

mSin1 Is Necessary for Akt/PKB Phosphorylation, and Its Isoforms Define Three Distinct mTORC2s

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Summary

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that participates in at least two distinct multiprotein complexes, mTORC1 and mTORC2 [1]. These complexes play important roles in the regulation of cell growth, proliferation, survival, and metabolism. mTORC2 is a hydrophobic motif kinase for the cell-survival protein Akt/PKB [2, 3] and, here, we identify mSin1 as a component of mTORC2 but not mTORC1. mSin1 is necessary for the assembly of mTORC2 and for its capacity to phosphorylate Akt/PKB. Alternative splicing generates at least five isoforms of the mSin1 protein [4], three of which assemble into mTORC2 to generate three distinct mTORC2s. Even though all mTORC2s can phosphorylate Akt/PKB *in vitro*, insulin regulates the activity of only two of them. Thus, we propose that cells contain several mTORC2 flavors that may phosphorylate Akt/PKB in response to different signals.

Results and Discussion

mSin1 Is a Novel Component of mTORC2 but Not mTORC1

To identify new components of the mammalian target of rapamycin (mTOR) complexes, we generated HEK-293T cells that stably express N-terminally tandem affinity purification (TAP)-tagged mLST8, a component of both mTORC1 and mTORC2, and used these to perform large-scale tandem affinity purifications [5]. Because we were interested in identifying components that were unique to mTORC1 or mTORC2, we also generated cell lines expressing TAP-tagged raptor. From these purifications, we identified, via tandem mass spectrometry, the protein mSin1 (mammalian stress-activated protein kinase [SAPK]-interacting protein) [6] as a novel component of mTORC2 but not mTORC1. To confirm the identity of

mSin1, we immunoblotted a small amount of each purification with a specific anti-human mSin1 antibody and showed that at least three isoforms of mSin1 are present in the TAP-mLST8 material (Figure 1A). To further characterize this interaction, we also performed immunopurifications with antibodies that specifically recognize mTOR, rictor, and raptor from total-cell extracts of HeLa and HEK-293T cells (Figure 1B). In agreement with the tandem affinity purifications, we verified that at least two endogenous mSin1 isoforms coimmunoprecipitate with endogenous mTOR and rictor but not with raptor.

mSin1, also known as MAPKAP1 (mitogen-activated protein kinase-associated protein 1), was originally identified (clone JC-310) in an expression screen for its ability to suppress the phenotypes generated in *S. cerevisiae* by the expression of a constitutively active human Ha-Ras mutant [7]. mSin1 likely belongs to a poorly conserved family of proteins that include Sin1 in *S. pombe*, AVO1 in *S. cerevisiae*, and RIP3 in *D. discoideum* [4]. Protein conservation among the Sin1 family members is mostly limited to a small region corresponding to residues 225 to 267 of the longest mSin1 protein. Contrasting with other species in which Sin1 has been studied, human mSin1 is unique in having five different protein isoforms, generated by alternative splicing [4] (Figure 2A). Importantly, AVO1 has been identified as a component of the yeast TORC2 [8], and RIP3 has been shown to bind to Pia (rictor), which is a TORC2 component in *D. discoideum* [9]. Although an interaction between mSin1 and mTOR was suspected, a previous attempt to show this association was unsuccessful [8], and thus our work provides the first evidence that endogenous mSin1 is a component of mTORC2 but not mTORC1.

We next asked whether mSin1 is also an mTORC2 component in eight different human cancer cell lines (Figure 1C). Different amounts of two mSin1 isoforms were detected in both mTOR immunopurifications and total-cell lysates made from all cell lines tested. Except for HeLa cells, these cell lines express low levels of isoform 2, which can only be seen after the enrichment through an mTOR immunopurification. These findings suggest that at least two mSin1 isoforms are expressed ubiquitously, though in different amounts, in several human cell lines. This is consistent with previous studies showing that several mSin1 isoforms exist in different human tissues [8, 10]. We conclude that at least two endogenous isoforms of mSin1 coimmunoprecipitate with mTOR and rictor but not raptor, and that, therefore, mSin1 is a component of mTORC2.

mSin1 Isoforms 1, 2, and 5 Assemble Separately into mTORC2 and Define Three Distinct Complexes

Human mSin1 is functionally related to yeast AVO1 even though they have diverged considerably. To determine potential functional differences between the isoforms, we used HEK-293T-derived stable cell lines expressing

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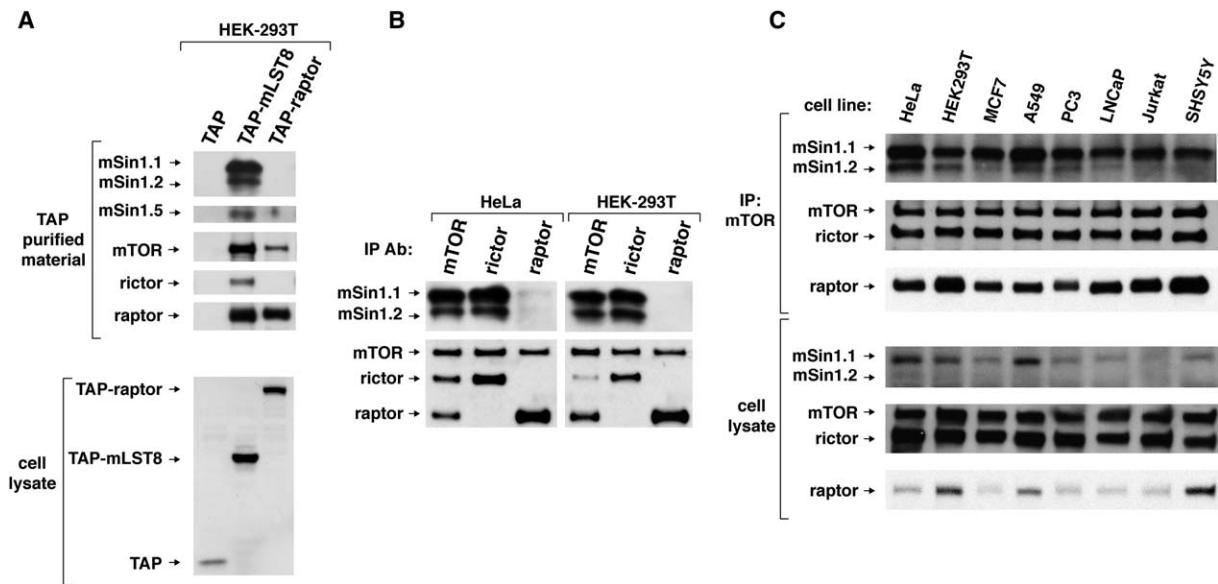


Figure 1. mSin1 Is a New Component of mTORC2 but Not mTORC1

(A) Detection by immunoblotting of indicated proteins in affinity purifications of TAP alone and of TAP-mLST8 and TAP-raptor fusion proteins. (B) mTOR, rictor, and raptor immunopurifications prepared from HeLa and HEK-293T cells were analyzed by immunoblotting for the levels of endogenous mSin1 and other mTORC1 and mTORC2 proteins, as indicated. (C) mTOR immunopurifications made from lysates of eight different human cancer cell lines were analyzed by immunoblotting for the levels of indicated proteins.

C-terminally myc-tagged versions of mSin1.1, 1.2, 1.4, and 1.5 isoforms. mSin1.3 was not included because we could not verify its expression by RT-PCR in mRNA obtained from HeLa and HEK-293T cells (Figure S1 in the Supplemental Data available online and data not shown). In immunoblot analysis of cells expressing recombinant isoforms, our mSin1 antibody recognized four different mSin1 bands with apparent molecular weights of approximately 80, 76, 55, and 52 kDa, which correspond respectively to isoforms 1, 2, 5, and 4 (Figure 2B). Interestingly, all isoforms have an apparent molecular weight that is higher than predicted. Additionally, isoform 5 shows a higher electrophoretic mobility than isoform 4, even though its predicted weight is less.

Using the stable cell lines expressing each myc-tagged mSin1 isoform or γ -tubulin, we performed immunopurifications with rictor, raptor, mTOR, and myc antibodies. We found that rictor immunopurifications contain isoforms 1, 2, and 5 (Figure 2C, left panel). mTOR immunopurifications also contain each of these isoforms (data not shown), whereas raptor immunopurifications do not (Figure 2C, right panel). In agreement with these findings, immunopurifications of myc-tagged isoforms 1, 2, and 5 coimmunoprecipitate mTOR and rictor but not raptor (Figure 2D, left panels). Isoform 4 does not coimmunoprecipitate with mTOR, rictor, or raptor. Our finding that isoform 5 coimmunoprecipitates mTOR and rictor confirmed our TAP results (Figure 1A) that isoform 5 can participate in mTORC2. It was not possible to detect it in the mTOR and rictor immunopurifications because isoform 5 migrates at a molecular weight similar to IgG and would have been masked by background bands in immunoblots (Figure 1B). The discovery that three mSin1 isoforms can participate in mTORC2 raised the question of whether the three

isoforms bind simultaneously or whether, instead, they bind exclusively to form three distinct mTORC2s, each defined by a different mSin1 isoform. To answer this question, we probed the myc immunopurifications of the mSin1 isoforms with an antibody for human mSin1. Our rationale was that if multiple isoforms bound simultaneously, we would see more than one mSin1 band in each of the myc immunopurifications. However, each myc immunopurification contained only a single mSin1 band, identical to the molecular weight of the myc-tagged isoform we expressed (Figure 2D, right panel). Our results suggest that there are at least three distinct mTORC2s, each of them defined by a specific mSin1 isoform, and uncover an unforeseen complexity of mTORC2 relative to other TORC2s. Inspection of the mSin1 isoform sequences (Figure 2A) revealed that the main difference between isoform 4 and the remaining isoforms, including the isoforms that assemble into mTORC2s, is the lack of the first 192 amino acid residues. This suggests that an unknown domain contained within the first 192 amino acids of the mSin1 proteins may be responsible for their assembly into mTORC2. Our unpublished data, showing that N-terminally myc-tagged mSin1 isoforms 1, 2, and 5 cannot enter mTORC2, also lead to the idea that the N-terminal region of the mSin1 proteins may be critical for binding to mTORC2 components during complex assembly. The use of N-terminally myc-tagged mSin1 isoforms may have hampered previous attempts to show that mSin1 is part of mTORC2 [8]. The potential importance of the N terminus was unexpected because all Sin1 ortholog proteins share a small region that does not overlap with the first 192 amino acid residues of mSin1 and could have been anticipated to be associated with their ability to bind to mTORC2.

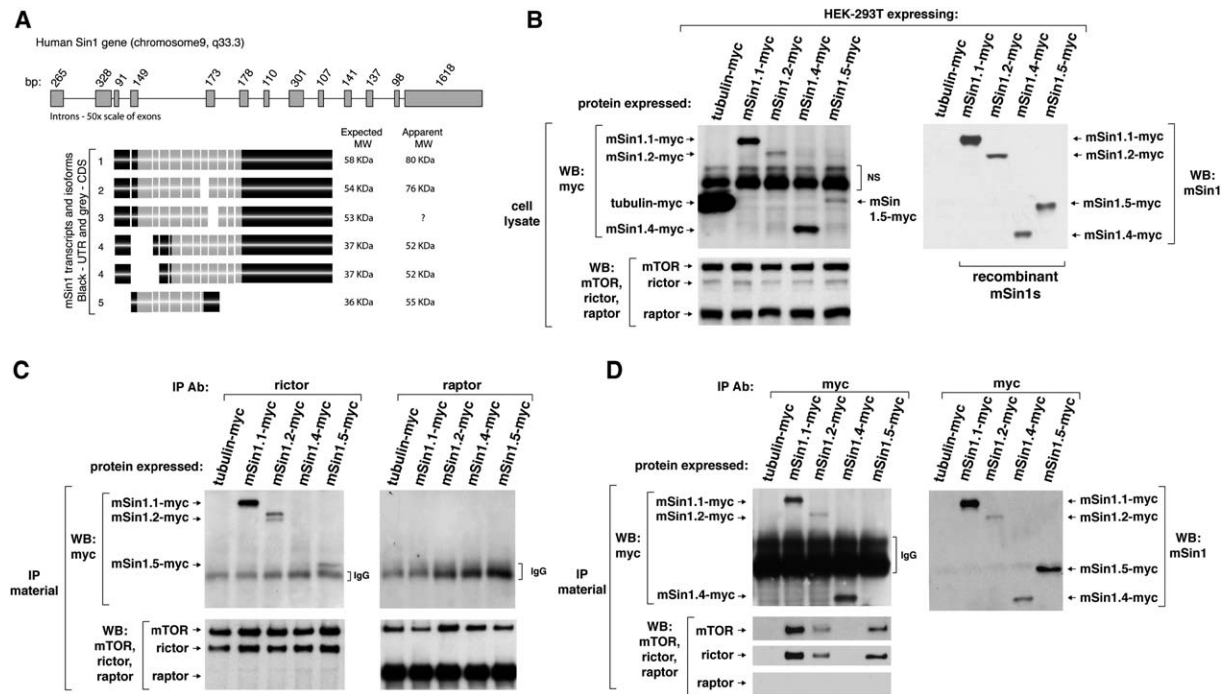


Figure 2. mSin1.1, mSin1.2 and mSin1.5, Three Different Protein Isoforms of mSin1, Define Three Distinct mTORC2s

(A) Schematic representation of the human Sin1 gene, transcript variants, and protein isoforms. Six mSin1 transcript variants, generated by alternative splicing, encode five different mSin1 protein isoforms. Two transcript variants encode for mSin1 isoform 4.
 (B) Detection of C-terminally myc-tagged mSin1.1, mSin1.2, mSin1.4, mSin1.5, and γ -tubulin levels in lysates of HEK-293T-derived cells stably expressing each recombinant protein by immunoblotting with an antibody to the myc tag (left panel) or to mSin1 (right panel).
 (C) Rictor and raptor immunoprecipitations made from lysates of cells described in (B) were analyzed by immunoblotting for the levels of myc-tagged proteins and other mTORC1 and mTORC2 proteins, as indicated.
 (D) Myc immunoprecipitations made from lysates of cells described in (B) were analyzed by immunoblotting with antibodies to the myc tag, mTOR, rictor, raptor (left panel), and mSin1 (right panel).

Recent studies provide genetic [11] and biochemical [12, 13] evidence suggesting that TOR complexes may work as dimers or even higher-order multimers. To understand whether the different mSin1-containing mTORC2s oligomerize with each other, we coexpressed, in HEK-293T cells, mSin1.1 tagged with an HA epitope along with one of the following proteins: mSin1.1-myc, mSin1.2-myc, mSin1.5-myc, or γ -tubulin-myc. In immunoprecipitations prepared with either the hemagglutinin (HA) or myc antibody, we detected the mSin1 isoform directly immunoprecipitated by the antibody as well as the mTOR and rictor components of mTORC2 (Figure S2). In no case did we detect the coexpressed mSin1 isoform that was tagged with the epitope recognized by the antibody not used in the immunoprecipitation. These results suggest that under our purification conditions mTORC2 is not a dimer and are consistent with our assertion that only one mSin1 protein can participate in mTORC2 at a time. Overall, our data support the idea that even though made with similar components, mTORC2 might be more complex than other TORC2s because it exists in at least three different formats.

Sin1 Is Essential for the Phosphorylation of the Hydrophobic-Motif Site of Akt/PKB

Because mTORC2 phosphorylates the hydrophobic-motif site Serine-473 of Akt/PKB [2], we asked whether mSin1 is required for this process. To test this hypothesis, we used short-hairpin-mediated RNA interference

to knock down mSin1 in both HEK-293T and HeLa cells (Figure 3A). Knockdown of mSin1 with these two different shRNAs severely inhibited the phosphorylation of Ser473-Akt/PKB without affecting that of Thr389 of S6K1. We observed the same effect by knocking down rictor, whereas a control shRNA had no effect on Akt/PKB. Interestingly, the knockdown of mSin1 also leads to a reduction in rictor protein levels, suggesting that mSin1 is required for either rictor expression or stabilization and therefore assembly into mTORC2. This is likely to be the main reason why rictor is absent from mTOR immunoprecipitations made from mSin1-knocked-down cells (Figure 3A, bottom panels). We also found a reduction in mSin1 protein levels in lysates made from rictor-knocked-down cells. Decreased rictor protein levels in the absence of mSin1, and vice versa, suggest a codependence between the two components for complex formation and have previously been observed for TORC2 components in yeast [12].

To test whether the role of Sin1 in the phosphorylation of Akt/PKB is evolutionarily conserved, we performed similar knockdown experiments with two different double-stranded RNAs that target the dSin1 transcript in *Drosophila* Kc167 cells (Figure 3B). dsRNA to dSin1 completely abolishes the phosphorylation of the hydrophobic-motif site Ser-505 of dAkt/PKB. This was still true when the dAkt/PKB-suppressor, dPTEN, was also knocked down, underscoring the importance of dSin1 in dAkt/PKB phosphorylation.

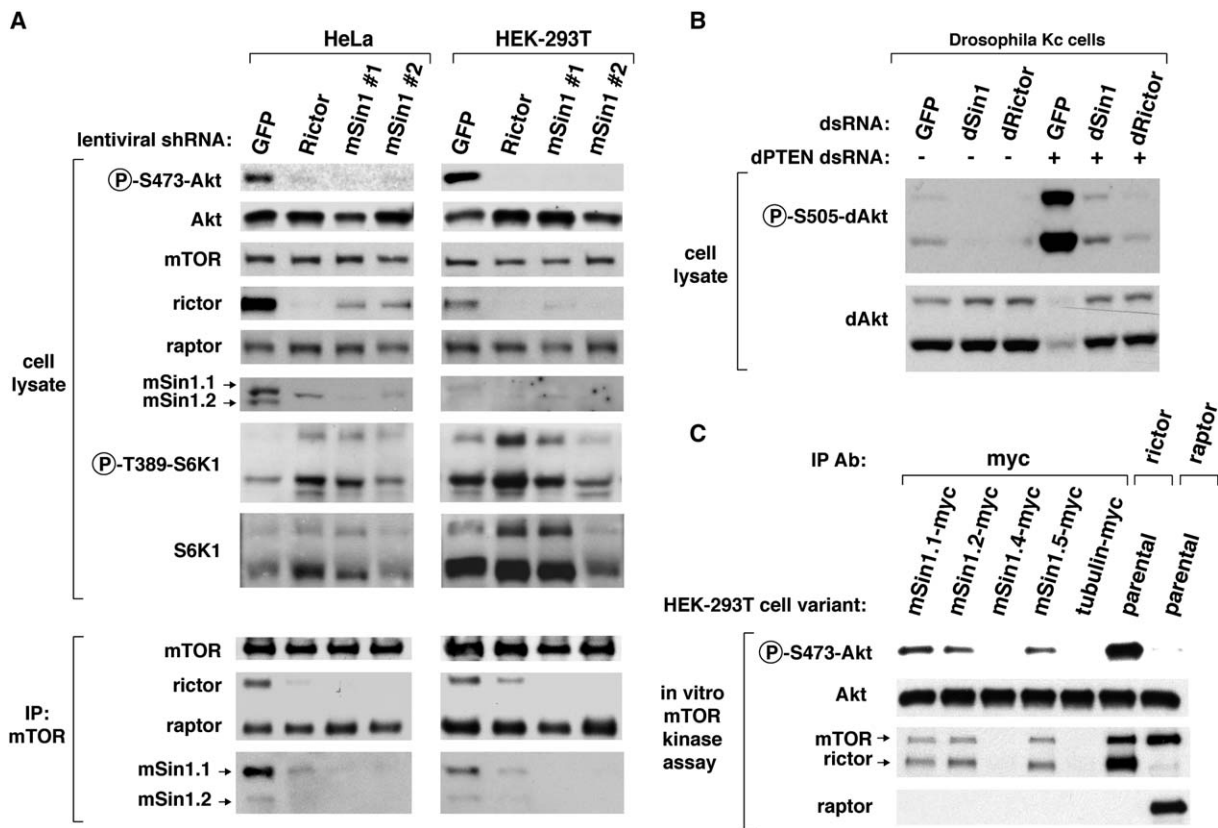


Figure 3. mSin1 Is Required for mTORC2 Assembly and Phosphorylation of the Hydrophobic-Motif Site (Ser473) of Akt/PKB
 (A) mSin1 was silenced in HeLa and HEK-293T cells with indicated shRNAs, and cell lysates were analyzed by immunoblotting for the levels of indicated proteins and phosphorylation states (upper panels). mTOR immunoprecipitations made from lysates of shRNA-expressing cells were also analyzed by immunoblotting for the levels of mSin1, mTOR, rictor, and raptor (lower panels).
 (B) dsRNA-mediated gene silencing of dSin1 in *D.melanogaster* Kc167 cells, performed individually or simultaneously with dPTEN gene silencing. Lysates of dsRNA-treated cells were analyzed by immunoblotting for the levels of indicated proteins and phosphorylation states.
 (C) Three distinct and mSin1-defined mTORC2s have the ability to phosphorylate the hydrophobic motif of Akt/PKB in vitro. Myc immunoprecipitations of each mTORC2, made from lysates of HEK-293T cells stably expressing each myc-tagged mSin1 isoform, were used to phosphorylate in vitro full-length Akt1/PKB1. Levels of phospho-Ser473-Akt/PKB and other indicated proteins were detected by immunoblotting.

Because our shRNAs target most, but not all, mSin1 isoforms simultaneously, we cannot determine whether different isoforms play different roles in mediating Akt/PKB phosphorylation. Because of extensive sequence overlap between mSin1 transcript variants (Figure 2A), it is not possible to silence each isoform individually with RNAi. To investigate possible functional differences between the isoforms, we had to use a different approach. Using cell lysates from HEK-293T cells stably expressing mSin1 isoforms, we tested myc immunoprecipitates for their ability to phosphorylate full-length Akt/PKB in vitro (Figure 3C). Rictor and raptor immunoprecipitations were also included as positive and negative controls, respectively. Through this system we were able to show that complexes containing mSin1 isoforms 1, 2, and 5 can directly phosphorylate Akt/PKB, just like complexes containing rictor, but not raptor. Interestingly, the levels of Akt/PKB phosphorylated in vitro were similar among the different mSin1-containing complexes, even though mSin1 isoforms 1, 2, and 5 are present at different levels. This suggests that the levels of mTOR, as the catalytic subunit of mTORC2, most likely determine the activity of the complex.

Our findings provide in vivo biochemical and physiological evidence that is consistent with an mSin1-containing mTORC2 (that exists in three forms) being the hydrophobic motif kinase of Akt/PKB and that this function is conserved between species. In agreement with this, a study of the *Dictyostelium* signaling pathways involved in chemotaxis and signal relay showed that Akt/PKB purified from rip3 (mSin1) null cells had less activity than in wild-type cells [9].

Insulin Activates Only Two of the Three mTORC2s
 Having shown that three mSin1 isoforms can be components of three distinct mTORC2s, all with the ability to phosphorylate the hydrophobic-motif site Ser473 of Akt/PKB, and considering that Ser473 phosphorylation is stimulated by insulin [14], we next determined whether the activity of all three mTORC2s is insulin sensitive. To answer this question, we performed in vitro kinase assays with immunoprecipitations of each mSin1 isoform from lysates of HeLa cells grown overnight under serum starvation or stimulated with 100 nM insulin for 20 min (Figure 4A and Figure S3B). We found that only mSin1.1- and mSin1.2-containing mTORC2s modulate

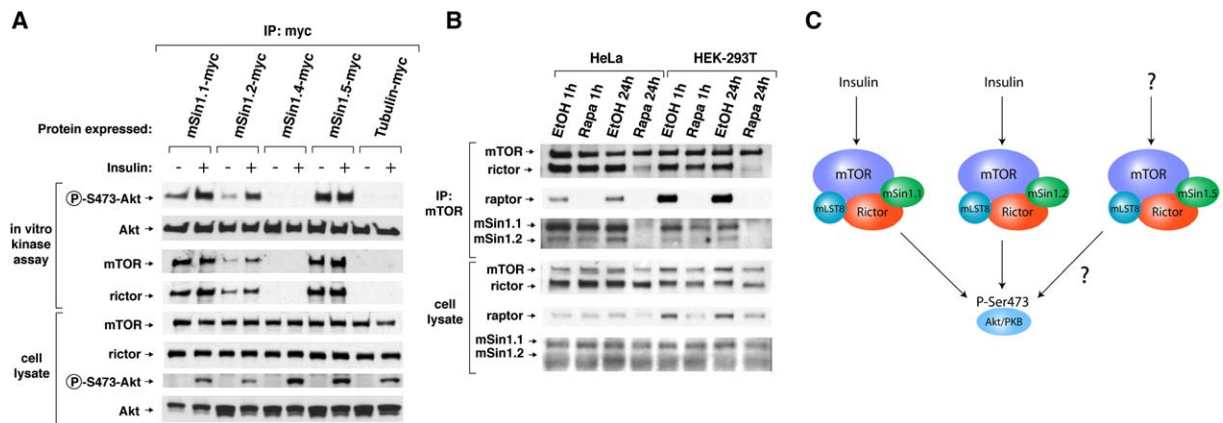


Figure 4. Regulation of mSin1-Containing mTORC2s by Insulin and Rapamycin

(A) mTORC2s containing mSin1.1 and mSin1.2, but not mSin1.5, phosphorylate the hydrophobic motif of Akt/PKB in response to insulin. Myc immunoprecipitations of each of the mSin1-containing mTORC2s, made from lysates of HeLa cells stably expressing each myc-tagged mSin1 isoform and grown under serum starvation or insulin-stimulated conditions, were used to phosphorylate in vitro full-length Akt1/PKB1. Levels of in vitro phosphorylated (Ser473) Akt/PKB, as well as other indicated proteins, were detected by immunoblotting.

(B) Chronic rapamycin affects mSin1 assembly into mTORC2. HEK-293T and HeLa cells were treated for 1 hr or 24 hr with 100 nM rapamycin. Cell lysates (lower panels) and mTOR immunoprecipitations (upper panels) made from cell lysates were analyzed by immunoblotting for the levels mSin1 and other indicated proteins.

(C) Proposed model for three distinct mSin1-containing mTORC2s.

their activity in response to insulin. Identical in vitro kinase assays performed with rictor immunoprecipitations also showed that insulin activates the in vitro phosphorylation of Ser473 of Akt/PKB by mTORC2 (Figure S3A). Recently, it has been suggested that phosphatidylinositol (3,4,5)-trisphosphate (PIP3,4,5) may regulate the capacity of mTORC2 to phosphorylate Akt/PKB [3]. However, the addition of PIP3,4,5 to in vitro mTOR kinase reactions did not lead to any alteration in the capacity of mTORC2 to phosphorylate Akt/PKB (Figure S3C). Surprisingly, the mTORC2 containing mSin1.5 is insensitive to serum starvation and insulin stimulation, as shown by its constitutive capacity to phosphorylate the Ser473 site of Akt/PKB under both conditions. It is known that Akt/PKB phosphorylation is regulated by PIP3,4,5-driven membrane localization [14], and even though the mSin1.5-containing mTORC2 retains activity toward Akt/PKB in the absence of insulin, it may not be localized where it has access to Akt/PKB. It is also possible that the normal role of mSin1.5-containing mTORC2 is not to respond to insulin and that it regulates Akt/PKB in response to other signals or signals to other molecules altogether. In *S. pombe*, Sin1 interacts with Sty1 (the fission-yeast ortholog of mammalian SAPKs, JNK, and p38) and is required for Sty1-mediated activation of the stress-induced transcription factors Atf1 and Pap1. It is thus possible that the mSin1.5-containing mTORC2 plays a role in stress signaling. Recent studies [10, 15] have shown that mSin1 isoforms 1 and 5 can interact with proteins of the mammalian SAPK signaling cascade, where mSin1 seems to have an inhibitory effect. Although Sin1 seems to be implicated in stress signaling, contradictory results obtained in fission-yeast and mammalian studies make the role of Sin1 in stress responses yet to be established. Our results open the possibility that different mSin1-containing mTORC2s may regulate Akt/PKB in response to different signals, but this remains to be proven.

Chronic Rapamycin Affects mSin1 Assembly into mTORC2

Chronic rapamycin treatment has recently been shown to affect the assembly of mTORC2 [16]. The mTOR-rictor interaction was severely disrupted in several human cancer cell lines grown in the presence of rapamycin for at least 24 hr. Because we have shown that mSin1 is an mTORC2 component, we wanted to understand whether the mSin1-mTOR interaction is also sensitive to chronic rapamycin treatment. For that, we challenged both HEK-293T and HeLa cells with acute or chronic rapamycin treatments (100 nM), of 1 hr and 24 hr, respectively. We observed that after 24 hr, rapamycin disrupts the interaction between endogenous mTOR and the mSin1.1 and mSin1.2 isoforms (Figure 4B). This supports the idea that even though rapamycin cannot bind to mTOR when assembled into mTORC2, it can bind to mTOR when assembled into mTORC1 or before assembling into any of the complexes. As mTORC2 turnover proceeds, the amount of free mTOR available to bind to mSin1 and the other mTORC2 components is strongly reduced, thus explaining the mTORC2 decay after long-term exposure to rapamycin. Our data indicate that the loss of mSin1 expression leads to a loss of rictor expression (Figure 3A), but the disruption of mTORC2 by long-term rapamycin treatment has no effect on rictor expression. One possible explanation for this is that mSin1 and rictor make within cells a weak interaction that is required to maintain their stability but is independent of mTOR. When cells are chronically treated with rapamycin, rictor and mSin1 are displaced from mTOR, but still may be available to interact with each other, although we currently have no evidence for this. On the other hand, when the expression of either mSin1 or rictor is knocked down by RNAi, this putative interaction would no longer be possible. It is also possible that mSin1 and/or rictor have mTOR-independent roles that are not affected by rapamycin but are lost when the

expression of either is reduced by RNAi. Finally, we cannot exclude the possibility that long-term treatment with rapamycin disrupts mTORC2 because rapamycin inhibits the synthesis of a short-lived protein that is necessary for complex assembly.

With this work, we propose that the multiple isoforms of mSin1 (mSin1.1, mSin1.2, and mSin1.5) define several unique mTORC2s, adding a functional complexity that seems to be absent in TORC2 signaling in other species. Additionally, our findings suggest that different mSin1 isoforms might mediate Akt/PKB (Ser473) phosphorylation in response to different upstream signals. Specifically, the activity of the mSin1.5-containing mTORC2 is not affected by insulin stimulation and might even not be associated with growth-factor signaling to Akt/PKB. However, we have not yet identified which stimuli do signal to Akt/PKB (Ser473) through this complex. Furthermore, the fact that both mSin1.1- and mSin1.2-containing mTORC2s regulate Ser473-Akt/PKB phosphorylation does not exclude the possibility that in the presence of other signals they might perform differently, thus supporting the idea of different mTORC2s being involved in the phosphorylation of the hydrophobic-motif site of Akt/PKB in response to specific upstream signals.

Supplemental Data

Supplemental Data include Experimental Procedures and are available with this article online at: <http://www.current-biology.com/cgi/content/full/16/18/1865/DC1/>.

Acknowledgments

This work was supported by grants from the National Institutes of Health (RO1 CA103866 and RO1 AI047389) to D.M.S and awards from the Keck Foundation, Pew Charitable Trust to D.M.S as well as fellowships from Fundação para a Ciência e Tecnologia (FCT), Portugal, and Fundação Calouste Gulbenkian, Portugal, to M.A.F. We thank members of the Sabatini Lab for discussions.

Received: May 25, 2006

Revised: July 31, 2006

Accepted: August 1, 2006

Published online: August 17, 2006

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