Minireview

dRAGging Amino Acid-mTORC1 Signaling by SH3BP4

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Mammalian target of rapamycin complex 1 (mTORC1) is a master regulator of cell growth and autophagy. Its activity is regulated by the availability of amino acids and growth factors. The activation of mTORC1 by growth factors, such as insulin and insulin-like growth factor-1 (IGF-1), is mediated by tuberous sclerosis complex (TSC) 1 and 2 and Rheb GTPase. Relative to the growth factor-regulated mTORC1 pathway, the evolutionarily ancient amino acid-mTORC1 pathway remains not yet clearly defined. The amino acid-mTORC1 pathway is mediated by Rag GTPase heterodimers. Several binding proteins of Rag GTPases were discovered in recent studies. Here, we discuss the functions and mechanisms of the newly-identified binders of Rag GTPases. In particular, this review focuses on SH3 binding protein 4 (SH3BP4), the protein recently identified as a negative regulator of Rag GTPases.

INTRODUCTION

Mammalian target of rapamycin (mTOR), the evolutionarily-conserved Ser/Thr kinase, participates in two multiprotein complexes, termed mTORC1 and mTORC2, that contain raptor and rictor, respectively. mTORC1 is responsible for the regulation of cell growth, autophagy and metabolism in response to the availability of amino acids, glucose and growth factors (Hara et al., 2002; Jung et al., 2010; Kim et al., 2002; Loewith et al., 2002). mTORC1 is highly activated in many cancers, and its activity is deregulated in diabetes, obesity and aging (Goberdhan and Boyd, 2009; Harrison et al., 2009; Seldman et al., 2009; Um et al., 2004; Wullschlegel et al., 2006). Due to the key role of mTORC1 in cellular physiology, any factor that can regulate mTORC1 has gained clinical attention as a target to prevent cancer and other diseases. There are two major branches of pathways upstream of mTORC1: the insulin/IGF1-regulated pathway and the amino acid-regulated pathway (Fig. 1). The growth factor-regulated pathway is subject to multiple, growth-enhancing alterations in cancer, such as mutations of the class I phosphoinositide 3-kinase (PI3K) subunits, phosphatase and tensin homolog (PTEN), the oncogenic Akt, and TSC genes. Relative to the well-established growth factor signaling limb, the amino acid-regulated mTORC1 pathway remains not clearly

defined and its contribution to tumorigenesis has not been explored.

The insulin/IGF1-regulated pathway is activated when insulin or IGF-1 binds to its receptor on the plasma membrane. Insulin and IGF-1 are circulating hormones that reflect the status of the nutrition in the whole body in multicellular organisms. Their binding to the receptors triggers recruitment of insulin receptor substrates (IRS) to insulin/IGF1 receptors. The insulin/IGF1 receptors have a tyrosine kinase domain in their cytoplasmic region that catalyzes phosphorylations of IRS. The phosphorylations enable IRS to recruit PI3K via Src homology 3 (SH3) domain-mediated interaction. The recruited PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits two key cellular signaling kinases, Akt (also called protein kinase B, PKB) and phosphoinositide-dependent kinase-1 (PDK1), to the plasma membrane where PDK1 phosphorylates Akt. The serine/threonine protein kinase Akt is a positive regulator of mTORC1 signaling. Akt phosphorylates TSC2, which has a GTPase activating protein (GAP) activity toward the small GTPase called Rheb (Ras homolog enriched in the brain) (Inoki et al., 2002; Manning and Cantley, 2003; Tee et al., 2003; Zhang et al., 2003) (Fig. 1). Rheb binds and stimulates mTORC1 (Long et al., 2005; Sancak et al., 2007). The phosphorylation of TSC2 by Akt inhibits the GAP activity of TSC2 thereby releasing the inhibitory effect of TSC2 on mTORC1. Akt also phosphorylates PRAS40 (proline-rich Akt substrate 40 kDa), a component of mTORC1, which contributes to an increase in the mTORC1 activity (Sancak et al., 2007; Vander Haar et al., 2007).

The amino acid-regulated mTORC1 pathway is evolutionarily more ancient than the insulin/IGF1-regulated pathway. While the latter has evolved for multicellular organisms to regulate metabolism in the whole body, the former has evolved early in yeast enabling the individual cellular organisms to monitor the availability of nutrients in the environment for growth and survival. Amino acids, especially the branched chain amino acids such as leucine, are potent inducers of mTORC1 activation. The amino acid-regulated mTORC1 pathway is mediated by Rag GTPase heterodimers and their associated proteins on the lysosome (Kim and Guan, 2009; Kim et al., 2008; Sancak et al., 2008; 2010). Rag GTPases are evolutionarily conserved throughout eukaryotes. Higher eukaryotes have four members of Rag

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Received September 19, 2012; revised December 4, 2012; accepted December 4, 2012; published online December 26, 2012

Keywords: mTOR, mTORC1, Rag GTPases, SH3BP4

GTPases: RagA, RagB, RagC and RagD (Sekiguchi et al., 2001). Rag GTPases exist in heterodimeric complexes consisting of RagA or RagB and RagC or RagD (Dubouloz et al., 2005; Hirose et al., 1998; Kim et al., 2008). In mammalian cells, the activity of Rag GTPase complexes depends on whether RagA and RagB are bound to GTP or GDP. The Rag GTPase complex containing GTP-bound RagA or RagB activates mTORC1 signaling, whereas the complex containing GDP-bound RagA or RagB does not activate mTORC1 (Kim et al., 2008; Sancak et al., 2008).

The regulation of mTORC1 by Rag GTPases is conserved in yeast, *Drosophila* and mammals. In yeast, Gtr1p and Gtr2p, which are orthologues of RagA and RagC, form a heterodimeric complex playing a similar role as that played by mammalian Rag GTPase complexes (Binda et al., 2009; Dubouloz et al., 2005; Valbuena et al., 2012). Gtr1p and Gtr2p exist in a large multiprotein complex termed the EGO complex together with Ego1p and Ego3p. The EGO complex binds and activates TORC1 in response to amino acid availability (Binda et al., 2009; Dubouloz et al., 2005). Ego1p and TORC1 preferentially interact with GTP-bound forms of Gtr1p, and this interaction is important for TORC1 activation. In *Drosophila*, the larval fat body expressing the active form of RagA has a larger cell size even under nutrient starved conditions (Kim et al., 2008). This suggests that *Drosophila* Rag GTPases may play a similar role as that played by mammalian Rag GTPases.

Rag GTPases are localized on the lysosome and recruit mTORC1 to the lysosome in response to amino acids (Sancak et al., 2008; 2010). RagA and RagB recruit mTORC1 *via* binding to raptor under amino acid-enriched conditions (Binda et al., 2009; Sancak et al., 2010). Once mTORC1 is recruited to the lysosome, it is activated by Rheb that resides on the lysosomal membrane (Sancak et al., 2010).

How Rag GTPases are regulated by amino acids remains a key question in the field. This review will highlight the recent findings that have contributed to our understanding of Rag GTPase regulation by amino acids. We will start with MAP4K3 (mitogen-activated protein kinase kinase kinase 3) that functions upstream of Rag GTPases and is activated by amino acids. We will review three recently-identified regulators of Rag GTPases: Ragulator, vacuolar H(+)-adenosine triphosphatase (v-ATPase), and leucyl-tRNA synthetase (LRS). Next, we will briefly review the recent reports describing intimate crosstalks between mTORC1 and the autophagy elements. The remaining part will focus on SH3BP4, which negatively regulates Rag GTPases under amino acid starvation, with a highlight on its regulatory mechanism and potential role as a tumor suppressor.

REGULATORS OF RAG GTPASES

MAP4K3 is an upstream regulator of Rag GTPases

MAP4K3 is a Sterile20 (Ste20) kinase family member involved in the yeast mating pheromone response pathway. MAP4K3 is a positive regulator of amino acid-mTORC1 signaling (Findlay et al., 2007; Yan et al., 2010). Amino acids stimulate the activity of MAP4K3 in *Drosophila* and mammalian cells (Findlay et al., 2007). The stimulatory effect of MAP4K3 on mTORC1 signaling depends on Rag GTPases, indicating that MAP4K3 functions upstream of Rag GTPases (Yan et al., 2010). In *Drosophila*, MAP4K3 physically binds to Rag GTPases, and this binding is regulated by amino acids (Bryk et al., 2010). *Drosophila* MAP4K3 has been shown to interact with TORC1 and regulate the assembly and the activation of TORC1 (Resnik-Docampo and de Celis, 2011). Flies lacking MAP4K3 are reduced in TOR

activity and retarded in growth (Bryk et al., 2010). Overexpression of constitutively active RagA in *Drosophila* resulted in tissue overgrowth even in a map4k3-mutant background, suggesting that Rag GTPases are downstream of MAP4K3 (Bryk et al., 2010). Amino acids induce the phosphorylation of MAP4K3 at Ser170 (Yan et al., 2010). This phosphorylation is important for the activation of MAP4K3 and mTORC1 signaling (Yan et al., 2010). Under amino acid starvation, protein phosphatase 2A (PP2A) binds to MAP4K3 through T61 epsilon, a targeting subunit of PP2A, and dephosphorylates MAP4K3 at Ser170 (Yan et al., 2010), thereby inactivating MAP4K3 and mTORC1.

Ragulator is a GEF of Rag GTPases

Rag GTPases interact with a lysosomal multiprotein complex termed Ragulator that consists of MP1, p14, p18, HBXIP and C7orf59 (Bar-Peled et al., 2012; Sancak et al., 2010). Ragulator is necessary for the localization of Rag GTPases to the lysosomal surface. Through interaction with Ragulator, Rag GTPases can be present on the lysosomal membrane regardless of their nucleotide bound state or the availability of amino acids (Sancak et al., 2010). The interaction between Ragulator and Rag GTPases is important for activation of mTORC1 since Rag GTPases need to recruit mTORC1 to the lysosome for the activation of mTORC1 by Rheb. The interaction between Ragulator and Rag GTPases is enhanced under conditions when amino acid levels are high (Bar-Peled et al., 2012). Ragulator was determined to function as a guanine nucleotide exchange factor (GEF) for Rag GTPases (Bar-Peled et al., 2012). The GEF function facilitates the exchange of GDP for GTP of RagA or RagB resulting in the formation of the active Rag heterodimeric complex (Bar-Peled et al., 2012).

v-ATPase is a lysosomal protein complex that regulates Rag GTPases

v-ATPase is a multiprotein complex that is evolutionarily conserved throughout eukaryotes (Nelson et al., 2000). v-ATPase pumps protons into the lysosomes and acidifies the lysosomal lumen. A recent study has identified v-ATPase as a binding protein of the Ragulator-Rag GTPase complex and as an important component of the lysosome-associated machinery required for amino acid activation of mTORC1 (Zoncu et al., 2011). v-ATPase directly binds to Ragulator, but not to Rag GTPases. The interaction between v-ATPase and Ragulator is sensitive to the concentration of amino acids inside the lysosomal lumen. The interaction becomes weaker by amino acid supplementation in the medium. The loose interaction between v-ATPase and Ragulator under amino acid-enriched conditions somehow facilitates the recruitment of mTORC1 to the Rag GTPase complex on the lysosome probably *via* increasing the GEF activity of Ragulator.

Rag GTPase functions are linked to autophagy regulators

mTORC1 is a major regulator of autophagy induction (Jung et al., 2010). mTORC1 is also important for the termination of autophagy and reformation of lysosomes (Yu et al., 2010). Rag GTPases and mTORC1 are enriched in a specified area in the cytoplasm termed the TOR-autophagy spatial coupling compartment (TASCC) (Narita et al., 2011). Rag GTPase-mediated recruitment of mTORC1 to TASCC affects protein synthesis, cytokine secretion, and biogenesis of lysosome and autophagosome. The colocalization of Rag GTPases, mTORC1 and the autophagy machinery suggests an intimate crosstalk between the Rag GTPase-mTORC1 pathway and the autophagy pathway, which must be important for cells to coordinately

Fig. 1. Two branches of pathway upstream of mTORC1. Insulin/IGF1-regulated pathway and amino acid-regulated pathway. The leucine influx mediated by the amino acid transporters, such as SLC1A5 and SLC7A5/ SLC3A2, is described in a previous study (Nicklin et al., 2009). Rag GTPase complex is in association with Regulator and v-ATPase on the lysosomes. mTORC1 contains raptor, GβL, pras40, deptor and mTOR itself. Rag GTPase complex consists of RagA or RagB and RagC or RagD. Regulator consists of MP1, p14, p18, HBXIP, and C7orf59.

regulate the fundamental nutrient-responsive processes. Rag GTPases interact with p62/SQSTM1, which is involved in binding polyubiquitinated proteins and degraded through autophagy (Duran et al., 2011). The interaction is mediated by raptor, the key regulatory component of mTORC1. The binding of p62 to raptor facilitates the binding of mTORC1 to Rag GTPase complex under amino acid-enriched conditions. In the absence of p62, the activation of mTORC1 in response to amino acids is drastically impaired (Duran et al., 2011).

Leucyl-tRNA synthetase is a cytosolic leucine sensor that regulates Rag GTPases in response to leucine

Leucyl-tRNA synthetase (LRS) is an enzyme that is responsible for charging leucine on leucyl-tRNA. Recently, LRS was shown to interact with Rag GTPases and mediate leucine signaling to Rag GTPases (Han et al., 2012). The interaction is enhanced under leucine-enriched conditions and is crucial to activate mTORC1. Under leucine-enriched conditions, LRS is translocated to lysosome to interact and activate Rag GTPases. LRS has a preferential binding affinity toward GTP-charged RagD. LRS has a putative GTPase-activating protein (GAP) motif and a GAP activity toward RagD in response to leucine (Han et al., 2012). The conversion of GTP-charged RagD to GDP-charged RagD enhances the capacity of Rag GTPase complex to activate mTORC1. In yeast, LeuRS Cdc60p is an ortholog of mammalian LRS. Like the mammalian LRS, Cdc60p interacts with Gtr1p in an amino acid-dependent manner (Bonfils et al., 2012). The binding of Cdc60p to Gtr1p depends on the activity of Cdc60p to produce the charged tRNA^{Leu}, suggesting that Cdc60p mediates leucine-stimulated TORC1 activation and the leucyl charging on the tRNA is important for the activation. This study suggests that LRS is a conserved cytosolic protein that regulates Rag GTPase-mTORC1 signaling in response to leucine.

Vam6p is a GEF for Gtr1 GTPase

Vam6p/Vps39p (vacuolar protein sorting 39) is a component of homotypic fusion and vacuole protein sorting (HOPS) complex

and plays a role as a guanine nucleotide exchange factor (GEF) for Ypt7p, an orthologue of Rab7, in yeast (Binda et al., 2009; Wurmser et al., 2000). In yeast, deletion of *Vam6* suppresses the cellular capacity to recover from rapamycin-induced growth arrest. Vam6p is important for the binding of Ego1p to Gtr1p and for the activation of TORC1 (Binda et al., 2009; Dubouloz et al., 2005). Vam6p binds to Gtr1p and acts as a guanine nucleotide exchange factor (GEF) for Gtr1p (Binda et al., 2009), thus positively regulating amino acid-TORC1 signaling. In HeLa cells, knockdown of hVps39, the mammalian ortholog of Vam6p, suppressed the activation of mTORC1 by amino acids and insulin (Flinn et al., 2010). Whether the suppression is due to inhibition of Rag GTPases needs further investigation.

SH3BP4, a negative regulator of Rag GTPases

SH3BP4 is a recently-identified binding protein of Rag GTPases (Kim et al., 2012). SH3BP4 functions as a negative regulator of amino acid-mTORC1 signaling by inhibiting the formation of active Rag GTPase complex. SH3BP4 exists only in vertebrates, and it has a unique domain architecture. SH3BP4 contains one Src homology 3 (SH3) domain, three N-P-F amino acid repeat motifs for binding to the Eps15 homology domain, putative binding sites for proteins involved in clathrin-mediated endocytosis, and one death domain (DD) near the C-terminus (Fig. 2). The presence of both endosomal trafficking and DD moieties has not been seen in any protein other than SH3BP4 and its close homolog 7a5. This implies that SH3BP4 is involved in a novel type of cellular regulatory mechanisms.

How SH3BP4 inhibits Rag GTPase complex

SH3BP4 directly binds to the inactive Rag GTPase complex through its SH3 domain under amino acid starvation (Kim et al., 2012). The binding stabilizes the inactive complex and inhibits the formation of the active Rag GTPase complex (Kim et al., 2012). The binding of SH3BP4 to Rag GTPases abrogates the interaction between Rag GTPases and mTORC1, thus inhibiting the recruitment of mTORC1 to the lysosome. The association of SH3BP4 with RagB is enhanced by amino acid starvation

Fig. 2. Schematic representation of SH3BP4 domain architecture. SH3BP4 contains multiple domains typical of the Eps15-homology (EH) network family members, which regulate endocytosis and intracellular sorting. The domains include three N-P-F amino acid repeats, one SH3 domain, and one WxxF motif. SH3BP4 also contains a death domain (DD) close to the C-terminus and one ZU5 domain involved in protein-protein interaction. SH3BP4 has multiple phosphorylation sites including one mediated by AKT (Chiba et al., 2009).

tion. The stoichiometry of the interaction between SH3BP4 and the Rag GTPase complex has not been determined. Since SH3BP4 can bind to all the four Rag GTPases directly, it is possible that two molecules of SH3BP4 bind to the Rag GTPase heterodimeric complex. According to our study, the nucleotide-bound state of RagB could affect the binding of SH3BP4 to RagC (Kim et al., 2012). This suggests that the dimeric interaction between Rag GTPases might somehow regulate the binding of SH3BP4 to the Rag GTPase complex. The recently-determined crystal structures of the yeast Gtr1p^{GTP}-Gtr2p^{GTP} complex and the Gtr1p^{GTP}-Gtr2p^{GDP} complex imply a possibility that RagA or RagB might regulate RagC and RagD GTPase activity (Gong et al., 2011; Jeong et al., 2012). The biochemical consequence of SH3BP4 binding to Rag GTPases is inhibition of GTP hydrolysis activity of Rag GTPases (Kim et al., 2012). A detailed analysis of the interaction between Rag GTPases and SH3BP4 is necessary to better understand how they interact in the complex and how SH3BP4 inhibits Rag GTPases.

It remains unknown how SH3BP4 regulates Rag GTPases in relation to other regulators of Rag GTPases, such as MAP4K3, Regulator, v-ATPase, and LRS. Once amino acids are sensed by LRS, Rag GTPase binding to SH3BP4 may become loose and the Rag GTPase complex be activated by positive regulators to promote mTORC1 signaling. Alternatively, amino acids could affect the cellular localization or post-translational modification of SH3BP4 thereby affecting the affinity of its association with Rag GTPases. There are multiple possible modes of regulation through which SH3BP4 crosstalks with other regulators of Rag GTPases. SH3BP4 could interfere with the binding of LRS or Regulator to Rag GTPases thereby inhibiting the activation of Rag GTPases under amino acid starvation. Alternatively, LRS or Regulator could interfere with the binding of SH3BP4 to Rag GTPases thereby suppressing the inhibitory effect of SH3BP4 on Rag GTPases under amino acid-enriched conditions. Further study is required to clarify which regulatory mode operates in cells.

Cellular localization of SH3BP4

The cellular localization of SH3BP4 is exclusive to that of mTORC1 (Kim et al., unpublished data). SH3BP4 was distributed on the plasma membrane and perinuclear region where it colocalized with RagB under leucine-deprived conditions (Kim et al., 2012). SH3BP4 contains multiple domains typical of proteins in the Eps15-homology (EH) network family that regulate endocytosis and intracellular sorting, which include three NPF motifs (putative EH domain-binding sites), one SH3 domain, LIDL motif (a putative clathrin-binding motif), and one WxxF motif (a putative AP2 binding site) (Fig. 2). The multiple motifs for binding to endosomal trafficking proteins indicate that SH3BP4 may play a role in vesicle trafficking. SH3BP4 is also called transferrin receptor trafficking protein (TTP) (Tosoni et al., 2005)

since SH3BP4 has been shown to regulate transferrin receptor internalization (Tosoni et al., 2005). How the function to regulate transferrin receptor internalization is linked to Rag GTPase regulation remains unclear. It is possible that SH3BP4 regulates multiple GTPases involved in endosomal trafficking through which SH3BP4 could coordinate spatial and temporal processes of endocytosis and vesicle trafficking in response to amino acids and other cellular signals. SH3BP4 may be able to carry a cargo of membrane-associated proteins that regulate Rag GTPases. SH3BP4 binding proteins will provide the critical information about the mechanism by which SH3BP4 regulates and coordinates endosomal trafficking and mTORC1 signaling in response to amino acids.

How SH3BP4 function is regulated

Whether SH3BP4 is a leucine sensor remains unclear. Amino acid starvation still suppressed mTORC1 signaling even in SH3BP4-silenced cells (Kim et al., 2012). Thus, SH3BP4 might be a modulator rather than a sensor of leucine. Alternatively, like LRS, SH3BP4 could act as a leucine sensor in different cellular compartments. Amino acids were able to affect cellular localization of mTORC1, which depended on vesicle trafficking proteins (Flinn et al., 2010; Hennig et al., 2006; Li et al., 2010; Sancak et al., 2008). The function of SH3BP4 to regulate vesicular trafficking might be important for mTORC1 regulation in response to amino acids. As mentioned above, the leucine-sensitive interaction between SH3BP4 and Rag GTPases implies a conformational change or a post-translational modification of SH3BP4 that enables alteration of its interaction with Rag GTPases in response to amino acids. SH3BP4 contains multiple phosphorylation sites including one mediated by Akt (Fig. 2) (Chen et al., 2009; Chiba et al., 2009; Pan et al., 2008). Hence, it is possible that Akt regulates the activity of SH3BP4 via phosphorylation of SH3BP4. Other phosphorylations are located near SH3, LIDL and NPF domains and motifs. The multiple phosphorylation sites imply that SH3BP4 might integrate multiple signals toward the regulation of Rag GTPases and other cellular processes.

SH3BP4 is a potential tumor suppressor

Given the negative role of SH3BP4 in the mTORC1 pathway, its expression could be potentially an important factor contributing to cancer cell growth or tumorigenesis. The insulin/IGF1-regulated mTORC1 pathway has several tumor suppressors and oncogenes, whereas the amino acid-regulated mTORC1 pathway does not yet have any identified tumor suppressor or oncogenes (Fig. 3). According to the human cancer genome databases and the cancer genome atlas (TCGA), the *SH3BP4* gene locus on the chromosome 2q37.1-2q37.2 is frequently deleted in many human cancers (Beroukhi et al., 2007; 2010; Kim et al., 2012). In particular, several subtypes of cancer inclu-

Fig. 3. SH3BP4 is a potential tumor suppressor functioning in the amino acid-mTORC1 pathway. *SH3BP4* gene is deleted at a high frequency in many tumors from patients. SH3BP4 might play a similar role in the amino acid-mTORC1 pathway as that played by PTEN in the insulin/IGF1-mTORC1 pathway as a tumor suppressor.

ding breast, renal and non-small cell lung cancers and neuroblastoma showed high frequencies of deletion of *SH3BP4* locus with a statistical significance. The gene *SH3BP4* also shows loss of heterozygosity (LOH) in many tumors. Thus, SH3BP4 is a potential tumor suppressor that functions in the amino acid-mTORC1 pathway. The potential tumor suppressor function might be due to its negative effect on Rag GTPases, although we could not exclude other unknown functions of SH3BP4.

Another possible role of SH3BP4 as a tumor suppressor might be related to its regulation of cell death. SH3BP4 contains one DD motif near the C-terminus (Fig. 2). The DD motif is a protein interaction module composed of a bundle of six α helices. The DD motif binds to each other by forming oligomers. Some DD-containing proteins are involved in the regulation of apoptosis and inflammation through activation of caspases and NF- κ B signaling. SH3BP4 has one ZU5 domain that can bind to DD (Reddy et al., 2011). It is possible that DD may be involved in intramolecular interaction with the ZU5 domain, influencing the interaction of SH3BP4 with endosomal proteins or Rag GTPases. Cells expressing SH3BP4 exhibited fragmentation of nuclei in HeLa cells, which is a morphological characteristic of apoptosis (Kim et al., unpublished data). It would be interesting to study whether the apoptosis-inducing role of SH3BP4 could contribute to tumor suppression.

CONCLUSION

The Rag GTPase regulators described above have greatly enhanced our understanding of how amino acids regulate mTORC1. Despite the recent advance in our understanding, there remain many important questions with respect to the regulatory mechanism of the amino acid-regulated mTORC1 pathway. First, we need a more detailed analysis of interaction between the regulators and Rag GTPases to obtain the information on the stoichiometry and the structure. Second, the molecular basis for the regulatory interaction, such as post-translational modifications, may need to be identified. It is possible that phosphorylations of SH3BP4 might regulate the cellular localization and interactions of SH3BP4 or its inhibitory effect on Rag GTPases. Third, the cellular trafficking machinery that regulates SH3BP4 localization needs to be identified. SH3BP4 has

multiple binding motifs for endosomal trafficking proteins (Fig. 2). Amino acids may regulate the interactions mediated by these motifs thereby altering the cellular localization of SH3BP4. Fourth, SH3BP4 needs to be studied further with respect to its potential role in mediating cell death via its DD motif. Fifth, it needs to be clarified how SH3BP4 regulation of transferrin receptor internalization is related to its regulation of Rag GTPases.

Although SH3BP4 homologues are not found in lower eukaryotes, we do not exclude the possibility that a protein with a divergent sequence might play a similar role in lower eukaryotes as that played by SH3BP4. SH3BP4 and 7a5 are the only proteins in the human genome that contain both the endosomal trafficking domains and the death domain. The unique feature of the domain composition implies that SH3BP4 and 7a5 might regulate endosomal trafficking and cell death pathways through previously-unknown mechanisms. The engagement of SH3BP4 in multiple putative functions, such as endocytosis and cell death pathways, implies its important role in coordinating endosomal trafficking, cell growth and apoptosis.

ACKNOWLEDGMENTS

We thank the Kim's lab members for the comments on this review. We apologize to those authors whose works have not been cited in reference due to space limitations. This work was supported by the NIH (DK050456, DK083474 and GM097057).

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