RaLA Functions as an Indispensable Signal Mediator for the Nutrient-sensing System

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Cells sense nutrients present in the extracellular environment and modulate the activities of intracellular signaling systems in response to nutrient availability. This study demonstrates that RaLA and its activator RaLGDS participate in nutrient sensing and are indispensable for activation of mammalian target of rapamycin complex 1 (mTORC1) induced by extracellular nutrients. Knockdown of RaLA or RaLGDS abolished amino acid- and glucose-induced mTORC1 activation, as judged by phosphorylation of S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1. The amount of GTP-bound RaLA increased in response to increased amino acid availability. In addition, RaLA knockdown suppressed Rhee-induced S6 kinase phosphorylation, and the constitutively active form of RaLA induced mTORC1 activation in the absence of Rhee. These results collectively suggest that RaLGDS and RaLA act downstream of Rhee and that RaLA activation is a crucial step in nutrient-induced mTORC1 activation.

Nutrients such as amino acids and carbohydrates are available in the extracellular space and are vital for cell homeostasis. Cells are able to detect extracellular nutrients and modulate their intracellular signaling systems in response to changes in nutrient levels. Recent studies have shown that mammalian target of rapamycin complex 1 (mTORC1) functions as a critical intracellular component of the nutrient-sensing system and governs many aspects of cellular responses to changing nutrient levels (1–5). Signaling cascades initiated by growth factors and leading to mTORC1 activation have been extensively characterized. Growth factor stimulation results primarily in the phosphorylation of TSC2, a GTPase-activating protein (GAP) for Rhee GTPase, in a phosphoinositide 3-kinase- and protein kinase B (PKB)-dependent manner, followed by inactivation of TSC2 GAP activity (6–9). It has been proposed that the decreased GAP activity allows Rhee to stay in an “active” GTP-bound form, leading to mTORC1 activation. In contrast to the mechanism of the growth factor-initiated mTORC1 activation, little is known about the mechanism(s) by which mTORC1 activity is regulated by amino acid availability (10–16). Recent studies have shown that calmodulin and hVps34 play a role in the amino acid-sensing system (12) and that Rap GTPases escort mTORC1 to sites where they can activate the kinase (17, 18). However, most of the amino acid-sensing signaling machinery, including the amino acid sensors themselves, is unknown, and the mechanism(s) underlying the nutrient-sensing process remains to be identified.

RaLA and RaLB GTPases belong to the Ras superfamily and are known to participate in multiple cellular processes through binding to diverse effector proteins. RaLA effectors described to date include RaL-binding protein 1, Sec5, Exo84, filamin, and ZO-1-associated nucleic acid-binding protein (19–22); these interactions are indicative of crucial roles for RaLA in cell migration, membrane dynamics, and transcriptional regulation. RaLB is highly homologous to RaLA and shares an identical effector domain with RaLA. However, these two proteins are believed to have distinct functions in cells (23–25); RaLB participates in apoptotic signaling, whereas RaLA is predominantly involved in proliferation-related signaling, particularly anchorage-independent proliferation (26, 27). In addition, an accumulating body of evidence suggests that both RaLA and RaLGDS, an activator of RaLA, are crucial for Ras-induced oncogenic transformation of cells and that Ras in fact binds to RaLGDS and induces RaLA activation (23, 24, 26, 28). These observations suggest that the RaLGDS–RaAL signaling unit plays a role in cell proliferation; however, how RaLA controls cell proliferation and participates in oncogenic transformation remains unknown.

In this report, we show that RaLA is activated in response to extracellular amino acids and that both RaLGDS and RaLA are indispensable for nutrient-induced mTORC1 activation. The
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RalGDS-RalA signaling unit functions downstream of Rheb and participates selectively in the nutrient-sensing system.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa cells (ATCC CCL-2) were maintained as described previously (29). Amino acid-free RPMI1640 medium (30) and glucose-free RPMI1640 medium (Invitrogen) were used for amino acid and glucose deprivation, respectively. Amino acid and glucose stimulation were performed by the addition of normal RPMI1640 medium and RPMI1640 medium containing 25 mM glucose, respectively. Serum starvation was carried out in RPMI1640 medium containing 0.1% fatty acid-free bovine serum albumin (Sigma). Transfection of plasmid DNA was carried out using either FuGENE HD (Roche Applied Science) or Lipofectamine 2000 (Invitrogen). Lipofectamine RNAi Max (Invitrogen) was used for transfection of siRNA; a mixed siRNA mixture was used unless otherwise specified. Transfections were performed according to the manufacturers’ protocols.

Plasmids and siRNAs—SMARTpool siRNA against human Rheb was purchased from Dharmacon. Other siRNAs (Stealth RNAi), including negative control siRNA, were purchased from Invitrogen: RALA RNAi 1 (HS109033), RALA RNAi 3 (HS109035), RALB RNAi 1–3 (HS109036 to -109038), RALGDS RNAi 1 (HS109039), and RALGDS RNAi 2 (HS109040).

Full-length human RalB (NP_005605, amino acids 1–184) cDNA was amplified from HeLa cell cDNA by PCR and cloned into the EcoRI site of pCAGGS (31) to generate RalB/pCAGGS. RalB mutations S16H (tct to cat) and S20N (tcc to tgg) were introduced by site-directed mutagenesis using a PCR-based strategy. Full-length human RalA (NP_005393, amino acids 1–206) cDNA was amplified from HeLa cell cDNA by PCR with the addition of Myc tag sequence at the 5’-end, followed by cloning into the EcoRI/Sall site of pCMV5 (gift from David W. Russell) to generate Myc-RalA/pCMV5. The Q72L mutation (cag to ctc) was introduced by site-directed mutagenesis employing a PCR-based strategy. The coding region of Sec5-RBD was amplified from the Sec5-RBD/pGEX vector (32) (gift of Dr. Hisanori Horiuchi; Kyoto University) and tagged with a FLAG sequence at the 5’-end by PCR, followed by cloning into the EcoRI/Sall site of pCMV5 (gift from David W. Russell) to generate Myc-RalA/pCMV5. 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~10 times higher affinity for RalB than for RalA (according to the manufacturer’s data sheet). We monitored phosphorylation of human p70S6K at Thr389 as a measure of mTORC1 activity throughout this study, since the Thr389 residue has been identified as a direct mTORC1 phosphorylation site (33–35). When cells were incubated in amino acid-depleted medium, phosphorylation of Thr389 was reduced to an almost undetectable level within 2 h. Thr389 phosphorylation became detectable 5–10 min after the readdition of amino acids and reached a plateau within 60 min in HeLa cells (data not shown). RalA knockdown robustly (~90%) inhibited S6K phosphorylation induced by the addition of amino acids, whereas knockdown of RalB showed a relatively low level (~30%) of suppression of S6K phosphorylation (Fig. 1, A and D). A similar effect was observed when two individual siRNAs were used to knock down RalA (supplemental Fig. 1), indicating that the inhibition of mTORC1 activation was not due to an “off-target” effect of the RNAi. This conclusion was further supported by the rescue experiment in which an RNAi-resistant RalA mutant, bearing silent mutations at codons 150 and 151, restored Thr389 phosphorylation under conditions of endogenous RalA knockdown (supplemental Fig. 2).

Knockdown of either RalA or RalB also showed inhibitory effects on glucose-induced S6K phosphorylation that were of similar magnitude to those observed for amino acid-induced inhibition (Fig. 1, B and D), suggesting that both the amino acid- and glucose-sensing systems share the Ral GTPase-dependent core signaling machinery. In contrast, when cells were stimulated by insulin, which is known to activate mTORC1 in a phosphoinositide 3-kinase/PKB-dependent manner, RalA knockdown resulted in partial (~20%) inhibition of S6K phosphorylation, and RalB knockdown resulted in no inhibition (Fig. 1, C and D). It is noteworthy that incubation of the cells in the presence of insulin for 15 min resulted in a level of S6K phosphorylation that was almost comparable with that observed in a 30-min incubation with either amino acids or glucose. This partial inhibition caused by the RalA knockdown might be a reflection of the requirement for a nutrient signal for growth factor-induced mTORC1 activation as described previously (1). In addition, the effects of RalA and RalB knockdown on the phosphorylation of 4EBP1, another well-characterized readout for mTORC1 activation, were very similar to their effects on S6K phosphorylation (Fig. 1, A–C). These results suggest that the nutrient-sensing system employs Ral GTPases to activate mTORC1 and that RalA is an indispensable component, whereas the phosphoinositide 3-kinase/PKB-dependent mTORC1 activation pathway does not depend to a significant extent on Ral GTPases.

The Nutrient-sensing System Requires the RalGDS-RalA Signaling Unit—RalGDS is a well-characterized upstream activator of the Ral GTPases in context of its relevance for cell proliferation. We therefore used RNAi to inactivate RalGDS in HeLa cells in order to analyze its role in the amino acid-sensing system. Unfortunately, we were unable to confirm the knockdown using commercially available anti-RalGDS antibodies to detect endogenous RalGDS; however, two distinct siRNAs against RalGDS successfully reduced expression of the RalGDS transcript (Fig. 2A). Cells transfected with these siRNAs showed reduced S6K phosphorylation at Thr389 upon the addition of amino acids to the medium (Fig. 2B). Because Ral GTPases appear to preferentially participate in the nutrient-induced mTORC1 activation pathway, we next tested whether the RalGDS contribution was selective for the nutrient-sensing system. As shown in Fig. 2, C and D, results of the RalGDS knockdown were fundamentally consistent with those observed for RalA knockdown (i.e. robust inhibition of amino acid- and glucose-induced mTORC1 activation). These findings strongly suggest that RalGDS participates in the nutrient-sensing system, presumably as an activator of Ral GTPases. In contrast, insulin-induced mTORC1 activation was not inhibited by RalGDS knockdown (Fig. 2, C and D), indicating that insulin induces mTORC1 activation in a RalGDS-independent manner. Hao et al. (36) recently

FIGURE 1. Ral GTPase knockdown inhibits nutrient-induced mTORC1 activation. HeLa cells were transfected with siRNAs against the indicated targets. After 2 days in culture, cells were deprived of amino acids (A), glucose (B), or serum (C) for 2 h. Cells were then exposed to amino acids (A), glucose (B), or 2.0 μg/ml bovine insulin (C) for the indicated times, followed by immunoblot analysis. D, quantification of the phospho-S6K (normalized to total S6K) immunoblots shown in A–C, as described under “Materials and Methods.” Values represent the mean ± S.D. of 3–5 independent experiments. Calculated p values (Student’s t test) are presented.
reported that RalGDS forms a complex with PKB, a critical component in growth factor-regulated mTORC1 activation, and promotes PKB activation in breast cancer cell lines. We are not able to completely explain the conflict between those findings and the discrepancy in the requirement for RalA and RalGDS in insulin-induced mTORC1 activation. However, taken together, these results suggest that RalGDS has more diverse effects on signaling systems than RalA and potentially provides a feedback mechanism for RalA action under some conditions.

**RalA Is Activated in Response to Amino Acid Availability**—The observation that knockdown of either RalA or RalGDS, which facilitates GDP-GTP exchange of RalA, inhibited the amino acid-sensing system prompted us to ask whether RalA was activated in response to amino acid availability. We assayed RalA activation in cells using the Sec5-RBD protein, which selectively binds to the GTP-bound form of Ral GTPases and, therefore, can be used as an “active” Ral GTPase probe (32). Fig. 3, A and B, show that levels of the active, GTP-bound form of RalA increased following a 15-min incubation of cells with amino acids, reaching a plateau within 40 min. In contrast, RalB activation was not observed under these conditions (data not shown). In addition, overexpression of Sec5-RBD, which may inhibit RalA-dependent signals by binding toward active RalA, clearly suppressed amino acid-induced Thr389 phosphorylation of S6K (Fig. 3, C and D). In contrast, insulin-induced Thr389 phosphorylation was not inhibited by Sec5-RBD overexpression (Fig. 3, C and D). On the basis of these results, we conclude that nutrient-induced activation of RalA is a critical step for the activation of mTORC1.

**RalA Functions as a Downstream Component of Rheb**—Rheb is the small GTPase that plays a crucial role in the mTORC1 activation machinery. Although the action of Rheb remains controversial, an accumulating body of evidence places Rheb upstream of and in proximity to mTORC1 (11, 37, 38). Our question arising here is whether RalA acts upstream or downstream of Rheb in the nutrient-sensing system, because RalA may exert its effect on diverse processes, such as cytoskeletal reorganization and membrane dynamics (19–22), which potentially influence the nutrient uptake process. In fact, a Ral effector, the exocyst complex, is involved in vesicle transport (20, 39, 40); Exo70, an exocyst component, plays a role in targeting of the glucose transporter 4 to the plasma membrane (41, 42). Therefore, we first
RalA restored the activity (Fig. 4). We were not able to detect direct association of either RalA or RalGDS with mTORC1 (data not shown). These results indicate that RalA alone would not be sufficient to induce mTORC1 activation, and another factor(s) must be required. This hypothesis is supported by the observation that Q72L-RalA did not fully restore S6K phosphorylation in cells when Rheb was depleted (Fig. 4C). Taken together, these results suggest that RalA promotes the action of Rheb to activate mTORC1 in response to extracellular nutrients, although further study will be required to unravel the underlying biochemistry (Fig. 5).

Rheb was shown to bind directly to and activate mTORC1 (11, 38) (Fig. 5). In addition, a recent report (44) demonstrated that Rheb binds to FKBP38, an endogenous inhibitor of mTORC1, and sequesters it from mTORC1 (Fig. 5). Therefore, we next asked whether RalA acts on FKBP38 to regulate mTORC1 activity. To address this question, we first tested the interaction between ectopically expressed Myc-RalA and FLAG-FKBP38; however, we were unable to detect the interaction of these molecules in HeLa cells (supplemental Fig. 5). In addition, it should be noted that we could not reproduce the inhibitory effect of FKBP38 on mTORC1 activity. Both S6K and 4EBP1 phosphorylation remained unaffected by overexpression of FKBP38 (supplemental Fig. 5). Further, knockdown of FKBP38 affected neither S6K nor 4EBP1 phosphorylation (supplemental Fig. 5). These observations are supported by a recent report (44), which argued that FKBP38 did not inhibit mTORC1 signaling. Taking these results together, we conclude that RalA participates in mTORC1 regulation independently of FKBP38 (Fig. 5).

Growth factor signaling cascades also include Rheb as an indispensable regulator of mTORC1 activity. However, in contrast to the pathway employed by nutrient-sensing systems, growth factor-induced mTORC1 activation employs TSC2, a Rheb-GAP, as a critical mediator (45, 46). Since the precise mechanism by which cells discriminate between nutrient- and growth factor-induced Rheb signals remains unknown, it would be difficult to incorporate a specific mechanism for the action of RalA in mTORC1 activation into a simple scheme. However, our observation that growth factor-induced mTORC1 activation does not require RalA (Fig. 1) suggests that RalA, which is activated as a result of growth factor stimulation and TSC2-inactivation, drives a pathway distinct from the nutrient-dependent pathway to activate mTORC1. S16H-Rheb might activate both RalA-dependent and RalA-independent signals, presumably due to its hyperactive characteristics (43), providing an explanation for the observation that depletion of

FIGURE 3. RalA is activated in response to amino acid availability, and the activation is required for nutrient-induced mTORC1 activation. A and B, HeLa cells were deprived of amino acids for 2 h, followed by exposure to amino acids for the indicated times. The RalA activation assay was then performed as described under “Materials and Methods.” Values represent the mean ± S.D. of four independent experiments. Calculated p values (Student's t test) are presented. C, HeLa cells were transfected with the DNA construct as indicated. After 1 day in culture, cells were deprived of amino acids or serum for 2 h. Cells were then exposed to amino acids or 2 μg/ml bovine insulin for the indicated times followed by immunoblot analysis. D, quantification of the phospho-S6K (normalized to total S6K) immunoblots shown in C, as described under “Materials and Methods.” Values represent the mean ± S.D. of 3–5 independent experiments.

investigated the effect of RalA and RalGDS knockdown on nutrient uptake and confirmed that neither knockdown inhibited uptake of amino acids or glucose (supplemental Fig. 3).

Ectopic expression of the hyperactive Rheb mutant S16H resulted in a robust increase in S6K phosphorylation (Fig. 4, A and B), consistent with a previous report (43). RalA knockdown resulted in significant (40–50%) suppression of S6K phosphorylation in S16H-Rheb-expressing cells (Fig. 4, A and B), raising the possibility that RalA functions downstream of Rheb. In order to explore this possibility, we next asked whether RalA expression induced mTORC1 activation in the absence of Rheb. Knockdown of Rheb abolished amino acid-induced mTORC1 activation, and expression of the constitutively active Q72L-RalA mutant partially restored mTORC1 activation (Fig. 4C). The dominant negative S20N-Rheb mutant also inhibited amino acid-induced mTORC1 activation by ~30%, and Q72L-RalA restored the activity (Fig. 4D). In addition, it should be noted that neither RalA nor RalGDS knockdown altered the “activation” (guanine nucleotide-binding) status of Rheb (supplemental Fig. 4). These observations collectively suggest that RalA primarily functions as a downstream component of Rheb in the amino acid-sensing system and that RalA requires Rheb to achieve full activation of mTORC1.

Cross-talk Occurs between RalA and Rheb in mTORC1 Activation—We then asked how RalA transduces the signal from Rheb to mTORC1. One plausible mechanism is direct activation of mTORC1 by RalA; however, we were unable to detect direct activation of immunopurified mTORC1 by recombinant RalA protein (data not shown). Further, we were not able to detect direct association of either RalA or RalGDS with mTORC1 (data not shown). These results indicate that RalA alone would not be sufficient to induce mTORC1 activation, and another factor(s) must be required. This hypothesis is supported by the observation that Q72L-RalA did not fully restore S6K phosphorylation in cells when Rheb was depleted (Fig. 4C). Taken together, these results suggest that RalA promotes the action of Rheb to activate mTORC1 in response to extracellular nutrients, although further study will be required to unravel the underlying biochemistry (Fig. 5).
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**FIGURE 4. RaLA functions downstream of Rheb.** A, HeLa cells were transfected with control siRNA or siRNA against RaLA. After 1 day in culture, cells were further transfected with empty pCAGGS or Rheb[S16H]/pCAGGS plasmid DNA. After 1 day in culture, cells were deprived of amino acids for 2 h. Cells were then exposed to amino acids for the indicated times, followed by immunoblot analysis. B, quantification of the phospho-S6K (normalized to total S6K) immunoblot shown in A, as described under “Materials and Methods.” Bars represent differences between two independent experiments. C, HeLa cells were transfected with control siRNA or siRNA against Rheb. After 1 day in culture, cells were further transfected with empty pCMV5 or RaLA(Q72L)/pCMV5 plasmid DNA. After 1 day in culture, cells were deprived of amino acids for 2 h. Cells were then exposed to amino acids for the indicated times, followed by immunoblot analysis. Quantification of the phospho-S6K (normalized to total S6K) immunoblot was performed as described under “Materials and Methods.” The first row of lane description in the figure represents averages of and differences between duplicate determinations in a typical study. D, HeLa cells were transfected with equal amounts of RaLA expression construct (pCMV5 empty vector or RaLA[Q72L]/pCMV5) and Rheb expression construct (pCAGGS empty vector or Rheb[S20N]/pCAGGS). After 1 day in culture, cells were deprived of amino acids for 2 h. Cells were then exposed to amino acids for the indicated times followed by immunoblot analysis. Quantification of the phospho-S6K (normalized to total S6K) immunoblot was performed as described under “Materials and Methods.” The first row of lane description in the figure represents averages of and differences between duplicate determinations in a typical study. Original unprocessed gel images are presented in supplemental Fig. 6.

**FIGURE 5. Model for the action of RaLGDS and RaLA.** Extracellular nutrients activate RaLGDS to promote guanine nucleotide exchange of RaLA. Activated RaLA then facilitates the action of Rheb to activate mTORC1 in a FKBP38-independent manner. In contrast, growth factor stimulation activates Rheb primarily through the inactivation of TSC2. Rheb then activates mTORC1 in a RaLA-independent manner.

RaLA did not result in complete inhibition of S6K phosphorylation in S16H-Rheb-expressing cells (Fig. 4, A and B).

**Biological Significance of RaLA as a Component of the Nutrient-sensing System**—RaLGDS and RaLA are known to be potential oncogenic factors that are required for Ras-induced transformation of cells. A number of studies have indicated that these two molecules are essential but not necessarily sufficient for the transformation (23, 24, 26, 28); however, the exact reason for the RaLGDS-RaLA requirement has been poorly understood to date. This study has demonstrated that the RaLGDS-RaLA signaling unit plays a critical role in nutrient-sensing systems that drive mTORC1 activation in the regulation of diverse intracellular processes. In order to achieve accelerated proliferation, a hallmark of transformed cells, cells require large amounts of extracellular nutrients, such as amino acids for protein synthesis and carbohydrates as a major energy source. In addition, networks of nutrient-initiated intracellular processes, such as protein synthesis and ATP production, should be up-regulated simultaneously; however, up-regulation of these systems alone may not directly induce transformation. Intriguingly, these characteristics also apply to the requirement for RaLA and RaLGDS in cell proliferation and oncogenic transformation. Although further studies will be required to confirm the biological significance of RaLGDS and RaLA in vivo, our findings on the participation of RaLGDS-RaLA in the mTORC1 activation pathway provide novel insight into roles of RaLGDS and RaLA in cell proliferation and oncogenic transformation.

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