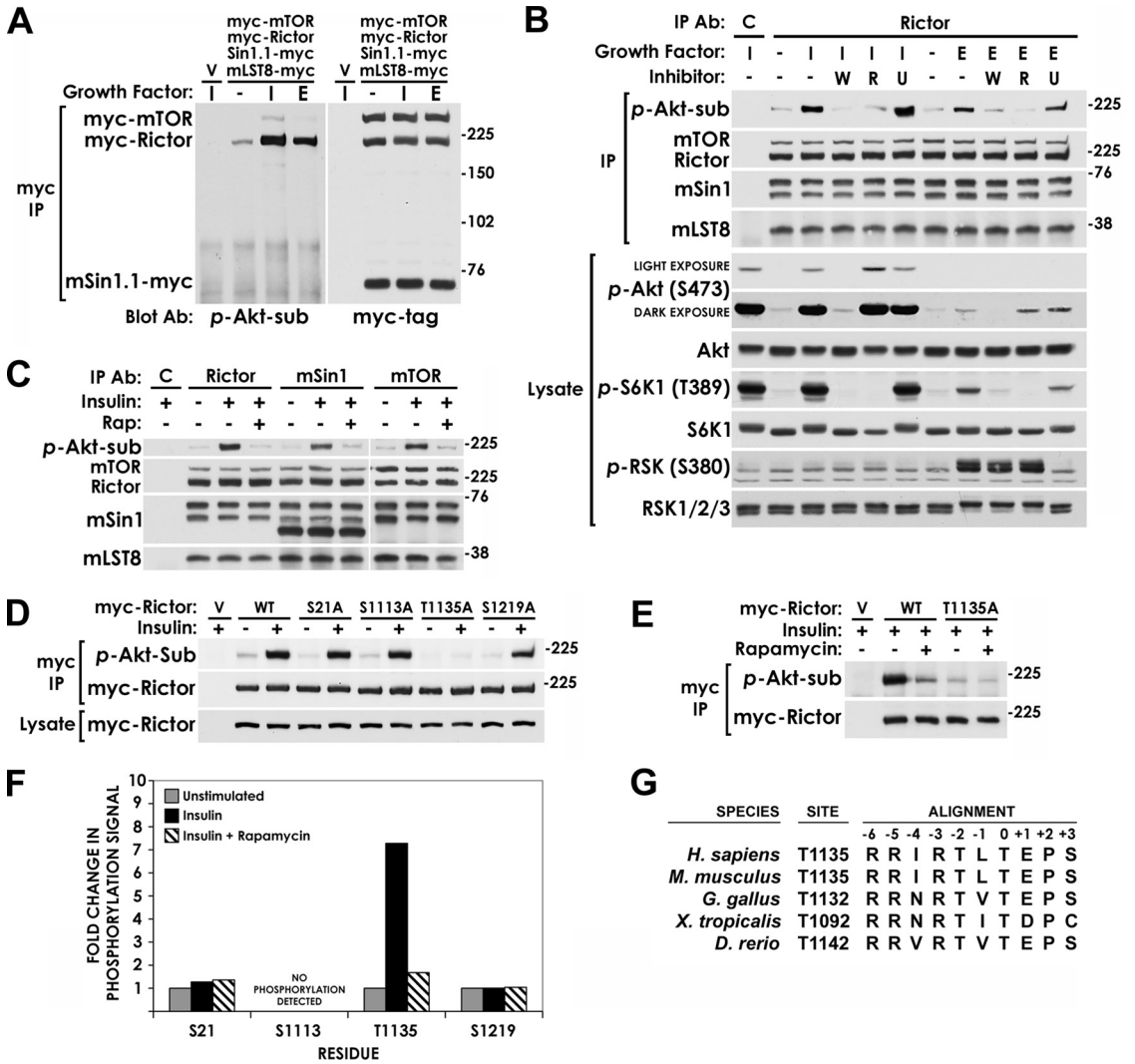

FIG. 2. Rictor is phosphorylated on T1135 in an insulin-responsive and rapamycin-sensitive manner. (A) Insulin and EGF stimulate phosphorylation of Rictor on a motif recognized by the phospho-Akt substrate antibody (*p*-Akt-sub). HEK-293E cells were transfected with empty vector (V) or cotransfected with myc-tagged mTOR, Rictor, mSIN1.1, and mLST8. After 16 h of serum starvation, cells were either left unstimulated (lanes −) or were stimulated for 30 min with insulin (250 nM) (lanes I) or EGF (25 ng/ml) (lanes E). Anti-myc immunoprecipitates (myc IP) were immunoblotted with either the phospho-Akt substrate antibody or the myc epitope antibody (Blot Ab). (B) The phosphorylation of endogenous Rictor on a motif recognized by the phospho-Akt substrate antibody is wortmannin and rapamycin sensitive. HEK-293E cells were serum starved for 16 h; pretreated with DMSO (lanes −), wortmannin (100 nM) (lanes W), or rapamycin (20 nM) (lanes R) for 5 min or U0126 (10 μM) (lanes U) for 1 h; and stimulated with either insulin (100 nM) or EGF (20 ng/ml) for 30 min, where indicated with I and E, respectively. Whole-cell lysates or immunoprecipitates of endogenous Rictor were immunoblotted with the indicated antibodies. (C) Rictor within mTORC2 is phosphorylated on a motif recognized by the phospho-Akt substrate antibody. HEK-293E cells were serum starved for 16 h and treated with insulin and/or rapamycin, where indicated, as described for panel B. Immunoprecipitates of endogenous Rictor, mSIN1, or mTOR were immunoblotted with the indicated antibodies. Note that the faster-migrating band detected in the mSIN1 immunoprecipitations that is not in those of Rictor or mTOR is either an isoform of mSIN1 that does not associate with mTORC2 in these cells or another protein with which the antibody cross-reacts. (D) A T1135A mutant eliminates insulin-stimulated phosphorylation of Rictor detected by the phospho-Akt substrate antibody. HEK-293E cells were transfected with empty vector (V), wild-type myc-Rictor (WT), or the indicated myc-Rictor phosphorylation site mutants. Cells were serum starved for 16 h before 30 min of stimulation with insulin (100 nM), where indicated. Whole-cell lysates and anti-myc immunoprecipitates were immunoblotted with either the phospho-Akt substrate antibody or the myc-epitope antibody. (E) Residual recognition of Rictor-T1135A by the phospho-Akt substrate antibody is not rapamycin sensitive. HEK-293E cells were transfected and treated as described for panel D but, where indicated, were pretreated for 5 min with rapamycin (20 nM). (F) Quantification of Rictor phosphorylation at four residues in response to insulin and rapamycin using an LC/MS/MS total ion current. HEK-293E cells were transfected with wild-type myc-Rictor, serum starved for 16 h, pretreated with rapamycin (20 nM) for 5 min, and stimulated with insulin (200 nM) for 30 min, as indicated. myc-Rictor was immunoprecipitated and separated on SDS-PAGE gels. Coomassie blue-stained bands were excised, proteolyzed, and subjected to targeted LC/MS/MS analyses. The relative phosphorylation levels, determined by quantification of the LC/MS/MS total ion current of the corresponding tryptic (S21, T1135, and S1219) or chymotryptic (S1113) peptides, are shown normalized to levels in the unstimulated sample. Although a significant number of S1113-containing peptides were detected, none were found to be phosphorylated. (G) The sequence surrounding human Rictor-T1135 is highly conserved among vertebrates. Sequences from the corresponding Rictor orthologs are aligned. See Materials and Methods for GenBank accession numbers. Numbers at the right of blots are molecular masses (in kilodaltons).

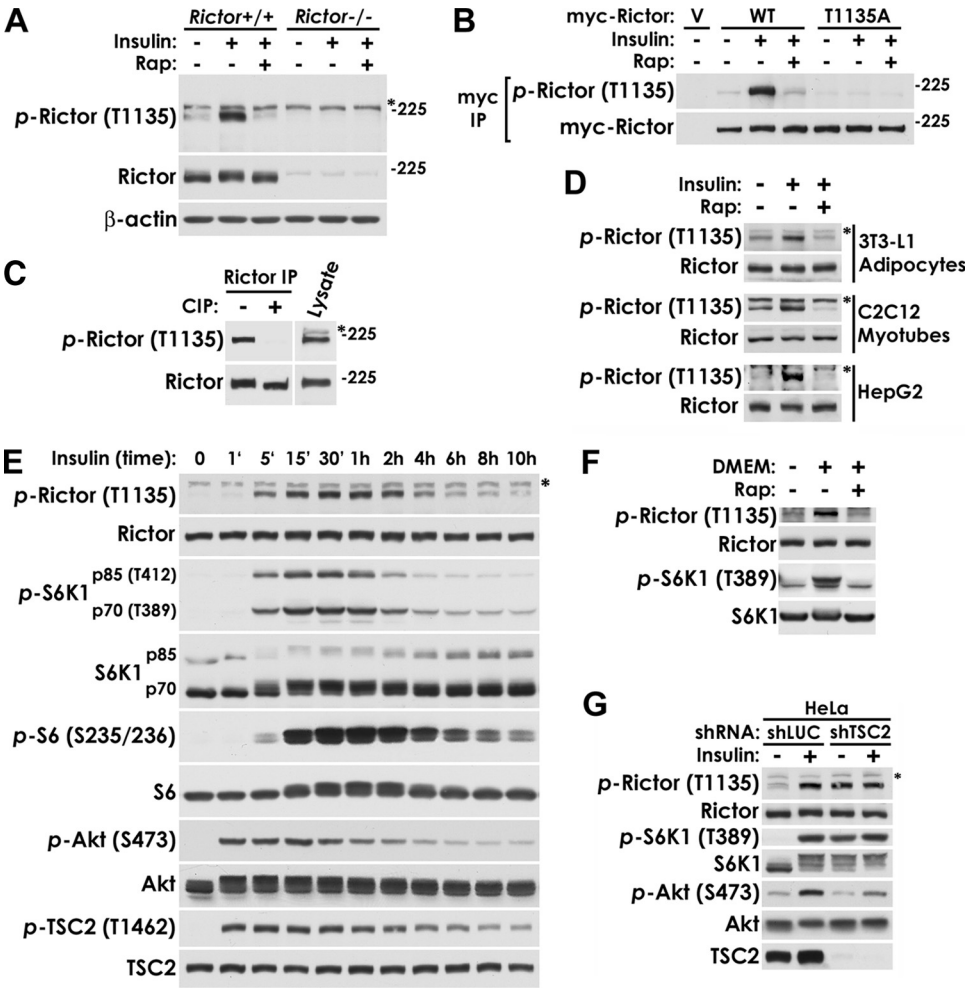

FIG. 3. mTORC1-dependent phosphorylation of Rictor on T1135. (A) Confirmation of endogenous Rictor-T1135 phosphorylation [*p*-Rictor (T1135)] with a phospho-specific antibody. Littermate-derived *Rictor*$^{+/+}$ and *Rictor*$^{-/-}$ MEFs were pretreated with rapamycin (20 nM) for 5 min followed by insulin (100 nM) for 30 min. Immunoblots of the indicated proteins are shown. *, nonspecific band. (B) Demonstration of the specificity of the Rictor phospho-T1135 antibody. HEK-293E cells were transfected as described for Fig. 2D, and anti-myc immunoprecipitates (myc IP) were immunoblotted with the indicated antibodies. V, empty vector; WT, wild type. (C) Phosphatase treatment of Rictor eliminates recognition by the phospho-T1135 antibody. Endogenous Rictor was immunoprecipitated from HEK-293E cells growing in full serum, treated with calf intestinal phosphatase (CIP), and immunoblotted with the phospho-T1135 and total Rictor antibodies. *, nonspecific band. (D) Rictor-T1135 is phosphorylated in a variety of cell types. 3T3-L1 adipocytes, C2C12 myotubes, and HepG2 cells were serum starved for 16 h, pretreated with rapamycin (20 nM) for 5 min, and stimulated with insulin (100 nM) for 30 min where indicated. Whole-cell lysates were immunoblotted for phospho-Rictor-T1135 and total Rictor. *, nonspecific band. (E) Phosphorylation of Rictor-T1135 in response to insulin at sequential time points. HEK-293E cells were serum starved for 16 h and stimulated for the indicated lengths of time. Whole-cell lysates were immunoblotted with the indicated antibodies. *, nonspecific band. (F) Amino acid refeeding stimulates Rictor-T1135 phosphorylation. HEK-293E cells were serum starved for 16 h, incubated in Dulbecco's phosphate-buffered saline for an additional 2 h, and then stimulated with fresh DMEM or DMEM with rapamycin (20 nM) for 30 min. (G) Constitutive phosphorylation of Rictor on T1135 in *TSC2*-deficient cells. HeLa cells stably expressing shRNAs targeting firefly luciferase (shLUC) or *TSC2* (shTSC2) were serum starved for 16 h and, where indicated, were stimulated with insulin (100 nM) for 30 min. Lysates were immunoblotted with the indicated antibodies. *, nonspecific band. Numbers to the right of blots are molecular masses (in kilodaltons).

T1135 is phosphorylated by S6K1. To facilitate the characterization of the mechanisms regulating the phosphorylation of endogenous Rictor at T1135, we obtained a phospho-specific antibody to this site. To validate the specificity of this antibody, we compared littermate-derived *Rictor*$^{+/+}$ and *Rictor*$^{-/-}$ MEFs (15). This antibody recognized a protein of approximately 200 kDa in insulin-stimulated *Rictor*$^{+/+}$ MEFs that was absent in *Rictor*$^{-/-}$ MEFs, and rapamycin reduced the presence of this band to the level without stimulation (Fig. 3A). It is worth pointing out that there is a prominent nonspecific band also present in the *Rictor*$^{-/-}$ MEFs that is recognized by this antibody and is present at various levels in different cell lines. As expected, this antibody does not recognize the T1135A mutant of Rictor (Fig. 3B) or endogenous Rictor pretreated with a phosphatase (Fig. 3C). Using this phospho-Rictor antibody, we first determined whether the phosphorylation of T1135 was regulated similarly in other cell types. Indeed, this site is phosphorylated in an insulin-responsive and rapamycin-sensitive manner in all cell types tested, including 3T3-L1 adipocytes, C2C12 myotubes, and HepG2 cells (Fig. 3D). Over a time

TABLE 2. Top 10 predicted S6K phosphorylation sites on Rictor[a]

Rank	Site[b]	Amino acid at position[c]:											Score[d]	Phosphorylation[e]	Coverage[f]
		−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5			
1	T1135	R	I	R	T	L	T	E	P	S	V	D	0.5331	Yes	+
2	S1219	K	I	R	S	Q	S	F	N	T	D	T	0.5431	Yes	+
3	S1035	S	S	R	H	N	S	E	S	E	S	V	0.6433	No	+
4	S1111	N	K	K	H	R	S	S	S	D	P	K	0.6869	No	+
5	T1133	N	R	R	I	R	T	L	T	E	P	S	0.6970	No	+
6	S1037	R	H	N	S	E	S	E	S	V	P	S	0.6970	No	+
7	S1113	K	H	R	S	S	S	D	P	K	G	G	0.8045	No	+
8	S21	R	G	R	N	D	S	G	E	E	N	V	0.8161	Yes	+
9	T210	R	G	G	L	N	T	I	L	K	N	V	0.8235	No	+/−
10	S1408	L	Q	R	S	S	S	V	R	S	M	V	0.8332	No	−

[a] An S6K1 position-specific scoring matrix developed by B. E. Turk (61) was used in conjunction with the Scansite program (40; http://scansite.mit.edu/) to predict sites on Rictor that may be phosphorylated by S6K1.
[b] Residue numbering for full-length human Rictor (GenBank accession no. AAS79796).
[c] Amino acid sequence centered around the phosphorylatable residue (0).
[d] Scansite score for each site. Sites with lower scores are predicted to be better recognized by S6K1.
[e] Whether phosphorylation was detected by LC/MS/MS analyses.
[f] Coverage of the human Rictor sequence achieved in multiple LC/MS/MS analyses. +, a significant number of peptides were detected; +/−, few peptides were detected; −, no peptides were detected.

course in HEK-293E cells, phosphorylation of Rictor follows kinetics similar to those of Akt and S6K1 (and their respective substrates TSC2 and S6) but more closely follows S6K1 signaling (Fig. 3E). Like the mTORC1-dependent phosphorylation of S6K1, amino acid refeeding following a 2-h starvation stimulates phosphorylation of Rictor-T1135, even in the absence of growth factors (Fig. 3F). In cells deficient for the TSC2 tumor suppressor, a critical negative regulator of mTORC1, mTORC1 and S6K become constitutively active (reviewed in reference 24). Again, consistent with the mTORC1-dependent nature of Rictor-T1135 phosphorylation, HeLa cells with stable shRNA-mediated knockdown of TSC2 (Fig. 3G), as well as *Tsc2* null MEFs (data not shown), display constitutive growth factor-independent phosphorylation of this site.

The sequence context of Rictor-T1135 and the clear regulation of its phosphorylation by mTORC1 suggested that S6K might be directly responsible for its phosphorylation (9). In support of this idea, an in silico survey of the primary sequence of Rictor with the Scansite program (40; http://scansite.mit.edu) using a position-specific scoring matrix based on the peptide substrate specificity of S6K1 (36, 61) reveals that T1135 is the best predicted S6K1 phosphorylation site on Rictor (Table 2). In fact, among the top 10 predicted sites, only S21 and S1219, which were found to not be regulated by mTORC1 (Fig. 2F), were detected as phosphorylated residues in our LC/MS/MS analyses. Therefore, we tested the ability of S6K1 to phosphorylate wild-type Rictor, the Rictor-T1135A mutant, or Rictor with the T1135A mutation in combination with the S21A, S1113A, or S1219A mutation (Fig. 4A). Only S6K1 isolated from insulin-stimulated cells could phosphorylate wild-type Rictor on a site recognized by the phospho-Akt substrate antibody. As in our cell culture experiments, the T1135A mutation was sufficient to eliminate the recognition of Rictor by the phospho-motif antibody in these S6K1 kinase reactions. Using the specific phospho-T1135 antibody, we confirmed that S6K1 can directly phosphorylate this residue in an insulin-responsive and rapamycin-sensitive manner (Fig. 4B). Expression of a rapamycin-resistant mutant of S6K1, but not wild-type S6K1, renders the phosphorylation of Rictor-T1135 in cells resistant to rapamycin (Fig. 4C). Finally, to confirm the dependence of Rictor-T1135 phosphorylation on S6K in cells and to differentiate between the S6K1 and S6K2 isoforms, we used siRNAs to knock down the expression of S6K1 and S6K2. S6K2 has been found previously to be the primary kinase responsible for S6 phosphorylation in MEFs and some other cell types (43, 56). Interestingly, we found that S6K1 knockdown has a much more pronounced effect on insulin-stimulated phosphorylation of S6 (both S235/236 and S240/244) than does S6K2 knockdown in HEK-293E cells (Fig. 4D). However, in these same cells grown in full serum, both S6K1 and S6K2 contribute significantly to the phosphorylation of these sites on S6 (Fig. 4E). Despite these differences in S6 regulation by the two S6Ks in these cells, siRNAs targeting S6K1, but not S6K2, robustly and specifically reduce the phosphorylation of Rictor-T1135 under basal conditions and in response to either insulin or serum (Fig. 4D and E). Furthermore, the S6K1-specific siRNAs do not affect the levels of other growth factor-stimulated AGC kinases, including Akt, SGK1, and RSK (Fig. 4D). Therefore, mTORC1-mediated activation of S6K1 leads to direct phosphorylation of the mTORC2 component Rictor on T1135.

The T1135A mutant of Rictor promotes increased phosphorylation of Akt at S473 in cells. The existence of a phosphorylation site on Rictor controlled by S6K1 presents the intriguing possibility that a relatively direct mTORC1-dependent feedback or cross talk mechanism exists to regulate mTORC2 signaling. To determine the role of this phosphorylation event on mTORC2 function, we first examined the effects of the T1135 site on mTORC2 stability and kinase activity. There was no discernible difference in the levels of endogenous mTORC2 components coimmunoprecipitating with the T1135A mutant of Rictor and with the wild type (Fig. 5A), suggesting that this phosphorylation site does not grossly affect mTORC2 assembly or stability. To determine whether the intrinsic kinase activity of mTORC2 differs between complexes immunoprecipitated with wild-type or Rictor-T1135A, we performed in vitro kinase assays using kinase-dead Akt as a substrate. Both the basal and insulin-stimulated kinase activities of mTORC2 were indistin-

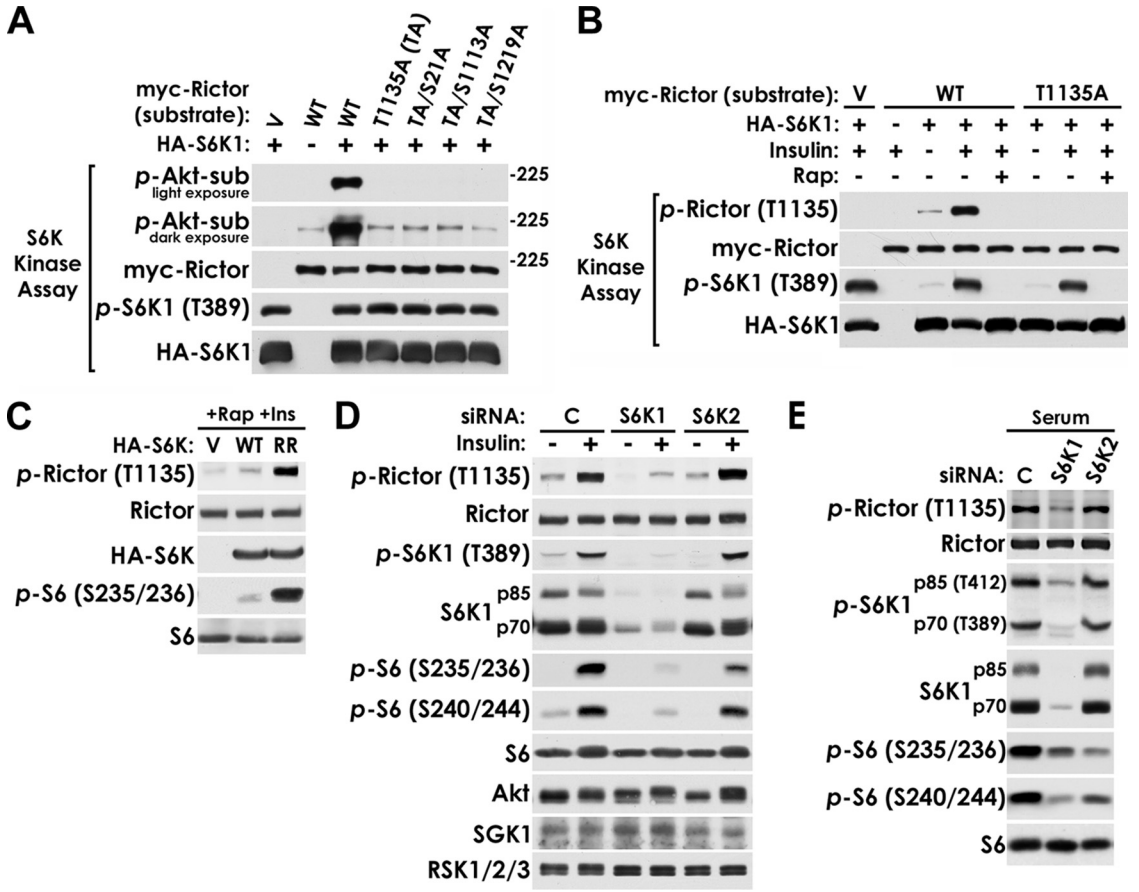

FIG. 4. S6K1 phosphorylates Rictor on T1135. (A) In vitro kinase assay demonstrating that T1135 is required for the phosphorylation of Rictor by S6K1 on the site(s) detected by the phospho-Akt substrate antibody (*p*-Akt-sub). In vitro kinase assays were performed with HA-S6K1 immunoprecipitated from insulin-stimulated HEK-293E cells. The Rictor substrate was immunoprecipitated from serum-starved, rapamycin-treated HEK-293E cells transfected with empty vector (V), wild-type myc-Rictor (WT), or myc-tagged versions of Rictor with the T1135A mutation alone or in combination with mutations at the indicated sites. The contents of the kinase reaction mixtures were immunoblotted with the indicated antibodies. (B) In vitro kinase assay demonstrating that S6K1 can directly phosphorylate Rictor-T1135. HA-S6K1 was immunoprecipitated from serum-starved HEK-293E cells that were left unstimulated, stimulated with insulin (100 nM) for 30 min, or pretreated with rapamycin (20 nM) for 5 min prior to insulin stimulation, as indicated. The Rictor substrate was obtained as described for panel A. (C) Rictor-T1135 phosphorylation becomes rapamycin resistant in cells expressing a rapamycin-resistant mutant of S6K1. HEK-293E cells were transfected with empty vector (V), wild-type S6K1 (WT), or a rapamycin-resistant mutant of S6K1 (RR). Cells were starved for 16 h and pretreated with rapamycin (20 nM) for 10 min prior to a 15-min stimulation with insulin (100 nM). Lysates were immunoblotted with the indicated antibodies. (D) S6K1 is required for the insulin-stimulated phosphorylation of T1135 in cells. HEK-293E cells were transfected with control (C) nontargeting siRNAs or siRNAs targeting either S6K1 or S6K2. Cells were serum starved for 16 h and either left unstimulated or stimulated with insulin (33 nM) for 30 min. Lysates were immunoblotted with the indicated antibodies. (E) S6K1 is required for phosphorylation of T1135 in cells growing in full serum. HEK-293E cells were transfected with siRNAs as described for panel D and grown in full serum, with a change of medium 16 h prior to lysis. Numbers to the right of blots are molecular masses (in kilodaltons).

guishable between complexes containing wild-type Rictor and those containing Rictor-T1135A (Fig. 5A). To test whether the loss of T1135 phosphorylation might affect mTORC2 function in cells, despite normal mTORC2 assembly and in vitro kinase activity, we examined the phosphorylation of an HA-Akt construct coexpressed with either wild-type Rictor or Rictor-T1135A in HEK-293E cells. Interestingly, over a time course of insulin stimulation, expression of the Rictor-T1135A mutant led to a modest but reproducible increase in the level of Akt-S473 phosphorylation compared to that with wild-type Rictor (Fig. 5B).

In order to further confirm this observation and determine whether this effect of Rictor-T1135A extends to endogenous Akt, we repeated this analysis in *Rictor*$^{-/-}$ MEFs, which are devoid of mTORC2 activity and Akt-S473 phosphorylation (15). As predicted, the introduction of either wild-type Rictor or Rictor-T1135A into these cells rescues the phosphorylation of Akt sites (T450 and S473) known to be controlled by mTORC2 (Fig. 6A). Importantly, as in the above-described experiments, *Rictor*$^{-/-}$ cells expressing Rictor-T1135A exhibit an increase in insulin-stimulated Akt-S473 phosphorylation relative to that of cells expressing the same levels of wild-type Rictor. Surprisingly, insulin-induced phosphorylation of the activation loop of Akt (T308) is also somewhat increased in cells expressing the T1135A mutant. While this site is not dependent on mTORC2 activity per se, this result is consistent with previous structural studies demonstrating that, in normal cells, S473 phosphorylation stabilizes the active state of Akt

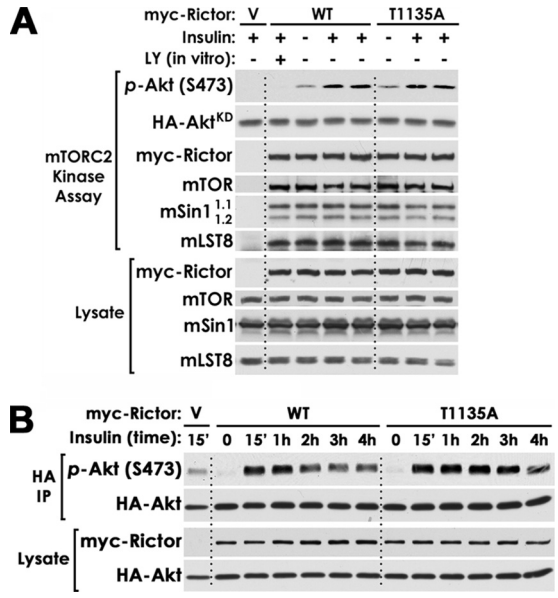

FIG. 5. The Rictor-T1135A mutant promotes increased phosphorylation of HA-Akt without altering the assembly or in vitro kinase activity of mTORC2. (A) Phosphorylation of T1135 does not affect mTORC2 assembly or in vitro kinase activity. mTORC2 was immunoprecipitated from HEK-293E cell lysates with myc-tagged Rictor (WT) or Rictor-T1135A following a 16-h serum starvation and, where indicated, stimulation for 30 min with insulin (100 nM). To demonstrate the specificity of the reaction, myc immunoprecipitates from vector (V)-transfected cells were used, and the mTOR kinase domain inhibitor LY294002 (15 μM; LY) was added to the indicated kinase reaction mixture prior to the addition of ATP. HA-tagged, kinase-dead Akt (HA-Akt^{KD}) was used as a substrate and was immunoprecipitated from serum-starved, wortmannin-treated (100 nM, 15 min) cells. The contents of the kinase reaction mixtures were immunoblotted with the indicated antibodies. (B) Expression of Rictor-T1135A increases phosphorylation of Akt at S473 [*p*-Akt (S473)] in HEK-293E cells. HA-Akt was coexpressed with empty vector (V), Rictor (WT), or Rictor-T1135A and was immunoprecipitated from cells that were serum starved for 16 h and then stimulated with insulin (100 nM) for the indicated times. HA immunoprecipitates (HA IP) and lysates were immunoblotted with the indicated antibodies.

and leads to sustained T308 phosphorylation (68, 69). This is also reflected in elevated Akt signaling, as detected by increased phosphorylation of T1462 on TSC2, a site specifically targeted by Akt (27, 35). In contrast, the mTORC2-dependent but growth factor-insensitive phosphorylation of Akt at its turn motif (T450) is indistinguishable from that of wild-type-Rictor- and Rictor-T1135A-expressing cells.

The finding that Akt phosphorylation on S473 but not T450 is affected by the T1135 site on Rictor led us to analyze the effects of the T1135A mutant on two other known mTORC2 targets, SGK1 and PKCα. Due to specificity issues with antibodies against the hydrophobic motif of SGK1 (S422), its activation state is commonly assessed by analyzing the phosphorylation of NDRG1, a highly specific substrate of SGK1 (12, 38). While both wild-type Rictor and Rictor-T1135A increased insulin-stimulated phosphorylation of NDRG1 when expressed in *Rictor*$^{-/-}$ MEFs, no differences in the levels of phosphorylation were detected, nor were there significant differences in SGK1 protein levels (Fig. 6B). The mTORC2-dependent phosphorylation of PKCα on both its turn motif (T638) and its hydrophobic motif (S657) occurs constitutively and is required for PKCα stability (8, 26, 39). Wild-type Rictor and the T1135A mutant increase PKCα protein levels to the same extent when expressed in *Rictor*$^{-/-}$ cells (Fig. 6B). Therefore, as with Akt-T450 phosphorylation, these other mTORC2 targets do not appear to be affected by the T1135 site on Rictor. However, in this same experiment, expression of the T1135A mutant, once again, led to increased phosphorylation of both Akt-S473 and TSC2-T1462 in response to insulin.

Studies of mTORC1-dependent feedback effects on Akt phosphorylation have often focused on the consequences of prolonged rapamycin treatment, which elicits both transcriptional and posttranslational effects on IRS-1 (e.g., see references 17 and 55). As Rictor-T1135 phosphorylation is acutely sensitive to rapamycin, we decided to compare the effects on Akt-S473 phosphorylation of short-term rapamycin treatment and expression of the T1135A mutant. In *Rictor*$^{-/-}$ MEFs expressing wild-type Rictor, a 5-min pretreatment with rapamycin led to a significant increase in insulin-stimulated Akt-S473 phosphorylation (Fig. 6C). Expression of the T1135A mutant in these cells resulted in a similar increase in Akt phosphorylation in response to insulin, even in the absence of rapamycin. However, rapamycin further increased Akt phosphorylation in the Rictor-T1135A-expressing cells, especially at the 30 min time point (Fig. 6C, see quantitation), suggesting the involvement of additional mTORC1-dependent feedback mechanisms affected by short-term treatments with rapamycin. To examine the contributions from feedback mechanisms affecting IRS-1, we examined the phosphorylation of a known rapamycin-sensitive site on IRS-1 (S307) (6, 14, 47). In response to insulin, cells expressing wild-type Rictor or the T1135A mutant exhibited similar levels of phosphorylated S307 on IRS-1, and this phosphorylation was blocked by acute rapamycin treatment, which also induced a mobility shift in IRS-1 indicative of dephosphorylation of serine residues (Fig. 6C). These findings are consistent with mTORC1-dependent feedback mechanisms regulating both IRS-1 and Rictor acting in parallel to affect the threshold of Akt activation.

Rictor-T1135 phosphorylation creates a 14-3-3 binding site. To gain further insight into the molecular consequences of Rictor-T1135 phosphorylation, we analyzed the effects of an acidic residue substitution (T1135D) as a potential mimetic of phosphorylation. Interestingly, we found that expression of the Rictor-T1135D mutant in *Rictor*$^{-/-}$ MEFs results in an increase in Akt-S473 phosphorylation similar to that detected for the Rictor-T1135A mutant (Fig. 7A). This result suggests that the T1135D mutant fails to substitute for the regulatory effects of phosphorylation at this site. Importantly, the sequence context in which the T1135 residue lies predicts that phosphorylation of this residue generates a consensus mode 1 14-3-3 binding motif, and acidic residues are poor substitutions for phosphorylation in mediating such binding (67). Indeed, in pulldown assays with recombinant GST–14-3-3, we found that wild-type Rictor can associate with 14-3-3 and that the T1135A mutant is greatly diminished in its binding (Fig. 7B). As residual binding of 14-3-3 to the Rictor-T1135A mutant was detected in this assay, we mutated all other predicted mode 1 and mode 2 14-3-3 binding sites on Rictor in combination with T1135A. However, all of the double mutants were indistinguishable from the T1135A mutant alone, and additional 14-

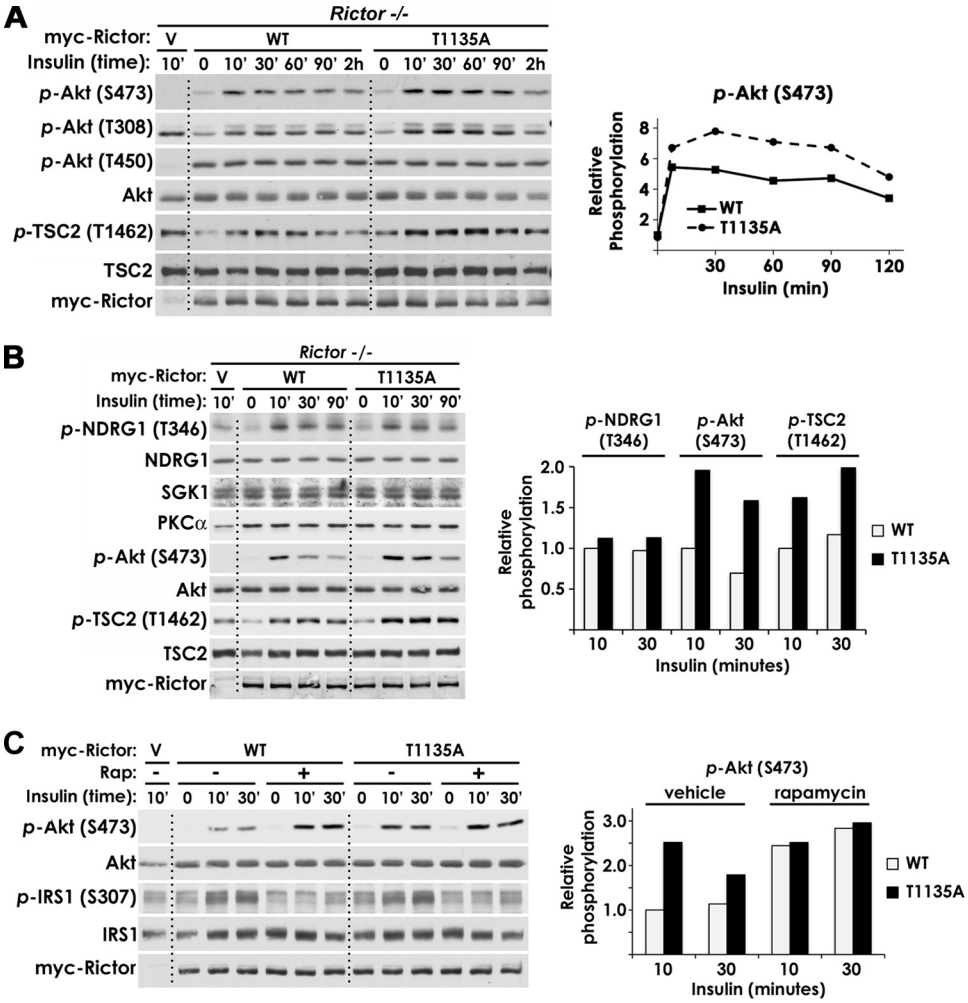

FIG. 6. Rictor-T1135A promotes increased phosphorylation of endogenous Akt on S473 [*p*-Akt (S473)] in *Rictor*$^{-/-}$ MEFs. (A) Introduction of Rictor-T1135A into *Rictor*$^{-/-}$ cells leads to increased phosphorylation of endogenous Akt. *Rictor*$^{-/-}$ MEFs were transfected with empty vector (V), Rictor (WT), or Rictor-T1135A, and confluent cells were serum starved for 16 h and stimulated with insulin (100 nM) for the indicated times. Lysates were immunoblotted with the indicated antibodies. Phosphorylation intensity in immunoblots was quantified using ImageJ software. Phospho-Akt (S473) [*p*-Akt (S473)] levels were divided by both total Akt and total Rictor levels and are graphed relative to levels at the zero time point for WT-Rictor-expressing cells. (B) Introducing Rictor-T1135A into *Rictor*$^{-/-}$ cells does not affect total SGK1 levels, phosphorylation of the SGK1 substrate NDRG1, or total PKCα levels compared to those with the wild type. Cells were transfected and treated as described for panel A. Lysates were immunoblotted with the indicated antibodies. Phosphorylation intensity in immunoblots was quantified as described for panel A, was normalized to the total levels of phosphorylation of both the indicated protein and of Rictor, and is shown relative to the level of phosphorylation at the 10 min time point for WT-Rictor-expressing cells. (C) The increase in insulin-induced Akt-S473 phosphorylation in T1135A-expressing cells compares in magnitude to the increase in Akt-S473 phosphorylation resulting from a short rapamycin pretreatment. *Rictor*$^{-/-}$ MEFs were transfected as described for panel A. Confluent cells were serum starved for 16 h, pretreated with rapamycin (20 nM) for 5 min, and stimulated with insulin (100 nM) for 10 or 30 min where indicated. Lysates were immunoblotted with the indicated antibodies. Phosphorylation in immunoblots was quantified as described for panel B and are shown relative to the quantity at the 10 min time point for untreated WT-Rictor-expressing cells.

3-3 binding sites on Rictor or an associated protein remain unknown. Finally, in coimmunoprecipitation experiments, we found that wild-type Rictor can associate with 14-3-3 in cells, and this is mediated largely by phosphorylation of Rictor at T1135 (Fig. 7C).

DISCUSSION

In this study, we show that Rictor is regulated by multisite phosphorylation in a C-terminal region, with a sequence conserved only in vertebrate orthologs of Rictor. If we combine previous phospho-proteomic data with the 21 unique high-confidence sites identified in this study, Rictor appears to be phosphorylated on as many as 37 different sites, primarily Ser or Thr residues within a putative regulatory region (summarized in Table S2 in the supplemental material). While we focus here on the regulation of sites likely to be phosphorylated by AGC family kinases, the identities of the remaining sites will greatly facilitate future studies of the regulation and functions of Rictor and mTORC2. We identify T1135 as a major growth factor-regulated site on Rictor that is sensitive to rapamycin. It has been previously reported that rapamycin

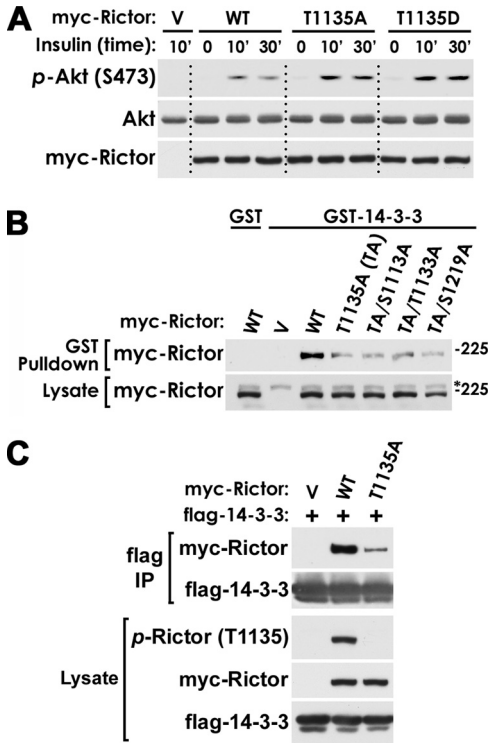

FIG. 7. Rictor-T1135 phosphorylation creates a 14-3-3 binding site. (A) Like Rictor-T1135A, Rictor-T1135D promotes increased phosphorylation of Akt-S473 [*p*-Akt (S473)]. *Rictor*$^{-/-}$ MEFs were transfected with empty vector (V), wild-type myc-Rictor (WT), myc-Rictor-T1135A, or myc-Rictor-T1135D, and confluent cells were serum starved for 16 h and stimulated with insulin (100 nM) for the indicated times. Lysates were immunoblotted with the indicated antibodies. (B) Rictor-T1135 can mediate 14-3-3 binding in vitro. HEK-293E cells were transfected with empty vector (V), wild-type myc-Rictor (WT), myc-Rictor-T1135A, or double mutants of Rictor-T1135A in combination with second-candidate 14-3-3 binding sites. GST pulldown assays were performed on the corresponding lysates using recombinant GST or GST–14-3-3. Pulled-down proteins and lysates were immunoblotted with the indicated antibodies. (C) Rictor-T1135 can mediate 14-3-3 binding in cells. *Rictor*$^{-/-}$ MEFs were cotransfected with Flag–14-3-3 and empty vector (V), myc-Rictor (WT), or myc-Rictor-T1135A. Anti-Flag immunoprecipitates (flag IP) and lysates from cells grown in full serum were immunoblotted with the indicated antibodies. Numbers at the right of blots are molecular masses (in kilodaltons).

treatment causes an electrophoretic mobility shift in Rictor to a faster-migrating form, suggestive of dephosphorylation (1, 46). However, these experiments were done using prolonged treatment with rapamycin, which results in diminished assembly of mTORC2 (51), thereby eliminating any Rictor phosphorylation site that is dependent on complex formation. Acute rapamycin treatment (e.g., 5 to 15 min), which eliminates T1135 phosphorylation, does not affect mTORC2 stability, nor does it cause a mobility shift on Rictor immunoblots. We identified several proline-directed phosphorylation sites in our LC/MS/MS analyses that could be sites of mTOR-mediated phosphorylation of Rictor within mTORC2 (i.e., autophosphorylation of the complex), and these sites might be primarily responsible for the previously reported shifts in Rictor mobility in response to prolonged rapamycin.

Our data demonstrate that the T1135 residue on Rictor is an S6K1-specific substrate. It remains possible that additional AGC family kinases can phosphorylate this site under specific conditions. However, the facts that Rictor-T1135 phosphorylation is acutely sensitive to rapamycin in numerous cell lines and that S6K1 knockdown blocks phosphorylation in response to both insulin and full serum suggest that S6K1 is the primary T1135 kinase in vivo. This site is phosphorylated on Rictor within mTORC2 and stimulates binding of 14-3-3 to Rictor. However, the precise molecular effects of this regulation are unknown. This site does not affect complex integrity or the intrinsic kinase activity of mTORC2, but a nonphosphorylatable mutant of Rictor (T1135A) reproducibly increases the ability of mTORC2 to phosphorylate Akt on S473 in cells. While this phosphorylation event is certainly not an on/off switch for mTORC2, it appears to play a role in mTORC2 regulation within cells, at least for its phosphorylation of Akt. It was somewhat surprising to find that this site does not affect other known mTORC2 targets, such as SGK1, PKCα, or the turn motif on Akt (T450). The sparse nature of our current understanding of growth factor-mediated activation of mTORC2 and its spatial regulation of Akt and its other substrates limits our ability to define the molecular mechanism of this regulatory effect at this time. However, our findings suggest that Rictor-T1135 phosphorylation and subsequent 14-3-3 binding specifically dampen the ability of mTORC2 to phosphorylate Akt on S473 in response to growth factors.

Our results are consistent with S6K1-mediated phosphorylation of Rictor on T1135 being a mechanism of feedback regulation or cross talk between mTORC1 and mTORC2. Like S6K-mediated regulation of IRS-1 upstream of PI3K, Rictor-T1135 phosphorylation represents another mechanism by which the status of mTORC1 activation leads to feedback regulation of Akt signaling (see the model in Fig. 8). Our direct comparison of the acute effects of rapamycin to the effects of the Rictor-T1135A mutant on Akt phosphorylation suggests that the mTORC1-dependent feedback regulation of mTORC2 acts in concert with the well-documented effects on IRS-1. While the effects of the T1135A mutant on Akt-S473 phosphorylation are modest, they are comparable in scale to the effects of rapamycin, which should block all mTORC1-mediated feedback mechanisms. The parallel and cooperative nature of these two feedback mechanisms is illustrated by the fact that rapamycin treatment of cells expressing the Rictor-T1135A mutant inhibits IRS-1 feedback and results in a further increase in insulin-stimulated Akt phosphorylation.

While feedback mechanisms affecting IRS-1 have been intensely studied for years, the effects of individual phosphorylation sites on insulin-stimulated Akt activation have not been thoroughly examined. A few studies have overexpressed phosphorylation site mutants of IRS-1 affecting specific mTORC1- or S6K1-regulated sites, in otherwise wild-type backgrounds, but only rarely have the effects of these mutants on Akt phosphorylation been analyzed. One study, which found IRS-1-S1101 to be phosphorylated by S6K1, detected a modest increase in insulin-stimulated Akt-S473 phosphorylation upon overexpression of an S1101A mutant in CHO cells (59). We are aware of only one study that has reconstituted an *Irs-1* null cell line with a mutant affecting a residue known to be phosphorylated by S6K1 (13). Surprisingly, in this particular study, expression of this mutant (IRS-1-S302A) actually decreased

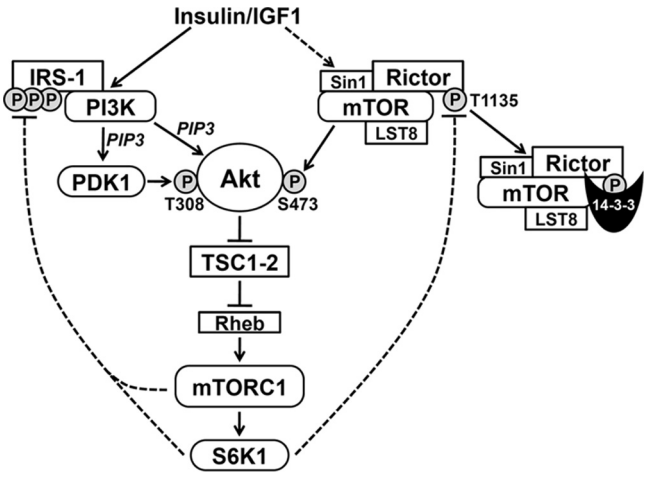

FIG. 8. Model of mTORC1-dependent feedback mechanisms affecting insulin-stimulated activation of Akt. Insulin and IGF1 stimulate IRS-1 binding to PI3K, thereby activating PI3K and increasing its production of phosphotidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits PDK1 and Akt to the plasma membrane, where PDK1 phosphorylates Akt-T308. Through an unknown mechanism, insulin and IGF1 also activate mTORC2, which phosphorylates Akt-S473, leading to full activation of Akt. Activated Akt directly phosphorylates and inhibits TSC2, leading to Rheb activation and subsequent stimulation of mTORC1 activation. Both mTORC1 and its downstream target S6K1 can phosphorylate specific serine residues on IRS-1, and this feedback mechanism negatively regulates IRS-1 signaling to PI3K. As shown in the current study, S6K1 also directly phosphorylates the Rictor subunit of mTORC2 on T1135 and stimulates 14-3-3 binding to Rictor. S6K1-mediated phosphorylation of Rictor negatively regulates the ability of mTORC2 to phosphorylate Akt-S473 in cells. Therefore, mTORC1 activation triggers two parallel negative-feedback mechanisms affecting the levels of insulin/IGF1-stimulated Akt phosphorylation and activation.

insulin-stimulated Akt-S473 phosphorylation relative to that in wild-type-reconstituted cells. Given our current knowledge of IRS-1 feedback mechanisms, it seems feasible that Rictor-T1135 phosphorylation contributes to the acute effects of mTORC1 signaling on Akt activation to an extent similar to that of IRS-1 serine phosphorylation. Whether additional mTORC1-mediated feedback mechanisms affecting PI3K-Akt signaling exist is unknown. It is interesting to note that neither T1135 on Rictor nor the specific inhibitory phosphorylation sites on IRS-1 are conserved in the *Drosophila* orthologs of these proteins. However, *Drosophila* TORC1 and S6K have been shown to exert negative regulatory effects on Akt, such that their knockdown increases Akt phosphorylation on its hydrophobic motif (45, 52). This suggests that there are alternative TORC1-dependent feedback mechanisms in *Drosophila* or that yet another mechanism that is conserved in *Drosophila* exists in mammalian cells. Given the complexity of signaling within the PI3K-mTOR signaling network, it would not be surprising if additional feedback mechanisms exist.

In cell lines lacking the tuberous sclerosis complex (TSC) tumor suppressors (TSC1 and TSC2), in which mechanisms of mTORC1-driven insulin resistance have been studied, chronic activation of mTORC1 causes a dramatic reduction in both IRS-1 transcript and protein levels, resulting in an inability of insulin to significantly stimulate PI3K activation (17, 54, 55, 72). However, these cells also exhibit a loss of mTORC2 kinase activity due to a role for the TSC1-TSC2 complex in activating mTORC2 (23, 25). Although we detect growth factor-independent phosphorylation of Rictor-T1135 in *Tsc2*-deficient cells due to constitutive S6K1 activation, the T1135 site does not substantially affect mTORC2 kinase activity or signaling to SGK1 or PKCα, which are all attenuated by loss of the TSC1-TSC2 complex (23, 25). Furthermore, the effects of the TSC1-TSC2 complex on mTORC2 are largely independent of its effects on mTORC1 signaling. Therefore, while it might contribute to the overall block in Akt activation in TSC-deficient cells, Rictor-T1135 phosphorylation is unlikely to be a major mechanism underlying the severe attenuation of mTORC2 activity upon loss of the TSC1-TSC2 complex.

Our knowledge of mTORC2 regulation and function has lagged greatly behind that of mTORC1. Fundamental questions remain regarding mechanisms of mTORC2 assembly, activation, and interaction with its downstream substrates. Furthermore, it seems likely that we are just scratching the surface with regard to mTORC2 functions and, perhaps, mTORC2-independent functions of Rictor. It is possible that phosphorylation of T1135, and/or other sites identified in our study, will be important for the regulation of these other putative functions. Given the shear number of phosphorylation sites identified on just this one subunit of mTORC2, its regulation is likely to be quite complex and perhaps vary significantly dependent on specific stimuli and settings.

ACKNOWLEDGMENTS

We are indebted to J. Huang for reagents and helpful discussion, L. M. Freimark and X. Yang for technical assistance, and J. Blenis, L. C. Cantley, D. A. Guertin, D. M. Sabatini, and R. J. Shaw for critical reagents. We thank B. E. Turk for sharing the position-specific scoring matrix for S6K1.

This work was supported through T32 training grant ES07155 (C.C.D.) and NIH grants R01-CA122617 (B.D.M.) and P01-CA120964 (B.D.M. and J.M.A.).

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