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*

Ryota Kunita‡, Asako Otomo§, Hikaru Mizumura‡, Kyoko Suzuki, Junko Showuchi-Miyata§, Yoshiko Yanagisawa‡, Shinji Hadano‡§, and Joh-E Ikeda‡§**

From the ¶Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, (Tokai University School of Medicine), the §Department of Molecular Neuroscience, The Institute of Medical Sciences, Tokai University, Isehara, Kanagawa 259-1193, Japan and the Department of Paediatrics, Faculty of Medicine, University of Ottawa, Ontario K1H 8M5, Canada

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), 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 paraplegia (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, cytoskeleton...
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-oligomerization 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 (MFP 1012–1651) was raised with the purified His-tagged mouse ALS2_1012–1651 fragment, followed by affinity purification using an antigen-coupled Sepharose column. MFP 1012–1651 allowed the detection of both mouse and human ALS2 proteins (data not shown). All other reagents were from commercial sources and of reagent grade.

Plasmid Constructs—All the DNA 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 and pB42AD-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 pCleeoFLAG-ALS2_L (full-length), pCleeoFLAG-ALS2_1018–1657, and pGEXP-Rab5A were also utilized (30). Plasmid constructs expressing deletion mutant forms of ALS2, including pCleeoFLAG-ALS2_1018–1657 (Δ1515–1531), and pCleeoFLAG-ALS2_L (Δ1280–1657) were generated by a PCR-based method. pCleeoFLAG-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_1018–1657 and pEGFP-ALS2_L (Δ1280–1657) 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 using 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 [pGAL-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-b-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 pCleeoFLAG-ALS2 constructs and/or pCleeoHA-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, and Complete protease inhibitor (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 5% milk in Tris-buffered saline (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (TBS-T) for 1 h at room temperature in a humid chamber. The membranes were incubated with the rabbit polyclonal antibody (MFP 1012–1651) (1:3000), followed by horse-radish 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 released into 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.

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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 test. 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 pcDNA/FLAG constructs. The small GTPase Rab5A was also prepared as previously described (30). 4 pmol of the [3H]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 [3H]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 MgCl2, 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. 1A, 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. 1A, parts b and c). These results indicate that ALS2 could be self-associated. But 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. 1A) 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. 1B). 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
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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_1018–1654, ALS2_1233–1657, ALS2_1100–1657, ALS2_1100–1657 (Δ1280–1335), and ALS2_1018–1657 (Δ1515–1554), completely lacking the conventional VPS9 domain, still interacted with HA-tagged ALS2_L, 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_1018–1654, ALS2_1233–1657, ALS2_1100–1657, ALS2_1100–1657 (Δ1280–1335), and ALS2_1018–1657 (Δ1515–1554). We also prepared the expression plasmid encoding the Trio_DH/PH domain (aa 1233–1628) as a negative control (Fig. 4A). Immunoprecipitation of the 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. 4B), as in the case of FLAG-tagged ALS2_L against HA-tagged ALS2_L (Fig. 2A). 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).
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. 5A). Furthermore, ALS2_1100–1657, lacking the N-terminal two copies of eight consecutive MORN motifs, still maintained even higher Rab5GEF activity (Fig. 5A). 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. 5A). 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 (∆1280–1335), which lost the ability to interact with ALS2_L (Fig. 4B), exhibited no catalytic activity (Fig. 5A). 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 (∆1515–1531), 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
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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. Rab5GEF activity in the ALS2 fragments such as ALS2_1233–1657, ALS2_1100–1657 (∆1280–1335), and ALS2_1018–1657 (∆1515–1531) resulted from a decrease in their binding affinity to Rab5, we conducted in vitro Rab5A associated 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 (∆1280–1335), 1018–1657 (∆1515–1531), 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.3 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. 5B). 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. 5B). 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. 5B), suggesting that the MORN and VPS9 domains are not essential for Rab5A binding (Fig. 5B). 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 (∆1280–1335), 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 (∆1515–1531), an oligomerization-prone and Rab5GEF-defective mutant (Fig. 5A), 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 vitro, 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 transected 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.-I. Ikeda, unpublished results.
homo-oligomerized (data not shown). As shown in Fig. 6A, expression of EGFP_695–1657 resulted in enlarged endosomes and most of them were EEA1-positive vesicles (Fig. 6C), 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. 6D), 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 constitutive 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 on 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. 7A), and the other could be that eight ALS2 molecules form an octagon-like structure (Fig. 7B). 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
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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-ALS2-associated Rab5GEF activity reside within the C-terminal region-mediated interaction. 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 also play significant roles in 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 region-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 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 the present study, eight ALS2 molecules form an octagon-like structure through the C-terminal region-mediated interaction.

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.
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-modulated 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 oligomerization, 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, 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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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

Ryota Kunita, Asako Otomo, Hikaru Mizumura, Kyoko Suzuki, Junko Showguchi-Miyata, Yoshiko Yanagisawa, Shinji Hadano and Joh-E Ikeda

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