Nutrients and growth factors in mTORC1 activation

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Abstract

Growth factors and nutrients regulate the mTORC1 [mammalian (or mechanistic) target of rapamycin complex 1] by different mechanisms. The players that link growth factors and mTORC1 activation have been known for several years and mouse models have validated its relevance for human physiology and disease. In contrast with the picture for growth factor signalling, the means by which nutrient availability leads to mTORC1 activation have remained elusive until recently, with the discovery of the Rag GTPases upstream of mTORC1. The Rag GTPases recruit mTORC1 to the outer lysosomal surface, where growth factor signalling and nutrient signalling converge on mTORC1 activation. A mouse model of constitutive RagA activity has revealed qualitative differences between growth-factor- and nutrient-dependent regulation of mTORC1. Regulation of mTORC1 activity by the Rag GTPases *in vivo* is key for enduring early neonatal starvation, showing its importance for mammalian physiology.

Growth factors and nutrients

Since the establishment of a link between growth factors and mTORC1 [mammalian (or mechanistic) target of rapamycin complex 1] activation via the interaction of Akt and the TSC (tuberous sclerosis complex) more than a decade ago [1,2], our understanding of the mechanism and players involved has increased substantially. In contrast, the picture of how nutrients activate mTORC1 is far less complete and we are just beginning to add pieces to a puzzle that remained virtually unknown until 2008, with the identification of the Rag GTPases as a direct link between amino acids and mTORC1 [3,4].

Although both growth factors and nutrients culminate in the activation of mTORC1, the means by which each input does this suggests co-operation in their ability to trigger mTORC1-based responses. Growth factor signalling drives kinase activation of mTORC1 through a process that starts at the plasma membrane with the transduction of a signal evoked by protein hormones such as insulin, via tyrosine kinase receptors and activation of PI3K (phosphoinositide 3-kinase). PI3K, in turn, activates Akt, which phosphorylates and inhibits TSC [1,2], a complex with GAP (GTPase-activating protein) activity towards the Rheb GTPase [5–7], responsible for direct kinase activation of mTORC1. Nutrient signalling operates in a different manner: when the Rag GTPases were discovered, the originally puzzling observation that the activity of purified mTORC1 was not affected *in vitro* by the Rag GTPases led to the realization that a different mechanism was responsible for Rag-dependent activation of mTORC1. Upon nutrient sufficiency, the Rag GTPases interact with and recruit mTORC1 to the outer lysosomal surface, where the Rag and Rheb GTPases reside [4,8], allowing mTORC1 kinase activation by the latter. This mechanistic insight explains why both nutrient and growth factor inputs cannot substitute for each other and must simultaneously occur in a cell to achieve activation of mTORC1, and shows that these inputs co-operate to activate mTORC1. Logically, a cell that would trigger anabolism and increase its mass needs to engage cellular processes that are energetically expensive and regulated by mTORC1. Hence such co-operation warrants that the cell will fully commit to it when (i) long-range growth factor signals are present, and (ii) local nutrient sensing by the Rag GTPases assures the availability of building blocks and energy.

There are, however, some aspects of Rheb- and Rag-dependent regulation of mTORC1 that are similar. In particular, they both reside at the lysosomal surface, and they are both GTPases that change their nucleotide state upon physiological fluctuations of their upstream signals. In the case of Rheb, activation of TSC leads to Rheb loading with GDP and, conversely, TSC inactivation by Akt leads to Rheb loading with GTP and activation of mTORC1. The four members of the Rag GTPase family behave differently from most GTPases, as they exist as obligate dimers, where RagA (or RagB) interacts with RagC (or RagD), and their nucleotide state is opposite. Nutrient availability leads to the loading of RagA/B with GTP and RagC/D with GDP, and its absence loads RagA/B with GDP and RagC/D with GTP. The role of RagA/B compared with RagC/D is not identical, as single amino acid substitutions in RagA/B that mimic a constitutive loading with GTP, leads to constitutive recruitment (and activation) of mTORC1 regardless of the loading status of RagC/D.

Key words: growth factor, mammalian target of rapamycin (mTOR), nutrient.

Abbreviations used: E, embryonic day; mTORC1, mammalian (or mechanistic) target of rapamycin complex 1; PI3K, phosphoinositide 3-kinase; TSC, tuberous sclerosis complex; v-ATPase, vacuolar H^+-ATPase.

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Navigating upstream

The discovery of the Rags also constituted a handle that allowed further interrogation of how nutrients activate mTORC1. The Rag GTPases, which have no lipid modification to allow them be membrane-bound, are tethered to the lysosomal surface by constitutive interaction with the multiprotein complex Ragulator [9] that is anchored to the lysosomal surface and is necessary and sufficient to recruit the Rag proteins. In addition to its role as a scaffold for Rag–mTORC1 interaction, the Ragulator complex also exerts a regulatory role, as it works as a GEF (guanine-nucleotide-exchange factor) for the Rag GTPases [10]. A complete picture of how fluctuations in nutrients affect the loading of the Rag GTPases is far from complete, but we have identified v-ATPase (vacuolar H^+-ATPase) as a key regulator upstream of the Rag GTPases. v-ATPase, in addition to its prime function of maintaining the pH gradient between the cytoplasm and the lysosome at the expense of ATP, engages into interactions with Rag–Ragulator that are sensitive to nutrient levels. Furthermore, the activity of v-ATPase is critical for recruitment and activation of mTORC1 [11]. The identity of the direct nutrient sensor and how it is connected to mTORC1 recruitment by the Rag GTPases is still unknown, but at least part of this sensing seems to occur in a lysosomal inside-out manner that involves v-ATPase.

mTORC1 regulation in a physiological setting

Tuberous sclerosis is the prime example of a human syndrome driven by constitutive mTORC1 activity. It is caused by germline mutations in *TSC1* or *TSC2* and causes benign and malignant tumours, cysts, seizures, mental retardation and other symptoms [12,13]. Proteus and Proteus-like syndromes, neurofibromatosis and von Hippel–Lindau disease are also caused by genetic alterations in regulators of mTORC1 activity that operate in the growth factor branch [14]. Furthermore, sporadic mutations in these genes are also key players in tumorigenesis. Nutrient-dependent regulation of mTORC1 is also associated with a human syndrome caused by a germline mutation in one of the Ragulator proteins [15], implicating that both main axes upstream of mTORC1 are at the core of pathogenesis of human disease.

Although cultured cells have taught us valuable aspects of mTORC1 biology, in order to model human disease, experimental mouse-driven approaches are required, and some efforts to manipulate mTORC1 activity in mice have been pursued. Loss of function of mTORC1 key components leads to embryonic lethality [16–18], which precludes deeper insight *in vivo*, so conditional deletions followed those studies (reviewed in [19]). Because most human syndromes of deregulated mTORC1 consist of increased activity, gain-of-function mouse strains may prove invaluable as tools for understanding and intervening human disease. As for human syndromes, the prime mouse model of hyperactive mTORC1 is loss of TSC1 or TSC2 [20–22]. TSC-deficient mice die at E (embryonic day) 9.5–11.5 with hyperactive mTORC1, severe anomalies and cells derived from them undergo senescence. Conditional deletion of TSC function in adult mice has recapitulated most characteristics of tuberous sclerosis, including neuronal defects and cancer, as well as its response to the mTORC1 inhibitor rapamycin.

Modelling constitutive RagA activity

Given the recent discovery of genes involved in nutrient-dependent regulation of mTORC1, mouse models are just underway. In a recent study, we generated a mouse strain with constitutive RagA activity [23]. We introduced a single nucleotide substitution in the endogenous RagA gene, which renders a glutamine to leucine change in amino acid 66 in the RagA protein sequence. This point-mutant form of RagA protein is constitutively bound to GTP ($RagA^{GTP}$) and allowed us generate mice with constitutive nutrient-dependent activation of mTORC1 in every cell. We first obtained MEFs (mouse embryonic fibroblasts) either $RagA^{+/+}$ or that harboured one copy of the $RagA^{GTP}$ allele ($RagA^{GTP/+}$), and analysed mTORC1 activity in those cells. To our surprise, in spite of normal expression of the mutant allele, regulation of mTORC1 activity in $RagA^{GTP/+}$ cells was normal, suggesting that the presence of the wild-type allele was somehow compensating for the effect of the $RagA^{GTP}$ allele. This turned to be true also in mouse tissues. When we intercrossed $RagA^{GTP/+}$ mice, $RagA^{GTP/GTP}$ embryos at E13.5, in spite of having complete insensitivity to amino acid deprivation in culture, were macroscopically indistinguishable from $RagA^{+/+}$ embryos. This result is in sharp contrast with $Tsc1^{-/-}$ or $Tsc2^{-/-}$ embryos, which succumb at ~E10.5 with serious anomalies, and cells derived from them undergo rapid p53-dependent senescence in culture, to a point where culturing these cells is almost impossible. $RagA^{GTP/GTP}$ cells proliferated in culture with kinetics similar to those of $RagA^{+/+}$ cells. Moreover, $RagA^{GTP/GTP}$ embryos developed normally and were born with Mendelian ratios and with minimal phenotypic defects. The different outcomes of constitutive growth-factor-dependent activation (death at E10.5 and premature senescence) and nutrient-dependent activation of mTORC1 (normal embryonic development) may underlie different causes. One possibility is that oscillations of growth factors during embryonic development must be accurately detected and cells in the embryo must properly respond to them, whereas oscillations in nutrients do not occur. Provided that trans-placental supply of nutrients is supposed to be steady during embryonic development, this possibility seems reasonable. In addition, there may be an intrinsic difference in the extent to which growth factors and nutrients can activate mTORC1. Indeed, even though mTORC1 activity in $RagA^{GTP/GTP}$ mice and cells was insensitive to amino acid withdrawal, maximal activity was comparable with that of $RagA^{+/+}$ cells in the presence of amino acids and growth factors. This contrasts with mTORC1 activity in TSC-deficient cells, which show a severalfold increase in

maximal mTORC1 activity. This difference may constitute an alternative explanation for the substantial difference between TSC-deficiency and RagA constitutive activity in the developing embryo. Whether this is related to the intrinsic nature of each type of stimulus (regulating recruitment compared with kinase activation) is not clear, but raises the question of how much TSC-deficient mice and cells, great models for tuberous sclerosis syndrome, mimic physiological states associated with deregulation in mTORC1 activity, such as nutrient overload, early stages of Type 2 diabetes and aging.

Nutrient crisis and death of RagA$^{GTP/GTP}$ newborns

In spite of being barely distinguishable from wild-type littermates, RagA$^{GTP/GTP}$ mice succumb during the first day of life. As expected, interruption of the maternal supply of nutrients at birth led to a profound fall in levels of circulating glucose and amino acids in mice of all genotypes, but mTORC1 activity was reduced only in wild-type newborns, and not in RagA$^{GTP/GTP}$ littermates. This is consistent with the critical role of mTORC1 in regulating starving/feeding responses [24]. Upon prolonged starvation in isolation following Caesarean section, RagA$^{GTP/GTP}$ newborns were unable to recover from this reduction in circulating nutrients, and succumbed to fatal hypoglycaemia within ~15 h. In contrast, RagA$^{+/+}$ mice lived for ~24 h and endured their starvation state by mobilizing internal energetic sources, reflected by their recovery from the initial hypoglycaemia. Glycogen is a critical source of energy after birth, but RagA$^{GTP/GTP}$ mice showed no alterations in glycogen levels or impaired consumption after birth. Gluconeogenesis is also critical, but, again, RagA$^{GTP/GTP}$ newborns were able to execute gluconeogenesis when gluconeogenic substrates were present. Because mice are born without significant amounts of fat, they rely on amino acids as substrates for gluconeogenesis. Hence we hypothesized that the fatal hypoglycaemia was secondary to a reduction in circulating amino acids to be used as substrates for gluconeogenesis. Importantly, free amino acids are the main product of autophagy, which is triggered immediately after birth, and autophagy-deficient mice share characteristics observed in RagA$^{GTP/GTP}$ newborns, including early lethality and reduced circulating amino acid levels [25,26]. We tested whether RagA$^{GTP/GTP}$ newborns had impairment in the induction of autophagy after birth, and, indeed, they showed a striking defect in their ability to induce autophagy. As a consequence, circulating amino acids were reduced, and the reduction in gluconeogenic amino acids failed to fuel a significant amount of gluconeogenesis, leading to a hypoglycaemic state that became fatal as soon as glycogen reserves were exhausted. This argues that nutrient signalling upstream of mTORC1 is the key regulator of autophagy in mammals, a surprising finding provided the multiple regulators of autophagy described in cultured cells.

Rag GTPases beyond amino acids

Considering that the Rag GTPases regulate mTORC1 activation by amino acids, the fact that RagA$^{GTP/GTP}$ newborns had high mTORC1 activity in spite of a significant reduction in amino acid levels was not surprising. What was unexpected was that mTORC1 activity in RagA$^{GTP/GTP}$ newborns was also insensitive to profound hypoglycaemia. This led us to consider that the Rag GTPases could also regulate mTORC1 by glucose levels. Indeed, RagA$^{GTP/GTP}$ cells in culture were resistant to amino acid deprivation, glucose deprivation or deprivation of both glucose and amino acids. Furthermore, deprivation of glucose, as that of amino acids, led to dispersion of mTORC1 in the cytoplasm in wild-type cells, but mTORC1 was constitutively recruited to the lysosomal surface, regardless of amino acid or glucose levels in RagA$^{GTP/GTP}$ cells. Glucose, as shown previously for amino acids, regulated the physical interactions of the v-ATPase with the Ragulator complex, implicating the same machinery in sensing amino acids and glucose. Whether the lysosomal nutrient-sensing machinery detects both nutrients independently or, in contrast, detects the levels of a common intermediate, awaits further research, but conceptually, this finding implies that the Rag GTPases behave as global nutrient regulators of mTORC1, conveying global information about the nutritional status of the cell. Several nodes seem to converge on the Rag GTPases, as anti-hyperglycaemic biguanide drugs also regulate Rag function [27], as well as 2-oxoglutarate (α-ketoglutarate) [28].

Concluding remarks

The discovery of the proteins involved in mTORC1 regulation by nutrients has opened a new area of research, still in its infancy. The identification of the sensor (or sensors), the mechanism of convergence of the lysosomal inside-out sensing compared with a potential cytoplasmic sensing, the existence of additional roles of the Rag GTPases upstream of mTORC1 and why they show this unique dimeric nature for GTPases, are all outstanding questions awaiting answers. Provided the numerous human syndromes associated with PI3K/mTORC1 genetic defects, it will be no surprise to find the involvement of the Rag/mTORC1 pathway in more of these. In addition, deregulation in nutrient sensing, as occurring in RagA$^{GTP/GTP}$ newborns, will almost certainly prove relevant for human diseases as cancer, neurodegeneration, diabetes and aging. Additional mouse models of deregulated nutrient sensing will certainly teach us additional aspects of physiology that are critically regulated by this pathway, which may be similar to or different from those where growth factors are involved.

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