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Are alsin and spartin novel interaction partners?

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ABSTRACT

Mutations in ALS2 gene/alsin are associated with recessive forms of motor neuron disorders including Juvenile Amyotrophic Lateral Sclerosis (JALS), Infantile-onset Ascending Hereditary Spastic Paraplegia (IAHSP) and Juvenile Primary Lateral Sclerosis (JPLS). In this study, we show that alsin and another MND-linked protein, spartin are related to each other both at mRNA and protein levels in Neuro2a cells. We observed significant alterations in spartin expression in alsin knock-down conditions. We further found that both proteins colocalize in N2a cells and spartin isoform-a precipitates with alsin in the same protein complex. In the light of these results we suggest that alsin and spartin may interact each other physically.

1. Introduction

Motor neuron disorders (MNDs) are a subgroup of neurodegenerative diseases, categorized according to the selective degeneration of motor neurons and leading to spasticity, muscle weakness, atrophy or paralysis [1]. In the last 20 years more than 30 genes have been associated with different MNDs [2]. *ALS2* is one of those genes which is a causative factor for a number of recessive MNDs, including Juvenile Amyotrophic Lateral Sclerosis (JALS), Infantile-onset Ascending Hereditary Spastic Paraplegia (IAHSP) and Juvenile Primary Lateral Sclerosis (JPLS) [3–6].

Although motor neuron disorders have late-onset characteristics, in the case of *ALS2* mutations, the symptoms get started at very early ages. This suggests that *ALS2*/alsin protein has an important role in motor neuron integrity [7]. However, the exact function of alsin is not very well known, thus alsin-linked motor neuron degeneration is still a mystery. In this study we aim to gain insights into alsin's function and alsin-linked motor neuron degeneration by investigating its relations/interactions with other MND-related proteins. Since alsin causes both upper and lower motor neuron degeneration, four candidate proteins were chosen depending on the type of MND they give rise to. While spartin and spastin were selected due to their upper MN specificity, HspB1 and HspB8 were investigated because of their lower MN implications [8]. This paper presents the results on spartin and the *SGP20* gene.

ALS2 gene with 33 introns and 34 exons, resides on chromosome 2q33. There are two splicing variants, a long and a short form, coding for 1657 and 396 amino acids, respectively [4]. Till today, 19 mutations were reported in *ALS2* gene, resulting in either premature termination of translation or substitution of an evolutionary conserved amino acid in the ALS2 protein/alsin, leading to loss of its function. Alsin is a ubiquitously expressed protein with the highest levels in central nervous system. Although the 3-D structure of the protein is not known, based upon sequence homology it has been proposed that alsin is composed of three putative guanine nucleotide exchange factor (GEF) domains: the regulator of chromosome condensation 1 (RCC1) domain, B-cell lymphoma (Dbl) homology and pleckstrin homology domain (DH/PH domain), and the vacuolar protein sorting 9 (VPS9) domain. In addition, there are 8 MORN (membrane occupation and recognition nexus) motifs between DH/PH and the VPS9 domains. In spite of having diverse GEF domains, alsin has specific GEF activity for only RAB5 GTPase, a key regulator of endocytosis during endosome fusion and trafficking, via its VPS9 domain [9]. In addition to the RAB5 GEF activity, with MORN motifs which are important tools for providing the physical interaction with micropinosomes and endocytic vesicles, alsin is implicated in endocytotic processes [10]. There are also few studies with alsin knock-out (KO) mice showing abnormalities in endocytosis. For instance, cortical and cerebellar granule neurons derived from alsin KO mice exhibit disturbances in endosomal transport of insulin-like growth factor 1 (IGF1) and brain-derived neurotrophic factor (BDNF) [11]. In addition, alsin is also implicated in the fusion of epidermal growth factor (EGF) endosomes [21]. Apart from its GEF activities, alsin has roles in neurite outgrowth, neuroprotection and NF-κB pathway via its DH/PH domain [12–14].

The *SPG20* gene coding for spartin is causative for Troyer Syndrome (TRS), an autosomal recessive and complicated form of Hereditary Spastic Paraplegia [15]. So far, two mutations were

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identified in the *SPG20* gene resulting in premature stop codons leading to spartin loss of function of [16]. Spartin has two isoforms and is composed of two conserved domains a PRS (plant-related senescence) domain and an MIT (microtubule-interacting and endosomal trafficking) domain. Via the PRS domain, which shares a strong homology with some plant-specific gene sequences, spartin binds to the outer membrane of the mitochondria and adjusts Ca^{2+} uptake [17]. On the other hand the MIT domain is a common region for several proteins with well-established functions in endosomal trafficking [18]. Although harboring an MIT domain, the exact function of spartin in endocytosis is not defined yet; however, many indirect implications in EGFR trafficking were reported [19,20].

The study aims to investigate the interactions of alsin and spartin at mRNA level by quantitative real time PCR and at protein level by immunolocalization and immunoprecipitation.

2. Materials and methods

2.1. Cell lines and transfection

Neuro2a (N2a) cells, purchased from ATCC (USA), were grown as described earlier [14]. An shRNA sequence designed against alsin's DH/PH domain and fused into a pSilencer™ 2.1-U6 hygro vector (Ambion Company, USA) was used to generate Alsin knock-down stable cell lines. Transfection of N2a cells with the vector was performed with FuGENE®HD Transfection Reagent (Roche, Germany) according to manufacturer's instructions. In the case of N2a control cell lines, the same vector with a control shRNA sequence was used.

2.2. Quantitative real time PCR (qRT-PCR)

After total RNA isolation by High Pure RNA Isolation Kit (Roche, Germany) expression levels of *Als2* and two isoforms of *Spg20* (*Spg20a* and *Spg20b*) genes were quantified by using the Light Cycler 2.0 (Roche, Germany) and the RNA Master SYBR Green 1 kit (Roche, Germany). The primer sets below were adopted from Harvard Primer Bank, USA.

Spg20a: Forward: 5′ AGCGGCGCTCAGAGATGGAGA 3′
Reverse: 5′ TCCTTCTGCCCTAACTCGTCCGT 3′
Spg20b: Forward: 5′ GCAGCAAGGATGCCCGCCATAAA 3′
Reverse: 5′ GTGGGCCACTTTCTCGCTCCATTCA 3′
Als2: Forward: 5′ TCCAGTTCTTGCTATGAGTCTCT 3′
Reverse: 5′ GGAATCCGTCATTTTCCCAGG 3′
β-Actin: Forward: 5′ GGCTGTATTCCCCTCCATCG 3′
Reverse: 5′ CCAGTTGGTAACAATGCCATGT 3′

All qRT-PCR experiments were repeated at least for three times independently and the data were normalized with β-actin expression levels. In order to analyze statistical significance, Student *t*-test was applied.

2.3. Immunofluorescence studies

Immunofluorescence studies were carried out as described earlier [14]. In order to obtain neurite-like extensions, the cells were grown in differentitation medium including, 1% Fetal Bovine Serum (Gibco, USA), 100 U penicillin–streptomycin (Gibco, USA), 1% GlutaMAX (Gibco, USA) and 1% non-essential amino acid (NEAA) (Gibco, USA) with retinoic acid (Sigma, USA) for 3 days. Then, the cells were incubated with 1:250 dilution of goat anti alsin (Novus, USA) and 1:250 rabbit anti spartin (sc-98961, Santa Cruz, USA) in PBS. Next day, secondary antibodies, 1:1000 dilution of chicken anti-rabbit IgG Red (sc-2862, Santa Cruz, USA) and 1:1000 dilution of donkey anti-goat IgG FITC (sc-2024, Santa Cruz, USA) were applied on cells. Following nuclear staining with DAPI (Santa Cruz, USA) and mounting of the cells on slides, immunofluorescence imaging was performed by using a Leica DM1 4000B fluorescence microscope with a monochromatic CDD camera.

2.4. Co-immunoprecipitation and Western Blotting

Protein samples were isolated by using RIPA lysis buffer (Santa Cruz, USA) from N2a cells that were grown in 75 cm^2 cell culture flasks until reaching 100% cell confluency. For immunoprecipitations, protein samples were first incubated with rabbit anti-Als2 (SAB4200137, Sigma, USA) and rabbit Octa antibody (sc-807, Santa Cruz, USA) (as a negative control) for 1 h on ice. Then, they were mixed with protein G PLUS-Agarose and incubated at 4 °C on a rotating shaker. In the final step, the mixtures were washed with lysis buffer for 3 times. In Western Blotting, rabbit anti-spartin antibody (sc-98961, Santa Cruz, USA) and goat anti-rabbit IgG (whole molecule) peroxidase (Sigma Aldrich, USA) were used to detect both isoforms of spartin on detection films.

3. Results

3.1. Als2 mRNA levels in Als2 knock-down stable cell lines

Since *Als2* mutations result in loss of function of the protein, in order to mimic the same conditions in N2a cells, we aimed to generate an Als2 knock-down stable cell line. A 73.40% decrease was measured in the relative amount of alsin mRNA in alsin knock-down cells as compared to control cells (Fig. 1). The *p*-value <0.0001 showed that the result was statistically significant.

3.2. Two different isoforms of spartin give rise to opposite results in Als2 knock-down stable cell lines

Since the exact functions of spartin's isoforms are not known, two different primer sets were designed, considering possible functional differences. In *Als2* KD background, the *Spg20* isoform-a mRNA level was found to be increased by 48%. As opposed to this result, the *Spg20* isoform-b exhibited a 32% decrease (Fig. 2).

3.3. Alsin colocalizes with spartin in N2a cells

Since the results in literature are very controversial regarding alsin's and spartin's cellular distributions, immunofluorescence

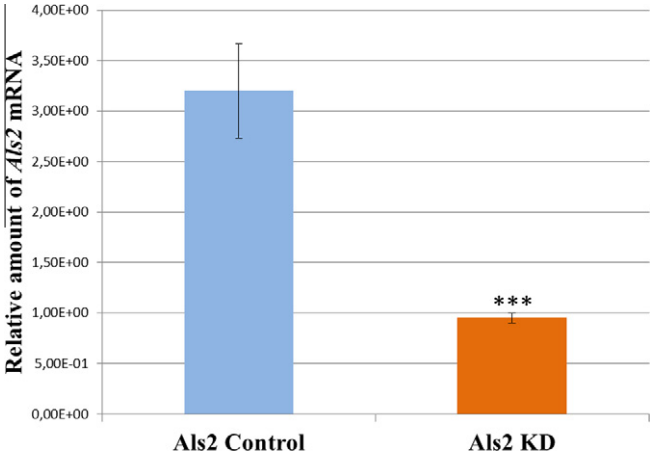

Fig. 1. 73.40% decrease in the knock-down level of *Als2* mRNA (***$p < 0.0001$).

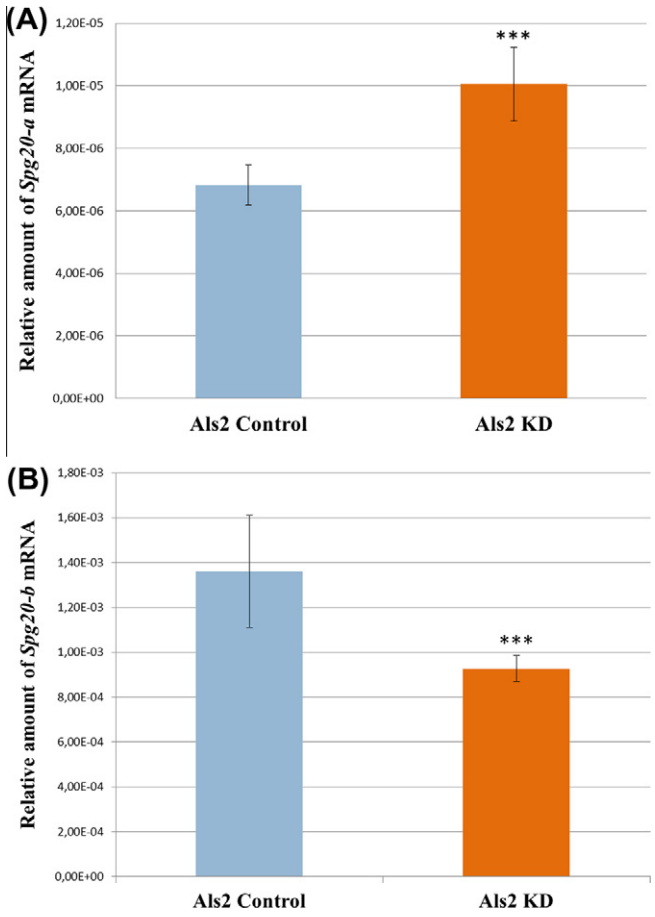

Fig. 2. (A) Alterations at *Spg20* isoform-a mRNA levels: 48% increase (***$p < 0.0001$). (B) Alterations at *Spg20* isoform-b mRNA levels: 32% decrease (***$p < 0.0001$).

studies were carried out to clarify the localizations of the endogenous forms of these proteins in differentiated N2a cells. Both proteins showed a general diffused pattern of staining in the cell body and neurite-like extensions with strong signals deriving from the peri-nuclear region of the cells, showing that alsin and spartin are colocalizing in N2a cells (Fig. 3).

3.4. Immunoprecipitation studies show that alsin and spartin are precipitating at the same protein complex

The observation of alsin's and spartin's co-localization in N2a cells implies that these two proteins may be present in the same protein complex. In order to confirm their co-existence, immunoprecipitation analyses were performed by using protein lysates isolated from wild type N2a cells. From three experimental groups, the first one is the positive control group containing the total protein lysate. Two distinct bands at 75 kDa and 85 kDa, representing both spartin isoforms, were observed after Western Blot analysis (Fig. 4A). The second experimental group (negative control) was the protein lysate which was precipitated with an unrelated antibody and G protein beads. After blotting with anti-spartin antibody, no bands representing spartin were observed (Fig. 4B). The third group was the protein lysate precipitated with an alsin antibody and the G protein beads. When the spartin antibody was applied on the Western Blot membrane, a distinct band for the isoform-a was detected (Fig. 4C). These results imply that alsin and spartin isoform-a are residing in the same protein complex.

4. Discussion

MNDs are a heterogeneous group of neurodegenerative disorders, sharing common mutated genes/proteins with roles in pathologic mechanisms. In order to understand the pathways involved in disease, relations between proteins leading to MNDs when mutated, and their exact functions have to be clarified. In this study, we aim to gain new insights into alsin's function by investigating its relations/connections with spartin, another MND-linked protein. When the changes in transcription levels of spartin isoforms were measured in alsin knock-down conditions, contradictory results (48% increase for isoform-a and 32% decrease in isoform-b) were obtained. This difference, possibly due to functional differences of both spartin isoforms, has not been reported in the literature so far. At protein level, immunofluorescence and immunoprecipitation experiments were carried out, in order to investigate the physical connections of alsin and spartin. In differentiated N2a cells, it was found that alsin and spartin colocalize in the cytoplasm; further, the isoform-a of spartin precipitates with alsin in the same protein complex when subjected to IP. These results suggest that there may be a possible physical interaction between alsin and spartin.

Functional studies on spartin and alsin have revealed that both proteins are implicated in EGFR (epidermal growth factor receptor) trafficking. For instance, EPS15 (epidermal growth factor receptor substrate 15) is defined as an interaction partner of spartin [19]. Furthermore, it has been shown that the internalization rate of the EGFR is altered with spartin expression levels in HeLa cells [20]. On the other hand, in embryonic fibroblasts derived from alsin knock-out mice models, it has been observed that there was a delay in fusion of EGF endosomes, which are also smaller in size, compared to wild type cells [21]. In addition, two isoforms of

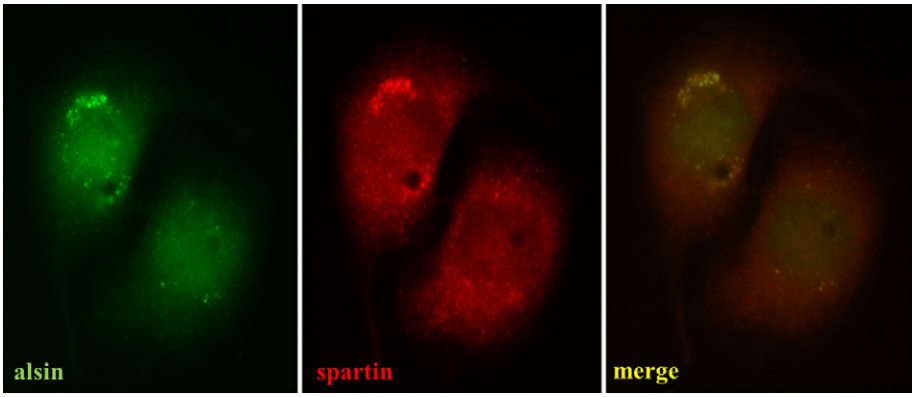

Fig. 3. Endogenous alsin and spartin were found to be co-localized in the peri-nuclear region of differentiated wild type N2a cells.

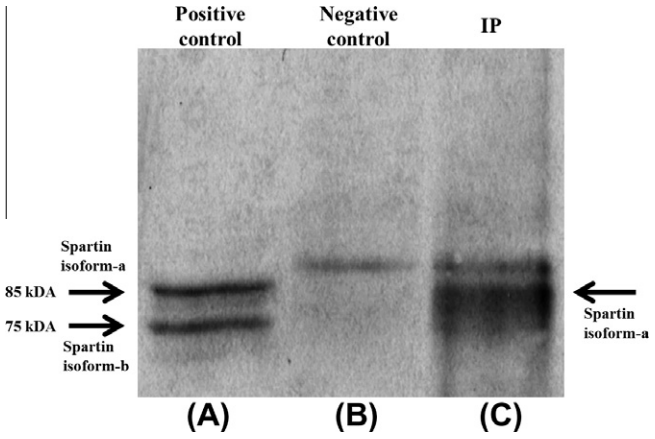

Fig. 4. Western Blot analysis of total protein lysate and immunoprecipitates of wild type N2a cells (A) Two spartin isoforms (A and B) at 85 kDa and 75 kDa, respectively. (B) Negative control (C) Spartin isoform-a at 85 kDa.

RAB5 GTPase which are getting activated by alsin's GEF activity are essential tools for EGF endocytosis, supporting alsin's role in EGFR metabolism [22]. EGFR trafficking is an important pathway which can be lethal if it is not controlled in a proper way. The activation of EGFR triggers several signal transduction pathways that control many cellular mechanisms including cell proliferation, differentiation and apoptosis. In order to control all of these crucial cellular activities, EGFR also starts a negative feedback mechanism in order to remove EGF-EGFR complexes from the plasma membrane by endocytosis [23].

Considering the above direct and indirect implications of alsin and spartin with EGFR trafficking, these proteins may have similar roles in assisting the cell to control some of the important activites that are governed by EGF-EGFR trafficking. To the best of our knowledge this is the first study, which reports a connection of alsin with spartin. The confirmation of these results and a thorough understanding of the functions of these two MND-related proteins in EGFR trafficking would give us more information about endocytosis-linked motor neuron degeneration.

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