C．研究結果

Tunisia 型 *Als2* ターゲティングベクターをマウス 129/Ola 由来 ES 細胞株 E14.1 に導入し、相同組み換え体を 14 クローン同定した。このうち 6 クローンについて、キメラマウス作製を行い、2 クローン（17C6 および 21B5）より生殖系列キメラが得られた。交配によりヘテロ変異マウスが得られ、さらにヘテロ変異マウス同士を交配することによりホモ変異マウスを得た。ホモ変異マウスは遺伝子およびタンパク質レベルで ALS2 の発現が失われていることから、*Als2* 遺伝子欠損マウスの作出に成功したことを確認した。12-18 ヶ月齢まで加齢させて観察を行ったが、*Als2* 遺伝子欠損マウスはヒトの筋萎縮性側索硬化症様の病態は示さなかった。

D．考察

Tunisia 型 *Als2* 遺伝子欠損マウスを加齢させてもヒトの筋萎縮性側索硬化症様の病態が認められないことから、（1）ヒトと異なりマウスには ALS2 の機能を相補する機構が存在する、（2）マウスの遺伝的背景の影響を受ける（今回の解析に用いたマウスは 129/Ola と C57BL/6J の F2 交雑系）、（3）神経細胞の生存期間とマウス／ヒトの寿命の違い（マウスの寿命は 1.5ー2 年ほど）などの可能性が考えられた。そのため、戻し交配により遺伝的背景を均一化する、神経細胞に対して刺激または負荷をかけるなど検討を行うことが必要であると考えられる。

E．結論

Tunisia 型 *Als2* 遺伝子欠損マウスの作出に成功したが、ヒトの筋萎縮性側索硬化症様の病態は認められず、単純に ALS モデルとなり得ないことが明らかとなった。

F．健康危険情報

特になし

G．研究発表

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H．知的財産権の出願・登録状況

１．特許取得　なし
２．実用新案登録　なし
３．その他　なし

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書
ALS2 蛋白の分子動態解析
研究分担者　成宮　周
京都大学医学研究科　神経・細胞薬理学　教授

研究要旨

ALS2 には、Rho ファミリー低分子量 GTP 結合蛋白質の活性化モチーフとして知られる DH/PH ドメインが存在する。このドメインの機能とこの欠損が筋萎縮性索硬化症の症状発現に果たす役割を検討するため、これが活性化すると思われる Rho 情報伝達の神経系での役割の検討を行った。既に前年度の研究で、Rho が下流分子の使い分けにより軸索の退縮にも伸長にも働くことを見出した。前者では Rho から ROCK (Rho キナーゼ)に至る経路が、後者では、Rho から mDia に至る経路が主として働く。今年度は、ROCK の神経系での役割を探るため昨年度の ROCK-II 遺伝子欠損マウスに続き、ROCK-I 遺伝子欠損マウスを作製したが、これまでのところ脳脊髄において神経構築の異常は観察されていない。また、昨年度には、mDia が真直ぐなアクチン線維の形成に働いていること、また、分裂期、間期を問わず微小管の安定化に働くことを示したが、今年度は、これら作用の細胞遊走における役割を解析した。また、ALS２と同様の DH-PH 領域をもつ RhoGDP-GTP 交換因子 Ect2 の分裂期における役割を解析した。

A．研究目的

ALS2 は、分子内に DH (Dbl homology) domain と PH (Pleckstrin homology) domain が tandem に並んだ構造をもつが、これは Rho ファミリー低分子量G蛋白質より GDP を遊離し GTP に交換する Rho 活性化因子の指紋モチーフである。このことより、ALS2 は、なんらかの Rho ファミリー蛋白質を活性化して働いている可能性が強い。本分担研究の目的は、この仮説を検証し、ALS2 によって活性化される Rho ファミリー蛋白質のメンバーを同定、これが神経細胞で働く機能を明らかにし、ALS2 分子の異常と筋萎縮性側索硬化症の病態との関連を明らかにすることである。

B．研究方法（倫理面への配慮）

１．ROCK-I 遺伝子の protein kinase domain の一部をコードする exon 3 と 4 の大半を b-galactosidase-Neomycin-resistance gene で置換し、targeting vector を作製した。これを用いて gene targeting を行い、ROCK-I 遺伝子欠損マウスを作製、解析を行った。この研究は京都大学の実験動物に対するガイドラインの下で行った。

２．Rho 情報伝達の下流で、神経突起伸長を行う mDia ファミリー蛋白質のうち、mDia1 の細胞移動における役割を、ラット C6 グリオーマ細胞で RNAi を用いて検討した。

３．HeLa 細胞を用いて分裂期での Ect2 の役割を RNAi を用いて解析した。

C．研究結果と考察

１．Rho 経路の神経系での役割を解析する目的で、ROCK-I 遺伝子について targeting を行い、欠損マウスを作製した。ROCK-I 欠損マウスは、

閉眼異常と臍ヘルニアを呈し、この異常がそれぞれの部位におけるアクトミオシン束の形成不全によることが明らかになった。このことから、ROCK-I が発生期におけるこれらの部位での細胞間を結びつけるようなアクトミオシンリングの形成に関与し組織閉鎖を行わしめていることが明らかとなった。一方、神経系では、HE 染色、ゴルジ染色などでのこれまでの解析では神経構築、神経走行の異常は認められなかった。

2．ラット C6 glioma 細胞を transwell、Dunn chamber, wound healing など様々な細胞移動のアッセイ系に供し、mDia1 を RNAi でノックダウンすることにより、その機能を解析した。その結果、mDia1 が細胞の極性の発現に関与し、また、細胞接着斑の回転の調節を行っていることを明らかにした。

3．Rho family GEF である Ect2 の細胞分裂期における役割を、Ect2 の RNAi および dominant negative 体の発現などにより解析した。その結果、Ect2 は分裂前期から中期にかけては Cdc42 の調節因子として核分裂に、分裂後期から終期にかけては Rho の調節因子として働くことを明らかにした。この結果は、同一の分子であっても、それが会合する分子や化学修飾によって異なった Rho GTPase に働く可能性を明らかにしたものである。

D．結論

本年度の研究から、Rho 蛋白による細胞機能制御について新しい知見を得ることができた。これは、神経細胞形態や機能の制御に通じるものであるが、今後これらの作用と ALS2 の機能発現の間を詰めていくことが必要である。

E．健康危険情報

特になし

F．研究発表

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G. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

III. 研 究 成 果 一 覧

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IV.　研究成果の刊行物・別刷

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Homo-oligomerization of ALS2 through Its Unique Carboxyl-terminal Regions Is Essential for the ALS2-associated Rab5 Guanine Nucleotide Exchange Activity and Its Regulatory Function on Endosome Trafficking*

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Mutations in the *ALS2* gene have been known to account for a juvenile recessive form of amyotrophic lateral sclerosis (ALS2), a rare juvenile recessive form of primary lateral sclerosis, and a form of hereditary spastic paraplegia (HSP), indicating that the ALS2 protein is essential for the maintenance of motor neurons. Recently, we have demonstrated that the ALS2 protein specifically binds to the small GTPase Rab5 and acts as a GEF (guanine nucleotide exchange factor) for Rab5. We have also shown that its Rab5GEF-requisite domain resides within the C-terminal 640-amino acid region spanning membrane occupation and recognition nexus motifs and the vacuolar protein sorting 9 domain. Transiently expressed ALS2 localized onto early endosomal compartments and stimulated endosome fusions in neuronal and non-neuronal cells in an Rab5GEF activity-dependent manner. These results indicate that the C-terminal region of ALS2 plays a crucial role in endosomal dynamics by its Rab5GEF activity. Here we delineate a molecular feature of the ALS2-associated function through the C-terminal region-mediated homo-oligomerization. A yeast two-hybrid screen for interacting proteins with the ALS2 C-terminal portion identified ALS2 itself. ALS2 forms a homophilic oligomer through its distinct C-terminal regions. This homo-oligomerization is crucial for the Rab5GEF activity *in vitro* and the ALS2-mediated endosome enlargement in the cells. Taken together, these results indicate that oligomerization of the ALS2 protein is one of the fundamental features for its physiological function involving endosome dynamics *in vivo*.

ALS2 was initially identified as a causative gene for a juvenile recessive form of amyotrophic lateral sclerosis (ALS2),[1] and a rare juvenile recessive form of primary lateral sclerosis (PLSJ) (1, 2). ALS2 is characterized by a loss of upper motor neurons and spasticity of limb and facial muscles occasionally associated with several signs of lower motor neuron defects (3), whereas PLSJ affects only upper motor neurons (4). Recently, several independent homozygous *ALS2* mutations have been found in families segregating an infantile-onset ascending hereditary spastic paralysis (IAHSP) (5–7) and a single family of a recessive complicated hereditary spastic paraplegia (HSP) (8). Thus, *ALS2* mutations account for a number of juvenile recessive motor neuron diseases, indicating that the ALS2 protein plays an important role in the maintenance and/or survival of motor neurons.

The *ALS2* gene encodes a protein of 1657 amino acid residues (aa), which contains three putative guanine nucleotide exchange factor (GEF) domains (1, 2). The N-terminal half of the ALS2 protein shares significant homology with RCC1 (regulator of chromosome condensation 1) (9), and this region is referred to as an RCC1-like domain (RLD), which has been found in a number of proteins (10–13). Although RCC1 acts as a GEF for Ran (Ras-related nuclear) GTPase (9), the functions for RLD domains are still unclear. RLD is followed by a tandem organization of Dbl homology (DH) and pleckstrin homology (PH) domains, which is a hallmark for GEFs for Rho (Ras homologous member) GTPases (14). The C-terminal end of ALS2 harbors a vacuolar protein sorting 9 (VPS9) domain, which has been found in Rab5 (Ras-related in brain 5) GEFs, including Vps9 (15), Rabex-5 (16), RIN1 (17, 18), RIN2 (19), and RIN3 (20). In addition, eight consecutive membrane occupation and recognition nexus (MORN) motifs (21) were noted in the region between PH and VPS9 domains.

The small GTPases generally control a wide range of fundamental cellular processes, including nuclear transport, cy-

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[1] The abbreviations used are: ALS2, amyotrophic lateral sclerosis 2; PLS, primary lateral sclerosis; HSP, hereditary spastic paraplegia; IAHSP, infantile-onset ascending hereditary spastic paralysis; GEF, guanine nucleotide exchange factor; aa, amino acid residues; MORN, membrane occupation and recognition nexus; RLD, RCC1-like domain; DH, Dbl homology; PH, pleckstrin homology; VPS9, vacuolar protein sorting 9; Y2H, yeast two-hybrid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; EEA1, early endosome (auto) antigen 1; EGFP, enhanced green fluorescent protein; WT, wild type; HA, hemagglutinin; Rab5, Ras-related in brain 5; PFA, paraformaldehyde.

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toskeletal reorganization, transcription, cell migration, and membrane trafficking (22–28). They serve as binary switches, cycling between inactive GDP-bound and active GTP-bound states (28). GEFs are known to activate the small GTPases by stimulating the release of GDP in exchange for GTP (29). In light of conserved GEF domains of ALS2, it appears to act as an activator of particular small GTPases, thereby regulating specific cellular processes.

We have recently demonstrated that one of three conserved GEF domains, VPS9, functions as an essential element for the ALS2-associated Rab5GEF activity (30). Transiently expressed ALS2 localized onto early endosomal compartments and facilitated the enlargement of endosomes in primary cultured cortical neurons (30). Ectopic expression of the ALS2 fragment comprising aa 660–1657 lacking RLD (ALS2_660–1657), which functioned as a constitutive active form, induced the prominent enlargement of endosomes (30). However, two Rab5GEF-defective VPS9 mutants, including ALS2_660–1657 (P1603A) and ALS2_660–1657 (L1617A), showed no enlarged endosome phenotypes (30), implying that the Rab5GEF activity is primarily important. We have also shown that the functional domain for the Rab5GEF activity resides within the C-terminal 640-aa region spanning MORN and VPS9 domains. Based upon these findings, it is certain that the MORN/VPS9 region of ALS2 is one of the requisite domains for the ALS2-mediated endosome dynamics *in vivo*. However, the molecular mechanism by which the ALS2 C-terminal MORN/VPS9 region takes part in early stages of the endocytic pathway is largely unknown.

To gain an insight into the molecular functions inherent in MORN/VPS9 domains, we investigated the C-terminal domain-interacting proteins by a Y2H screen. Surprisingly, the ALS2 protein itself was identified as an interactor for the C-terminal region of ALS2. In this study, we delineate the molecular basis of the ALS2 self-interaction and its implication in the Rab5GEF activity as well as endosomal dynamics. Y2H showed that this self-interaction was mediated by two distinct C-terminal regions both of which were mapped within the MORN/VPS9 region. Immunoprecipitation and gel filtration analyses demonstrated that ALS2 homo-oligomerized in mammalian cells. The oligomerization-defective ALS2 mutant completely abolished the Rab5GEF activity *in vitro* and its endosomal localization in the cells, despite the intactness of its binding ability to Rab5A *in vitro*. Thus, the ALS2·Rab5 binding is not sufficient enough to activate Rab5, but rather ALS2 oligomer formation should be an important determinant for the ALS2-associated Rab5GEF activity. Collectively, these data strongly suggest that ALS2 homo-oligomerization is crucial for its physiological function involving endosome dynamics.

EXPERIMENTAL PROCEDURES

Antibodies and Materials—Monoclonal anti-FLAG (M2), anti-HA, anti-Rab5, and anti-early endosome antigen 1 (EEA1) antibodies were purchased from Stratagene, Sigma, BD Transduction Laboratories, and BD Biosciences, respectively. Anti-ALS2 rabbit polyclonal antibody (MPF 1012–1651) was raised with the purified His-tagged mouse ALS2_1012–1651 fragment, followed by affinity purification using an antigen-coupled Sepharose column. MPF 1012–1651 allowed the detection of both mouse and human ALS2 proteins (data not shown). All other regents were from commercial sources and of analytical grade.

Plasmid Constructs—All the cDNA expression constructs used in this study were obtained by subcloning the PCR or the reverse transcriptase-PCR-amplified fragments into the appropriate expression vectors. The DNA sequence of the insert as well as the flanking regions in each plasmid construct were verified by sequencing. For the Y2H assay, the PCR-amplified cDNA fragments of *ALS2* were subcloned into pLexA (Clontech) and pB42AD (Clontech) to generate pLexA-ALS2 constructs (bait) and pB42AD-ALS2 constructs (prey), respectively. For co-immunoprecipitation, gel filtration, *in vitro* GEF assay, and *in vitro* Rab5A binding experiments, the cDNA fragments of *ALS2* and *Trio* (aa 1233–1628), a *RhoGEF*, were subcloned into the modified pCI-neo Mammalian Expression Vector (Promega), allowing the production of the N-terminally FLAG- or HA-tagged proteins. The previously generated pCIneoFLAG-ALS2_L (full-length), pCIneoFLAG-ALS2_1018–1657, and pGEX6P-Rab5A were also utilized (30). Plasmid constructs expressing deletion mutant forms of ALS2, including pCIneoFLAG-ALS2_1018–1657 (Δ1515–1531), and pCIneoFLAG-ALS2_L (Δ1280–1335) were generated by a PCR-based method. pCIneoFLAG-ALS2_1100–1657 (Δ1280–1335) were generated by subcloning the reverse transcriptase-PCR-amplified *ALS2* splicing variant, which lacked the entire exon 25. For the subcellular localization studies, N-terminally enhanced green fluorescent protein (EGFP)-fused ALS2 proteins expression plasmids pEGFP-ALS2_695–1657, pEGFP-ALS2_695–1657 (Δ1280–1335), and pEGFP-*ALS2_695–1657* (Δ1515–1531) were generated by subcloning the PCR-amplified *ALS2* fragment, or deletion mutant forms of the same fragments, into pEGFP-C1 vector (Clontech).

Yeast Two-hybrid Assay—A Y2H screen was performed according to the manufacturer's instructions by utilizing the MATCHMAKER LexA Two-Hybrid System (Clontech). A human brain cDNA library (Clontech) in the pB42AD vector was screened with ALS2_1041–1351 as a bait. Briefly, the yeast strain, EGY48 [p8op-LacZ], was sequentially transformed with pLexA-ALS2_1041–1351 and then with a brain cDNA library. Co-transformants were selected on synthetic dropout (DO) media (Clontech) lacking uracil, histidine, tryptophan, and leucine, but including 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) by activation of both the leucine and the *LacZ* reporter genes.

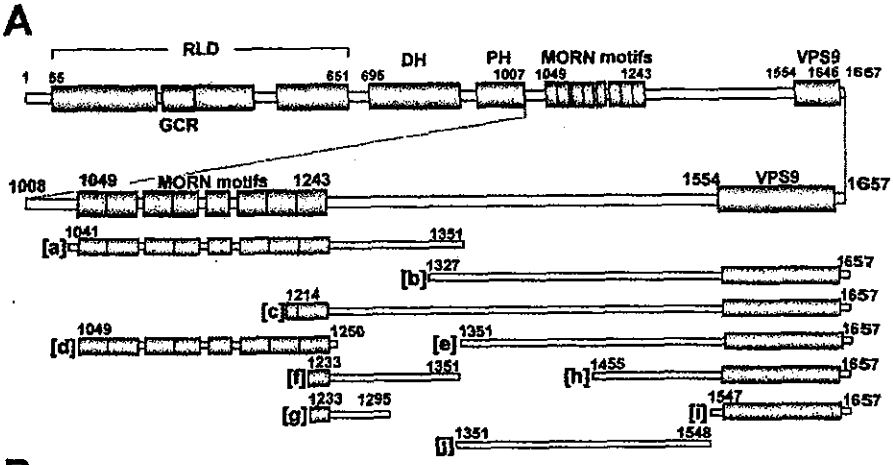
Cell Culture and Transfection—HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated 10% fetal bovine serum and antibiotics. Transfections were performed by using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions.

Co-immunoprecipitation—COS-7 cells were transfected with pCI-neoFLAG-ALS2 constructs and/or pCIneoHA-ALS2 constructs. Forty-eight hours after transfection, the cells were washed twice with 150 mM NaCl and lysed in buffer A consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 100 μM phenylmethylsulfonyl fluoride, and 1 tablet of Complete protease inhibitor mixture (Roche Applied Science)/50 ml of the buffer. After gently rotating for 1 h at 4 °C, supernatants were recovered by centrifugation at 12,000 × *g* for 15 min, followed by immunoprecipitation with Ezview™ Red ANTI-FLAG® M2 affinity gel (Sigma) (30). The M2 affinity gels were washed three times with the ice-cold buffer A containing 0.1% IGEPAL CA-630 instead of 1% IGEPAL CA-630. Appropriate amounts of the immunoprecipitates were used for Western blot analysis with either the anti-FLAG M2 or anti-HA antibodies.

Western Blot Analysis—Protein samples in Laemmli SDS-sample buffer were separated by SDS-PAGE and electrophoretically transferred onto the polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked with 10% skim milk in TBST (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) for 2 h and probed with the anti-FLAG M2 antibody (1:3000), anti-HA antibody (1:3000), or anti-ALS2 polyclonal antibody (MPF 1012–1651) (1:3000), followed by horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG sheep secondary antibody (Amersham Biosciences). Signals were visualized by the ECL Plus system (Amersham Biosciences) and BioMax x-ray films (Kodak).

Preparation of FLAG-tagged ALS2 and Trio Proteins—FLAG-tagged ALS2 fragments and Trio DH/PH domain (aa 1233–1628) were prepared as described previously (30). The N-terminally FLAG-tagged ALS2 or Trio proteins bound to the anti-FLAG M2-beads were re-suspended in the appropriate buffers (described below in each section) for the gel filtration, GEF assay, or *in vitro* Rab5A binding experiments. A portion of the proteins was subjected to Western blot analysis with anti-FLAG antibody or SDS-PAGE analysis, followed by staining with Coomassie Brilliant Blue to estimate the amount of conjugating FLAG-tagged proteins on the beads.

Gel Filtration—FLAG-tagged ALS2_L and FLAG-tagged ALS2_1018–1657 proteins on the beads were re-suspended in buffer B consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% IGEPAL CA-630 and subsequently eluted with buffer B containing 500 ng/ml 3×FLAG peptide (Sigma) for 1 h at 4 °C. ~30 pmol of FLAG-tagged ALS2_L or FLAG-tagged ALS2_1018–1657 was applied to a Superdex 200 column (HR 10/30, Amersham Biosciences) that was equilibrated with buffer B beforehand. Elution was carried out at 4 °C at a flow rate of 0.3 ml/min with a fraction volume of 0.5 ml. Fractions were subjected to Western blot analysis with anti-ALS2 polyclonal antibody (MPF 1012–1651). The elution profile of the column was calibrated with the sizing stand-



B

pLexA \ pB42AD	b	c	e	h	i	j
a	+++	+++	+++	-	-	+
d	-	-	-	-	-	-
e	A.A.	A.A.	A.A.	A.A.	A.A.	A.A.
f	+++	+++	+++	-	-	+
g	N.D.	N.D.	+	N.D.	N.D.	N.D.

FIG. 1. **ALS2 self-interacts in yeast cells.** *A*, schematic representation of ALS2 deletion mutants used. ALS2 contains the RLD, DH/PH, MORN, and VPS9 domains. The *alphabetical letters* in *brackets* indicate names of the fragments. The *numbers* represent the amino acid positions. Fragment *a* represents the bait for the Y2H screen. Both fragments *b* and *c* represent two independent positive clones, which were originally isolated by the Y2H screen. *B*, summary of the self-interaction of ALS2 analyzed by the Y2H test. Blue colonies grown on the Dropout (DO) media (Clontech) are classified into two categories by color intensities, intense blue (blue color appeared until second day of screening, +++) and significant but weak blue (blue color appeared until fourth day, +). "−" represents the results showing no growth on DO media, indicating no interaction. N.D., not determined; A.A., autonomous activation (it is impossible to use in Y2H test).

ards (Amersham Biosciences) of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and ovalbumin (43 kDa).

GEF Assay—The *in vitro* GEF assay was conducted as previously described (30). Briefly, the N-terminally FLAG-tagged ALS2 proteins were purified by the immunoprecipitations from COS-7 cells transfected with the appropriate pCIneoFLAG constructs. The small GTPase Rab5A was also prepared as previously described (30). 4 pmol of the [^{3}H]GDP-preloaded Rab5A was subjected to the *in vitro* GDP/GTP exchange reaction in the presence of FLAG M2 gel beads alone or FLAG M2 beads conjugating 2 pmol of the immunoprecipitated FLAG-tagged ALS2 proteins. The percentages of bound [^{3}H]GDP on Rab5 after 1-h incubation at 30 °C were calculated.

In Vitro Rab5A Binding to the FLAG-tagged ALS2 Proteins—Purified Rab5A (4 pmol) was incubated with FLAG M2 beads conjugating 4 pmol of the immunopurified FLAG-tagged ALS2 proteins in 100 μl of the modified GEF buffer consisting of 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 1.5 mM CHAPS, 0.1% (w/v) skim milk for 2 h at 30 °C. After washing four times with the same buffer without skim milk, the FLAG M2 beads were mixed with SDS-PAGE sample buffer, boiled for 5 min, and subjected to Western blot analysis with anti-Rab5 or anti-FLAG M2 antibodies.

Immunocytochemistry and Confocal Microscopy—Immunofluorescence studies were carried out as previously described (30). In brief, HeLa cells transfected with pEGFP-ALS2 constructs were washed with PBS (−) twice, fixed with 4% paraformaldehyde (PFA) in PBS (−) for 30 min, followed by permeabilization with 0.5% (w/v) Triton X-100 in PBS (−) for 30 min. Anti-EEA1 monoclonal antibody, diluted in PBS (−) containing 1.5% normal goat serum and 0.05% Triton X-100, were added to the fixed cells and incubated for 12 h. Alexa 594-conjugated goat anti-mouse IgG (1:500, Molecular Probes) was utilized as a secondary antibody to detect endogenous EEA1. Finally, images of serial optical sections with 1- to 2-μm thickness were captured and analyzed by Leica TCS_NT confocal-microscope systems (Leica).

RESULTS

Identification of the ALS2 Self-interaction—As we have previously described, the ALS2 protein is thought to involve in endosomal dynamics *in vivo* through the Rab5GEF activity mediated by the C-terminal MORN/VPS9 region of ALS2. The Rab5GEF-defective mutants of ALS2 lost their functions (30). However, it is still unclear how the MORN/VPS9 domains play the role in endosomal dynamics. To gain more insight into the molecular functions inherent in MORN/VPS9 domains, we screened a human brain cDNA library for the C-terminal domain-interacting proteins by the Y2H system. A screen with ALS2_1041–1351 (Fig. 1*A*, *part a*) as a bait isolated two independent clones that strongly interacted with ALS2_1041–1351. Both of these two clones contained the partial cDNAs encoding the C-terminal parts of the ALS2 protein (Fig. 1*A*, *parts b* and *c*). These results indicate that ALS2 could be self-associated. Bait *e* auto-activation is not suitable for the Y2H screen.

ALS2 Self-interaction in Yeast Cells—To confirm the self-interaction and, at the same time, to define minimum required regions for self-interaction in yeast cells, we generated various truncated ALS2-expressing constructs (Fig. 1*A*) and used them in the Y2H test. As a result, we found that two distinct C-terminal regions of ALS2 that, when coded by fragment *f*-deleting consecutive MORN motifs and fragment *e*, were essential for the strong self-interaction in yeast (Fig. 1*B*). Fragment *j*, deleting the VPS9 domain, exhibited a weak but significant interaction with fragment *f*. On the other hand, deletion of the N-terminal portion of fragment *e*, *i.e.* fragment *h*, completely abolished the interaction. Furthermore, fragments *d* or *i*, containing either MORN motifs or the VPS9 domain, solely showed no interactions with any fragments tested. All together, these results suggest that two distinct consecutive regions of ALS2, aa 1233–1351 and aa 1351–1454, are indispensable for the ALS2 self-interaction in yeast cells, whereas both MORN motifs and the VPS9 domain are dispensable for that.

Self-interaction of the ALS2 Protein in Mammalian Cells—To investigate whether the ALS2 proteins interact with each other in mammalian cells, we conducted co-immunoprecipitation experiments. We transfected pCIneoFLAG-ALS2_L (full-length) along with pCIneoHA-ALS2_L into COS-7 cells and then performed co-immunoprecipitation using the anti-FLAG

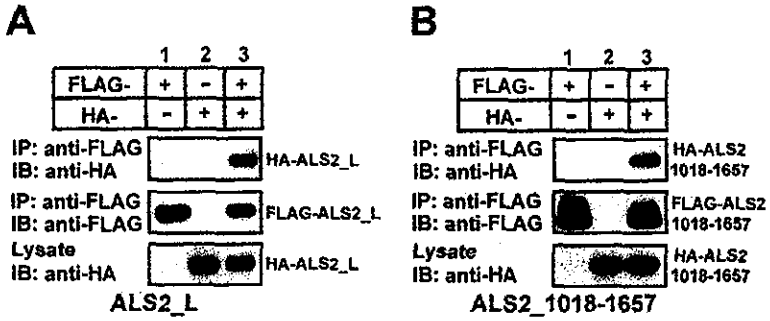


FIG. 2. **ALS2 self-interacts in mammalian cells.** *A*, Western blot analyses of immunoprecipitates (*top* and *middle*) and lysates (*bottom*) derived from COS-7 cells that were co-transfected with two selected expression plasmids with a combination of pCIneoFLAG-ALS2_L and pCIneoHA_empty (*lane 1*), pCIneoFLAG_empty, and pCIneoHA-ALS2_L (*lane 2*), or pCIneoFLAG-ALS2_L and pCIneoHA-ALS2_L (*lane 3*). *IP*, antibody used for immunoprecipitation; *IB*, antibody used for Western blot. *B*, Western blot analyses of immunoprecipitates (*top* and *middle*) and lysates (*bottom*) derived from COS-7 cells transiently co-transfected with two selected plasmids as in *A* except that pCIneoFLAG-ALS2_1018–1657 and pCIneoHA-ALS2_1018–1657 instead of ALS2_L constructs were used.

M2 beads. As shown in Fig. 2*A*, HA-tagged ALS2_L was efficiently co-immunoprecipitated with FLAG-tagged ALS2_L. FLAG-tagged ALS2_L was also detected in the pellets together with HA-tagged ALS2_L, when immunoprecipitated with anti-HA antibody (data not shown). Consistent with the results of the Y2H screen, HA-tagged ALS2_1018–1657, which contained all of the required region for the self-interaction in yeast, was also co-immunoprecipitated with FLAG-tagged ALS2_1018–1657 (Fig. 2*B*). These results demonstrate that ALS2 proteins interact with each other through their C-terminal region, suggesting that ALS2s could either homo-dimerize or homo-oligomerize in mammalian cells.

Homo-oligomerization of the ALS2 Protein—To determine whether the ALS2 protein existed as a homo-oligomerized or homo-dimerized form in the cells, we conducted a gel exclusion analysis. We prepared the FLAG-tagged ALS2_L and ALS2_1018–1657 proteins from COS-7 cells transfected with each expression plasmid. Purified ALS2 proteins were subjected to a gel filtration column, and the eluted fractions were analyzed by Western blot analysis with anti-ALS2 polyclonal antibody (MPF 1012–1651). As shown in Fig. 3, FLAG-tagged ALS2_L (~180 kDa in SDS-PAGE) and ALS2_1018–1657 (~75 kDa in SDS-PAGE) were eluted at apparent peak molecular masses of ~1,200 kDa and ~600 kDa, respectively (Fig. 3, *A* and *B*). These molecular masses indicate that ALS2 is likely to form a homophilic oligomer (presumably octamer) rather than a dimer in native conditions. Because ALS2_1018–1657 (MORN/VPS9 region) lacking the N-terminal RLD and DH/PH domains also oligomerized in the same manner as the full-length ALS2 (ALS2_L), the C-terminal region of ALS2 might be the region requisite for oligomerization. It is also noteworthy that monomeric forms for FLAG-tagged ALS2_L and ALS2_1018–1657 were not visible in the Western blots (Fig. 3, *A* and *B*). Furthermore, no stoichiometrically co-purified proteins with FLAG-tagged ALS2_L and ALS2_1018–1657 were detected in silver stainings of the SDS-PAGE gels (data not shown). Taken together, these results strongly suggest that ALS2 exists as very stable homophilic oligomers, presumably octamers, *in vivo*.

The Regions Responsible for the ALS2 Homo-oligomerization in Mammalian Cells—Next, to confirm the responsible regions for the oligomerization in mammalian system, we generated the expression vectors encoding various FLAG-tagged deletion mutants of ALS2, including ALS2_1018–1554, ALS2_1233–1657, ALS2_1100–1657, ALS2_1100–1657 (Δ1280–1335), and ALS2_1018–1657 (Δ1515–1531). We also prepared the expression plasmid encoding the Trio_DH/PH domain (aa 1233–1628) as a negative control (Fig. 4*A*). Immunoprecipitation of the

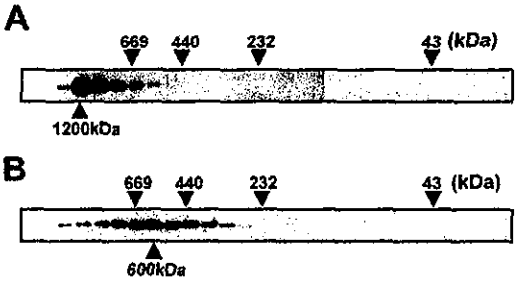


FIG. 3. **ALS2 homo-oligomerizes in mammalian cells.** *A*, Western blot analysis of the gel-fractionated FLAG-tagged ALS2_L. FLAG-tagged ALS2_L was purified from COS-7 cells transfected with pCIneoFLAG-ALS2_L and applied to a Superdex 200 gel filtration column as described under "*Experimental Procedures*." The fractions were analyzed by Western blot analysis with the anti-ALS2 antibody (MPF 1012–1651). Molecular masses of the sizing standards are shown at the *top of the panel*. Molecular sizes of the elution peak for FLAG-tagged ALS2_L (~1200 kDa) are shown at the *bottom of the panel*. Note that the signals of ALS2_L on the Western blot were detected asymmetrically due to the high molecular mass of the ALS2 complex (reaching to void volume). *B*, Western blot analysis of the gel-fractionated FLAG-tagged ALS2_1018–1657. All experiments were performed as in *A* except that FLAG-tagged ALS2_1018–1657 was used instead. Molecular size of the elution peak for FLAG-tagged ALS2_1018–1657 is ~600 kDa as shown at the *bottom*.

lysates, which were prepared from COS-7 cells ectopically expressing HA-tagged ALS2_L (*k* in Fig. 4) together with one of the FLAG-tagged deletion mutants or Trio_1233–1628 (*l*, *m*, *n*, *o*, *p*, *q*, and *r* in Fig. 4), with anti-FLAG M2 beads revealed that the HA-tagged ALS2_L proteins were most efficiently co-immunoprecipitated by FLAG-tagged ALS2_1018–1657 (*l*) and ALS2_1100–1657 (*o*) (Fig. 4*B*), as in the case of FLAG-tagged ALS2_L against HA-tagged ALS2_L (Fig. 2*A*). This indicated that ALS2_1100–1657 essentially contained all the requisite regions for oligomerization. In addition, both FLAG-tagged ALS2_1233–1657 (*n*), lacking the MORN motifs, and ALS2_1018–1554 (*m*), completely lacking the conventional VPS9 domain, still interacted with HA-tagged ALS2_L, respectively. Thus, the region flanked by the MORN motifs and the VPS9 domain is most likely to mediate the ALS2 oligomerization, whereas both the MORN motifs and the VPS9 domain, which are essential for the ALS2-associated Rab5GEF activity (30), are rather dispensable for oligomerization, consistent with the results obtained by the Y2H test (Fig. 1).

To determine the responsible regions for the ALS2 oligomerization within the region flanked by MORN motifs and VPS9 domain, we tested two additional deletion mutants for co-immunoprecipitation. FLAG-tagged ALS2_1018–1657 (Δ1515–

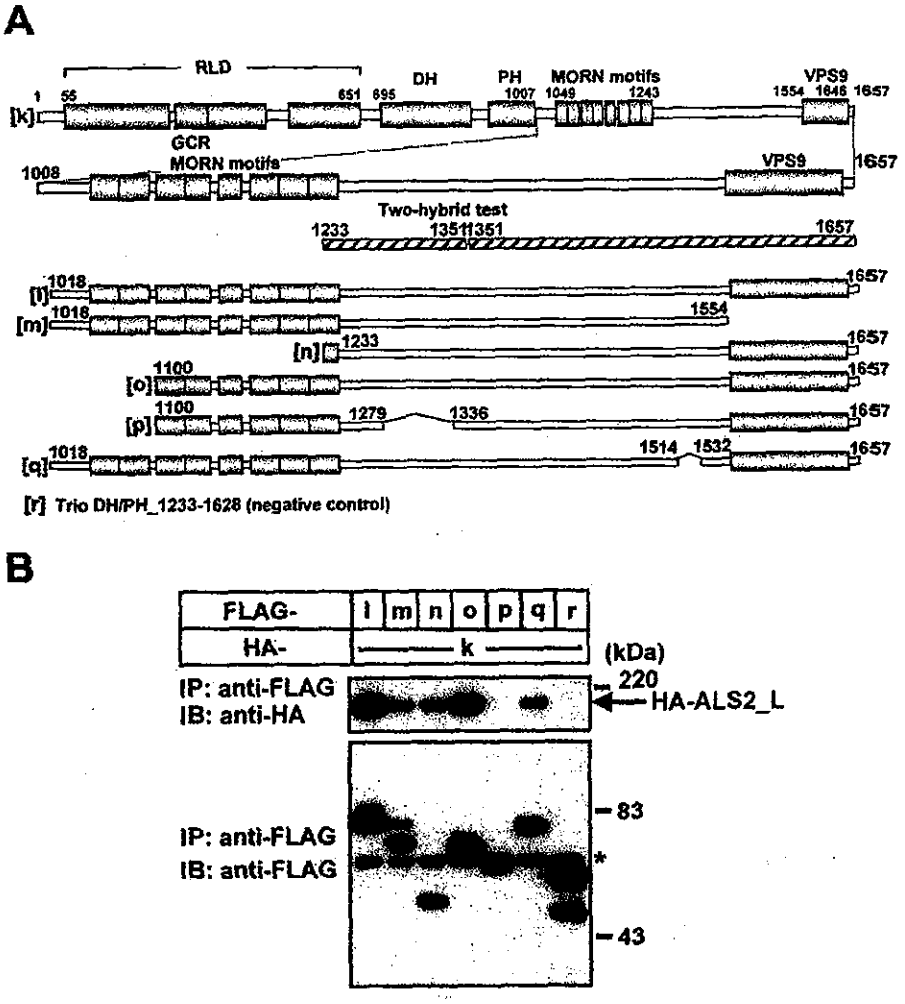


FIG. 4. **ALS2 interacts with each other through the region flanked by the MORN motifs and the VPS9 domain in mammalian cells.** *A*, schematic representation of the full-length ALS2 protein and its deletion mutants used in this experiment. The *alphabetical letters* in *brackets* indicate names of the fragments. The *numbers* represent the amino acid positions. Two *right-hatched boxes* represent regions responsible for the strong self-interaction, which are defined by the Y2H test. Trio_DH/PH domain was used as a negative control (fragment *r*). *B*, Western blot analyses of immunoprecipitates (*top* and *bottom*) derived from COS-7 cells transfected with the expression plasmid for HA-tagged ALS2_L (*k*) together with one of the expression plasmids for ALS2 mutants as indicated at the *top* of the *panel* (*l*, *m*, *n*, *o*, *p*, *q*, or *r*). Note that the HA-tagged ALS2_L protein were not precipitated with the fragments *p* and *r* (negative control) under the conditions in which each of the FLAG-tagged ALS2 deletion mutants was readily detected after the immunoprecipitation (*bottom*). *IP*, antibody used for immunoprecipitation; *IB*, antibody used for Western blot. An *asterisk* represents the signals derived from the immunoglobulin heavy chains (*bottom*).

1531) (*q*) lacking 17 residues (aa 1515–1531) corresponding to one of the evolutionally conserved region among ALS2 and a recently identified ALS2-homologous protein, ALS2CL (ALS2 C-terminal-like),[2] in vertebrates still showed the significant interaction with HA-tagged ALS2_L. In stark contrast, FLAG-tagged ALS2_1100–1657 (Δ1280–1335) (*p*), which was encoded by the naturally occurring ALS2 splicing variant lacking the entire exon 25,[2] totally lost the ability to bind with HA-tagged ALS2_L, implying that aa 1280–1335 are crucial for the ALS2 self-interaction. The result was also consistent with those in the Y2H tests, in which the aa 1233–1351 portion, covering the exon 25-coded region, was critical for the ALS2 self-interaction in yeast (Fig. 3). Taken together, our results indicate that this aa 1280–1335 comprises a novel functional domain that is essential for the ALS2 oligomerization.

Requisiteness of the ALS2 Oligomerization for Its Rab5 GEF Activity in Vitro—To examine whether the oligomerization of ALS2 through its C-terminal region is mandatory in its catalytic Rab5GEF activity, we conducted the *in vitro* GEF assay. We purified the N-terminally FLAG-tagged various wild type and mutant ALS2 proteins from COS-7 cells transfected and assayed the *in vitro* GDP/GTP exchange activities of them. We detected a trace amount of the endogenous ALS2 protein in immunoprecipitates from COS-7 cells expressing the oligomerization-prone ALS2 fragments (data not shown). However, limited amounts of co-immunoprecipitated endogenous ALS2 had no significant effect on the GEF activity *in vitro* (data not shown).

As we have previously shown (30), ALS2_L and ALS2_1018–1657, both of which stably oligomerized (in this work), stimulated GDP dissociation on Rab5A (Fig. 5*A*). Furthermore, ALS2_1100–1657, lacking the N-terminal two copies of eight consecutive MORN motifs, still maintained even higher Rab5GEF activity (Fig. 5*A*). On the other hand, another oligomerization-prone ALS2 mutant, ALS2_1233–1657, lacking all the intact eight MORN motifs, completely lost its GEF activity (Fig. 5*A*). Thus, at least six repeats of the MORN motifs, which were dispensable for oligomerization, were required for the Rab5GEF activity. Our preliminary study using a series of N-terminally truncating mutants generated by the deletion of eight consecutive MORN motifs one by one revealed that the presence of more than four copies of MORN motifs are required for its GEF activity (data not shown).

Notably, ALS2_1100–1657 (Δ1280–1335), which lost the ability to interact with ALS2_L (Fig. 4*B*), exhibited no catalytic activity (Fig. 5*A*). Similarly, even in the context of the full-length ALS2 protein, deletion of aa 1280–1335 abolished its GEF activity (Fig. 5A). We also found that ALS2_1018–1657 (Δ1515–1531), which contained the intact MORN motifs, the VPS9 domain, and all the essential regions for the oligomerization still lost their Rab5GEF activity, indicating that the 17 conserved amino acid residues are also crucial for the ALS2-associated Rab5GEF activity. These results strongly suggest

[2] S. Hadano, A. Otomo, K. Suzuki, R. Kunita, Y. Yanagisawa, J. Showguchi-Miyata, H. Mizumura, and J.-E. Ikeda, unpublished results.

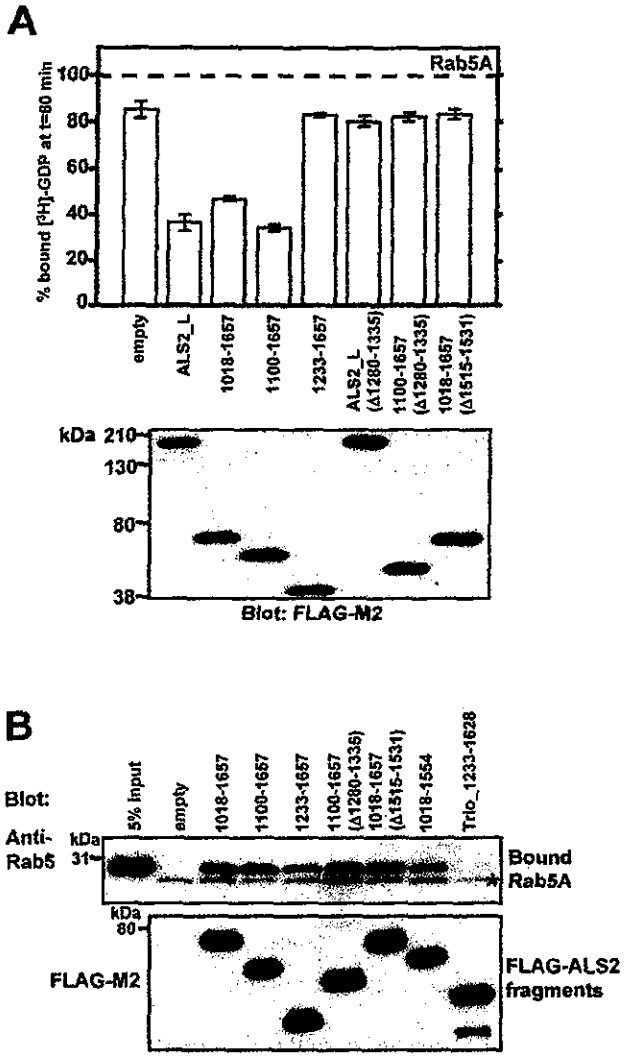


FIG. 5. **Homo-oligomerization of ALS2 is fundamental for the ALS2-associated Rab5GEF activity, but not for the binding to Rab5A.** *A*, analysis of the ALS2-associated Rab5GEF activity. *In vitro* [^{3}H]GDP dissociation assay on Rab5A in the presence of the FLAG M2 beads alone or the FLAG M2 beads conjugating FLAG-tagged ALS2 or ALS2 mutants as indicated was performed as described under "Experimental Procedures." The percentages of bound [^{3}H]GDP remaining on Rab5A after 1-h incubation at 30 °C are presented (*top*). Each value represents the mean ± S.D. of at least three independent assays. Western blot analysis of the FLAG-tagged ALS2 mutants used in this GEF assay was conducted using the anti-FLAG M2 antibody, showing the equal amount of protein in each fragment used (*bottom*). *B*, *in vitro* Rab5A binding to FLAG-tagged ALS2 mutants. A nucleotide free form of Rab5A was incubated with FLAG M2 beads alone or FLAG M2 beads conjugating one of the FLAG-tagged ALS2 mutants as indicated at the *top* of the *upper panel*. The bound Rab5A was detected by Western blot using anti-Rab5 antibody (*top*). Empty beads and beads conjugating FLAG-tagged Trio_1233–1628 were used as negative controls. Western blot analysis of the FLAG-tagged ALS2 mutants used in the *in vitro* Rab5A binding experiment was conducted (*bottom*). An *asterisk* represents the signals derived from the immunoglobulin light chains (*top*).

that, although there are several functionally important elements, including the intact VPS9 domain, the MORN motifs, and evolutionally conserved residues, the region spanning aa 1280–1335, which comprises one of the essential sequences for the ALS2 homo-oligomerization, is indeed essential for its Rab5GEF activity *in vitro*.

In Vitro Rab5A Binding to the Oligomerization-prone and -supine ALS2 Fragments—To examine whether loss of Rab5GEF activity in the ALS2 fragments such as ALS2_1233–1657, ALS2_1100–1657 (Δ1280–1335), and ALS2_1018–1657 (Δ1515–1531) resulted from a decrease in their binding affinity to Rab5, we conducted *in vitro* Rab5A·ALS2 fragments binding experiments. The *in vitro* binding assays were performed using the FLAG M2 beads conjugating the FLAG-tagged ALS2_1018–1657, 1100–1657, 1233–1657, 1100–1657 (Δ1280–1335), 1018–1657 (Δ1515–1531), or Trio_1233–1628 in the presence of Rab5A. We used the nucleotide-free form of Rab5A in this study, because ALS2 could bind to the nucleotide-free form of Rab5A much more potently than to the GDP- or GTP-bound forms (30).[2] ALS2_1018–1657, a catalytically active Rab5GEF, interacted with Rab5A (Fig. 5), consistent with our previous results (30). As expected, another active Rab5GEF, ALS2_1100–1657, also strongly bound to Rab5A, whereas empty beads as well as beads conjugating Trio_1233–1628 did not interact with Rab5A (Fig. 5*B*). Thus, catalytically active ALS2 fragments could possess the binding ability to their substrate, Rab5A.

To our surprise, all four Rab5GEF-defective ALS2 fragments still retained the binding abilities to Rab5A (Fig. 5*B*). Both ALS2_1233–1657, lacking the intact MORN motifs, and ALS2_1018–1554, lacking the catalytic VPS9 domain, still bound to Rab5A with slightly weaker affinities (Fig. 5*B*), suggesting that the MORN and VPS9 domains are not essential for Rab5A binding (Fig. 5*B*). However, this does not exclude the possibility that the VPS9 domain of ALS2 independently binds to Rab5, thereby modulating its Rab5GEF activity. Even more surprisingly, ALS2_1100–1657 (Δ1280–1335), a mutant lacking the abilities for both homo-oligomerization and the Rab5GEF activity, indistinguishably bound to Rab5A. This result indicates that even oligomerization is not an essential feature for Rab5A binding, rather it might be required for maintaining the proper conformation to exhibit the catalytic activity. Finally, ALS2_1018–1657 (Δ1515–1531), an oligomerization-prone and Rab5GEF-defective mutant (Fig. 5*A*), also bound to Rab5A with relatively higher affinity. Collectively, it is obvious that a defect in the Rab5GEF activity in a number of mutated ALS2 fragments is not simply due to a loss of its binding affinity to Rab5A, indicating that the interaction of ALS2 with Rab5 is not a sole determinant for the ALS2-associated Rab5GEF activity.

Endosome Enlargement Induced by a Constitutive Active Form of ALS2 in a Rab5GEF Activity- and Oligomerization-dependent Manner—To delineate the functional significance of the ALS2 oligomerization as well as the ALS2-associated Rab5GEF activity *in vivo*, we investigated the effects of overexpression of either a constitutive active form of ALS2 or those with an internal deletion on the cellular phenotypes in HeLa cells. Previously, we have shown that ALS2_660–1657 consisting of DH/PH/MORN/VPS9 domains evokes unleashed endosome enlargement in mammalian cells (30). We recently found that ALS2_695–1657 could also function as a constitutively active form and exhibit even higher activity eliciting endosome enlargement. In fact, this fragment could frequently induce almost nuclear-sized endosomes by 48 h after transfection in COS-7 cells.[3] Therefore, we decided to use ALS2_695–1657 instead of ALS2_660–1657 as a constitutively active form in this study and to assess the effect of mutations in ALS2_695–1657 on the degree of endosome enlargement.

First, we transfected the expression plasmid encoding the N-terminally EGFP-fused ALS2_695–1657 (WT) in HeLa cells. We confirmed that the N-terminally EGFP-fused ALS2 also

[3] R. Kunita, A. Otomo, H. Mizumura, K. Suzuki, S. Hadano, and J.-E. Ikeda, unpublished results.