様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　２９　年　　５　月　２３　日現在

機関番号：　１３９０１
研究種目：　若手研究(B)
研究期間：　2015～2016
課題番号：　１５Ｋ２１０６１

研究課題名（和文）Study on the mechanism of Girdin-mediated amino acid signaling and regulation of mTORC1 activity in cancer cells

研究課題名（英文）Study on the mechanism of Girdin-mediated amino acid signaling and regulation of mTORC1 activity in cancer cells

研究代表者

翁　良（Weng, Liang）

名古屋大学・医学系研究科・特任助教

研究者番号：２０７２９２８０

交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：本研究ではアミノ酸トランスポーター複合体の構成因子である4F2hcと多機能ハブ分子でアクチン結合分子であるGirdinが結合し、本複合体がmTORC1（mechanistic target of rapamycin complex 1）の活性を抑制することを見出した。Girdinと4F2hcの結合はMAPKによるGirdinのリン酸化に依存し、その結果、4F2hcがリソソームに移行することを示した。これによる細胞表面の4F2hcの減少は細胞内のグルタミン量の低下およびmTORC1活性の低下に結びつく可能性も示した。本機序は、アミノ酸シグナルの脱感作を制御している可能性が示唆された。

研究成果の概要（英文）：Desensitization, which involves the downregulation of cell surface receptors, is a common negative regulatory process for cell signalings. Amino acid signaling mediated by the activation of mechanistic target of rapamycin complex 1 (mTORC1) is fundamental to cell growth and metabolism. However, how cells desensitize to amino acid signaling is unknown. Here, we show that interaction between 4F2 heavy chain (4F2hc), a subunit of multiple amino acid transporters, and the multifunctional hub protein Girdin downregulates mTORC1 activity. 4F2hc interacts with Girdin in mitogen-activated protein kinase- and amino acid signaling-dependent manners to translocate to the lysosome. The resultant decrease in cell-surface 4F2hc leads to lowered cytoplasmic glutamine content, which desensitizes cells to amino acid signaling. These findings uncovered the mechanism underlying desensitization of amino acid signaling, which may play a role in tightly regulated cell growth and metabolism.

研究分野：　実験病理学

キーワード：　アミノ酸シグナル　mTORC1　Girdin

１．研究開始当初の背景

Cells respond to extracellular stimuli through multiple signaling pathways, which govern and coordinate various cellular activities. Because of their vital roles, these signaling pathways need to be precisely controlled so that cells can respond appropriately to the external cues and maintain cell homeostasis. Signaling pathways mediated by receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) have a similar negative regulatory mechanism called “adaptation” or “desensitization” to adjust their sensitivity to signals, in which cells endocytose the receptors, which leads to their destruction in the lysosomes.

In recent years, mechanisms by which amino acids function to control several cellular processes have attracted considerable attention. Amino acids activate the mechanistic target of rapamycin complex 1 (mTORC1), which is a signaling complex composed of a conserved serine-threonine kinase mTOR, Raptor, mammalian lethal with SEC13 protein 8 (mLST8), Deptor, and the proline-rich Akt substrate of 40 kDa (PRAS40). mTORC1 is essential for multiple biological processes such as cell growth, anabolism, and autophagy. Notably, mTORC1 signaling is frequently deregulated in several diseases, including cancer and diabetes, which makes it an attractive target for drug discovery.

The mTORC1 pathway integrates both amino acid and growth factor signaling by distinct mechanisms in which several small guanosine triphosphatases (GTPases) play important roles. For example, growth factor stimulation activates mTORC1 via the GTPase Rheb, whereas amino acids activate mTORC1 through the heterodimeric Rag, adenosine diphosphate ribosylation factor-1 (Arf1), and Rab1 GTPases. Among these, Rag GTPases, which are tightly regulated by the Guanine nucleotide exchange factor (GEF) Ragulator and the GTPase-activating protein (GAP) GATOR downstream of amino acid sensors including Castor or sestrin 2, are the most studied. The activated Rag GTPases induce the translocation of mTORC1 to the lysosomes and its activation.

We previously identified Girdin as a substrate for the Akt kinase and an actin-binding protein, which is a multifunctional protein that interacts with several proteins and is involved in cell migration and neuroblast differentiation. Recently, we found that Girdin regulates endocytosis via its function as a GAP for dynamin 2. Intriguingly, Girdin has also been reported to be involved in cell-size control and the regulation of the phosphatidilinositol-3 kinase/Akt pathway, both of which are closely related to mTORC1 activity, suggesting that it may participate in the mTORC1 signaling pathway.

２．研究の目的

Although the mechanisms by which cells transmit amino acid signaling to mTORC1 have been elucidated, it remains unknown whether cells possess a desensitization mechanism for amino acid signaling as observed for the RTK and GPCR signaling pathways.

３．研究の方法

To investigate the role of Girdin in mTORC1 signaling, we employed affinity column chromatography to isolate Girdin-interacting proteins. To this end, glutathione sepharose beads coated with purified glutathione S-transferase (GST) protein or fused with Girdin amino-terminal (NT) domain (GST-NT) were incubated with whole cell lysate from HeLa cells. The interacting proteins eluted by high-salt solutions were analysed by mass spectrometry, which identified 4F2hc as a Girdin-interacting protein. The interaction of 4F2hc with the Girdin NT domain was confirmed by co-immunoprecipitation (co-IP) and western blot analysis.

Next, we investigated the mechanism underlying the 4F2hc/Girdin interaction downstream of serum and amino acid stimulation. An in vitro kinase assay was performed to check on whether Girdin is phosphorylated by MAPK. IP and Ni-NTA beads pulldown were employed to test the ubiquitination of 4F2hc. Girdin phosphorylation sites mutants and 4F2hc ubiquitination sites mutants were generated to confirm the effect of MAPK-induced phosphorylation and amino acids-induced ubiquitination of 4F2hc on Girdin/4F2hc interaction.

To see the effect of Girdin and 4F2hc on mTORC1 signaling, RNAi and CRISPR/Cas9 were used to deplete Girdin expression. Amino acids-induced mTORC1 activation was checked by Western blot analysis. GFP-LC3 was transfected into the cells to monitor the effect of Girdin and 4F2hc on autophagy.

Immunofluorescence analysis was used to further test the effect of Girdin on 4F2hc internalization. In addition, cell surface proteins were isolated to verify the role of Girdin in the translocation of 4F2hc to lysosome. Finally, we isolated cell cytosolic and lysosomal fraction to detect the amino acids contents by amino acids analyzer.

４．研究成果

By affinity column chromatography and mass spectrometry analysis, we identified 4F2hc as a Girdin interacting protein (Figure 1). Girdin amino-terminal (NT) domain was responsible for the association with 4F2hc. Furthermore, 4F2hc interacted with Girdin in mitogen-activated protein kinase (MAPK)- and amino acid signaling-dependent manners. MAPK-mediated phosphorylation of Girdin Ser233 and Ser237, which occured downstream of several growth factors, was critical for its interaction with 4F2hc. In addition, amino acids-induced ubiquitination of 4F2hc also played an important role in its interaction with Girdin. Mutation either Girdin phosphorylation sites or 4F2hc ubiquitination sites abolished their interaction.

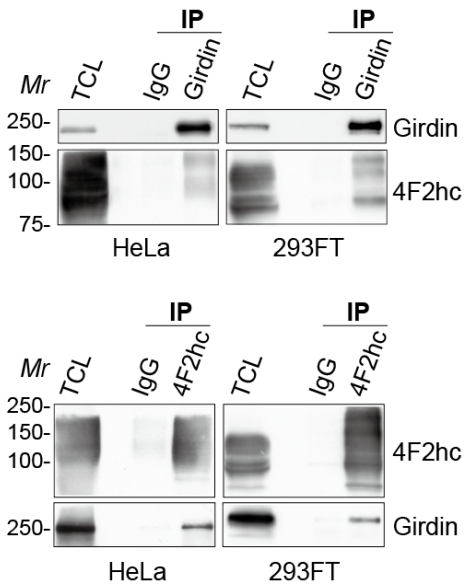

Figure 1. Girdin interaction with 4F2hc. Immunoprecipitation data indicated the interaction of endogenous Girdin and 4F2hc proteins in HeLa and 293FT cells.

Unfortunately, we failed to develop anti-Girdin pSer233/237 antibody, which was probably due to the complicated conformation of Girdin-NT domain. Therefore, we could not check the localization of pGirdin Ser233/237 in cells.

Considering that 4F2hc is a component of multiple heterodimeric amino acid transporters, we next asked whether the 4F2hc/Girdin complex regulates mTORC1 activity（Figure 2）. We found Girdin negatively regulated amino acid signaling through 4F2hc in several different cell lines, including 293FT cells, HeLa cells and MEFs. Consistently, Girdin depletion augmented amino acid-induced mTORC1 activation and inhibits amino acid deprivation-induced autophagy.

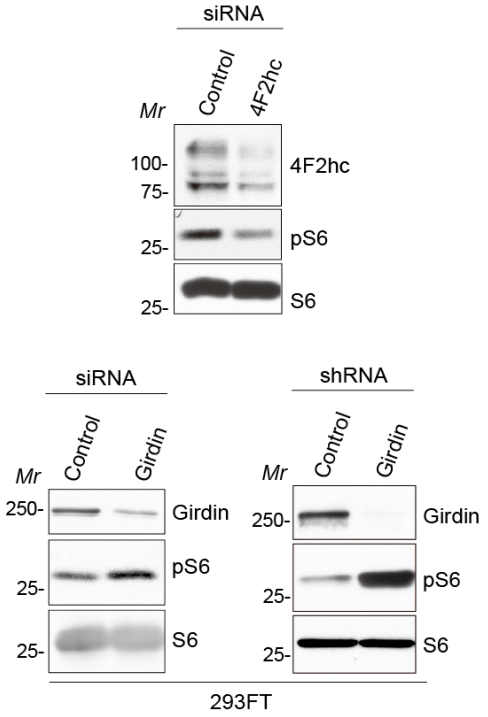

Figure 2. Girdin and 4F2hc regulated the activity of mTORC1 complex. Lysates from cells transduced with the indicated siRNA or shRNA were subjected to Western blot analysis. Note that pS6 representes the activity of mTORC1.

To further investigate the mechanism underlying the involvement of Girdin/4F2hc in amino acids signaling, we examined the effect of Girdin on 4F2hc internalization. The expression of WT Girdin, but not the AA mutant, promoted 4F2hc translocation to the lysosomes after amino acid stimulation (Figure 3). Consistently, cell fractionation showed that Girdin overexpression and depletion decreased and increased the cell-surface level of 4F2hc, respectively. Together, these results suggested that the

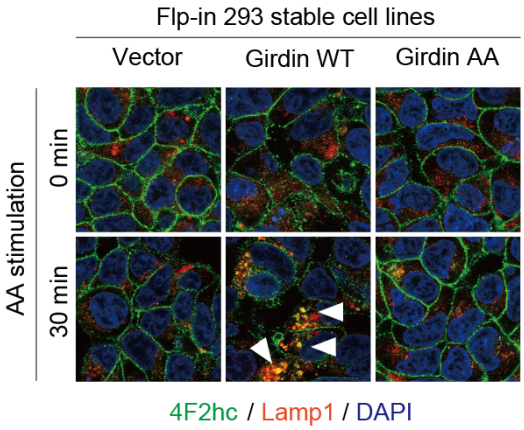

Figure 3. The effect of Girdin WT (wild type) and its unphosphorylatable mutant (AA) on the internalization of 4F2hc was shown. Arrowheads indicate the colocalization of 4F2hc and Lamp1 that is a maker of the lysosome.

4F2hc/Girdin interaction promotes the translocation of 4F2hc from the plasma membrane to the lysosome.

Considering that Girdin downregulated the cell-surface level of 4F2hc via internalization and previous reports showing that amino acids in both the cytosol and the lysosomes are crucial for mTORC1 activation, we next asked whether the 4F2hc/Girdin complex regulates mTORC1 activity through the modulation of intracellular amino acids contents. To this end, we comprehensively measured amino acids in the cytosol and in lysosomes. The overexpression of Girdin and 4F2hc led to a significant decrease in Gln, but not Leu or Arg, in the cytosol, whereas the lysosomal contents of Gln were comparable between the groups. These data suggested that Girdin and 4F2hc modulate amino acid signaling through the regulation of cytosolic Gln uptake. Supporting this notion, Girdin depletion significantly up-regulated mTORC1 activation in cells stimulated with Gln.

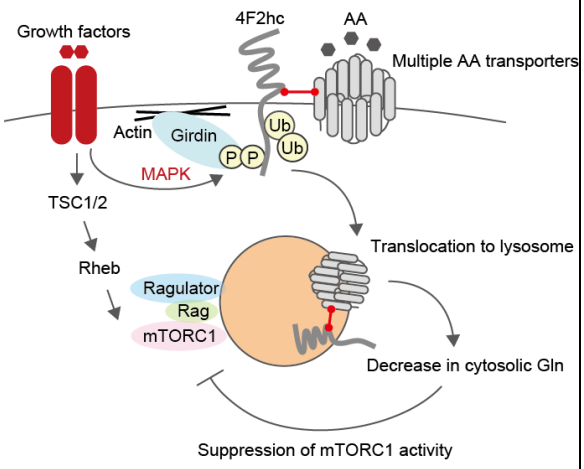

Figure 4. Schematic illustration showing the negative regulation of mTORC1 activity by Girdin/4F2hc protein complex.

In summary, this study, for the first time, showed that amino acid signaling has its own desensitization process, which is required for the cooperation between 4F2hc and Girdin. Girdin down-regulates the cell surface level of 4F2hc via endocytosis, which subsequently decreases cytosolic Gln content to desensitize cells to amino acid stimulation. The study also showed a crosstalk between the MAPK and amino acid signalling pathways, which extends our understanding on amino acid signaling. In addition, our work revealed that cytosolic glutamine content is the most critical driver factor for mTORC1 activation.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 3 件）

①Maeda K, Enomoto A, Hara A, Asai N, Kobayashi T, Horinouchi A, Maruyama S, Ishikawa Y, Nishiyama T, Kiyoi H, Kato T, Ando K, Weng L, Mii S, Asai M, Mizutani Y, Watanabe O, Hirooka Y, Goto H, Takahashi M. Identification of Meflin as a Potential Marker for Mesenchymal Stromal Cells. Sci Rep. 6:22288, 2016.（査読有）

②Muramatsu A, Enomoto A, Kato T, Weng L, Kuroda K, Asai N, Asai M, Mii S, Takahashi M. Potential involvement of kinesin-1 in the regulation of subcellular localization of Girdin. Biochem Biophys Res Commun. 463:999-1005, 2015.（査読有）

③Ichimiya H, Maeda K, Enomoto A, Weng L, Takahashi M, Murohara T. Girdin/GIV regulates transendothelial permeability by controlling VE-cadherin trafficking through the small GTPase, R-Ras. Biochem Biophys Res Commun. 461:260-7, 2015.（査読有）

〔学会発表〕（計 3 件）

①榎本 篤、翁 良、韓 一梵、浅井 直也、三井 伸二、高橋 雅英. 脳腫瘍幹細胞における mTORC1 活性制御とその分子機構の解析. 第 62 回日本病理学会秋期特別総会、金沢市文化ホール（石川県金沢市）、 2016 年 11 月 11 日（ポスター）

②Weng L、Han Y、Enomoto A、Kitaura Y、Asai N、Shimomura Y、Takahashi M：Amino acid signaling is desensitized by MAPK-mediated regulation of the 4F2hc-Girdin complex. 第 75 回日本癌学会学術総会、パシフィコ横浜（神奈川県横浜市）、2016 年 10 月 8 日（ポスター）

③Weng L, Enomoto A, Asai N, Kato T, Watanabe T, Kaibuchi K, Takahashi M. Regulation of cargo-selective endocytosis by dynamin 2 GTPase-activating protein girdin. The 6th EMBO meeting, The International Convention Centre（英国バーミンガム）、2015 年 9 月 6 日（ポスター）

６．研究組織
(1)研究代表者
翁 良（Weng Liang）
名古屋大学・大学院医学系研究科・特任助教
研究者番号：20729280

(2)研究分担者
なし