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Molecular Organization of Target of Rapamycin Complex 2*

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The target of rapamycin (TOR), a highly conserved serine/threonine kinase, plays a central role in the control of eukaryotic cell growth. TOR exists in two functionally and structurally distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 controls cell growth via a rapamycin-sensitive signaling branch regulating translation, transcription, nutrient uptake, ribosome biogenesis, and autophagy. TORC2 controls the organization of the actin cytoskeleton through a rapamycin-insensitive signaling branch and in yeast consists of the six proteins AVO1, AVO2, AVO3, BIT61, LST8, and TOR2. Here we have focused on the characterization of TORC2. Our studies suggest that TORC2 is oligomeric, likely a TORC2-TORC2 dimer. AVO1 and AVO3 bind cooperatively to the N-terminal HEAT repeat region in TOR2 and are required for TORC2 integrity. AVO2 is a nonessential peripheral protein associated with AVO1 and AVO3. LST8 binds separately to the C-terminal kinase domain region in TOR2 and appears to modulate both the integrity and kinase activity of TORC2. TORC2 autophosphorylates sites in AVO1 and AVO3, but TORC2 kinase activity is not required for TORC2 integrity. We have demonstrated that mammalian TOR is also oligomeric. The architecture of TORC2 is discussed in the context of TORC2 assembly and regulation.

Target of rapamycin (TOR)[1] is a highly conserved serine/threonine kinase that controls cell growth in response to nutrients. TOR controls its various growth-related readouts via two distinct signaling branches, a rapamycin-sensitive and a rapamycin-insensitive branch (1, 2). The TOR protein consists of several distinct domains. The N-terminal half of TOR comprises ~20 tandemly repeated HEAT repeats (named for Huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1) (3). HEAT repeats mediate protein-protein interactions and are required for localization of TOR to the plasma membrane (4). The HEAT repeats are accompanied by a so-called FAT domain that possibly serves as a scaffold or protein interaction domain (5, 6). Following the FAT domain are the FKBP-rapamycin binding domain, the kinase catalytic domain, and a C-terminal FATC domain. Because TOR contains a number of domains that may mediate protein-protein interactions, TOR has been proposed to exist in a multiprotein complex. Indeed, TOR is found in two complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2), both of which are at least partly conserved (7–12). These two complexes, which contain both common and distinct proteins, account for the specificity of TOR signaling. TORC1 mediates the rapamycin-sensitive signaling branch that positively regulates anabolic processes such as translation and ribosome biogenesis and negatively regulates catabolic processes such as RNA degradation, autophagy, and other degradative pathways (13–15). TORC2 signaling is rapamycin-insensitive and is required for the organization of the actin cytoskeleton. In *Saccharomyces cerevisiae*, TORC2 signals to the actin cytoskeleton by activating the RHO1 GTPase switch (16, 17). Upon activation via the exchange factor ROM2, RHO1 interacts with and activates PKC1. Activated PKC1 subsequently regulates the organization of the actin cytoskeleton by activating the mitogen-activated protein kinase cascade consisting of BCK1, MKK1/2, and MPK1 (18). TORC2 also signals to the actin cytoskeleton via the two recently described substrates SLM1 and SLM2 (19).

TORC1 in yeast consists of KOG1, TCO89, LST8, and either TOR1 (TORC1-A) or TOR2 (TORC1-B). TORC2 is composed of the proteins AVO1, AVO2, AVO3, LST8, TOR2, and the recently described BIT61 (7, 20, 21). AVO1 is a 131-kDa (apparent molecular mass 165 kDa) essential protein. Analysis of *avo1* mutant cells revealed that AVO1 acts positively in TORC2. Similar to *tor2* mutant cells, AVO1-depleted cells exhibit a defect in polarization of the actin cytoskeleton. Furthermore, hyperactivation of the RHO1 GTPase or the PKC1-mitogen-activated protein kinase pathway suppresses an *avo1* mutation (7). AVO3, a conserved 164-kDa protein (known as mAVO3 or rictor in mammals), is also essential and mediates TOR signaling to the actin cytoskeleton in yeast and mammals (11, 12). *AVO3* (*TSC11*) was originally identified genetically as a suppressor of a mutation in *CSG2*, a gene involved in sphingolipid biosynthesis, suggesting a link between sphingolipids and TORC2 signaling in yeast (22). LST8, a highly conserved and essential protein (known as mLST8 or GβL in mammals), is composed almost entirely of seven WD40 domains. As LST8 associates with both TORC1 and TORC2, *lst8* mutant cells resemble mutants defective in TORC1 or TORC2 (7, 23, 24). AVO2 and BIT61 perform nonessential functions in TORC2 (7, 21). SLM1 and SLM2, the recently identified substrates of TORC2, bind to TORC2 via AVO2, suggesting that AVO2 might serve as an adaptor protein (19).

The specific molecular functions of the individual proteins within the two TOR complexes are largely unknown. We took a biochemical approach to study the role of the different proteins that form TORC2. We provide evidence that AVO1 and AVO3, but not AVO2, act as scaffold proteins important for the integrity of TORC2. LST8, which binds to the C-terminal half of TOR2, appears to be involved in modulating both the integrity of TORC2 and the kinase activity of TOR2.

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[1] The abbreviations used are: TOR, target of rapamycin; mTOR, mammalian TOR; HA, hemagglutinin.

TABLE I
Strains

Strain	Genotype
JK9–3da	*MATa leu2–3,112 ura3–52 trp1 his4 rme1 HMLa*
JK9–3dα	*MATα leu2–3,112 ura3–52 trp1 his4 rme1 HMLa*
TB50a	JK9–3da *HIS4 his3*
TB50α	JK9–3dα *HIS4 his3*
RL25–1c	TB50a [*kanMX4*]-*GAL1p-3HA-AVO1*
RL39–1a	TB50a *AVO2–3HA*-[*kanMX4*]
RL42–1c	TB50a *AVO3–3HA*-[*kanMX4*]
RL58–1a	TB50a *LST8–3HA*-[*kanMX4*]
RL59–2d	TB50a *LST8–13myc*-[*kanMX4*]
RL69–1c	TB50a *AVO1–3HA*-[*kanMX4*]
RL74	TB50a/α *LST8–3HA*-[*kanMX4*]/*LST8–13myc*-[*kanMX4*]
RL125a	TB50a [*kanMX4*]-*GAL1p-3HA-LST8*
RS61–5b	TB50a [*kanMX4*]-*GAL1p-3HA-AVO3*
SW62–7a	TB50a *HA-TOR2 AVO3–13myc*-[*kanMX4*]
SW63–1d	TB50α *HA-TOR2 LST8–13myc*-[*kanMX4*]
SW65–11c	TB50a [*HIS3MX6*]-*GAL1p-AVO3 AVO1–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW67–3b	TB50α [*HIS3MX6*]-*GAL1p-AVO3 AVO1–3HA*-[*kanMX4*] *LST8–13myc*-[*kanMX4*]
SW68–3b	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2 AVO3–13myc*-[*kanMX4*]
SW69–4d	TB50a [*HIS3MX6*]-*GAL1p-AVO1 LST8–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW70	TB50a *3HA-TOR2*
SW72–5a	TB50a [*HIS3MX6*]-*GAL1p-AVO3 3HA-TOR2 AVO2–13myc*-[*kanMX4*]
SW73–4b	TB50a [*HIS3MX6*]-*GAL1p-AVO1 AVO3–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW74–11b	TB50a [*HIS3MX6*]-*GAL1p-AVO1 LST8–3HA*-[*kanMX4*] *AVO3–13myc*-[*kanMX4*]
SW75–10c	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2 LST8myc*-[*kanMX4*]
SW76–5b	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2 AVO2–13myc*-[*kanMX4*]
SW77–5d	TB50a [*HIS3MX6*]-*GAL1p-AVO3 3HA-TOR2 LST8–13myc*-[*kanMX4*]
SW78–3a	TB50a [*HIS3MX6*]-*GAL1p-AVO3 LST8–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW80–1d	TB50a [*HIS3MX6*]-*GAL1p-TOR2 AVO2-TAP*-[*kanMX4*]
SW84–1d	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2*
SW94–1a	TB50a [*HIS3MX6*]-*GAL1p-TOR2 AVO3–13myc*-[*kanMX4*]
SW100–1a	TB50a [*HIS3MX6*]-*GAL1p-LST8 3HA-TOR2*
SW102–8b	TB50a *avo2::kanMX4 3HA-TOR2 AVO3–13myc*-[*kanMX4*]
SW103–2d	TB50α *avo2::kanMX4 3HA-TOR2 LST8–13myc*-[*kanMX4*]
SW104–3c	TB50a [*kanMX4*]-*GAL1p-LST8 3HA-TOR2 AVO3–13myc*-[*kanMX4*]
SW105–3d	TB50α [*HIS3MX6*]-*GAL1p-TOR2 AVO1–3HA*-[*kanMX4*] *AVO3–13myc*-[*kanMX4*]
SW106–5a	TB50a [*HIS3MX6*]-*GAL1p-TOR2 AVO1–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW107–8c	TB50a [*HIS3MX6*]-*GAL1p-TOR2 AVO3–3HA*-[*kanMX4*] *AVO2–13myc*-[*kanMX4*]
SW108–4a	TB50α [*kanMX4*]-*GAL1p-LST8 3HA-TOR2 AVO2–13myc*-[*kanMX4*]
SW109–3d	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2 LST8–13myc*-[*kanMX4*] *KOG1-TAP*-[*HIS3MX6*]
SW110	TB50a/α *AVO1–3HA*-[*kanMX4*]/*AVO1-TAP*-[*KlTRP1*]
SW111	TB50a/α *AVO2–3HA*-[*kanMX4*]/*AVO2–13myc*-[*kanMX4*]
SW112	TB50a/α *AVO3–3HA*-[*kanMX4*]/*AVO3–13myc*-[*kanMX4*]
SW121–5c	TB50a [*HIS3MX6*]-*GAL1p-AVO3 2myc-TOR2 AVO1–3HA*-[*kanMX4*]
SW123–1c	TB50a [*HIS3MX6*]-*GAL1p-3HA-TOR2*
SW125	TB50a/α *3HA-TOR2 2myc-TOR2*
SW126–1c	TB50a *3HA-TOR2 KOG1-TAP*-[*HIS3MX6*]
SW127–1a	TB50a [*kanMX4*]-*GAL1p-AVO3 3HA-TOR2 KOG1-TAP*-[*HIS3MX6*]
SW128–5a	TB50a [*HIS3MX6*]-*GAL1p-AVO1 3HA-TOR2 KOG1-TAP*-[*HIS3MX6*]
SW129–5c	TB50a [*kanMX4*]-*GAL1p-LST8 3HA-TOR2 KOG1-TAP*-[*HIS3MX6*]
SW133	TB50a/α [*kanMX4*]-*GAL1p-LST8/lst8::kanMX4 3HA-TOR2/2myc-TOR2*
SW137–4b	TB50a [*HIS3MX6*]-*GAL1p-TOR2 tor1::kanMX4 LST8–13myc*-[*kanMX4*]

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The *S. cerevisiae* strains and plasmids used are listed in Tables I and II, respectively. All strains are isogenic derivatives of JK9–3da or TB50a. Rich media, YPD or YPGal/Gly, and synthetic complete media, SD or SGal/Gly, were as described previously (25, 26). Nitrogen starvation experiments were performed with synthetic media as previously described (27). For TOR2, AVO1, AVO3, and LST8 depletion experiments, cells from logarithmically growing SGal/Gly or YPGal/Gly cultures were inoculated into S.D. or YPD medium, respectively. Glucose cultures were incubated with aeration for 15 h before cells were harvested for analysis (7).

Genetic Techniques—Restriction enzyme digests and ligations were performed according to standard methods. All enzymes and buffers were obtained commercially (Roche Diagnostics). *Escherichia coli* strains MH1 and DH5α were used for propagation and isolation of plasmids. Yeast transformation was performed by the lithium acetate procedure (28). PCR cassettes were used to generate gene deletions and modifications as described (29, 30).

Gel Filtration—Yeast extracts from cells expressing HA-tagged TOR2 were prepared as described below. Gel filtration was performed using a Superose 6 HR 10/30 column (Amersham Biosciences) as previously described (7). Elution profiles of tagged TOR2 were analyzed by immunoprecipitation/Western analysis and compared with the elution profile of known standards.

Mammalian Tissue Culture, Transfections, Immunoprecipitation—Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum. Cells were transfected using jetPEI transfection reagent (Qbiogene) following the manufacturer's instructions and harvested after 24 h. Lysate preparation and immunoprecipitation were performed as previously described (12).

Immunoprecipitations and TAP Pulldowns—A 200-ml culture was grown in YPGal/Gly or YPD at 30 °C for 15 h to an A_{600} of 0.8, harvested by centrifugation, and washed with ice-cold water. The pellet was resuspended in 2 ml of lysis buffer (1× phosphate-buffered saline, 10% glycerol, 0.5% Tween 20, plus inhibitors 10 mM NaF, 10 mM NaN_3, 10 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 × Roche complete inhibitor mixture). Cells were lysed by vortexing 5 × 30 s with glass beads using a FastPrep machine (Savant Instruments). Extracts were cleared with a 5-min, 2900 × *g* spin. An aliquot of extract containing 3 mg of protein was adjusted to 1 ml with lysis buffer plus inhibitors. To immunoprecipitate an epitope-tagged protein, 1 μl of either concentrated 12CA5 (anti-HA) or 9E10 (anti-Myc) tissue culture supernatant was added and the tubes were rotated for 1 h at 4 °C.

TABLE II
Plasmids

Plasmids	Description
pHA-TOR2	Expresses HA-tagged TOR2 from TOR2 promoter; pRS314::*3HA-TOR2* (*CEN, TRP1*) (35)
pHA-TOR2-KD	Expresses HA-tagged TOR2 kinase-dead from TOR2 promoter; pRS314::*3HA-TOR2*^{D2298E} (*CEN, TRP1*) (35)
pSW62	Expresses HA-tagged TOR2-(1290–2474) from *TOR2* promoter. Cloned as 4.1-kb SacI-PstI fragment into YEplac195::*TOR2* promoter *ATG-3HA* (*2μ, URA3*)
pSW67	Expresses HA-tagged TOR2-(1–1390) from *TOR2* promoter. Cloned as 4.7-kb BamHI-PstI fragment into YEplac195::*3HA CYC1* terminator (*2μ, URA3*)
pSW75	Expresses HA-tagged LST8 from *LST8* promoter. Cloned as 1.7-kb SacI-PstI fragment into YCplac111::*3HA CYC1* terminator (*CEN, LEU2*)
pAN54	Expresses Myc-tagged TOR2 from *TOR2* promoter; YCplac111::*2myc-TOR2* (*CEN, LEU2*)
pHA-mTOR	Expresses HA-tagged mTOR from the CMV promoter (36)
pmyc-mTOR	Expresses Myc-tagged mTOR from the CMV promoter (8)

Afterward, 20 μl of protein G-Sepharose slurry (Sigma) was added, and the tubes were rotated for an additional 2 h at 4 °C. For TAP pulldowns, 20 μl of IgG-Sepharose 6 fast flow (Amersham Biosciences) was added, and tubes were rotated for 3 h at 4 °C. Beads were collected by centrifugation, washed four times with 1 ml of lysis buffer, and resuspended in 2× SDS-PAGE sample buffer. For coimmunoprecipitations, the sample was split in half and each aliquot was subjected to SDS-PAGE; one aliquot was used for expression control and the other half to detect the coimmunoprecipitated protein. After electrophoresis, the proteins were electroblotted onto nitrocellulose, blocked in 5% milk in 1× phosphate-buffered saline, 0.1% Tween 20, and incubated with primary antibody (12CA5 or 9E10 1:10000 in blocking buffer or anti-Protein A 1:5000 in blocking buffer) overnight at 4 °C. The membranes were washed and incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The tagged proteins were detected using ECL reagents (Amersham Biosciences).

Kinase Assays—Cells expressing HA-TOR2 in different mutant backgrounds maintained on YPD or YPGal/Gly medium were inoculated into YPGal/Gly liquid media and cultured overnight. Per strain (three assays), 3 liters of YPD was inoculated and grown at 30 °C for 15 h to an A_{600} of 0.8 and then chilled on ice for 30 min. Cells were collected by centrifugation, washed once in ice-cold water, and lysed with a Bead Beater (Biospec Products) in ~20 ml of lysis buffer (1× phosphate-buffered saline, 10% glycerol, 0.5% Tween 20 plus inhibitors 10 mM NaF, 10 mM NaN_3, 10 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1× Roche complete inhibitor mixture). The lysate was cleared with a 5-min, 2900 × *g* spin. Lysates were normalized to ~20 ml and ~250 mg of protein per strain (three kinase reactions) and passed over 250 μl of Sepharose CL-4B (Sigma):Protein A-Sepharose (Amersham Biosciences) (3:1) that had been previously equilibrated in lysis buffer. To the flowthrough was added 150 μl of anti-HA cross-linked to Protein A-Sepharose beads. This mixture was rotated for 3 h at 4 °C, after which the beads were collected in a column and washed with 30 ml of lysis buffer. Antibody beads were split equally among three tubes. To each aliquot, 1.5 μg of 4E-BP1 (Stratagene) in 50 μl of kinase buffer (lysis buffer with 20% glycerol) and 6 μl of 10× buffer (40 mM $MnCl_2$, 100 mM dithiothreitol, 10× Roche complete inhibitor mixture-EDTA, 100 mM NaN_3, 100 mM NaF, 100 mM *p*-nitrophenylphosphate, 100 mM β-glycerophosphate) were added. The kinase reaction was started with the addition of 4 μl of ATP mix (1.2 mM ATP, 2.5 μCi/μl [γ-^{32}P]ATP (3000 Ci/mmol) in kinase buffer). Tubes were mixed (1200 rpm) at 30 °C for 10 min. The reactions were terminated with the addition of 15 μl of 5× SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE (5–20%). The top of the gel containing TOR2 was cut and subjected to immunoblotting, while the lower part was Coomassie Blue stained. Radioactivity was quantified using GeneSnap software (SynGene). Kinase reactions using the kinase-dead version of TOR2 were performed as described (19). The activity of immunopurified TORC2 was compared with the activity of mock purified TORC2.

RESULTS

TORC2 Is an Oligomer—Gel filtration of yeast extracts reveals that TORC2 exists as two species, one of 1.5–2 MDa and a second of 0.7–0.8 MDa (7) (Fig. 1*A*). The combined molecular masses of TOR2 and its TORC2 partner proteins yield a mass of 0.78 MDa. These findings suggest that TORC2 may exist in a multimeric state. To determine whether the components of TORC2 indeed interact with themselves, pairwise coimmunoprecipitation experiments were performed with strains expressing heterologously tagged versions of TOR2, AVO1, AVO2, AVO3, or LST8. As shown in Fig. 1*B*, each of the examined components of TORC2 coimmunoprecipitated with its differently tagged copy, suggesting that TORC2 exists in an oligomeric state. Considering the molecular masses of the two species observed by gel filtration, TORC2 most likely forms a dimer.

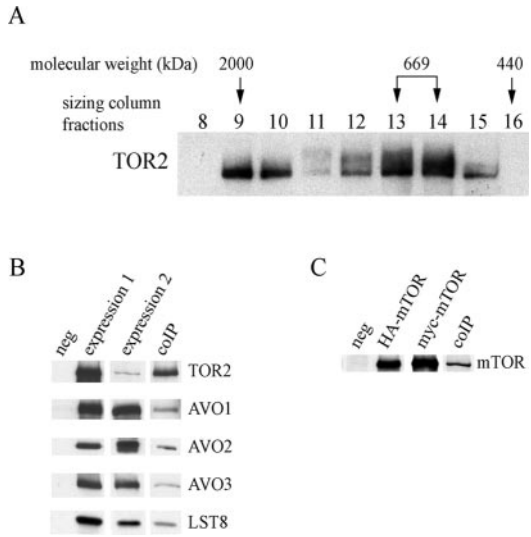

FIG. 1. **TORC2 is an oligomer.** *A*, gel filtration elution profile of TOR2. Clarified extracts prepared from cells expressing HA-TOR2 (SW70) were loaded onto a Superose 6 sizing column. 1-ml fractions were collected, and HA-TOR2 was immunoprecipitated and visualized by immunoblotting. The elution patterns of known molecular mass standards are indicated. *B*, components of TORC2 interact with themselves. Lysates from cells expressing HA-TOR2 and Myc-TOR2 (SW125), AVO1-HA and AVO1-TAP (SW110), AVO2-HA and AVO2-Myc (SW111), AVO3-HA and AVO3-Myc (SW112), or LST8-HA and LST8-Myc (RL74) were subjected to immunoprecipitation with anti-Myc or pull down with IgG-Sepharose and probed with anti-HA to test for coimmunoprecipitation (*coIP*). Expression of HA-tagged proteins (*expression 1*) and Myc- or TAP-tagged proteins (*expression 2*) are shown. As a negative control (*neg*), lysates from cells expressing HA-TOR2 (SW70), AVO1-HA (RL69–1c), AVO2-HA (RL39–1a), AVO3-HA (RL42–1c), or LST8-HA (RL58–1a) were subjected to immunoprecipitation with anti-Myc or pull down with IgG-Sepharose and probed with anti-HA. *C*, mTOR interacts with itself. Human embryonic kidney 293 cells were cotransfected with plasmids expressing HA-mTOR and Myc-mTOR. Protein extracts were prepared, and Myc-mTOR was immunoprecipitated with anti-Myc antibody and probed with anti-HA to test for coimmunoprecipitation of HA-mTOR (*coIP*). Expression of HA- (*HA-mTOR*) and Myc-tagged (*Myc-mTOR*) mTOR are shown. As a negative control (*neg*), lysates from human embryonic kidney 293 cells transfected with empty vector and pHA-mTOR were subjected to immunoprecipitation with anti-Myc and probed with anti-HA.

Nutrient limitation does not affect the integrity of TORC2 as measured by coimmunoprecipitation of different subunits (7).

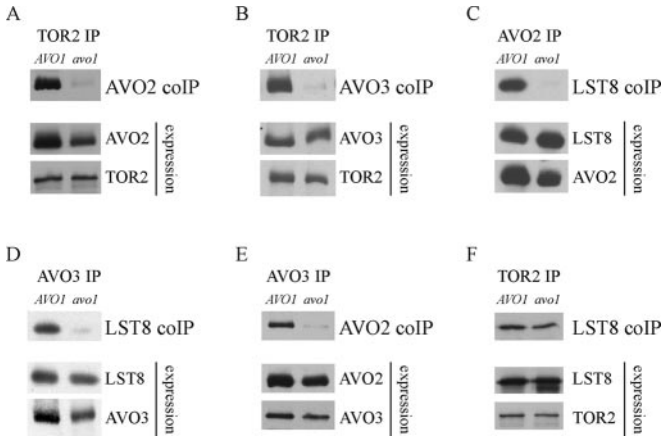

FIG. 2. **AVO1 is important for the structural integrity of TORC2.** Lysates from cells expressing *GAL1p-AVO1* (*AVO1* under control of the galactose promoter) and HA- or Myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-Myc. Cells expressing AVO1 (*AVO1*) (cells grown in galactose medium) were compared with AVO1-depleted cells (*avo1*) (cells grown for 15 h in glucose medium). Immunoprecipitates were probed with anti-Myc or anti-HA to detect a coimmunoprecipitated partner protein (*coIP*, *top panel*). The expression levels of the HA- or Myc-tagged TORC2 components (*expression*) are shown in the *middle* and *bottom panels*. *GAL1pAVO1 HA-TOR2 AVO2-myc* (SW76–5b) (*A*), *GAL1pAVO1 HA-TOR2 AVO3-myc* (SW68–3b) (*B*), *GAL1pAVO1 LST8-HA AVO2-myc* (SW69–4d) (*C*), *GAL1pAVO1 LST8-HA AVO3-myc* (SW74–11b) (*D*), *GAL1pAVO1 AVO3-HA AVO2-myc* (SW73–4b) (*E*), *GAL1pAVO1 HA-TOR2 LST8-myc* (SW75–10c) (*F*).

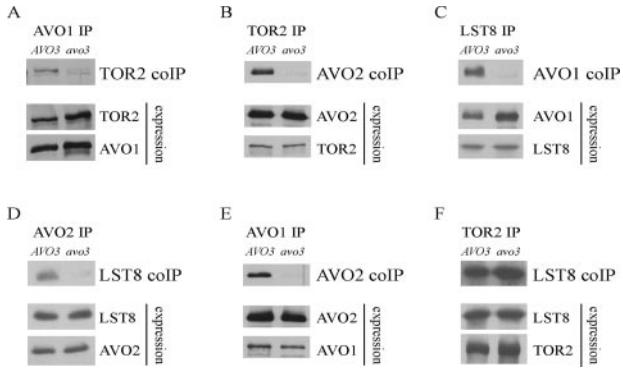

FIG. 3. **AVO3 is important for the structural integrity of TORC2.** Lysates from cells expressing *GAL1p-AVO3* (*AVO3* under control of the galactose promoter) and HA- or Myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-Myc. Cells expressing AVO3 (*AVO3*) (cells grown in galactose medium) were compared with AVO3-depleted cells (*avo3*) (cells grown for 15 h in glucose medium). Immunoprecipitates were probed with anti-Myc or anti-HA to detect a coimmunoprecipitated partner protein (*coIP*, *top panel*). The expression levels of the HA- or Myc-tagged TORC2 components (*expression*) are shown in the *middle* and *bottom panels*. *GAL1pAVO3 AVO1-HA myc-TOR2* (SW121–5c) (*A*), *GAL1pAVO3 HA-TOR2 AVO2-myc* (SW72–5a) (*B*), *GAL1pAVO3 AVO1-HA LST8-myc* (SW67–3b) (*C*), *GAL1pAVO3 LST8-HA AVO2-myc* (SW78–3a) (*D*), *GAL1pAVO3 AVO1-HA AVO2-myc* (SW65–11c) (*E*), *GAL1pAVO3 HA-TOR2 LST8-myc* (SW77–5d) (*F*).

However, this assay would not detect whether TORC2 is regulated by association of TORC2 monomers, as different subunits would remain associated within a monomer. To investigate a possible role of TORC2-TORC2 oligomerization in TORC2 regulation, we examined whether heterologously tagged TOR2 or LST8 coimmunoprecipitated in cells that were starved for nitrogen (60 min). The interaction of Myc-TOR2 with HA-TOR2 or of LST8-Myc with LST8-HA was unaffected by nitrogen starvation (data not shown). Likewise, the molecular mass of TORC2 as determined by gel filtration was not affected by nutrient conditions (data not shown). Thus, TORC2 appears to exist in a dimeric or higher order state that is unaffected by nutrient limitation. However, the signaling cues that regulate TORC2 are unknown. Although ample evidence has been presented that TORC1 is regulated by nutrient cues, TORC2 is only assumed to be regulated in response to nutrient conditions. The significance of TORC2 oligomerization remains to be determined.

To investigate whether mammalian TOR (mTOR) also oligomerizes, we examined whether Myc-mTOR coimmunoprecipitates with HA-mTOR in an extract prepared from human embryonic kidney 293 cells coexpressing the two differently tagged versions of mTOR. Myc- and HA-mTOR efficiently coimmunoprecipitated, indicating that, similar to TOR2 in yeast, mTOR oligomerizes in mammalian cells (Fig. 1*C*). It remains to be determined whether mTOR oligomerizes in mTORC1, mTORC2, or both. Pretreatment of cells with rapamycin (200 nM, 30 min) did not affect the mTOR-mTOR interaction (data not shown).

AVO1 and AVO3 Are Important for the Structural Integrity of TORC2—To study the contribution of TORC2 components to TORC2 architecture, we depleted cells of individual TORC2 components and assayed, by coimmunoprecipitation, the interactions of the remaining subunits. Because most components of TORC2 are essential, we conditionally depleted cells of an essential partner by placing the corresponding gene under the control of the *GAL1* promoter, which is active in galactose- and inactive in glucose-containing media. After growing cells in glucose medium for 15 h, the protein under control of the *GAL1* promoter was no longer detectable by immunoblotting (data not shown). Cells depleted for a protein are referred to as mutant cells. The expression levels of the analyzed, remaining proteins did not vary upon depletion of any single TORC2 component.

To investigate the role of AVO1 in the architecture of TORC2, interactions between the remaining TORC2 proteins were analyzed in an *avo1* mutant. As shown in Fig. 2, upon depletion of AVO1, AVO2 and AVO3 no longer interacted with TOR2 (Fig. 2, *A* and *B*), LST8 (Fig. 2, *C* and *D*), or with each other (Fig. 2*E*). Conversely, TOR2 and LST8 still interacted in the absence of AVO1 (Fig. 2*F*). Because TOR2 and LST8 are also components of TORC1-B, coimmunoprecipitation of these two proteins could reflect an interaction within TORC1-B and could thus be misleading. To determine whether the interaction between TOR2 and LST8 in *avo1* cells was due to contamination by TORC1-B, cell extracts were depleted of TORC1 by precipitating the TORC1-specific component KOG1. TAP-tagged KOG1 was precipitated by incubating the cell extracts with IgG-Sepharose prior to the coimmunoprecipitation experiment. When TORC1 was depleted, TOR2 still associated with LST8 in the absence of AVO1 (data not shown), indicating that this interaction was indeed TORC1-B-independent. Furthermore, TOR2 was found primarily in TORC2 (7) (data not shown). Thus, AVO1 is required for the binding of AVO2 and AVO3 to TOR2, LST8, and to each other but is not required for the interaction between TOR2 and LST8.

Cells depleted of AVO3 showed similar defects in TORC2 integrity as described above. In *avo3* mutant cells, AVO1 and AVO2 no longer associated with TOR2 (Fig. 3, *A* and *B*), LST8 (Fig. 3, *C* and *D*), or with each other (Fig. 3*E*). TOR2 still interacted with LST8 in the absence of AVO3 (Fig. 3*F*). The above observations combined with the fact that AVO2 is nonessential suggest that AVO2 is a peripheral protein that binds TOR2 via AVO1 and AVO3, although we cannot exclude the possibility that AVO1 and AVO3 cause a conformational change in TOR2 that allows AVO2 to bind directly to TOR2. Consistent with these models, AVO3 still interacted with TOR2 in an *avo2* mutant (Fig. 4*A*). Furthermore, deletion of *AVO2* did

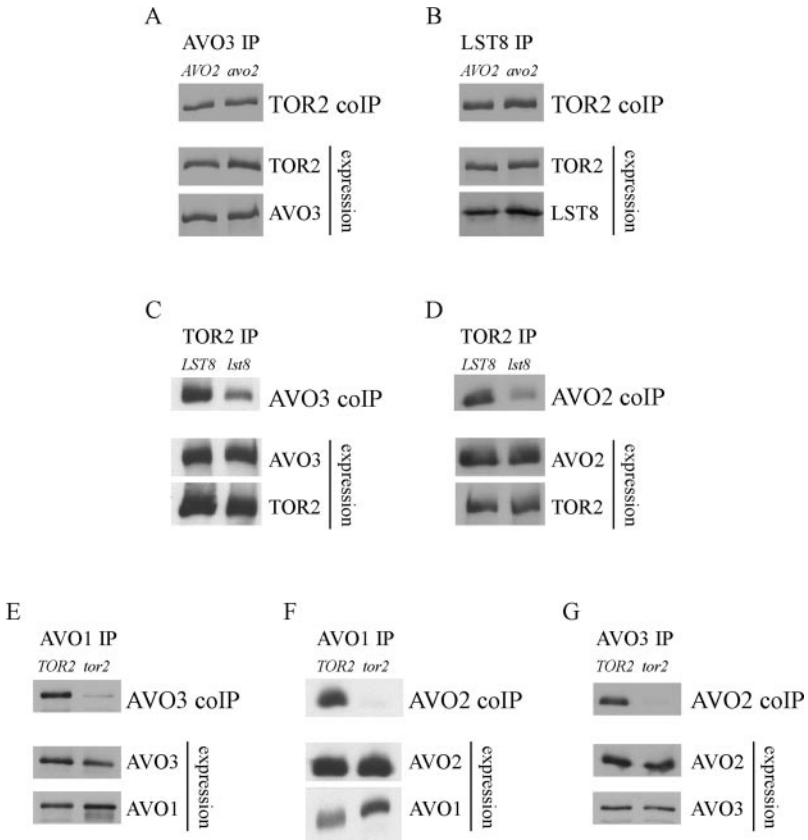

FIG. 4. **Role of AVO2, LST8, and TOR2 in the integrity of TORC2.** *A* and *B*, AVO2 is not required for AVO3 and LST8 to interact with TOR2. Lysates from wild-type (*AVO2*) or *avo2* mutant cells (*avo2*) expressing HA- or Myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-Myc. Immunoprecipitates were probed with anti-HA to detect a coimmunoprecipitated partner protein (*coIP*, *top panel*). The expression levels of the HA- or Myc-tagged TORC2 components (*expression*) are shown in the *middle* and *bottom panels*. *C* and *D*, depletion of LST8 destabilizes the interaction between AVO3 and AVO2 with TOR2. Lysates from cells expressing *GAL1p-LST8* (*LST8* under control of the galactose promoter) and HA- or Myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-Myc. Cells expressing LST8 (*LST8*) (cells grown in galactose medium) were compared with LST8-depleted cells (*lst8*) (cells grown for 15 h in glucose medium). Immunoprecipitates were probed with anti-Myc to detect a coimmunoprecipitated partner protein (*coIP*, *top panel*). The expression levels of the HA- or Myc-tagged TORC2 components (*expression*) are shown in the *middle* and *bottom panels*. *E–G*, TOR2 is required for the interaction between AVO1, AVO2, and AVO3. Lysates from cells expressing *GAL1p-TOR2* (*TOR2* under control of the galactose promoter) and HA- or Myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-Myc. Cells expressing TOR2 (*TOR2*) (cells grown in galactose medium) were compared with TOR2-depleted cells (*tor2*) (cells grown for 15 h in glucose medium). Immunoprecipitates were probed with anti-Myc to detect a coimmunoprecipitated partner protein (*coIP*, *top panel*). The expression levels of the HA- or Myc-tagged TORC2 components (*expression*) are shown in the *middle* and *bottom panels*. *HA-TOR2 AVO3-myc* (SW62–7a), *avo2 HA-TOR2 AVO3-myc* (SW102–8b) (*A*), *HA-TOR2 LST8-myc* (SW63–1d), *avo2 HA-TOR2 LST8-myc* (SW103–2d) (*B*), *GAL1pLST8 HA-TOR2 AVO3-myc* (SW104–3c) (*C*), *GAL1pLST8 HA-TOR2 AVO2-myc* (SW108–4a) (*D*), *GAL1pTOR2 AVO1-HA AVO3-myc* (SW105–3d) (*E*), *GAL1pTOR2 AVO1-HA AVO2-myc* (SW106–5a) (*F*), *GAL1pTOR2 AVO3-HA AVO2-myc* (SW107–8c) (*G*).

not affect the interaction between TOR2 and LST8 (Fig. 4*B*). Removal of LST8 destabilized but did not abolish the TOR2-AVO3 or the TOR2-AVO2 interaction (Fig. 4, *C* and *D*). Thus, AVO1 and AVO3 bind TOR2 cooperatively and are particularly important for the integrity of TORC2.

To investigate whether AVO1, AVO2, and AVO3 form a complex independent of TOR2, pairwise interactions between AVO1, AVO2, and AVO3 were examined in TOR2-depleted cells. As shown in Fig. 4, *E–G*, AVO1-AVO3 was significantly destabilized and the AVO1-AVO2 and AVO2-AVO3 interactions were abolished in TOR2-depleted cells. Thus, AVO1, AVO2, and AVO3 fail to form a complex in the absence of TOR2. In summary, AVO1, AVO3, and TOR2 are interdependent members of a TORC2 core complex. LST8 binds TOR2 independently of AVO1, AVO2, and AVO3. AVO2 is likely a peripheral protein associated with AVO1 and AVO3.

LST8 Binds to the C-terminal Kinase Domain Region in TOR2, and AVO3 Associates with the N-terminal HEAT Repeat Region in TOR2—To identify the regions in TOR2 that interact with AVO1, AVO3, and LST8, coimmunoprecipitation experiments were performed with plasmid-encoded deletion variants of TOR2. HA-tagged deletion variants of TOR2 were expressed in strains containing Myc-tagged partner proteins. LST8 interacted with the C-terminal half of TOR2 containing the FAT, FKBP-rapamycin binding, kinase, and FATC domains, but not with the N-terminal half of TOR2 containing the HEAT repeats (Fig. 5*A*). The binding of AVO1 and AVO3 to TOR2 appeared to be more complex as we failed to detect an interaction of these two proteins with either the N-terminal or the C-terminal half of TOR2. Because this could be due to interference by chromosomally encoded wild-type TOR2, wild-type TOR2 was depleted using a strain that contained chromosomal *TOR2* under the control of the *GAL1* promoter. In cells grown in glucose medium, we observed a weak interaction between AVO3 and the N-terminal half of TOR2 consisting of the HEAT repeats, but not between AVO3 and the C-terminal half of TOR2 (Fig. 5*B*). Using this approach, we did not detect an interaction between AVO1 and either of the TOR2 deletion variants (data not shown). AVO1 might bind, via multiple contacts, to various domains in TOR2. In summary, AVO3 seems to associate with the HEAT repeats of TOR2, whereas LST8 avidly binds the C-terminal half of TOR2.

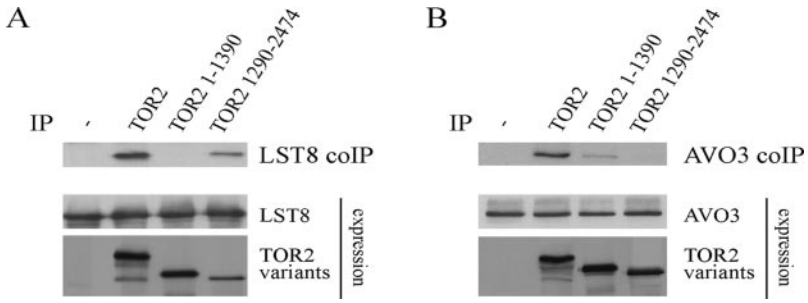

FIG. 5. **AVO3 and LST8 bind the N- and C-terminal regions of TOR2, respectively.** *A*, LST8 binds to the C-terminal kinase domain region in TOR2. Cells expressing Myc-tagged LST8 (RL59–2d) were transformed with empty vector (−) or plasmids expressing HA-tagged TOR2 (*TOR2*, pHA-TOR2), the N-terminal half of HA-tagged TOR2 (*TOR2-(1–1390)*, pSW67), or the C-terminal half of HA-tagged TOR2 (*TOR2-(1290–2474)*, pSW62). Protein lysates were prepared and immunoprecipitated with anti-HA. Immunoprecipitates were probed with anti-Myc to detect coimmunoprecipitated LST8 (*coIP*, *top panel*). The expression levels of LST8 and TOR2 variants (*expression*) are shown in the *middle* and *bottom panels*. *B*, AVO3 associates with the N-terminal HEAT repeat region in TOR2. Cells expressing *GAL1p-TOR2* (*TOR2* under control of the galactose promoter) and Myc-tagged AVO3 (SW94–1a) were transformed with empty vector (−) or plasmids expressing HA-tagged TOR2 (*TOR2*, pHA-TOR2), the N-terminal half of HA-tagged TOR2 (*TOR2-(1–1390)*, pSW67), or the C-terminal half of HA-tagged TOR2 (*TOR2-(1290–2474)*, pSW62). Cells were grown for 15 h in glucose medium (to repress expression of genomic *TOR2*). Protein lysates were prepared and immunoprecipitated with anti-HA. Immunoprecipitates were probed with anti-Myc to detect coimmunoprecipitated AVO3 (*coIP*, *top panel*). The expression levels of AVO3 and TOR2 variants (*expression*) are shown in the *middle* and *bottom panels*.

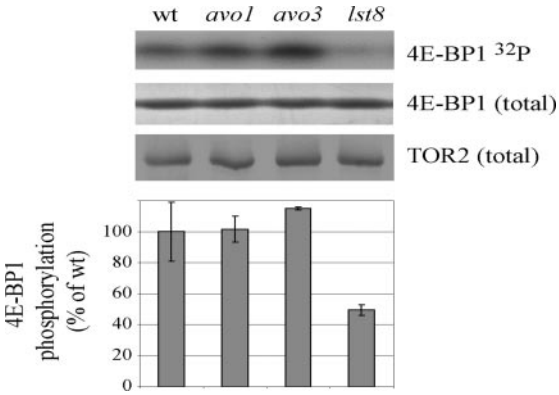

FIG. 6. **LST8, but not AVO1 or AVO3, is required for TOR2 kinase activity.** Wild-type cells expressing HA-TOR2 (SW70) and cells expressing *GAL1pAVO1* (SW84–1d), *GAL1pAVO3* (SW72–5a), or *GAL1pLST8* (SW100–1a) and HA-TOR2 were grown in glucose medium for 15 h. HA-TOR2 was immunoprecipitated from lysates of wild-type cells (*WT*) or AVO1 (*avo1*)-, AVO3 (*avo3*)-, or LST8 (*lst8*)-depleted cells and tested for 4E-BP1 kinase activity. The autoradiograph shows 4E-BP1 phosphorylation (*4E-BP1 ^{32}P*). Coomassie Blue-stained total 4E-BP1 protein and the immunoblot of total HA-tagged TOR2 protein are also depicted. 4E-BP1 phosphorylation was normalized to the amount of TOR2 added to the respective kinase assay and graphed. Kinase reactions were performed in triplicate; one of three independent experiments is shown.

LST8, but Not AVO1 or AVO3, Is Required for TOR2 Kinase Activity—To investigate the contribution of AVO1, AVO3, and LST8 to TORC2 kinase activity, the activity of immunopurified TORC2 lacking AVO1, AVO3, or LST8 was determined in an *in vitro* kinase assay using 4E-BP1 as a substrate (see "Experimental Procedures"). 4E-BP1, a physiological substrate for mTORC1, is unstructured and a nonphysiological substrate for a number of unrelated kinases, including TORC2 (19). The activity of TOR2 toward 4E-BP1 was determined by performing kinase reactions with immunopurified TOR2. The phosphorylation was normalized to the amount of TOR2 present in the kinase reaction as determined by immunoblotting. In wild-type and AVO1-, AVO3-, or LST8-depleted cells, the rate of 4E-BP1 phosphorylation was linear up to 30 min of incubation time (data not shown). Kinase activity of TOR2 immunopurified from *avo1* or *avo3* mutant cells was not significantly altered compared with TOR2 purified from wild-type cells (Fig. 6). However, TOR2 isolated from LST8-depleted cells exhibited 50% less kinase activity compared with TOR2 purified from wild-type cells. To exclude possible contamination by TORC1-B, which could also be present in the TOR2 immunoprecipitate, extracts were depleted of TORC1 as described above, prior to immunopurification of TOR2. Depletion of TORC1 did not significantly alter the results (data not shown), indicating that the TOR2-associated kinase activity was primarily, if not exclusively, TORC2 mediated. TOR2 is primarily associated with TORC2 (7).

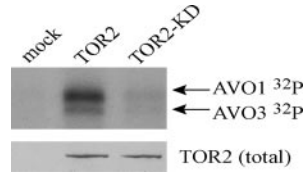

FIG. 7. **TORC2 autophosphorylates sites in AVO1 and AVO3.** Cells expressing *GAL1pTOR2* and TAP-tagged *AVO2* (SW80–1d) were transformed with empty vector (*mock*) or plasmids expressing HA-tagged TOR2 (*TOR2*, pHA-TOR2), or HA-tagged kinase-dead TOR2 (*TOR2-KD*, pHA-TOR2-KD). Cells were grown for 15 h in glucose medium (to repress expression of genomic *TOR2*). TORC2 was isolated via TAP-tagged AVO2 using IgG-Sepharose beads and subjected to *in vitro* kinase assays. The autoradiograph shows AVO1 and AVO3 phosphorylation (*AVO1 ^{32}P* and *AVO3 ^{32}P*). Total HA-tagged TOR2/TOR2-KD protein visualized by immunoblotting is also depicted.

Because AVO1 or AVO3 depletion, which results in the disruption of TORC2 integrity, did not change TOR2-mediated phosphorylation of 4E-BP1, we conclude that AVO1 and AVO3 are not required for intrinsic TORC2 catalytic activity. In contrast, LST8 depletion caused a decrease in 4E-BP1 phosphorylation, suggesting that LST8 may modulate TOR2 kinase activity. These effects on kinase activity are consistent with the above findings that LST8 binds TOR2 in or around the C-terminal kinase domain, whereas AVO1 and AVO3 bind the N-terminal region of TOR2.

TORC2 Autophosphorylates Sites in AVO1 and AVO3—AVO1 and AVO3 are phosphorylated during an *in vitro* kinase assay with purified TORC2 (Fig. 7). To determine whether this is autophosphorylation by TORC2 or phosphorylation by a contaminating kinase, an *in vitro* kinase assay was performed with TORC2 containing a kinase-dead version of TOR2 (TOR2-KD). Kinase-dead TORC2 was isolated from cells after turning off expression of the endogenous wild-type copy of TOR2 as described previously (19). Kinase-dead TORC2 failed to phosphorylate AVO1 and AVO3 (Fig. 7), suggesting that TORC2 autophosphorylates sites in AVO1 and AVO3. Mass spectro-

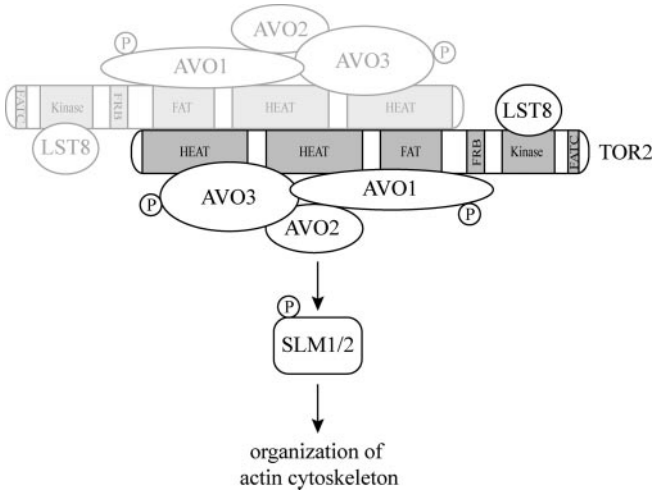

FIG. 8. **Model of TORC2 architecture.** TORC2 is oligomeric, likely a TORC2-TORC2 dimer. AVO1 and AVO3 bind cooperatively to the HEAT repeat region in TOR2 and mediate binding of AVO2 to the complex. AVO2 is a peripheral protein and might serve as adaptor protein for TORC2 substrates, such as SLM1 and SLM2, which signal to the actin cytoskeleton. LST8 binds to the C-terminal kinase domain region in TOR2 and appears to modulate the intrinsic kinase activity of TOR2. TORC2 autophosphorylates sites in AVO1 and AVO3.

metric analysis of isolated TORC2 indicates that AVO1 and AVO3 are also phosphorylated *in vivo* on many sites.[2] The significance of AVO1 and AVO3 phosphorylation remains to be determined. AVO1 and AVO3 phosphorylation could be important for the regulation of TORC2 kinase activity or for mediating signaling to downstream effectors. Interestingly, isolations of wild-type and kinase-dead TORC2 routinely yielded similar amounts of the two complexes (Fig. 7), suggesting that the kinase activity of TOR2 is not required for TORC2 integrity.

DISCUSSION

We have analyzed the molecular interactions and functions of the proteins within TORC2. A model summarizing our findings on the molecular organization of TORC2 is shown in Fig. 8. The protein-protein interactions in TORC2 appear to be complex. The assembly of a given protein into TORC2 may involve binding to multiple partner proteins through various domains. This may account for the observation that directed two-hybrid binding assays failed to detect an interaction between any two components of TORC2 other than between AVO1 and the N-terminal region of AVO3 (data not shown). Furthermore, upon depletion of AVO1, AVO3, or TOR2, the interactions of the remaining two proteins with each other in any combination or with the nonessential peripheral protein AVO2 were abolished or significantly destabilized. AVO1 and AVO3 may bind cooperatively to the N-terminal HEAT repeat region of TOR2 to form a core complex that then associates with AVO2 and possibly BIT61. AVO1 and AVO3 thus perform a scaffold-like function. Although in our model we show that both AVO1 and AVO3 bind directly to TOR2, we cannot firmly conclude that their cooperative binding to TOR2 involves direct contacts between TOR2 and both AVO1 and AVO3. AVO2 may act as an adaptor protein for TORC2 substrates such as SLM1 and SLM2 (19). Our model suggests that the rapamycin resistance of TORC2 could be due to AVO1 masking the FKBP-rapamycin binding site in TOR2. This is based on the observations that AVO1, unlike AVO3, is unable to bind an isolated N-terminal fragment of TOR2 and that LST8 does not prevent FKBP-rapamycin binding in TORC1. LST8 binds to the C-terminal kinase domain region of TOR2 independently of AVO1, AVO2, and AVO3 and unlike the AVOs is required for the intrinsic kinase activity of TOR2. Our model on the organization of TORC2 is consistent with previous studies on TOR1-TOR2 and TOR2-TOR1 hybrids suggesting that TOR1 and TOR2 are functionally different based on their amino terminus (31).

LST8 is a subunit of both TORC1 and TORC2 and acts positively with TOR in each complex (7, 20, 32), suggesting that LST8 may perform a similar function(s) in TORC1 and TORC2. Our data suggest that LST8 bound to the C-terminal kinase domain region of TOR2 is required for intrinsic TOR2 kinase activity and for stability of TORC2. This is consistent with the function of mLST8 (mammalian ortholog of LST8; also known as GβL) in mammalian cells (7, 10). mLST8 binds to the kinase domain region of mTOR, and overexpression of mLST8 stimulates mTOR kinase activity, underscoring a conserved role for LST8 in modulation of TOR kinase activity (10). Furthermore, mLST8 stabilizes the interaction between mTOR and raptor in mTORC1 (10). LST8 was originally identified in a synthetic lethal screen with *sec13* and was shown to be required for transport of the general amino acid permease GAP1 from Golgi to the plasma membrane (23). However, the effect of LST8 on GAP1 sorting appears to be an indirect consequence of LST8 acting in the TOR signaling pathway (26, 32, 33). Furthermore, TOR1 is still associated with internal membranes in a *lst8* mutant, indicating that LST8 is not required for localization of at least TOR1 to membranes (32).

We have shown that TORC2 is oligomeric, most likely a TORC2-TORC2 dimer. The oligomerization of TORC2 accounts for the high molecular mass (1.5–2 MDa) of the complex. Oligomerization of TORC2 likely occurs via at least TOR2 because the self-association of LST8 (*i.e.* LST8-LST8) is disrupted in cells depleted for TOR2, and TOR2 still self-associates in AVO3- or LST8-depleted cells.[3] ATM, like TOR a member of the phosphatidylinositol kinase-related protein kinase family, is regulated by dimerization (34). By analogy, oligomerization of TORC2, and possibly TORC1, could also be an important mechanism for the regulation of TOR activity. Consistent with this notion, we find that only the high molecular mass, oligomeric form of TORC2 (Fig. 1*A*) exhibits kinase activity as assayed by 4E-BP1 phosphorylation.[3] However, until the upstream cues that regulate TORC2 are defined, the role of oligomerization in TORC2 regulation remains to be determined. The role of oligomerization may be conserved as mTOR is also oligomeric. We anticipate that the overall molecular organization of TORC2 is conserved.

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Molecular Organization of Target of Rapamycin Complex 2

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