Regulation of mTORC1 by the Rab and Arf GTPases

The mammalian target of rapamycin (mTOR) is a key cell growth regulator, which forms two distinct functional complexes (mTORC1 and mTORC2). mTORC1, which is directly inhibited by rapamycin, promotes cell growth by stimulating protein synthesis and inhibiting autophagy. mTORC1 is regulated by a wide range of extra- and intracellular signals, including growth factors, nutrients, and energy levels. Precise regulation of mTORC1 is important for normal cellular physiology and development, and dysregulation of mTORC1 contributes to hypertrophy and tumorigenesis. In this study, we screened Drosophila small GTPases for their function in TORC1 regulation and found that TORC1 activity is regulated by members of the Rab and Arf family GTPases, which are key regulators of intracellular vesicle trafficking. In mammalian cells, uncontrolled activation of Rab5 and Arf1 strongly inhibit mTORC1 activity. Interestingly, the effect of Rab5 and Arf1 on mTORC1 is specific to amino acid stimulation, whereas glucose-induced mTORC1 activation is not blocked by Rab5 or Arf1. Similarly, active Rab5 selectively inhibits mTORC1 activation by Rag GTPases, which are involved in amino acid signaling, but does not inhibit the effect of Rheb, which directly binds and activates mTORC1. Our data demonstrate a key role of Rab and Arf family small GTPases and intracellular trafficking in mTORC1 activation, particularly in response to amino acids.

The target of rapamycin (TOR) protein kinase regulates cell growth and cell size. The function of TOR in promoting cell growth is conserved from yeast to mammals. TOR forms two structurally and functionally distinct complexes, TORC1 and TORC2. Only TORC1, but not TORC2, is directly inhibited by rapamycin. The mammalian TORC1, mTORC1, directly phosphorylates and activates the ribosomal S6 kinase, S6K. Because direct kinase assay of mTORC1 is rather challenging, phosphorylation of S6K is the most frequently used readout for mTORC1 activation.

As a key cell growth regulator, TORC1 activity is tightly regulated by mitogenic growth factors, the availability of amino acids, and cellular ATP levels. Extensive studies have elucidated the signaling mechanisms of growth factors and cellular energy levels in mTORC1 activation. The TSC1 and TSC2 tumor suppressors are key upstream negative regulators of mTORC1. TSC1 and TSC2 form a complex and function as a GTPase-activating protein (GAP) to inhibit the Rheb GTPase, which can directly bind to and activate mTORC1. AKT, which is a protein kinase activated by numerous growth-stimulating signals, such as growth factors, can phosphorylate and inhibit TSC2 and thereby relieve the inhibitory effect of TSC2 on mTORC1.

Amino acids are the most potent stimulator of mTORC1. In the absence of amino acids, mTORC1 activation by insulin is severely compromised. We have recently shown that the Rag family GTPases play an essential role in mTORC1 activation in response to amino acid stimulation. In mammalian cells, the RagA or RagB forms heterodimers with either RagC or RagD, and the resulting heterodimers strongly bind to raptor in a manner depending on GTP binding of RagA or RagB. It has been suggested that amino acids modulate the subcellular translocation of mTORC1 through Rag GTPases. These results indicate that intracellular trafficking may be important in mTORC1 regulation. In this report, we demonstrate that members of the Rab and Arf family GTPases play important roles in mTORC1 activation.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Plasmids, and Chemicals—Anti-Drosophila S6 kinase antibody was provided by Mary Stewart (North Dakota State University, Fargo, ND). Anti-phospho Drosophila S6K, anti-S6K, anti-phospho S6K, anti-Akt, anti-phospho Akt, and anti-phospho 4EBP1 antibodies were from Cell Signaling. Anti-Myc, anti-HA, and anti-FLAG antibodies were from Santa Cruz Biotechnology, Covance, and Sigma, respectively. RagA/C constructs were made as described previously. Rab5, Rab7, Rab10, Rab11A, Rab22, Rab31, and Ran constructs were ob-
tained from Drs. X. Chen and A. Saltiel (University of Michigan). Rab5A was subcloned into pBABE-puro retroviral vector. All other DNA constructs, including HA-S6K, Myc-dEBP1, GST-Akt, and Myc-Rheb, were from laboratory stock. Insulin and brefeldin A were obtained from Sigma.

Cell Culture—Drosophila S2 cells (Invitrogen) were cultured in Drosophila serum-free medium (Invitrogen) supplemented with 18 mM l-glutamine and maintained at 28 °C. HEK293 cells and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. PC3 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum. Amino acid-containing or -free medium used for Drosophila S2 cells were made using Schneider’s Drosophila medium (Invitrogen) formulation as described previously (15). Amino acid-containing (DMEMK) or amino acid-free (DMEMK-AA) media used for HEK293 and HeLa cells were made using DMEM medium (Invitrogen, catalog number 12430) formulation.

RNA Interference—Drosophila RNA interference (RNAi) experiments were performed as described previously (17).

Transfection and Cell Lysis—Transfection was performed in serum-free conditions using Lipofectamine reagent (Invitrogen) as described by the manufacturer. Cells were lysed in SDS lysis buffer (1% SDS, 0.1 M Tris, pH 7.5).

Drosophila Genetics and Histology—Clonal knockdown of Arf1 in larval fat body cells was performed using the double-stranded RNA UAS line GD12522 from the Vienna Drosophila RNAi Center. This line was co-expressed with UAS-dicer to increase RNAi efficiency. Spontaneous flippase-mediated induction of GFP-marked cells and staining with Texas Red-phalloidin and LysoTracker Red (Invitrogen) were performed as described previously (15). Cell area measurements were determined from confocal images of fixed fat body tissues using Adobe Photoshop, as described previously (15). mCherry-Atg8a was expressed and analyzed as described previously (18).

RESULTS AND DISCUSSION

Knockdown of Rab and Arf Decreases TORC1 Activity in Drosophila S2 Cells—To search for GTases that may regulate TORC1, we used RNAi to knock down all putative small GTases predicted in the Drosophila genome. Examples of GTase knockdown on dS6K phosphorylation are shown in Fig. 1. Besides Ras, Rheb, and Rag, which are known to regulate TORC1, knockdown of several other small GTases also significantly decreased dS6K phosphorylation (Fig. 1). Ran GTase is essential for nuclear import and export (19). In cells treated with double-stranded RNA against Ran, both dS6K protein and phosphorylation were decreased dramatically, possibly due to a reduction of total cell numbers. However, the remaining dS6K displayed a fast migration, indicating that dS6K was dephosphorylated in Ran knockdown S2 cells.

We found that knockdown of Rab5 and Rab11 similarly decreased both dS6K protein and phosphorylation (Fig. 1). Due to a dramatic reduction in dS6K protein, it is unclear whether the relative dS6K phosphorylation was decreased by knockdown of these two GTases. However, knockdown of Rab1 caused a significant decrease of dS6K phosphorylation as indicated by both the phosphorylation-specific antibody and an increased electrophoretic mobility. Similar results were observed with Arf1 knockdown, and the effects of Rab1 and Arf1 knockdown on dS6K phosphorylation were similar to that caused by RagA knockdown. These results strongly indicate that Rab1 and Arf1 are important for TORC1 activity in Drosophila S2 cells.

Constitutive Activation of Rab Inhibits mTORC1—In mammalian cells, there are large numbers of Rab and Arf family GTases that often have overlapping functions in intracellular trafficking (20, 21). The data from Drosophila S2 cells indicate that intracellular vesicle trafficking may play a key role in TORC1 activation. To test this possibility, we overexpressed several constitutively active Rab GTases in 293 cells to determine their effect on mTORC1 in mammalian cells. As positive controls, expression of either constitutive RagALQ or RhebL64 increased S6K phosphorylation (Fig. 2A). We observed that expression of active mutants of Rab5, Rab7, Rab10, and Rab31 potently inhibited S6K phosphorylation. In contrast, expression of active Rab11 had little effect, whereas Rab22 had a minor inhibitory effect on S6K phosphorylation. The inhibitory effect of the Rab GTases on S6K phosphorylation was surprising because knockdown of these GTases in Drosophila S2 cells also decreased dS6K phosphorylation. However, it is well documented that disruption of GTP cycling of Rab proteins can disrupt normal cellular trafficking (20, 22). Our data suggest that normal intracellular trafficking is critical for proper mTORC1 activation.

To further elucidate the effect of Rab proteins on mTORC1, we picked Rab5 as a representative for further characterization. We tested the effects of wild type, constitutively active, and dominant negative Rab5 on the phosphorylation of S6K.

FIGURE 1. Rab and Arf proteins are indispensable in regulating TORC1 activity in Drosophila S2 cells. Drosophila S2 cells untreated (lane 1) or treated with the double-stranded RNA against individual genes (as indicated by the Drosophila genome CG numbers) were starved of amino acids for 1 h followed by amino acid stimulation for 30 min. Phosphorylation and protein levels of dS6K were determined by immunoblotting with the indicated antibodies. Signals detected by anti-phospho-dS6K (pdS6K) and anti-dS6K were quantified, and the ratio was calculated. The control ratio is set to be 1, and all other ratios are the comparison with the control. — means protein signal not detectable.
Several mammalian Rab proteins inhibit mTORC1 activity. A, constitutively active Rab proteins inhibit S6K phosphorylation. Each mammalian Rab construct was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. B, Rab5 regulates TORC1 but not TORC2 activity. Each indicated Rab protein construct was co-transfected with HA-S6K, Myc-4EBP1, or GST-Akt. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. Rab5WT, wild type Rab5. C, Rab5 regulates TORC1 activity in PC3 cells. PC3 cells stably express pBABE-puro vector; corresponding Rab5 constructs were generated, and the protein and phosphorylation levels were determined by immunoblotting with the indicated antibodies. AA+, amino acid stimulation; AA−, amino acid starvation. D, the effect of Rab5 on mTORC1 is dependent on its membrane localization and effector binding. Each Rab5 mutant construct was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. E, GAPex overexpression inhibits S6K phosphorylation. Either Rab5 or GAPex construct was transfected into HEK293 cells together with HA-S6K. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

and 4EBP1, two direct substrates of mTORC1 (23). We found that both the wild type and the constitutively active Rab5 strongly inhibited the phosphorylation of co-transfected S6K and 4EBP1, although the Rab5QL was more potent (Fig. 2B). The dominant negative Rab5TN also inhibited S6K phosphorylation but was significantly less effective than the active Rab5WT. In addition, the endogenous S6K phosphorylation was also inhibited by either Rab5QL or Rab5TN overexpression (supplemental Fig. S1). These data support a notion that too much or too little Rab5 activity inhibits mTORC1, suggesting that continuous intracellular trafficking is important for mTORC1 activation. We also determined the effect of Rab5 on AKT phosphorylation, which is phosphorylated by mTORC2 but not mTORC1 (24), and found that neither the constitutively active nor the dominant negative Rab5 affected AKT phosphorylation (Fig. 2B, supplemental Fig. S1), indicating that the effect of Rab5 is specific on mTORC1. In PC3 cells, which have elevated phosphatidylinositol 3-kinase pathway activity, Rab5 overexpression still inhibited S6K phosphorylation (Fig. 2C).

We tested whether Rab5 interacts with mTORC1 by co-immunoprecipitation. Our data showed that Rab5 could not co-immunoprecipitate with either Raptor or mTOR, whereas the positive control RagA-RagC interacted with both (supplemental Fig. S2). Immunofluorescence of transfected Rab5 showed that a significant fraction of endogenous mTOR co-localized with Rab5 (supplemental Fig. S3), indicating that Rab5 and mTOR can exist in the same subcellular compartment.

We then examined the importance of Rab5 membrane localization, which is required for its function in vesicle trafficking (25), in mTORC1 inhibition. Expression of the Rab5QL-2CS, which has the two C-terminal membrane-targeting essential cysteines substituted by serines and not associated with membrane (supplemental Fig. S4), failed to inhibit S6K phosphorylation (Fig. 2D), supporting that membrane localization is essential for Rab5 to influence mTORC1 activity. Next, we tested the importance of Rab5 signaling in mTORC1 inhibition. Mutations of Phe-57, Trp-74, or Tyr-89 of Rab5 are known to compromise Rab5 signaling in vivo (26). Our data showed that mutations of these residues abolished the ability of Rab5 to inhibit mTORC1 (Fig. 2D). Together, these observations further support the notion that a proper Rab5 signaling is important for physiological mTORC1 regulation.

To further investigate a role of Rab5 in mTORC1 regulation, GAPex, a guanine nucleotide exchange factor for Rab5 and Rab31 (27), was co-expressed with S6K1. We reasoned that hyper-activation of endogenous Rab5 might also inhibit mTORC1. Indeed, we observed that GAPex overexpression decreased S6K1 phosphorylation (Fig. 2E). The above data are consistent with a critical role of endogenous Rab5 and Rab31 in mTORC1 regulation.

Rab5 Specifically Affects Amino Acid- or Rag-induced but Not Glucose- or Rheb-induced mTORC1 Activation—It has been proposed that amino acids activate TORC1 possibly by affecting intracellular mTORC1 localization through Rag GTPases (16). We tested the effect of Rab5 on mTORC1 activation by amino acids. Co-expression of Rab5QL inhibited S6K phosphorylation in response to amino acid stimulation (Fig. 3A). In contrast to the amino acid stimulation, Rab5QL had no inhibitory effect on S6K phosphorylation when cells were stimulated by glucose (Fig. 3B). These results show that the effect of Rab5 is pathway-specific and consistent with the model that amino acids stimulate mTORC1 activation by regulating its intracellular localization.

Previous studies have shown that transient overexpression of Rheb-induced mTORC1 activation is insensitive to amino acid deprivation, whereas Rag mediates amino acid signals to regulate mTORC1 localization (16). We tested the relationship between Rab5 and RagA or Rheb. Co-expression of Rab5QL strongly inhib-
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|   | Rab5QL | Arf1QL |
|---|--------|--------|
| A | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| B | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |

**FIGURE 3.** The active Rab5QL blocks amino acid-stimulated but not glucose- or Rheb-induced mTORC1 activation. A, Rab5 inhibits mTORC1 activity induced by amino acids. Rab5 construct was co-transfected with HA-S6K into HEK293 cells. Cells were starved for amino acids (AA−) for 1 h followed by amino acid stimulation (AA+) for 30 min before harvesting. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. B, Rab5 does not affect glucose-induced mTORC1 activity. Rab5 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived of glucose for 1 h followed by glucose stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. C, Rab5 blocks Rag- but not Rheb-induced S6K phosphorylation. Rab5 was transfected into HEK293 cells with or without RagA/C or Rheb construct as indicated. S6K was included in the co-transfection. Phosphorylation and protein levels of the transfected proteins were determined by immunoblotting with the indicated antibodies.

**FIGURE 4.** Regulation of TORC1 by Arf1. A, Arf1 inhibits S6K phosphorylation. HA-S6K was co-transfected with increasing amounts of Rab5QL (50, 100, 200 ng) or Arf1QL (50, 100, 200 ng) into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. Signals detected by anti-pS6K, anti-HA-S6K, and anti-HA-Rab5QL were quantified, and the ratio was calculated. AA−, amino acid starvation; AA+, amino acid stimulation. B, brefeldin A (BFA) inhibits S6K phosphorylation. Mouse embryonic fibroblast cells were treated with the indicated concentrations or times of brefeldin A. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. C, down-regulation of Arf1 expression reduces cell size. Body cells expressing UAS-Arf1-RNAi (GFP-positive clone) have a decreased area when compared with surrounding wild type control cells (GFP-negative). D, 6-Diamidino-2-phenylindole (blue) labels the nuclei; phalloidin staining of F-actin (red) helps to visualize the cell boundary. D and E, Arf1 down-regulation induces autophagy. Clonal expression of UAS-Arf1-RNAi results in punctate staining of mCherry-Atg8a (D) and LysoTracker Red (E) under fed conditions.

|   | Rab5QL | Arf1QL |
|---|--------|--------|
| A | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| B | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| C | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| D | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| E | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |

|   | Rab5QL | Arf1QL |
|---|--------|--------|
| A | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| B | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| C | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| D | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |
| E | pS6K(T389) | HA-S6K |
|   | HA-Rab5QL | Myc-Arf1 |

**Arf1 Regulates mTORC1 in Both Drosophila and Mammalian Cells—** The RNA interference screen revealed that knockdown of Arf1 homolog in S2 cells significantly decreased dS6K phosphorylation (Fig. 1). Arf family GTPases are also involved in intracellular vesicle trafficking (23). We tested the effect of Arf1 in mammalian cells. Co-expression of the constitutively active Arf1QL inhibited S6K phosphorylation as strongly as Rab5QL (Fig. 4A). Similar to the results observed with Rab5 mutants, the constitutively active Arf1QL was more potent than the wild type or dominant negative Arf1TN in inhibiting S6K1 phosphorylation (Fig. 4A). We also found that a significant fraction of mTOR was co-localized with the transfected Arf1 (supplemental Fig. S3). On the other hand, Arf1 did not show a direct interaction with either Raptor or mTOR (supplemental Fig. S2) by our co-immunoprecipitation experiments.

Brefeldin A is a drug that inhibits Arf1 guanine nucleotide exchange factor, thereby inhibiting Arf1 activity (28, 29). We tested the effect of brefeldin A on S6K1 phosphorylation and found that it potently inhibited endogenous S6K phosphorylation in a dose-dependent manner (Fig. 4B). The inhibitory effect was rather rapid as an almost complete inhibition of S6K1 phosphorylation was observed with a 60-min brefeldin A treatment. These data support a role of endogenous Arf in mTORC1 regulation.

Two well characterized cellular functions of TORC1 are to promote cell growth and to inhibit autophagy (1, 30). We investigated the role of Arf1 in cell size regulation in Drosophila. Knockdown of Arf1 in clones of GFP-marked cells in the larval fat body resulted in a 40% reduction in cell area (Fig. 4C), consistent with a decreased TORC1 activity in these cells. In parallel, we examined the effect of Arf1 on autophagy, which is inhibited by high TORC1 activity. Arf1 knockdown caused a strong induction of autophagy, as indicated by punctate localization of mCherry-Atg8a and LysoTracker Red, which mark early (autophagosome) and late (autolysosome) stages of autophagy, respectively (Fig. 4, D and E). These data further support that Arf1 plays an important role in TORC1 regulation in vivo.
The TORC1 complex has an essential role in cell growth in response to various growth-promoting and inhibitory signals (1). Two GTPase families, Rheb and Rag, have previously been shown to stimulate mTORC1 activation (8, 9, 15, 16). However, the mechanisms of these GTPases in mTORC1 regulation are different. Rheb, acting downstream of TSC1-TSC2, binds to and activates mTORC1 (5–9). Therefore, the effect of Rheb on mTORC1 is direct. On the other hand, the Rag GTPases act downstream of amino acids to promote mTORC1 activation (15, 16). Although RagA and RagB bind directly to raptor with high affinity, they cannot directly activate mTORC1 without the help of Rheb (16). A model has been proposed that Rag GTPases may regulate the intracellular localization of mTORC1 in response to amino acids. When amino acids are sufficient, the active RagA and RagB recruit mTORC1 to subcellular compartments where Rheb is localized (16). Therefore, active Rag presents mTORC1 to a location where it can be activated by Rheb. This model suggests that intracellular trafficking would be important for mTORC1 activation.

In this study, we have discovered Rab and Arf GTPases as having an important function in mTORC1 activation. However, the roles of these GTPases in mTORC1 regulation are different from Rag or Rheb. Neither Rab5 nor Arf1 is sufficient to activate mTORC1, and they do not interact with mTORC1 (supplemental Fig. S2). Their effects on mTORC1 are likely to indirectly influence the subcellular localization of mTORC1 or its regulators. Consistently, both Rab and Arf are key regulators of intracellular trafficking. Unlike Rheb or Rag, which promote mTORC1 activation when in GTP-bound form and inhibit mTORC1 in GDP-bound form, overexpression of both the GTP-bound and the GDP-bound forms of Rab5 or Arf1 inhibits mTORC1. These results are consistent with an important role of GTPase cycling of Rab or Arf for proper intracellular vesicle trafficking. Overexpression of either GTP-bound or GDP-bound forms of these proteins might result in trapping of target proteins in a certain step and disruption of normal cycling. It is worth noting that Rab5 selectively blocks the stimulating effect of RagA but not Rheb on mTORC1 activation. Furthermore, Rab5 inhibits the effect of amino acids but not glucose on mTORC1 activation. These data support a model that intracellular trafficking is critical for amino acid signaling to mTORC1 activation that is mediated by the Rag GTPases. Future studies are needed to clarify the subcellular compartment where mTORC1 localizes.

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