Rapgef2, a guanine nucleotide exchange factor for Rap1 small GTPases, plays a crucial role in adherence junction (AJ) formation in radial glial cells through ERK-mediated upregulation…

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学位論文の内容要旨

Rapgef2, a guanine nucleotide exchange factor for Rap1 small GTPases, plays a crucial role in adherence junction (AJ) formation in radial glial cells through ERK-mediated upregulation of the AJ-constituent protein expression

低分子量 G 蛋白質 Rap1 のグアニンヌクレオチド交換因子 Rapgef2 は、ERK を介した adherence junction (AJ) 構成蛋白質の発現上昇により、放射状グリア細胞における AJ の形成に重要な機能を果たす。

神戸大学大学院医学研究科医科学専攻

分子生物学

（指導教員：片岡　徹　教授）

Maged Ibrahim Ibrahim Abu-Zeid Farag

Introduction

Cerebral cortical development requires highly orchestrated events involving proliferation, differentiation and migration of neural progenitors and neurons. During neurogenesis, radial glial cells (RGCs), located in the ventricular zone at the apical side of the neuroepithelium, function as neural progenitors and generate self-renewing RGCs and neurons as well as committed intermediate progenitor cells. Intermediate progenitor cells move to the subventricular zone, divide and differentiate into neurons. Newly-born neurons migrate to the intermediate zone, where they change their morphology from multipolar to bipolar and migrate along the RG fibers to the cortical plate. Finally, they undergo RG fiber-independent somal translocation to reach their final destinations.

Rap1 small GTPases, consisting of two isoforms Rap1A and Rap1B, play pivotal roles in regulation of cell proliferation, polarity and cell adhesion by cycling between GTP-bound active and GDP-bound inactive forms. Interconversion between the two forms is reciprocally catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins, where GEFs activate Rap1 in response to extracellular stimuli by catalyzing GDP release and thereby accelerating GTP loading. There exist more than ten GEFs specific for Rap1, including Rapgefs 1 to 6, which are regulated by distinct mechanisms and responsible for differential regulation of Rap1 activity in spacio-temporally defined and cell-type-specific manners. During the cerebral cortical development, Rap1 plays crucial roles in neuronal migration, particularly in multipolar migration, multipolar-bipolar transition and terminal translocation.

Rapgef2, also called RA-GEF-1 or PDZ-GEF1, and Rapgef6, also called RA-GEF-2, constitute a unique Rap1-GEF subfamily characterized by possession of the Ras/Rap-associating domain. Through association with the GTP-bound forms of Rap1 and M-Ras at the Ras/Rap-associating domains, they are recruited from the cytoplasm to the Golgi complex and the plasma membranes, respectively, and cause Rap1 activation. We found that dorsal telencephalon-specific *Rapgef2* conditional knockout (*Rapgef2*-cKO) mice develop an ectopic cortical mass (ECM) resembling that seen in subcortical band heterotopia and that additional knockout of *Rapgef6* in *Rapgef2*-cKO mice resulted in gross enlargement of the ECM. Moreover, *Rapgef2*-cKO mice displayed severe defects in formation of the apical surface structure, which is composed of an assembly of the endfeet of the apical RG fibers linked together by an array of AJs, resulting in earlier detachment and aberrant localization of RGCs around embryonic day (E) 14.5. In addition, Rapgef2 and Rap1 are implicated in multipolar-bipolar transition of post-mitotic neurons. However, little is known about the molecular mechanism underlying these functions of Rapgef2 and Rap1 during cortical development. In this study, we use a primary culture of RGCs established from the developing cerebral cortex to address this problem.

Methods and Results

Morphological and adherent properties of *Rapgef2*-f/f and *Rapgef2*-cKO RGCs

Developing cerebral cortices of E14.5 embryos were dissected from *Rapgef2*$^{flox/flox}$ (*Rapgef2*-f/f) and *Rapgef2*$^{flox/flox}$;*Emx1*$^{Cre/+}$ (*Rapgef2*-cKO) brains to establish neurosphere cultures. RGC cultures were established by dissociating the neuropheres and spreading the dissociated cells onto laminin/lysine-coated culture dishes. The establishment of primary

cultures was confirmed by the substantial reduction of *Rapgef2* mRNA and protein levels in *Rapgef2*-cKO cultures compared to *Rapgef2*-f/f cultures.

Comparison between the *Rapgef2*-f/f and *Rapgef2*-cKO neurosphere cultures revealed that *Rapgef2*-cKO neurospheres were much smaller in size than *Rapgef2*-f/f neurospheres. Moreover, *Rapgef2*-cKO RGCs had their RG fibers markedly shortened or almost disappeared compared to *Rapgef2*-f/f RGCs, and *Rapgef2*-cKO RGCs assumed more round morphology with a smaller size. These results suggested that Rapgef2-deficient cells had lower adherent capacity.

Reduction of the AJ protein expression in RGCs deficient in *Rapgef2*

To clarify the molecular mechanism underlying the disruption of the apical surface AJ structures in *Rapgef2*-cKO cerebral cortex, we compared the expression level of AJ-constituent proteins such as N-cadherin, ZO-1, E-cadherin, β-catenin and afadin, which were heavily concentrated on the apical surface structure, between *Rapgef2*-f/f and *Rapgef2*-cKO RGCs. The expression of N-cadherin, ZO-1, E-cadherin and β-catenin, but not afadin, was substantially reduced in *Rapgef2*-cKO RGCs at both the mRNA and protein levels. Moreover, immunostaining for the AJ proteins showed that while all *Rapgef2*-f/f RGCs were positive for AJ proteins as well as Rapgef2, most of *Rapgef2*-cKO RGCs, negative for Rapgef2, were stained very weakly for the AJ proteins.

Effects of knockdown and overexpression of Rapgef2, Rapgef6 and Rap1A on AJ protein expression

We examined the effects of siRNA-mediated knockdown of *Rapgef2*, *Rapgef6* and *Rap1A* on the AJ protein expression in *Rapgef2*-f/f RGCs. Transfection of the *Rapgef2* siRNA yielded a substantial population of cells that showed very low expression of both Rapgef2 and the AJ proteins, indicating that *Rapgef2* knockdown inhibited the AJ protein expression. Moreover, these cells assumed more round morphology and possessed shortened RG fibers compared to the cells expressing both Rapgef2 and the AJ proteins. We obtained similar results by the *Rap1A* siRNA treatment. On the contrary, the *Rapgef6* siRNA failed to inhibit the AJ protein expression and alter the cell morphology.

We next examined the effects of overexpression of Rapgef2, Rapgef6 and the constitutively active mutant, Rap1A^{G12V} on the AJ protein expression in *Rapgef2*-cKO RGCs. Transfection of the expression plasmids for Rapgef2, Rapgef6 and Rap1A^{G12V} all yielded substantial populations of cells that expressed AJ proteins simultaneously with Rapgef2, Rapgef6 and Rap1A^{G12V}, respectively. Moreover, these cells assumed less round morphology and resumed long RG fibers, which were similar to those of *Rapgef2*-f/f RGCs.

Roles of extracellular signal-regulated kinase (ERK) and c-jun in Rapgef2/Rap1A-dependent regulation of AJ protein expression

We finally examined the role of the Raf-MEK-ERK pathway in Rapgef2/Rap1A-dependent regulation of AJ protein expression because Rap1 had been known to activate this pathway through direct association with B-Raf in neurons. The phosphorylation levels of MEK and ERK were substantially diminished in *Rapgef2*-cKO RGCs compared to *Rapgef2*-f/f RGCs. Moreover, both the amounts of total and phosphorylated c-jun, a subunit of activator protein-1 transcription factor, were also diminished in *Rapgef2*-cKO RGCs compared to *Rapgef2*-f/f RGCs. Moreover, treatment of *Rapgef2*-f/f RGCs with Trametinib, a MEK inhibitor, inhibited the expression of not only c-jun but also AJ proteins at both the mRNA and protein levels. Also, treatment with AGE3482, an inhibitor of c-jun phosphorylation, inhibited the expression of the AJ proteins but not c-jun at both the mRNA and protein levels.

Discussion

Rap1 was shown to play pivotal roles in cadherin-mediated cell adhesion by controlling the assembly of the AJ complex. In this study, we have analyzed the molecular mechanism underlying the disruption of the apical surface AJs observed in developing *Rapgef2*-cKO cerebral cortex using primary cultures of RGCs isolated from E14.5 embryos and demonstrated a novel role of the Rapgef2-Rap1A-ERK-c-jun pathway in regulation of the expression of AJ proteins. Also, *Rapgef2*-cKO RGCs exhibit a decreased ability to form neurospheres in culture, suggesting an impairment of their adherent capacity. Because the AJ-constituent proteins are highly concentrated in the apical surface structure, downregulation of their expression observed in *Rapgef2*-cKO RGCs may well account for the disruption of the apical surface structure observed in *Rapgef2*-cKO cerebral cortex. Moreover, we have shown that *Rapgef2*-cKO RGCs assume more round morphology with a smaller size and have their RG fibers markedly shortened or almost disappeared, both of which are partly reversed by overexpression of Rapgef2 and Rap1A^{G12V}. These morphological alterations appear to bear a considerable resemblance to those observed *in vivo* for *Rapgef2*-cKO RGCs prematurely detached from the apical surface structure, which assume a round morphology and have their basal RG fibers heavily disorganized and their apical RG fibers disappeared.

Our results have also revealed that Rapgef6 plays only an auxiliary role in the physiological condition although it possesses a redundant function with Rapgef2 and is able to implement the Rapgef2 function when artificially overexpressed. This is consistent with our previous observation that additional knockout of *Rapgef6* in *Rapgef2*-cKO mice results in gross enlargement of the ECM.

We have also shown that the Rapgef2-Rap1A-ERK pathway regulates the AJ protein expression through upregulating c-jun expression in RGCs. However, the molecular mechanisms for transcriptional regulations of the expression of c-jun by activated ERK and of AJ proteins by c-jun are presently unknown and remain to be clarified in our future studies. It is noteworthy that rare inherited copy number variations of the *RAPGEF2* and *RAPGEF6* genes were reported to exhibit strong genetic association with schizophrenia. Further investigation on the functions of Rapgef2 and Rapgef6 may advance our understanding of the mechanism of the cerebral corticogenesis as well as the etiology of various central nervous system diseases.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第 2735 号	氏 名	Maged Ibrahim Ibrahim Abu-Zeid Farag
論文題目 Title of Dissertation	Rapgef2, a guanine nucleotide exchange factor for Rap1 small GTPases, plays a crucial role in adherence junction (AJ) formation in radial glial cells through ERK-mediated upregulation of the AJ-constituent protein expression 低分子量G蛋白質Rap1のグアニンヌクレオチド交換因子Rapgef2は、ERKを介したadherence junction（AJ）構成蛋白質の発現上昇により、放射状グリア細胞における AJ の形成に重要な機能を果たす		
審査委員 Examiner	主 査 Chief Examiner 的場尚 副 査 Vice-examiner 榎本秀樹 副 査 Vice-examiner 平島正則		

（要旨は１，０００字～２，０００字程度）

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Developing cerebral cortices of E14.5 embryos were dissected from *Rapgef2*$^{flox/flox}$ (*Rapgef2*-f/f) and *Rapgef2*$^{flox/flox}$;*Emx1*$^{Cre/+}$ (*Rapgef2*-cKO) brains to establish neurosphere cultures. RGC cultures were established by dissociating the neuropheres and spreading the dissociated cells onto laminin/lysine-coated culture dishes. Comparison between the *Rapgef2*-f/f and *Rapgef2*-cKO neurosphere cultures revealed that *Rapgef2*-cKO neurospheres were much smaller in size than *Rapgef2*-f/f neurospheres. Moreover, *Rapgef2*-cKO RGCs had their RG fibers markedly shortened and assumed more round morphology with a smaller size. To clarify the molecular mechanism for the disruption of the apical surface AJ structures in *Rapgef2*-cKO cerebral cortex, the expression levels of AJ-constituent proteins such as N-cadherin, ZO-1, E-cadherin, β-catenin and afadin were compared between *Rapgef2*-f/f and *Rapgef2*-cKO RGCs. The expression of N-cadherin, ZO-1, E-cadherin and β-catenin, but not afadin, was substantially reduced in *Rapgef2*-cKO RGCs at both the mRNA and protein levels. Moreover, immunostaining showed that while all *Rapgef2*-f/f RGCs were positive for both the AJ proteins and Rapgef2, most of *Rapgef2*-cKO RGCs, negative for Rapgef2, were stained very weakly for the AJ proteins.

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expression in *Rapgef2*-f/f RGCs were examined. Transfection of the *Rapgef2* siRNA yielded a substantial population of cells that showed very low expression of both Rapgef2 and the AJ proteins, indicating that *Rapgef2* knockdown inhibited the AJ protein expression. Moreover, these cells assumed more round morphology and possessed shortened RG fibers. Similar results were obtained by the *Rap1A* siRNA treatment. On the contrary, the *Rapgef6* siRNA failed to inhibit the AJ protein expression and alter the cell morphology. Next, the effects of overexpression of Rapgef2, Rapgef6 and the constitutively active Rap1A^{G12V} on the AJ protein expression in *Rapgef2*-cKO RGCs were examined. Transfection of the expression plasmids for Rapgef2, Rapgef6 and Rap1A^{G12V} all yielded substantial populations of cells that expressed AJ proteins simultaneously with Rapgef2, Rapgef6 and Rap1A^{G12V}, respectively. These cells assumed less round morphology and resumed long RG fibers.

Finally, the role of the Raf-MEK-ERK pathway in Rapgef2/Rap1A-dependent regulation of AJ protein expression was examined because Rap1 had been known to activate this pathway through direct association with B-Raf in neurons. The phosphorylation levels of MEK and ERK were substantially diminished in *Rapgef2*-cKO RGCs compared to *Rapgef2*-f/f RGCs. Moreover, both the amounts of total and phosphorylated c-jun, a subunit of AP-1 transcription factor, were also diminished in *Rapgef2*-cKO RGCs compared to *Rapgef2*-f/f RGCs. Moreover, treatment of *Rapgef2*-f/f RGCs with Trametinib, a MEK inhibitor, inhibited the expression of not only c-jun but also AJ proteins at both the mRNA and protein levels. Also, treatment with AGE3482, an inhibitor of c-jun phosphorylation, inhibited the expression of the AJ proteins at both the mRNA and protein levels.

Rap1 had been shown to play pivotal roles in cadherin-mediated cell adhesion by controlling the assembly of the AJ complex. The results of this study demonstrated a novel role of the Rapgef2-Rap1A in regulation of the expression of AJ proteins via activation of ERK-c-jun. Also, Rapgef2-deficient RGCs exhibit a decreased ability to form neurospheres in culture, suggesting an impairment of their adherent capacity. Because the AJ proteins are highly concentrated in the apical surface structure, downregulation of their expression observed in Rapgef2-deficient RGCs may well account for the disruption of the apical surface structure in *Rapgef2*-cKO cerebral cortex. Moreover, it was shown that Rapgef2-deficient RGCs assume more round morphology with a smaller size and marked regression of RG fibers, both of which are reversed by overexpression of Rapgef2 and Rap1A^{G12V}. These morphological alterations bear considerable resemblance to those of *Rapgef2*-cKO RGCs prematurely detached from the apical surface.

It is noteworthy that rare inherited copy number variations of the *RAPGEF2* and *RAPGEF6* genes were reported to exhibit strong genetic association with schizophrenia. Further investigation on the functions of Rapgef2 and Rapgef6 may advance our understanding of the mechanism of the cerebral corticogenesis as well as the etiology of various central nervous system diseases.

The candidate, having completed studies on the regulatory mechanism for the adherence junction formation in RGCs and having advanced the field of knowledge in the area of Developmental Neuroscience, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).