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ScienceDirect
Biochemical and Biophysical Research Communications 354 (2007) 491–497
BBRC
www.elsevier.com/locate/ybbrc

ALS2CL, a novel ALS2-interactor, modulates ALS2-mediated endosome dynamics

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Received 29 December 2006
Available online 11 January 2007

Abstract

ALS2, the causative gene product for a number of recessive motor neuron diseases, is a guanine-nucleotide exchange factor for Rab5, and acts as a modulator for endosome dynamics. Recently, we have identified a novel ALS2 homolog, ALS2CL, which is highly homologous to the C-terminal half of ALS2. In this study, we investigate the molecular features of ALS2CL and its functional relationship with ALS2. A majority of ALS2CL is present as a homo-dimeric form, which can interact with the ALS2-oligomer, resulting in the formation of the large ALS2/ALS2CL heteromeric complex. In cultured cells, overexpressed ALS2CL is colocalized with ALS2 onto membranous compartments. Further, ALS2CL dominantly suppresses the endosome enlargement induced by a constitutively active form of ALS2, and results in an extensive perinuclear tubulo-membranous phenotype, which are dependent upon the ALS2CL–ALS2 interaction. Collectively, ALS2CL is a novel ALS2-interacting protein and is implicated in ALS2-mediated endosome dynamics.

Keywords: ALS2CL; ALS2; Rab5; Guanine-nucleotide exchange factor (GEF); Endosome; Motor neuron disease; Interacting protein

The *ALS2* gene was initially identified as a causative gene for a number of juvenile recessive motor neuron diseases (MNDs) [1,2]. It encodes a novel 184 kDa protein, termed ALS2 or alsin, comprising three predicted guanine-nucleotide exchange factor (GEF) domains [1,2]; i.e., the regulator of chromosome condensation-like domain (RLD) [3], the Dbl homology and pleckstrin homology (DH/PH) domains [4], and the vacuolar protein sorting 9 (VPS9) domain [5]. In addition, eight consecutive membrane occupation and recognition nexus (MORN) [6] motifs are noted in the region between DH/PH and VPS9 domains. ALS2 activates the small GTPase Rab5, an endocytic/vesicle trafficking regulator [7], via its specific GEF activity inherent to the C-terminal MORN/VPS9 domain, and induces enlarged endosome in the cells [8,9]. It has also been shown that ALS2 forms homo-oligomeric complex that is crucial for its Rab5GEF activity and ALS2-mediated endosome enlargement [9]. Moreover, ALS2 binds to and stimulates Rac1 via its DH/PH domain [10–12], thereby protecting cultured motor neuronal cells from toxicity of mutant Cu/Zn-superoxide dismutase 1 (SOD1) [11,13], and facilitating neurite outgrowth in neuronal cell cultures [12].

Abbreviations: ALS2CL, ALS2 carboxy-terminal like; MNDs, motor neuron diseases; GEF, guanine-nucleotide exchange factor; RLD, RCC1-like domain; DH, Dbl homology; PH, pleckstrin homology; VPS9, vacuolar protein sorting 9; MORN, membrane occupation and recognition nexus; Y2H, yeast two-hybrid; EGFP, enhanced green fluorescent protein; aa, amino acid residues; pAb, polyclonal antibody; mAb, monoclonal antibody.

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.229

Recently, we reported the novel *ALS2* homologous gene, ALS2 C-terminal like (*ALS2CL*) and its murine ortholog (*Als2cl*) [14]. The *ALS2CL* gene encodes a 108 kDa protein, ALS2CL, comprising several domains and motifs including MORN and VPS9. ALS2CL exhibits rather strong Rab5-binding properties but a relatively weak Rab5GEF activity [14]. Co-expression of ALS2CL and Rab5 results in a unique tubulation phenotype of endosome compartments with significant colocalization of ALS2CL and Rab5 in HeLa cells. Thus, ALS2CL and ALS2 play overlapping but distinctive roles on the Rab5-mediated membrane dynamics in the cells, tempting to speculate that ALS2CL modulates the ALS2-mediated molecular and cellular functions.

In the present study, we investigated the molecular features of ALS2CL and its functional relationship with ALS2. Our studies reveal that ALS2CL is a novel ALS2-binding protein and modulates ALS2-mediated membrane dynamics. Thus, ALS2CL might act as a modulator in the ALS2- and Rab5-mediated membrane trafficking *in vivo*.

Materials and methods

Antibodies. Anti-ALS2CL rabbit polyclonal antibody (pAb) (CLHPF560–953) was raised by immunizing Japanese White rabbit with the recombinant peptide of human ALS2CL spanning 560–953 amino acids (aa), followed by affinity-purification using an antigen coupled sepharose column. Other antibodies used in this study are listed in Supplementary Materials and Methods.

Plasmid constructs. All cDNA clones used in this study were obtained by subcloning the PCR or the reverse transcriptase-PCR-amplified fragments into the appropriate expression vectors as described [8,14,15]. The DNA sequence of the insert as well as its flanking regions in each plasmid construct was verified by sequencing. For the antibody generation, the cDNA fragment of human *ALS2CL*, encoding the region spanning 560–953aa, was subcloned into pRSET Bacterial Expression Vector (Invitrogen), generating pRSETHis-hALS2CL_560–953. For the co-immunoprecipitation, gel filtration, and subcellular localization studies, the cDNA fragments of human *ALS2CL*, mouse *Als2cl*, and mouse *Als2* were subcloned into the modified pCI-neo Mammalian Expression Vector (Promega), allowing the production of the N-terminally FLAG- or HA-tagged human ALS2CL (hALS2CL), mouse ALS2 (mALS2), and mouse ALS2CL (mALS2CL) proteins, and their deletion mutants. Previously generated pEGFP-hALS2_L [8], and pEGFP-hASLS2_695–1657 [9] were also utilized.

Cell culture, transfection, and Western blot analysis. Details of these methods are available in Supplementary Materials and Methods.

Co-immunoprecipitation, gel filtration, and immunocytochemistry. Co-immunoprecipitation assay, gel filtration and immunofluorescence studies were conducted as previously described [8,9,15]. Details of these methods are available in Supplementary Materials and Methods.

Preparation of the ALS2/ALS2CL complex. COS-7 cells that were transfected with pCIneoFLAG-mALS2_1012–1651 or pCIneoHA-mALS2CL were lysed in buffer A consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2% Tween 20, and Complete protease inhibitor mixture (Roche) and immunoprecipitated using EZview™ Red ANTI-FLAG M2 or ANTI-HA affinity gel (Sigma). These affinity gels conjugating ALS2 or ALS2CL were washed three times with the ice-cold buffer B consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1% Tween 20. The N-terminally FLAG-tagged mALS2_1012–1651 protein on the ANTI-FLAG affinity gels were eluted with buffer B containing 500 μg/ml 3× FLAG peptide (Sigma) for 1 h at 4 °C. The eluted sample was mixed with the ANTI-HA affinity gel conjugated with N-terminally HA-tagged mouse ALS2CL, and incubated for 2 h at 30 °C. The affinity gels were washed three times with appropriate buffer, and subjected to gel filtration.

Results

Interaction of ALS2CL with ALS2

We have previously demonstrated that ALS2 forms a homophilic oligomer through its distinct C-terminal regions [9]. 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 half of ALS2 [14], tempting to speculate that ALS2CL can interacts itself and also interacts with ALS2. To confirm these possibilities, we generated various truncated ALS2CL-expressing constructs and used them in the Y2H tests (Fig. S1). As we expected, ALS2CL could self-associate, and two distinct regions of ALS2CL, 329–582aa and 652–953aa, are indispensable for its self-interaction in yeast cells, consistent with the previous finding that ALS2 can self-interact in a similar manner [9]. We also demonstrated that ALS2CL could interact with ALS2 by the Y2H assays. Interestingly, ALS2CL interacted with ALS2 through the regions between the central region (329–651aa) of ALS2CL and the C-terminal VPS9-containing region (1351–1657aa) of ALS2, but not the regions between the ALS2CL C-terminus VPS9-containing region and the ALS2 MORN-containing region in yeast (Fig. S1).

To confirm this interaction, we transfected the expression constructs of human ALS2 (pCIneo-FLAG_hALS2_L) along with human ALS2CL (pCIneoHA_hALS2CL) into COS-7 cells and performed co-immunoprecipitation using the ANTI-FLAG affinity gel. HA-tagged hALS2CL was efficiently co-immunoprecipitated with FLAG-tagged hALS2_L (Fig. 1A). HA-tagged hALS2_L was also co-immunoprecipitated with FLAG-tagged hALS2CL (Fig. 1B, lane 2). We also obtained similar results using mouse ALS2CL and ALS2 (Fig. S2). Next, to confirm the responsible regions for the interaction, we generated the expression constructs encoding various FLAG-tagged deletion mutants of ALS2CL. HA-tagged hALS2_L was co-immunoprecipitated with FLAG-tagged hALS2CL_329–953 (Fig. 1B, lane 8), but not with FLAG-tagged hALS2CL_1–582 (Fig. 1B, lane 5) or FLAG-tagged hALS2CL_560–953 (Fig. 1B, lane 11). These results indicate that ALS2CL, through the region of 329–953aa, interacts with ALS2 in mammalian cells. In addition, endogenous ALS2, albeit rather lower level, was also detected in the FLAG-immunoprecipitated pellets prepared from COS-7 cells singly overexpressing FLAG_hALS2CL using anti-ALS2 pAb (HPF1–680) (data not shown), supporting the molecular interaction between ALS2CL and ALS2 in the cells.