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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.

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 pCIneoFLAG-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-

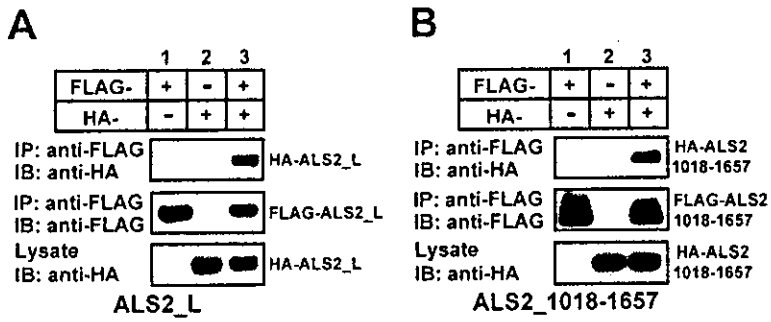

FIG. 2. **ALS2 self-interacts in mammalian cells.** *A*, Western blot analyses of immunoprecipitates (*top* and *middle*) and lysates (*bottom*) derived from COS-7 cells that were co-transfected with two selected expression plasmids with a combination of pCIneoFLAG-ALS2_L and pCIneoHA_empty (*lane 1*), 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

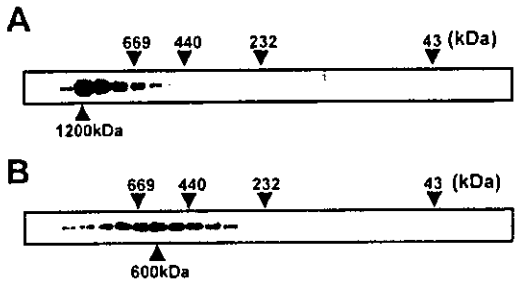

FIG. 3. **ALS2 homo-oligomerizes in mammalian cells.** *A*, Western blot analysis of the gel-fractionated FLAG-tagged ALS2_L. FLAG-tagged ALS2_L was purified from COS-7 cells transfected with pCIneoFLAG-ALS2_L and applied to a Superdex 200 gel filtration column as described under "Experimental Procedures." 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–

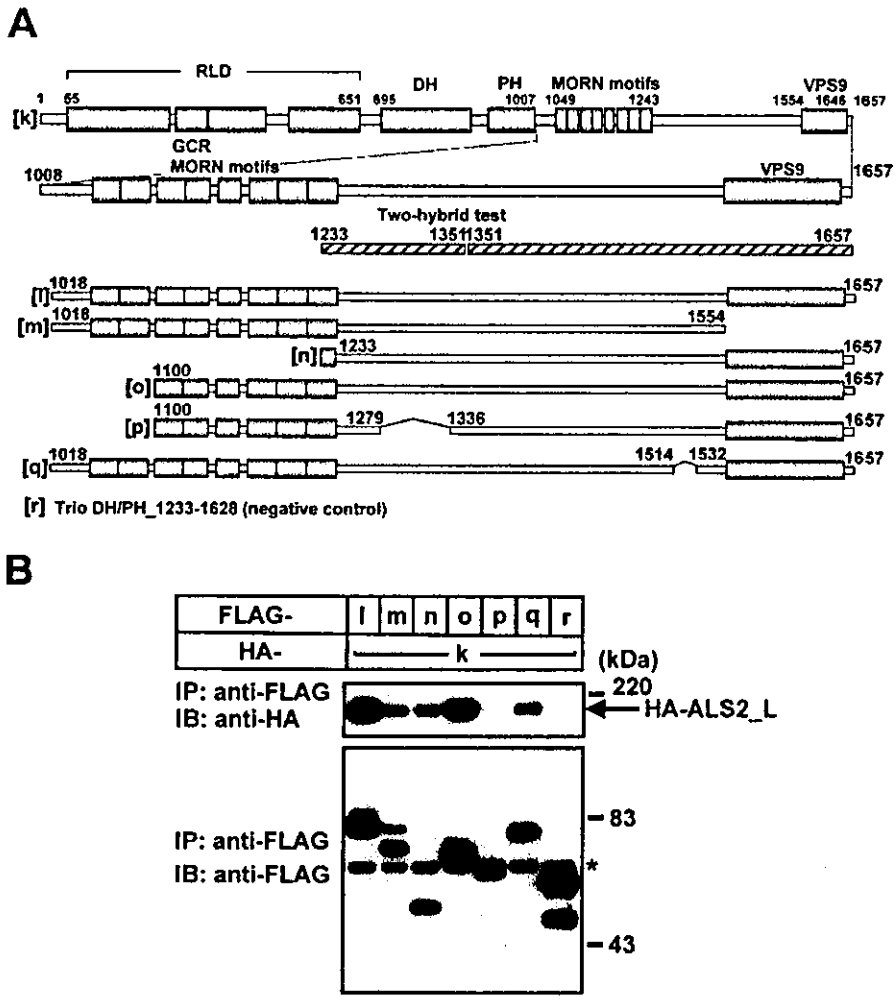

FIG. 4. **ALS2 interacts with each other through the region flanked by the MORN motifs and the VPS9 domain in mammalian cells.** *A*, schematic representation of the full-length ALS2 protein and its deletion mutants used in this experiment. The *alphabetical letters* in *brackets* indicate names of the 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. 5*A*). 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.

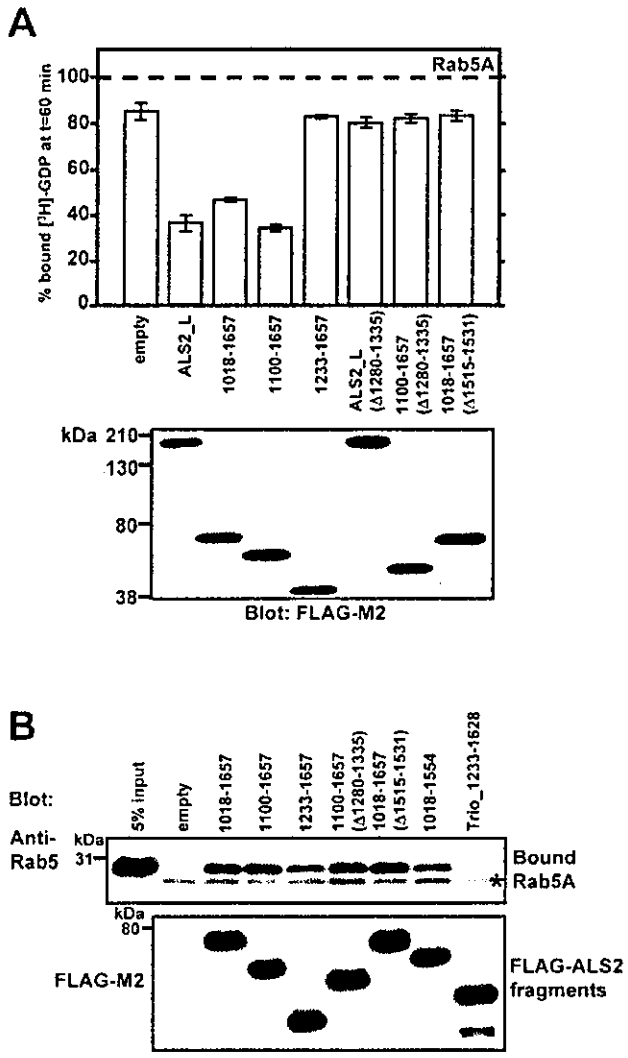

FIG. 5. **Homo-oligomerization of ALS2 is fundamental for the ALS2-associated Rab5GEF activity, but not for the binding to Rab5A.** *A*, analysis of the ALS2-associated Rab5GEF activity. *In vitro* [³H]GDP dissociation assay on Rab5A in the presence of the FLAG M2 beads alone or the FLAG M2 beads conjugating FLAG-tagged ALS2 or ALS2 mutants as indicated was performed as described under "Experimental Procedures." The percentages of bound [³H]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.

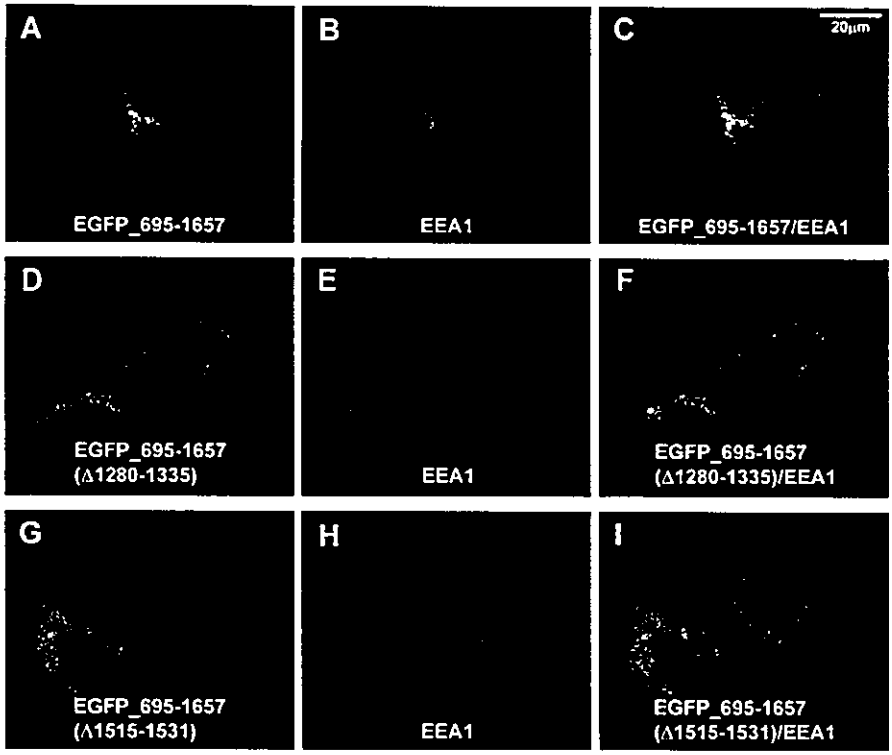

FIG. 6. **A constitutive active form of ALS2(EGFP_695–1657) induces the enlargement of the EEA1-positive compartments in a Rab5GEF/oligomerization-dependent fashion.** HeLa cells were transfected with pEGFP-ALS2_695–1657 (*A–C*), pEGFP-ALS2_695–1657 (Δ1280–1335) (*D–F*), or pEGFP-ALS2_695–1657 (Δ1515–1531) (*G–I*). Forty-eight hours after transfection, HeLa cells were labeled with anti-EEA1 antibody. The fluorescence of EGFP-ALS2 (*A*, *D*, and *G*) and Alexa-594 secondary antibody (*B*, *E*, and *H*) was detected and visualized by confocal microscopy. *Yellow stainings* in the *merged images* (*right*; *C*, *F*, and *I*) represent the overlapping signals between two different images (*left* and *middle*).

homo-oligomerized (data not shown). As shown in Fig. 6*A*, expression of EGFP_695–1657 resulted in enlarged endosomes and most of them were EEA1-positive vesicles (Fig. 6*C*), implying that EGFP_695–1657 enlarges early endosomes. Next, we evaluated the effect of the oligomerization-defective/Rab5GEF-defective ALS2 mutant, EGFP_695–1657 (Δ1280–1335), on the endosome phenotypes in HeLa cells. Notably, results showed that this mutation completely abolished the capabilities of the early endosome enlargement. Furthermore, this mutant ALS2 peptide also localized onto the vesicular structures (Fig. 6*D*), representing the EEA1-negative (Fig. 6, *D–F*) and Rab5A-negative compartments (data not shown). Overexpression of the EGFP_695–1657 (Δ1515–1531) oligomerization-prone/Rab5GEF-defective mutant also demonstrated that this mutant lost its ability to induce the endosome enlargement, consistent with our previous results using two VPS9-domain mutants, EGFP_660–1657 (P1603A) and EGFP_660–1657 (L1617A) (30). Collectively, these results indicate that the oligomerization of ALS2 requisite for the Rab5GEF activity is indeed crucial for the endosome enlargement induced by overexpression of constitute active forms of ALS2 in the cells, and that, in particular, the Rab5GEF activity associated with ALS2 might be an essential feature for the ALS2-associated physiological function on endosome trafficking.

DISCUSSION

We have previously demonstrated that the ALS2 protein is involved in endosomal dynamics through its intrinsic Rab5GEF activity mediated by the C-terminal MORN/VPS9 domains (30). In this study, we show for the first time that ALS2 forms a homo-oligomeric complex through its C-terminal region and that this oligomerization is essential for the ALS2-associated Rab5GEF activity and its regulatory function on endosome dynamics, such as endosome enlargement.

Our co-immunoprecipitation and gel filtration analyses revealed that the ectopically expressed FLAG-tagged ALS2 protein formed a very stable oligomer, presumably an octamer in the cells. Self-interaction was also detected when either the HA-tagged or EGFP-fused form of ALS2 was used (data not shown). Furthermore, untagged or even endogenous ALS2 was immunoprecipitated with epitope-tagged truncated ALS2 peptide carrying the C-terminal region (data not shown). These results strongly suggest that the endogenous ALS2 protein exists as an oligomeric form *in vivo*. In this study, we also determined the regions requisite for the ALS2 homo-oligomerization by co-immunoprecipitation and Y2H using various truncated or internally deleted ALS2 fragments. The results showed that two distinct non-overlapping regions, aa 1233–1351 and 1351–1548, mediated oligomerization by interacting with each other. These two regions reside within the region flanked by MORN motifs and VPS9 domain where no known motifs and/or domains have ever been assigned. Notably, the aa 1280–1335 region must contain the crucial residues determining the structural basis for the ALS2 oligomerization, because deletion of this portion completely abolished the oligomerization. Unfortunately, we have so far failed to obtain the sufficient amounts of the oligomerization-defective ALS2 proteins, including ALS2_L (Δ1280–1335) and ALS2_1100–1657 (Δ1280–1335), due to their inefficient expression as soluble proteins in the cells, and thus could not analyze these proteins by gel-filtration as to whether they existed as monomers in the cells. Based upon our results, two structural models for the ALS2 oligomerization could be possible (Fig. 7). One is that the dimerized ALS2 units in an anti-parallel fashion may form an octamer (Fig. 7*A*), and the other could be that eight ALS2 molecules form an octagon-like structure (Fig. 7*B*). Further experiments will clarify the *bona fide* tertiary structure for the oligomerized ALS2 protein complex.

In this study, we also obtained the important insights into the functional-structural relationship by characterizing the oligomerization of ALS2 and its associated Rab5GEF activity *in vitro*. We have previously shown that the C-terminal region spanning MORN and the VPS9 domain of ALS2 consists of the

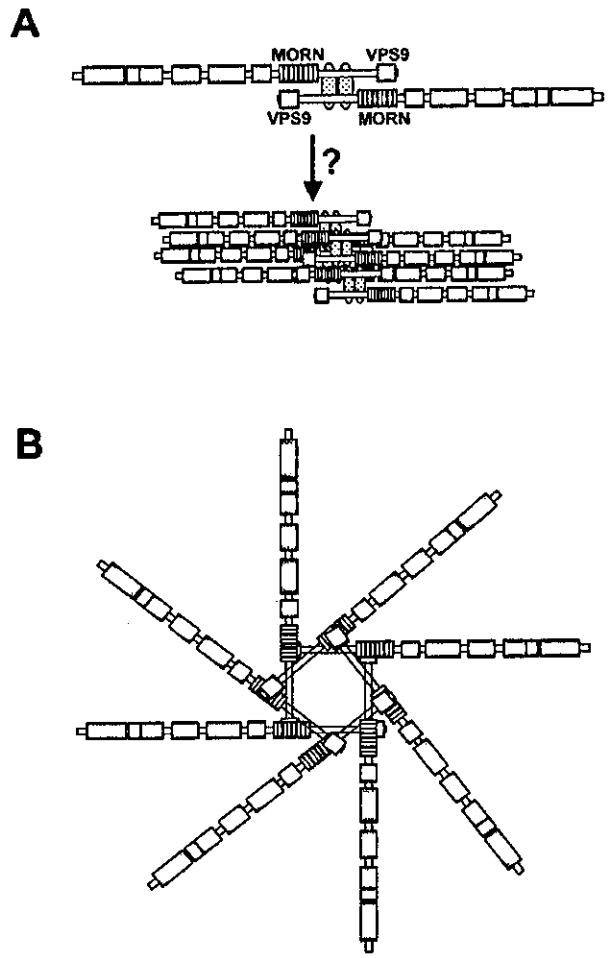

FIG. 7. **Two possible models for the ALS2 oligomeric structure.** Schematic representations of the ALS2 oligomers deduced from the present study. *A*, two ALS2 molecules that interact with each other in an anti-parallel manner (the dimer units) form oligomeric complexes by the yet to be identified mechanism. *Two dotted areas* represent the C-terminal region-mediated interaction. *B*, eight ALS2 molecules form an octagon-like structure through the C-terminal region-mediated interaction.

minimum required region for the ALS2-associated Rab5GEF activity (30). Present results in conjunction with the previous findings suggest that a number of key elements regulating the ALS2-associated Rab5GEF activity reside within the C-terminal region, including not only the MORN motifs and VPS9 domain (30) but also other regions, such as aa 1280–1335 and aa 1515–1531. Notably, one of these elements, corresponding to aa 1280–1335, was found to be the region requisite for the ALS2 oligomerization. This result indicates that the ALS2 homo-oligomerization can be one of the key determinants for the ALS2-associated Rab5GEF activity and, thus, a pivotal molecular feature underlying the physiological function for ALS2 *in vivo*. In fact, our *in vitro* GEF activity assay revealed that the oligomerization-defective ALS2 mutants completely lost their Rab5GEF activities. However, besides these oligomerization-defective mutants, all other Rab5GEF-defective ALS2 mutants still retained their ability to form the oligomers, suggesting that oligomerization itself is not sufficient to activate Rab5. Furthermore, all the normal as well as mutant ALS2 proteins examined bound to Rab5A, irrespective of whether they retained the Rab5GEF activity and/or the oligomerization capability or not, implying that ALS2·Rab5 binding is not also a sole determinant for the ALS2-associated Rab5GEF activity. Taken together, it is suggested that every element determining the structural content of the ALS2 protein complex, ALS2 oligomerization, and the formation of the ALS2·Rab5 complex, might be important for its normal Rab5GEF activity mediated by the C-terminal region of ALS2.

With regard to the minimum catalytic region for the ALS2-associated GEF activity, Topp *et al.* (31) have recently reported the contradictory result, in which the C-terminal portion of ALS2 fragment (ALS2_1360–1657 aa) lacking both MORN motifs as well as the region crucial for the oligomerization (determined in this study) possesses Rab5GEF activity *in vitro*. In our results, the oligomerization-defective/Rab5GEF-defective ALS2 protein, ALS2_695–1657 (Δ1280–1335), covering the entire ALS2_1360–1657 peptide, loses the ALS2-associated Rab5GEF activity. Although it is conceivable that this discrepancy could be a result of different assay conditions used, the answer for this question remains elusive.

Given the importance of the ALS2 self-interaction in the ALS2-associated Rab5GEF activity, ALS2 homo-oligomerization must also play significant roles on the modulation of endosome/vesicle trafficking, and thus the loss of oligomerization should exhibit significant impact on the endosomal phenotypes. Remarkably, we demonstrate here that the ALS2 mutant carrying the intact DH/PH domain but lacking one of the self-interacting regions, ALS2_695–1657 (Δ1280–1335), completely lost the normal endosomal (EEA1-positive) localization and enlarged endosome phenotypes, whereas the oligomerization-prone WT fragment (ALS2_695–1657) localized predominantly onto EEA1-positive compartments and induced the prominent enlargement. These results imply that homo-oligomerization is one of the fundamental molecular features for the ALS2 function *in vivo*. Nevertheless, it should be noted again that oligomer formation alone is not sufficient for the endosomal localization and function of ALS2, because the oligomerization-prone/Rab5GEF-defective mutant ALS2 also miss-localized in the cells. Since the oligomerization-defective ALS2 fragments, such as ALS2-695-1657 (Δ1280–1335), always exhibit no Rab5GEF activity, we cannot formally rule out the possibility that the loss of Rab5GEF activity rather than oligomerization itself resulted in the miss-localization of the ALS2_695–1657 (Δ1280–1335) protein at present.

Recently, several studies on the subcellular distribution and function of the ALS2 protein have been reported (31, 32). We have previously demonstrated the presence of regulatory elements and domains within the ALS2 molecule (30). The N-terminal RLD appears to suppress recruitment of ALS2 onto vesicles and/or endosomes, particularly in non-neuronal cells. By contrast, the DH/PH domains facilitate the endosomal localization of ALS2 and, at the same time, enhance the C-terminal MORN/VPS9 domain-mediated endosome fusions *in vivo*. Because this DH/PH-mediated enhancing effect is totally dependent on the intact Rab5GEF activity (see Fig. 6) (30), DH/PH appears to function as an upstream-regulatory element to the MORN/VPS9 domain-mediated function. In addition, the N-terminally truncated ALS2 mutant carrying the intact MORN-VPS9 region (ALS2_1018–1657), which retains both the oligomerization and full-Rab5GEF potencies, loses the prominent endosome phenotypes, indicating that the presence of DH/PH domains followed by the intact MORN/VPS9 region might be crucial for the regulation on endosome trafficking in the cells (30). Topp *et al.* (31) also presumed that DH/PH and VPS9 domains were mainly involved in the ALS2 membranous localization, consistent with our findings. Furthermore, they also reported that the DH/PH domain could directly interact with Rac1 and that overexpression of ALS2 along with Rac1 in Sf9 cells resulted in an increase in the level of GTP-bound form

of Rac1 (active Rac1) despite the fact that we and others have so far failed to show the ALS2-associated direct catalytic GEF activity on either Rac1, Cdc42, or RhoA (30, 31). These results suggest that ALS2 could indirectly activate the Rac1-mediated signaling pathway possibly through the interaction between ALS2_DH/PH and Rac1. However, it is still unclear whether the ALS2-mediated Rac1 activation contributes to the endosome dynamics. On the other hand, Yamanaka *et al.* (32) have reported a seemingly contradictory finding that endosomal localization of ALS2 is mediated through the RLD region. Recently, we found that localization of ectopically expressed ALS2 and its phenotypic effects on endosomes appeared to be different in different types of cultured cells (30). Because each study described above has utilized different types of cultured cells and conditions, the variable effect of the ALS2 domains on the subcellular localization of ALS2 observed might reflect the differences in the physiological conditions. Together, evidence has accrued to support the notion that subcellular localization and function of ALS2 are independently or cooperatively regulated by the domain-mediated protein-protein or protein-lipid interactions, which take place under a certain physiological condition.

In this study, we demonstrated the first molecular evidence for the homophilic oligomerization to be associated with Rab5GEF activity. To our knowledge, among the VPS9 domain-containing Rab5GEF family members, ALS2 is second only to RIN2, revealing its potencies to form homophilic oligomers. Saito *et al.* (19) reported that RIN2 might exist as a tetramer composed of anti-parallel linkage of two parallel dimers. However, it has yet to be determined whether tetramer formation of RIN2 is essential for its Rab5GEF activity or not. On the other hand, Rabex-5, one of the mostly characterized mammalian Rab5GEF, has been shown to interact with Rabaptin-5 (16, 33, 34). This heterophilic complex formation strongly enhanced the Rabex-5-associated Rab5GEF activity and was essentially required for the endosome fusions (35). These findings fuel speculation that the Rab5GEFs might function as a multiple protein complex *in vivo*, regardless of whether homo- or heterophilic complexes are formed. By contrast, several RhoGEFs, including β1PIX (36, 37), Dbl (38), RasGRF1 (39), RasGRF2 (39), p115RhoGEF (40, 41), LARG (41), and PDZ-RhoGEF (41), have already been shown to dimerize or oligomerize. Inhibition of oligomerization diminishes the *in vivo* GEF activity of Dbl (38) and abolishes β1PIX function *in vivo* (37). In the cases of p115RhoGEF, LARG, and PDZ-RhoGEF, deletion of the C-terminal parts that were required for oligomerization had no significant effect on the *in vitro* GEF activities but resulted in the drastic stimulation of *in vivo* functions (40, 41). Taken together, these findings suggest that oligomerization may be an important common molecular feature in the GDP/GTP exchanging reactions on the small GTPase, which are mediated by either some members of Rab5GEFs or RhoGEFs, albeit with fundamental differences in their physiological roles.

Thus far, nine independent homozygous *ALS2* mutations resulting in three distinct but clinically overlapping recessive motor neuron diseases, ALS2, PLSJ, and IAHSP/HSP, have been reported. All these mutations cause the disruption of the coding sequences, leading to the functionally defective truncated ALS2 proteins. Because a feature common to these mutations is the loss of intact VPS9 domain, dysregulation of endocytic trafficking due to the loss of the ALS2-associated Rab5GEF activity might underlie the pathogenesis for these disorders. In this study, we identified a novel alternative splicing variant that lacked the sequences encoded by entire exon 25. This novel variant is predicted to produce internally deleted ALS2 protein (aa Δ1280–1335), which is oligomerization- and Rab5GEF-defective. At the moment, the abundance of this variant is undetermined, and thus its biological relevance is also unclear. However, it is conceivable that this oligomerization-defective ALS2 could diminish the function of ALS2 by sequestering certain important ALS2 interactors *in vivo*, thereby being implicated in the dysregulation of normal endosome dynamics.

In summary, here we show that the ALS2 oligomerization is crucial for the ALS2-mediated modulation of endosome dynamics. These findings are an important step to fully understand the ALS2 functions on endosomal dynamics. In addition to such endosome-related ALS2 function, a recent study has also revealed that ALS2 protects the cultured neuronal cells from toxicity induced by mutated Cu/Zn-superoxide dismutase (42), suggesting the possible neuroprotective function for ALS2. Further studies on the normal function for ALS2 will give us more insight toward the comprehension of pathogenesis underlying a number of recessive motor neuron disease caused by loss of functional mutation in the *ALS2* gene.

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ALS2CL, the novel protein highly homologous to the carboxy-terminal half of ALS2, binds to Rab5 and modulates endosome dynamics ☆

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Abstract ALS2, the causative gene product for juvenile recessive amyotrophic lateral sclerosis (ALS2), is a guanine-nucleotide exchange factor for the small GTPase Rab5. Here, we report a novel *ALS2* homologous gene, ALS2 C-terminal like (*ALS2CL*), which encodes a 108-kD ALS2CL protein. ALS2CL exhibited a specific but a relatively weak Rab5-GEF activity with accompanying rather strong Rab5-binding properties. In HeLa cells, co-expression of ALS2CL and Rab5A resulted in a unique tubulation phenotype of endosome compartments with significant colocalization of ALS2CL and Rab5A. These results suggest that ALS2CL is a novel factor modulating the Rab5-mediated endosome dynamics in the cells.

Keywords: ALS2 carboxy-terminal like; Amyotrophic lateral sclerosis 2; Guanine-nucleotide exchange factor; Rab5; Endosome dynamics

1. Introduction

Amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), and hereditary spastic paraplegia (HSP) are neurological disorders, which are characterized by a progressive motor neuronal degeneration [1,2]. Recently, we and other groups have identified the loss-of-functional mutations in the *ALS2* gene, accounting for a number of juvenile recessive motor neuron diseases (MND) including a type of juvenile ALS (ALS2), juvenile-onset PLS (PLSJ), and infantile-onset ascending hereditary spastic paralysis (IAHSP) [3–7].

The *ALS2* gene encodes a novel 184 kD protein, termed ALS2 or alsin, comprising three predicted guanine-nucleotide exchange factor (GEF) domains [3,4]; i.e., regulator of chromosome condensation (RCC1) [8]-like domain (RLD), the Dbl homology and pleckstrin homology (DH/PH) domains [9], and a vacuolar protein sorting 9 (VPS9) domain [10,11]. In addition, eight consecutive membrane occupation and recognition nexus (MORN) motifs [12] are noted in the region between DH/PH and VPS9 domains [13]. Recently, we have identified the ALS2-associated Rab5-specific GEF activity that is mediated by the carboxy-terminal MORN/VPS9 domain of ALS2 and also shown that ALS2 localizes preferentially onto the early endosome compartments in neuronal cells [13]. Since Rab5 plays crucial roles in clathrin-mediated vesicle endocytosis, trafficking, and early endosomal membrane fusion [14], ALS2 may implicate in the vesicle/membrane dynamics in the cells through the activation of Rab5.

Here, we report the identification and characterization of the novel *ALS2* homologous gene, ALS2 C-terminal like (*ALS2CL*/*Als2cl*), and its gene product ALS2CL.

2. Materials and methods

2.1. Cloning of the ALS2 homologous genes

To identify the potential homologs/paralogs for *ALS2*/*Als2* genes, we conducted BLAST searches [15] on the public databases. Cloning of the full-length transcripts for the identified homologous genes, i.e., *ALS2CL* and *Als2cl*, was performed by a reverse transcriptase (RT)-polymerase chain reaction (PCR)-based method. Analysis of the predicted amino acid sequences, including motif/domain identification and multiple protein sequence alignment was conducted as described [3].

2.2. Northern blotting

Multiple human adult tissue Northern (MTN) blot (Clontech) was hybridized with [α-^{32}P]dCTP-labeled open reading frame (ORF) probes generated from the *ALS2CL* or *ACTB* (β-actin) cDNA in PerfectHyb hybridization solution (Toyobo) at 68 °C. Membranes were washed with 0.1× SSC containing 1% SDS at 65 °C and exposed to X-ray film (Bio-MAX, Kodak). The mouse adult tissue MTN blot (Clontech) was also hybridized with the *Als2cl* or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) cDNA.

☆ The cDNA sequences of *ALS2CL* and *Als2cl* have been deposited in DDBJ/EMBL/GenBank Database under Accession Nos. AB107015 and AB107016.

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Abbreviations: ALS, amyotrophic lateral sclerosis; PLS, primary lateral sclerosis; HSP, hereditary spastic paraplegia; MND, motor neuron disease; ALS2CL, ALS2 carboxy-terminal like; GEF, guanine-nucleotide exchange factor; RLD, regulator of chromosome condensation-like domain; DH, Dbl homology; PH, pleckstrin homology; MORN, membrane occupation and recognition nexus; VPS9, vacuolar protein sorting 9; EEA1, early endosome antigen-1; EGFP, enhanced green fluorescent protein

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2.3. Expression constructs

All cDNA expression constructs were generated by subcloning the RT-PCR amplified fragments into the appropriate vectors. For the GEF assay, four cDNA fragments; i.e., human ALS2_1018-1657aa (ALS2_1018-1657), human *ALS2CL* ORF (ALS2CL_human), and mouse *Als2cl* ORF (ALS2CL_mouse), were subcloned into pCI-neo Mammalian Expression Vector (Promega). For the subcellular localization studies, human and mouse ALS2CLs, which were fused amino-terminally with enhanced green fluorescent protein (EGFP), were generated using pEGFP-C1 (Clontech). The bacterial expression plasmids encoding the small GTPases and FLAG-tagged human Rab5A, which have been previously generated [13], were also utilized.

2.4. GEF assay and in vitro binding

GEF assay was conducted as previously described [13]. In vitro binding experiments were also performed as described [13] with several modifications. In brief, purified Rab5A (4 pmol), which was pre-loaded with either GDP or GTPγS, or nucleotide-free, was mixed with FLAG-M2 beads conjugating 4 pmol equivalent of the immunoprecipitated FLAG-tagged ALS2CL or ALS2 in 100 μl of a modified GEF buffer [25 mM Tris–HCl, pH 7.4, 100 mM NaCl, 20 mM $MgCl_2$, 1.5 mM CHAPS, and 0.1% (w/v) skimmed milk] containing 50 μM of either GDP or GTPγS, or without nucleotides, for 2 h at 30 °C. After washing with 4 × 1 ml of the same buffer, bound Rab5A was co-eluted with FLAG-ALS2CL protein by the addition of SDS–PAGE sample buffer and detected by Western blotting analysis using appropriate antibodies.

2.5. Cell culture, transfection, and microscopic observations

HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with plasmid constructs using Effectene Transfection Reagent (Qiagen). Immunocytochemical detection and image analysis were conducted by Leica TCS_NT confocal-microscope systems (Leica) as previously described [13].

3. Results

3.1. Identification of the human ALS2CL and mouse Als2cl genes

A BLAST search of the GenBank/DDBJ/EMBL database for potential *ALS2/Als2* paralogs and/or homologs revealed the presence of the genomic segments, mRNAs, and EST sequences, which encode a novel protein highly homologous to the carboxy-terminal half of the human and mouse ALS2 proteins, in human, mouse, and rat genome. Computational analyses of the DNA sequences by assembling the mRNA and EST sequences, followed by the comparison with genomic DNA sequences and mapping, demonstrated that human and mouse *ALS2/Als2* homologous genes comprised 26 exons and resided within approximately 24.5 kb of the genomic region on human chromosome 3p21.31 and the 23.2 kb genomic region on mouse chromosome 9F3, respectively (Fig. 1A). We designated these human and mouse genes as ALS2 C-terminal like; *ALS2CL* (HGNC approved symbol) and *Als2cl* (MGD nomenclature committee approved symbol), respectively. The sequences of the *ALS2CL* and *Als2cl* transcripts encompassed 4741 nt (ORF; 2862-nt long) (AB107015) and 5081 nt (ORF; 2859-nt long) (AB107016), and matched with the sequences for human transcript LOC259173_isoform 1 (NM_147129) and mouse RN49018 (NM_146228) [16], respectively (Fig. 1A and B).

3.2. Alternative splicing variants for ALS2CL and Als2cl

In the course of our database searches and RT-PCR based cloning of the transcribed DNA sequences, an extensive alternative splicing of the transcripts, giving rise to at least 17 minor variants of *ALS2CL*, was noted in human tissues (Fig. 1B), in addition to the major *ALS2CL* transcript. Analyses of the flanking DNA sequences for these variants identified the cryptic acceptor and donor consensus sequences (data not shown), suggesting that all these variants arose by alternative splicing. It was also evident that all these alternative splicing, resulting in premature stop codons, disrupted the coding frame for full-length *ALS2CL* (Fig. 1B). Physiological significances of these extensive splicing events were currently unknown.

We also identified five minor murine variants as follows: (1) alternative 5′-UTR exons '**a**' and '**b**' proceeded by exon 2 (Fig. 1A), (2) cryptic three nucleotides insertion (-27_-26insTAG), (3) transcript with intron 13, (4) transcripts with skipping exon 19, and (5) alternative exon '**c**' between exons 7 and 8 (RIKEN_5830412B02: with skipping exon 19, Fig. 1A).

3.3. Deduced amino acid sequences

Computational predictions have shown that major transcripts for both *ALS2CL* and *Als2cl* encode 108-kD proteins, ALS2CL [953 and 952 amino acids (aa), respectively], comprising several domains and motifs including PH, MORN, and VPS9 (Fig. 2A). Human and mouse ALS2CL proteins showed a high degree of sequence conservation to each other (81% identity and 88% similarity), and with the corresponding C-terminal region of human ALS2 (33% identity and 51% similarity) (Fig. 2B). Interestingly, phylogenetical analysis revealed that amino acid sequences for ALS2CL were much closer to that for fugu ALS2 than those for human and mouse ALS2 (data not shown).

3.4. Tissue distribution of the ALS2CL/Als2cl mRNA

Northern blot analysis revealed the *ALS2CL* transcript of an approximately 5 kb of the major mRNA in various adult human tissues with higher expression in both heart and kidney (Fig. 3A). It is also noted that the band-signals for the *ALS2CL* transcript were rather blurred, consistent with the presence of multiple alternative splicing variants. In contrast, a single 5 kb discrete band for the mouse *Als2cl* transcript was observed in variety of tissues in adult mice with highest in liver (Fig. 3B).

3.5. Assays of the ALS2CL-associated GEF activity

To analyze the ALS2CL-associated GDP/GTP exchanging activities, i.e., so-called GEF activities, on the small GTPases, in vitro GDP dissociation assays were conducted using nine bacterially produced small GTPases including Rab4A, Rab5A, Rab5B, Rab5C, Rab7, Rab11A, Rac1, RhoA, and Cdc42. The ALS2_1018-1657 peptide, which spans the minimum region associating with Rab5-GEF activity [13], was used as a positive control. The results showed that human ALS2CL exclusively catalyzed GDP dissociation on Rab5A, Rab5B, and Rab5C, as in the cases for ALS2_1018-1657 (Fig. 4A), suggesting that humanALS2CL retained selective catalytic activity with all the members of Rab5 family. However, the degree of activities for ALS2CL was significantly lower than that for ALS2_1018-1657 (Fig. 4A). Interestingly, mouse ALS2CL exhibited almost no Rab5-GEF activities (Fig. 4A).

To define the enzymatic kinetics for the Rab5GEF activity, we next carried out the GDP dissociation assay on Rab5A using a wide range of concentrations for either ALS2CLs or

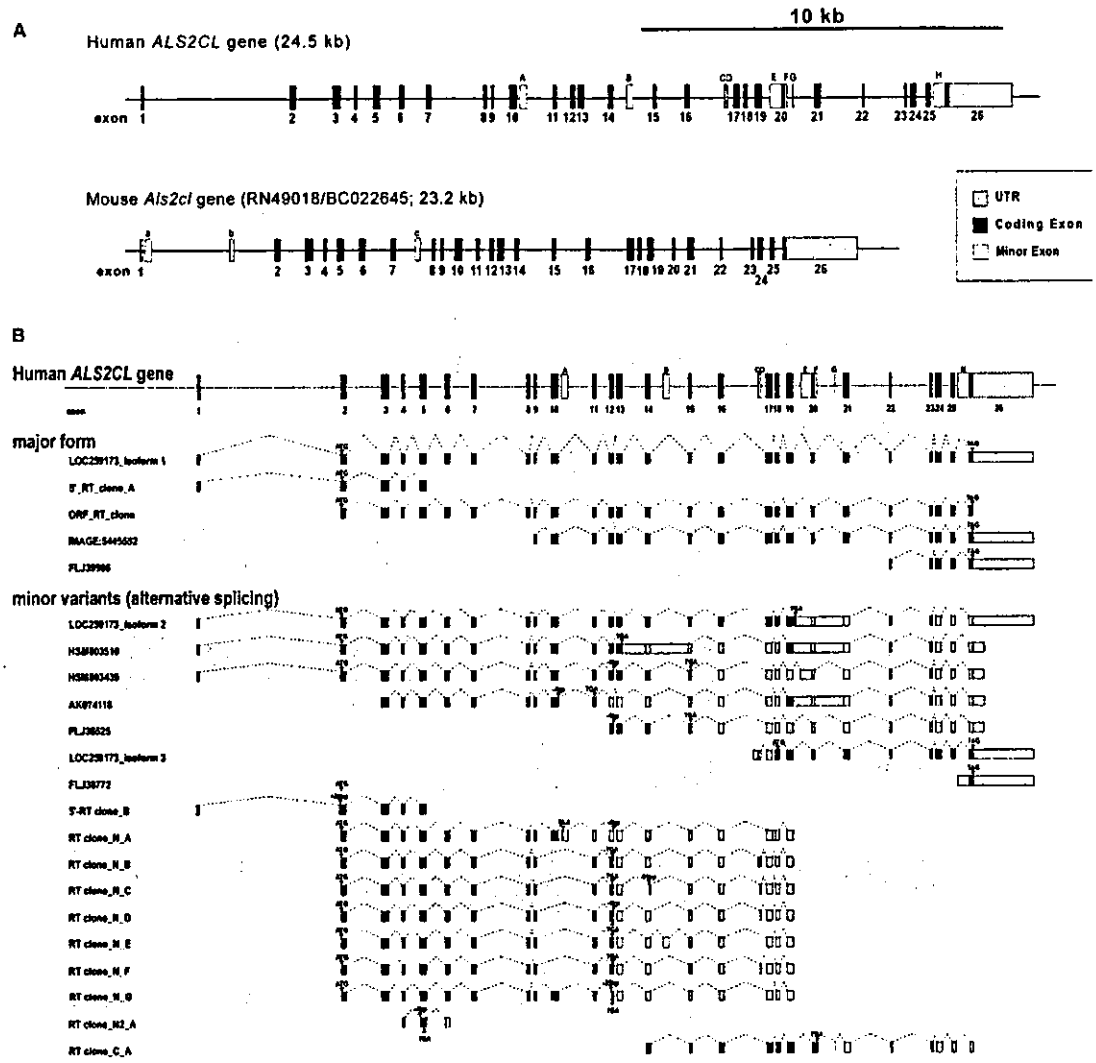

Fig. 1. Genomic organization of the human *ALS2CL* and mouse *Als2cl* genes. (A) Schematic representation of genomic organizations for human *ALS2CL* (upper) and mouse *Als2cl* (lower) genes. *ALS2CL* spans approximately 24.5 kb of the genomic region on human chromosome 3p21.31, while *Als2cl* covers approximately 23.2 kb of the region on chromosome 9F3. Both genes comprise 26 major exons. Black and gray boxes represent coding (translated) and non-coding (untranslated) region of major exons (exons 1–26), respectively. Minor alternative exons (A–H for human; a–c for mouse) are also shown as white boxes. (B) Exon organization of splicing variants for the human *ALS2CL* transcripts. A single major form and 17 differentially spliced minor variants are shown. The positions of translation initiation (ATG) and termination (TGA, TAG, or TAA) codons are shown.

ALS2_1018-1657 (final 0–1.6 μM) (Fig. 4B). Both ALS2_1018-1657 and human ALS2CL revealed the protein concentration-dependent Rab5-GEF activities with approximately eightfold lower dissociation constant with human ALS2CL (ALS2_1018-1657; ~25 nM vs. human ALS2CL; ~200 nM). However, mouse ALS2CL did not show any significant Rab5-GEF activities at any concentrations of the protein (Fig. 4B).

3.6. Interaction of ALS2CL and Rab5

To examine whether the ALS2CL proteins directly interact with Rab5, we conducted the in vitro binding assays using the FLAG-M2 pull-down experiments. The amino-terminally FLAG-tagged ALS2_1018-1657, human ALS2CL, and mouse ALS2CL were immunoprecipitated in the presence of recombinant Rab5A, which was pre-loaded with either GDP or GTPγS, or left unloaded with any nucleotides. Despite the fact that the ALS2CL proteins showed a low or negligible Rab5-GEF activity, they bound rather strongly to Rab5A (Fig. 5). Notably, the ALS2_1018-1657 peptide, which revealed the higher Rab5-GEF activity (Fig. 4), bound preferentially to the nucleotide-free form of Rab5A, whereas mouse ALS2CL, which exhibited no Rab5-GEF activity, interacted non-selectively with any forms of Rab5A. The Rab5A-binding properties for human ALS2CL demonstrated an intermediate preferentiality.

3.7. Subcellular localization of ALS2CL

To investigate the subcellular localization of ALS2CL, HeLa cells were transfected with plasmid expressing either human or mouse ALS2CL. Ectopically expressed amino-terminally

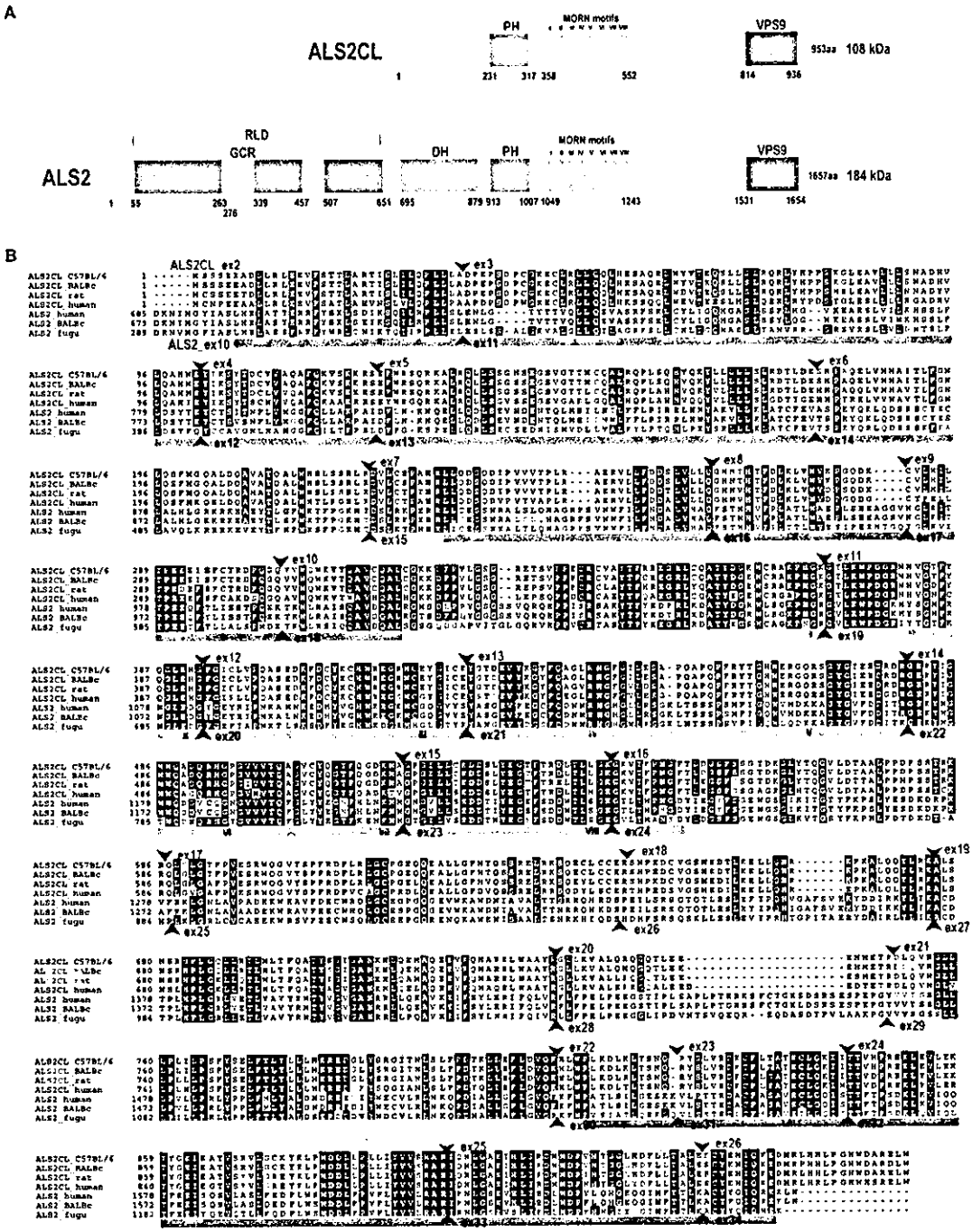

Fig. 2. Amino acid sequence analysis. (A) Schematic representation of the domains and motifs identified in the predicted human ALS2CL and ALS2 proteins. (B) ClustalW multiple amino acid sequence alignment of mouse (ALS2CL_C57BL/6; NP_666340, and ALS2CL_BALBc; this study), rat (ALS2CL_rat; XP_236654), and human (ALS2CL_human; this study) ALS2CL proteins, and the C-terminal portions of human (ALS2_human; NP_065970), mouse (ALS2_BALBc; NP_082993), and fugu (ALS2_fugu; SINFRUP00000067515) ALS2 proteins. Intron–exon boundaries for human *ALS2CL* and *ALS2* are also shown. Sequences are numbered with the initiation codon of each protein as #1.

FLAG-tagged (Fig. 6A) and EGFP-fused (Fig. 6B) human ALS2CL proteins localized throughout the cells with strong punctated stainings in cytoplasm. EGFP-fused mouse ALS2CL also showed similar distribution pattern (Fig. 6C). Co-localization analyses revealed that human ALS2CL partially overlapped with early endosome antigen 1 (EEA1) [17] immunopositive vesicles and/or early endosomes (Fig. 6D–F), but not with the late-endosomal/lysosomal markers, LAMP-1

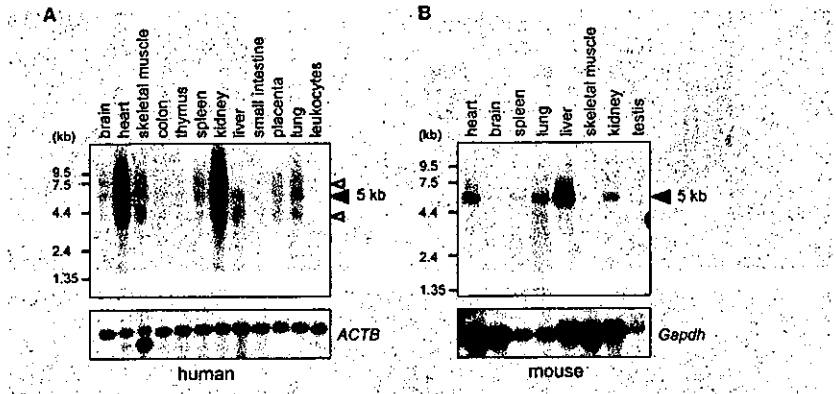

Fig. 3. Northern blot analysis. MTN blots were hybridized with *ALS2CL/Als2cl* (upper panel) cDNA clones. Filled and open arrowheads indicate the position of major and minor transcripts, respectively. The lower panel represents the same blots hybridized with (A) the human *ACTB* (β-actin) cDNA or (B) the mouse glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) cDNA to confirm RNA quality and relative loading. Positions of size-markers are shown on the left.

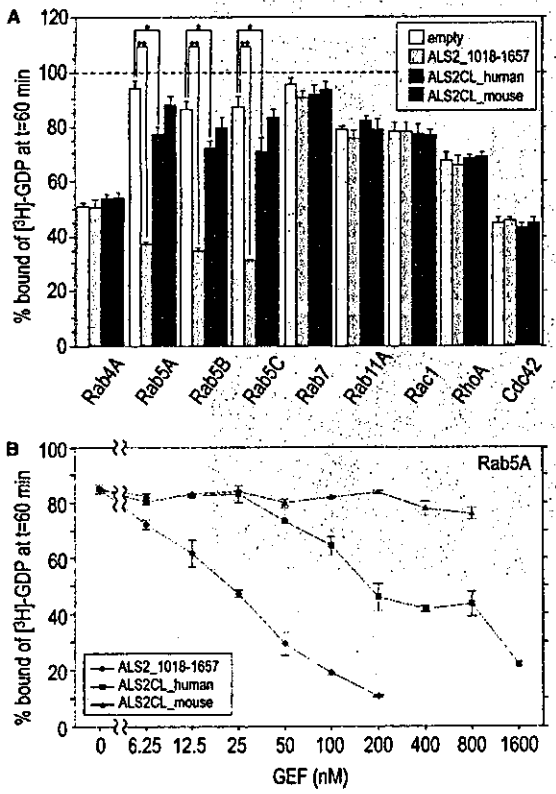

Fig. 4. GEF activity assays. (A) In vitro [^{3}H]GDP dissociation assay for FLAG-tagged ALS2 peptide (ALS2_1018-1657aa; light gray bars), human ALS2CL (ALS2CL_human; dark gray bars), mouse ALS2CL (ALS2CL_mouse; black bars), and FLAG-tag alone (empty; as a control, open bars) on nine different small GTPases. Each value represents the mean and standard deviation of at least three independent assays. Double asterisk, $P<0.001$; asterisk, $P<0.01$ in t tests. (B) In vitro [^{3}H]GDP dissociation assay on Rab5A in the presence of increasing amount of recombinant ALS2CL and ALS2 proteins. Each value represents means ± S.D. ($n=3$) of the percentage of [^{3}H]-GDP bound to Rab5A after 60 min in the presence of given amount either of ALS2_1018-1657 (diamonds), ALS2CL_human (squares), or ALS2CL_mouse (triangles).

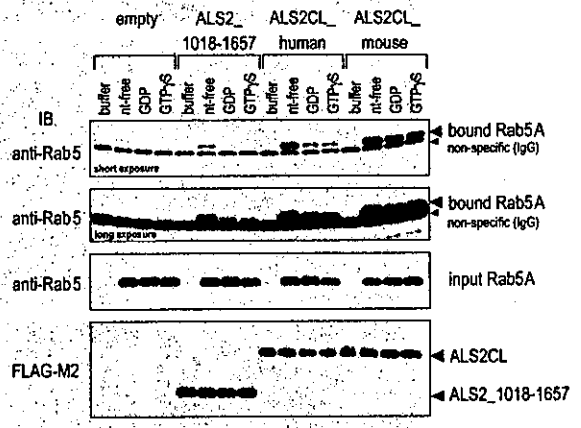

Fig. 5. In vitro Rab5A binding assay. Nucleotide free (nt-free), GDP bound (GDP), or GTPγS bound (GTPγS) forms of Rab5A were incubated with FLAG-M2 beads conjugating FLAG-tagged ALS2_1018-1657aa, human ALS2CL (ALS2CL_human), or mouse ALS2CL (ALS2CL_mouse), or with FLAG-M2 beads alone (empty) as a control. The bound and input Rab5A were detected by immunoblotting method using anti-Rab5 antibody. Western blotting analysis of the FLAG-tagged proteins was also conducted using the FLAG-M2 antibody.

and LAMP-2 [18], or Golgi marker GM130 [19] (data not shown). However, the degree of EEA1 overlapping was quite low (<10%), when compared with those for ALS2_660-1657, in which a high degree (~85%) of vesicular co-localization was observed [13].

3.8. Co-localization of ALS2CL and Rab5A

To investigate whether human ALS2CL co-localized with Rab5 in vivo, co-transfection of HeLa cells with expression constructs for EGFP-fused ALS2CL and FLAG-tagged Rab5A was conducted. The results showed that both proteins were significantly co-distributed onto the vesicular/membranous compartments in cytoplasm, particularly to the leading edges of the cells (Fig. 6G–I). Notably, co-expression of ALS2CL and Rab5A frequently (~40% of the co-transfected

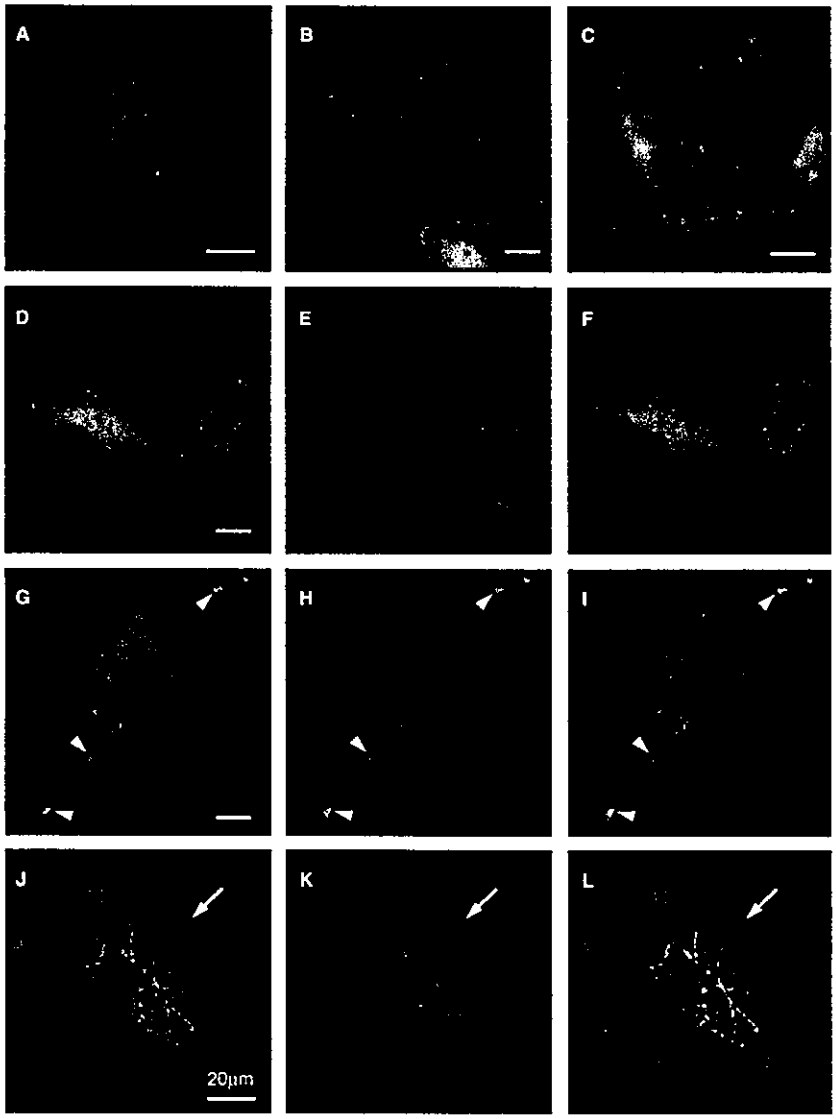

Fig. 6. Localization of ALS2CL in the cells. (A–C) Subcellular distribution of the ectopically expressed ALS2CL in HeLa cells (A, FLAG-tagged human ALS2CL; B, EGFP-fused human ALS2CL; C, EGFP-fused mouse ALS2CL). (D–F) Partial co-localization of the human ALS2CL onto EEA1-positive endosomes (D, EGFP-fused human ALS2CL; E, endogenous EEA1; F, merged image). (G–L) Co-transfection of the EGFP-fused human ALS2CL and FLAG-tagged Rab5A expression constructs in HeLa cells (G and J, EGFP-fused human ALS2CL; H and K, FLAG-tagged Rab5A; I and L, merged images). The representative images for the cells exhibiting vesicular (G–I) and tubular membranous (arrows; J–L) distributions are shown. Arrowheads indicate the leading edges of the cells.

cells) caused a striking effect on the morphology of endosomal compartments, showing a tubulation and/or elongation with accompanying extensive co-localization of ALS2CL and Rab5A (Fig. 6J–L).

4. Discussion

In this study, we newly identified the *ALS2* homologous gene, *ALS2CL* and *Als2cl*. *ALS2CL* and *Als2cl* map to the syntenic chromosomal regions on human chromosome 3p21 and mouse chromosome 9F3, respectively, both comprising 26 major exons. The intron–exon boundaries for *ALS2CL* and *Als2cl*, and those for the homologous regions of *ALS2*/*Als2* were nearly completely preserved. Further, analysis of the predicted amino acid sequences revealed a high level of sequence similarity throughout the entire region of ALS2CL and the C-terminal portion of the ALS2 proteins, indicating that *ALS2CL*/*Als2cl* and *ALS2*/*Als2* were evolutionally conserved genes evolved from a common ancestry of origin. Notably, *ALS2CL*/*Als2cl* lack several exons corresponding to those encoding N-terminal RLD for ALS2, suggesting that the N-

terminal portion of ALS2 can be independently evolved. In spite of such structural conservation, our results demonstrate that the molecular functions for ALS2CL and the C-terminal homologous region of ALS2 seem to be slightly different.

Previously, we have shown that enzymatic activity for the activation of Rab5 GTPase is retained in the C-terminal portion of human ALS2 comprising MORN/VPS9 domain, and that either deletion of MORN motifs or the mutation in the evolutionally conserved amino acid residues in VPS9 domain resulted in the loss of its catalytic activity [13]. In this study, we demonstrated that, although human ALS2CL showed a similar catalytic specificity on the small GTPases as that for human ALS2, the level of its Rab5-GEF activity was significantly lower than that for ALS2, or even at almost negligible levels. On the other hand, ALS2CL, especially an enzymatically almost-inactive mouse ALS2CL, exhibited a stronger binding to Rab5 than ALS2, in a nucleotide state-independent manner. These results suggest that ALS2CL may act as a functional modulator in the Rab5-mediated enzymatic reactions, whilst those actions are slightly different from that for ALS2.

We have previously also shown that overexpression of the C-terminal human ALS2 peptide containing 660–1657 aa, spanning a homologous region corresponding to entire ALS2CL, localizes onto the Rab5/EEA1-positive early endosomal compartments, and induces the striking enlargement of endosomes, which is mediated through the Rab5GEF activity inherent to the MORN/VPS9 region of ALS2 [13]. On the other hand, ectopically expressed ALS2CLs localize in both nucleus and cytoplasm with strong punctated stainings that are extensively overlapped with Rab5A but not with EEA1, suggesting that the ALS2CL proteins preferentially localize to the Rab5-positive/EEA1-negative vesicles and/or endosome sub-compartments. Further, co-expression of human ALS2CL and Rab5A caused unique morphological changes in endosomal compartments, which were characterized by membrane tubulation rather than enlargement. Taken together, these results again support the biochemical notion that ALS2CL and ALS2 mediate similar but slightly different molecular functions in the cells.

Several recent studies demonstrated that the similar tubular endosome phenotypes were elicited under certain experimental conditions, such as in the presence of wortmannin, an inhibitor for phosphatidylinositol-3 kinase [20], or brefeldin A, a potent inhibitor of trafficking through the secretory pathway [21], as well as by the overexpression of dominant positive Rab4 small GTPase [22]. As each of these treatments leads to the impairment of normal endosomal membrane trafficking [20–22], overexpression of human ALS2CL may also disturb the normal vesicle/membrane trafficking possibly through the actions associating with a lower Rab5-GEF activity with a higher Rab5 binding property for the ALS2CL proteins.

In summary, our results demonstrate that *ALS2CL/Als2cl* and *ALS2/Als2* are evolutionally conserved genes, and that their products, ALS2CL and ALS2, play overlapping but slightly distinctive roles on the Rab5-mediated vesicle/membrane trafficking and dynamics in the cells. Thus, it is possible that ALS2CL modulates the ALS2-mediated molecular and cellular functions either directly or indirectly, thereby implicating in the phenotypic variations seen in patients with ALS2/PLSJ/IAHSP. Further dissection of molecular and cellular functions for ALS2CL as well as those for ALS2 will give us additional insights into the pathogenesis for a number of MNDs caused by the *ALS2* mutations.

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<シンポジウム3—4>脊髄小脳変性症と運動ニューロン疾患

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池田　穣衛

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池田　稳衛

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はじめに

若年性筋萎縮性側索硬化症2型（ALS2）は常染色体劣性遺伝形式を示す上位運動ニューロン変性疾患である．ALS2の遺伝子座はヒト第2染色体長腕q33領域にマップされている．私達はこの領域から新規遺伝子ALS2(全長約80kbp，34個のエクソンをコードしている)を単離し，当該遺伝子のエクソン中にALS2患者に特異的な欠失変異（homozygous deletion mutation)をみいだした．これらの欠失変異はALS2遺伝子産物（ALS2蛋白質，推定1657アミノ酸残基，184kDa）の蛋白質コードフレームを壊し不完全長ALS2蛋白質の産生によるALS2機能喪失変異であることが明らかになった．これらのことから，私達はALS2遺伝子変異がALS2発症の主因であると同定した．その後，上位運動ニューロンの変性を病徴とするjuvenile primary lateral sclerosisやinfantile-ascending hereditary spastic paralysisにおいてもALS2遺伝子変異が同定され，今日までに独立した9家系においてそれぞれことなる9個のhomozygous ALS2 mutationが同定されている．このことから，上位運動神経変性の主因はALS2蛋白質の機能欠損であると考えられる．続いて，私達はALS2蛋白質が低分子G蛋白質Rab5を基質とするグアニンヌクレオチド交換因子（GEF）活性を有する事，ALS2蛋白質はこの活性を背景とする神経細胞シグナル伝達にかかわるエンドソーム分子動態にかかわる事を確認した．さらに，私達はALS2モデル動物としてALS2 KO mouseを作出しており，本稿ではALS2遺伝子機能と併せて上位運動神経変性の分子機序について考察する．

ALS2の臨床的特徴

ALS2は上位運動ニューロンの変性にともなう四肢，顔面，咽頭筋の痙攣，構音障害などの偽球麻痺症状，および進行性の四肢筋萎縮等の諸症状を特徴とする．発症年齢は平均6.9歳と若年発症型である一方，病状の進行はきわめて緩徐である．本疾患の初期症状は若年発症型の原発性側索硬化症(juvenile primary lateral sclerosis；JPLS）と酷似している[1]．

ALS2遺伝子と機能

筋萎縮性側索硬化症(amyotrophic lateral sclerosis；ALS)は大脳皮質錐体細胞から脊髄にいたる上位運動ニューロンおよび脊髄前角細胞から筋にいたる下位運動ニューロンが選択的に障害される進行性神経変性疾患である．ALS発症の分子機序は未だ明らかでなく，治療法も確立されていないのが現状である．ALS患者の大多数は弧発例であるが，5～10%の患者は家族性（遺伝性）ALSと考えられている．家族性ALS（FALS）については，優性遺伝形式をとるALS1，ALS3，ALS4，ALS6およびFTD-ALS，そして劣性遺伝形式をとるALS2，ALS5，およびType 2とX染色体連鎖を示すALSXがそれぞれ報告されている．ヒトゲノム解析を背景に，今日までに漸く2つのALS発症責任遺伝子の同定に成功している．最初に，Rosenら[2]は優性遺伝形式をとるALS1の責任遺伝子としてCu-Zn superoxide dismutase 1（SOD1）遺伝子を同定した．当初はSOD1変異による神経細胞内でのフリーラジカルの集積が運動神経変性をもたらす要因となっていると推定したが，その後の一連の解析によってALS1にみられる神経細胞死はむしろ変異SOD1タンパク質の異型機能（toxic gain-of-function）による奇形複合体形成によるのではないかと推測されるにいたっているがその本態はまだ不明である．

SOD1遺伝子に次ぐ2つ目のALS発症責任遺伝子として，私達は劣性遺伝形式をとる若年性筋萎縮性側索硬化症2型（ALS2）の発症責任遺伝子；ALS2遺伝子の同定に成功した[3][4]．ALS2患者にみいだされたALS2遺伝子変異はすべて当該遺伝子のエクソン中の1および2bpの欠失変異（homozygous deletion mutation)であることから，これらの欠失変異はALS2遺伝子産物（ALS2蛋白質，推定1657アミノ酸残基，184kDa）の蛋白質コードフレームを壊し不完全長ALS2蛋白質を作ると考えられる．したがって，ALS2にみられる運動ニューロンの変性はALS2機能喪失（loss-of-function)によると考えられる．その後，juvenile primary lateral sclerosis（JPLS）やinfantile-ascending hereditary spas-

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tic paralysis (IAHSP) においても ALS2 遺伝子変異が同定された．今日までに独立した9家系においてそれぞれことなる9個の homozygous ALS2 mutation（1bp あるいは 2，10bp の homozygous deletion によるフレームシフト変異およびナンセンス変異）[9]~[11]が同定されている．これらすべての患者例において，不完全長 ALS2 蛋白質が産生され，ALS2 正常機能が損なわれていると思われる．ALS では上位と下位の運動ニューロンが障害されるのに対して，PLS や HSP での神経変性は上位運動ニューロンに限定されている．したがって，ALS2 蛋白質の機能喪失が上位運動神経変性を特徴とする ALS2 や JPLS，IAHSP 発症の原因であることから，ALS2 蛋白質は上位運動神経の機能と生存に必須であるといえる．ALS2 蛋白質の機能としては，3種類の低分子 G 蛋白質の機能調節因子グアニンヌクレオチド交換因子（RanGEF，RhoGEF，Rab5GEF）機能ドメインの他に膜結合ドメインなどが推定されている．

私達は，ALS2 蛋白質が低分子 G 蛋白質 Rab5 を基質とするグアニンヌクレオチド交換因子（GEF）活性を有する事，この活性を背景とする神経細胞シグナル伝達にかかわるエンドソーム分子動態にかかわる事を確認した[9]．さらに，ALS2 蛋白質は発現の強弱はみとめられるものの，中枢神経系のみならず組織全体で幅広く発現している．このような現象は ALS2 蛋白質にかぎらず，ほとんどの遺伝性神経疾患責任遺伝子においても共通している発現パターンである．したがって，神経組織・細胞特異的な ALS2 蛋白質の機能を保障する共役因子の存在が想定される．一方，神経細胞内での ALS2 蛋白質は樹状突起に局在する傾向を示している．したがって，ALS2 蛋白質は上記機能を背景とする上位運動ニューロンに特異的な情報伝達あるいは物質輸送や機能維持に深くかかわっていると考えられる．一方，上記の独立した9家系の ALS2 遺伝子変異に共通した ALS2 蛋白質機能喪失は，ALS2 蛋白質の C-末端に同定された Rab5GEF 活性の消失にあると考えられる．

これらの活性調節機能の喪失（たとえば回収できなくなった上位運動ニューロンにユニークな glutamate 受容体を介した Ca イオンの過剰流入にともなう神経細胞障害）こそが ALS2 の発症，すなわち上位運動ニューロンを death-spiral へと導く最初のでき事であると推定される．

私達は ALS2 の分子病態解析を目的として ALS2 モデル動物の作出を試みた．ALS2 蛋白質のほぼ全長を欠失している Tunisia の ALS2 家系患者と同一の遺伝子型を持つ Als2 knock-out (KO) mouse を作出した．しかし，当該マウスは生後8カ月を過ぎた時点においても発症していない（他の4研究グループの Als2 KO mouse も発症していない）．上位運動神経変性モデル動物としてマウスの妥当性もうたがわれるが，ALS2 遺伝子変異が ALS2 を始めとして PLS や HSP の責任遺伝子であることから発症に第2の遺伝子の関与もうたがわれる．これらの可能性については，現在遂行している経時的な神経病理学的解析の結果を待って判定したい．

ま と め

孤発性，家族性を問わず ALS は "heterogeneous group of inexorable neurodegenerative disorders" である．したがって，ALS の発症と進行には複数の因子（遺伝子）の関与が想定される．これら複数の因子を同定し，それらの機能と分子環境を統合して理解する事が運動ニューロン変性の分子機序に基づいた本態治療を可能にする唯一の道である．運動神経の変性に直接あるいは間接的にかかわる因子を的確に把握するためには ALS をはじめ家族性運動神経変性疾患責任遺伝子の単離・同定が必須である．とくに，loss-of-function をともなう劣性遺伝形式をとる運動神経変性責任遺伝子こそが，遺伝子機能と神経細胞死を直接結びつける格好の因子である．遺伝子同定には，患者，神経内科医，分子医学研究者らの連携，膨大な DNA・遺伝子解析を敢行する努力と継続する意志，それを支えるチームワークと研究費が必要である．私達の ALS2 遺伝子単離・同定と一連の解析もその例外ではない．ALS2 蛋白質の分子動態・機能解析と ALS2 発症の分子機序の解明を通して，運動ニューロンをふくめた神経細胞系の生存・機能維持の分子基盤の理解を深め，ALS および神経変性疾患の本態治療法・治療薬の開発のための新たな展開が拓かれることを期待している．

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