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A mental retardation gene, motopsin/prss12, modulates cell morphology by interaction with seizure-related gene 6

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ABSTRACT

A serine protease, motopsin (prss12), plays a significant role in cognitive function and the development of the brain, since the loss of motopsin function causes severe mental retardation in humans and enhances social behavior in mice. Motopsin is activity-dependently secreted from neuronal cells, is captured around the synaptic cleft, and cleaves a proteoglycan, agrin. The multi-domain structure of motopsin, consisting of a signal peptide, a proline-rich domain, a kringle domain, three scavenger receptor cysteine-rich domains, and a protease domain at the C-terminal, suggests the interaction with other molecules through these domains. To identify a protein interacting with motopsin, we performed yeast two-hybrid screening and found that seizure-related gene 6 (sez-6), a transmembrane protein on the plasma membrane of neuronal cells, bound to the proline-rich/kringle domain of motopsin. Pull-down and immunoprecipitation analyses indicated the interaction between these proteins. Immunocytochemical and immunohistochemical analyses suggested the co-localization of motopsin and sez-6 at neuronal cells in the developmental mouse brain and at motor neurons in the anterior horn of human spinal cords. Transient expression of motopsin in neuro2a cells increased the number and length of neurites as well as the level of neurite branching. Interestingly, co-expression of sez-6 with motopsin restored the effect of motopsin at the basal level, while sez-6 expression alone showed no effects on cell morphology. Our results suggest that the interaction of motopsin and sez-6 modulates the neuronal cell morphology.

1. Introduction

Motopsin (prss12), which is also known as neurotrypsin, is a serine protease secreted by neuronal cells in various brain regions, such as the hippocampus, cerebral cortex, and cranial nerve nuclei [1,2]. This protease is important for neuronal functions because the loss of motopsin function in humans causes severe nonsyndromic mental retardation [3]. Mice lacking motopsin show a decreased number of dendritic spines and reduced phosphorylation of cAMP-responsive element-binding protein (CREB) in the hippocampal neurons, which is induced by social interaction [4]. Furthermore, although axonal injury of facial nerves transiently reduces the expression levels of motopsin mRNA, the recovery of neuronal function accompanies the restoration of the expression of motopsin mRNA, which suggests the involvement of motopsin in neuronal functions [5]. Time-lapse imaging has revealed that depolarization causes the exocytosis of motopsin from presynaptic vesicles [6]. In extracellular space, motopsin cleaves a proteoglycan, agrin [7,8]. As agrin is important for the formation and maintenance of excitatory synapses [9,10], these reports suggest that motopsin may modulate neuronal plasticity via the modification of agrin function.

In a previous report, we indicated that motopsin is detected in the dendrites or in the somatic body of neurons under normal expression levels [2]. The expression of motopsin mRNA in the cerebral cortex culminates around the second week after birth [11,12], and then gradually decreases through the lifetime of an individual. This temporal expression pattern raises the additional possibility that motopsin is involved in the development of the cerebral cortex. The mosaic structure of motopsin, which consists of a signal sequence at the N-terminus followed by a proline-rich domain adjacent to a kringle (proline-rich/kringle) domain, three scavenger receptor cysteine-rich (SRCR) domains, and a protease domain at the C-terminus, suggests that motopsin interacts with other molecules through these domains. Motopsin secreted at

Abbreviations: Ade, adenine; ANOVA, analysis of variance; CUB, C1r/C1s, urinary EGF, and bone morphogenetic protein; DIV, days *in vitro*; EGFP-F, farnesylated enhanced green fluorescent protein; IPTG, isopropyl β-D-1-thiogalactopyranoside; P, postnatal day; PBS-T, phosphate-buffered saline containing 0.3% Triton X-100; SD, minimal synthetic dropout medium; SCR, short consensus repeat; SRCR, scavenger receptor cysteine-rich; T-rich, threonine-rich; X-α-gal, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside.

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synaptic clefts appears to be captured around the synapses [6]. To identify motopsin-interacting proteins, we screened a mouse embryonic cDNA library using a yeast two-hybrid system. We found that the proline-rich/kringle domains of motopsin bound to a transmembrane protein, seizure-related gene 6 (sez-6), which consists of a threonine-rich (T-rich) domain, five short consensus repeat (SCR) domains, two C1r/C1s, urinary EGF, and bone morphogenetic protein (CUB)-like domains, and a transmembrane domain followed by a short cytoplasmic domain at the C-terminal [13]. Biochemical and immunohistochemical analyses indicated that sez-6 is a candidate protein interacting with motopsin in the developing mouse brain.

2. Materials and methods

2.1. Vector construction

The plasmid vectors used in this study were constructed as described in the Supplemental information and listed below:

pGBK/motopsinS711A: a bait vector expressing motopsinS711A fused with DNA-BD domain.
pGAD/sez-6: a prey vector expressing sez-6 fused with DNA-AD domain.
pET43/proline-rich/kringle: an expression vector to express the NUS-tagged proline-rich/kringle domain of motopsin in *Escherichia coli* (*E. coli*).
pTricHis/SCR/CUB: an expression vector to express the $(His)_6$-tagged SCR/CUB domains of sez-6 in *E. coli*.
pEF1/motopsin: an expression vector to express motopsin without any tag in mammalian cells.
D-HA/motopsin: an expression vector to express HA-tagged motopsin in mammalian cells.
pcDNA/EGFP-F: an expression vector to express farnesylated enhanced green fluorescent protein (EGFP-F) as a control vector.
pcDNA/Sez-6/EGFP-F: an expression vector to express myc-tagged sez-6 and EGFP-F simultaneously in mammalian cells.
pcDNA/motopsin/EGFP-F: an expression vector to express motopsin and EGFP-F simultaneously in mammalian cells.
pcDNA/Sez-6/motopsin/GFP-F: an expression vector to express motopsin, myc-tagged sez-6, and EGFP-F simultaneously in mammalian cells.

2.2. Two-hybrid screening

Yeast two-hybrid screening was performed using MATCHMAKER Gal4 Two-Hybrid System 3 (Clontech Lab., Inc., Mountainview, CA) according to the instruction manual. Since protease activity may be toxic to the host cell, we used a mutant lacking protease activity by substituting Ser^{711}, which is essential for the proteolytic activity, with Ala (S711A) as a bait protein. Yeast AH109 was transformed by the bait vector pGBK/motopsin S711A, and mated with Y187 strain pretransformed using the mouse E17 cDNA library and plated on minimal synthetic dropout medium lacking adenine, leucine, histidine, and tryptophan (SD/-Ade/-Leu/-His/-Trp). Growing colonies were replated onto SD/-Ade/-Leu/-His/-Trp containing 20 μg/mL 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-gal). Complementary DNA from blue colonies was sequenced using T7 primer with an automatic sequencer (ABI 310 sequencer, Applied Biosystems, Foster city, CA).

To clarify the binding domain of motopsin and sez-6, yeast AH109 was transformed by a set of bait and prey vectors, which expressed various kinds of motopsin and sez-6 mutants, respectively, and were assayed on an SD/-Ade/-Leu/-His/-Trp/X-α-gal plate.

2.3. Pull-down assay

To prepare a bait protein, an expression vector, pET43/proline-rich/kringle or pET43.1a(+) as a control, was introduced into *E. coli*, Rosetta gami (Merk KGaA, Darmstadt, Germany). The recombinant protein was induced by incubation with 1 mM β-D-1-thiogalactopyranoside in the medium for 3 h. The inclusion body was extracted by sonication in ice-cold lysis buffer (1% Triton X-100, 50 mM Tris–Cl (pH 7.5), 100 mM NaCl) containing protease inhibitors and recovered by centrifugation at $10{,}000 \times g$ at 4 °C for 15 min [15]. The cell lysate was applied to 50 μL of S-protein agarose (Merck KGaA) to bind the proline-rich/kringle domains or NUS-tag protein to the agarose beads.

To prepare SCR/CUB domains of sez-6 fused with an HA-tag, pTricHis/SCR/CUB was introduced into *E. coli*, DH5α. The induction of the recombinant protein and cell lysis were performed as above. Inclusion bodies were recovered by centrifugation and dissolved in 20 mM Tris–Cl (pH 7.5), 150 mM NaCl, and 8 M urea, and then applied to the S-protein agarose possessing NUS-tagged proline-rich/kringle domains or only NUS tag. The agarose was washed with 20 bed volumes of 20 mM Tris–Cl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100. The bound proteins were eluted in 60 μL of SDS-sample buffer (50 mM Tris–Cl, pH 6.8, 2% SDS, 10% glycerol, 50 mM 2-mercaptoethanol, 0.1% bromophenol blue) and detected by Western blotting using anti-HA tag antibody (Life Technologies Corp., Carlsbad, CA).

2.4. Cell cultures

The preparation and culture of primary hippocampal neurons were performed as described elsewhere [2,14]. The cells were cultured in serum-free Neurobasal medium containing B27 supplement (Life Technologies Co.) at a density of 3×10^4 cells/cm^2 on a glass slide with four glass-surface wells in water-repellant print (Matsunami Glass Ind., Ltd., Osaka, Japan), which was coated with poly-D-lysine and laminin. At 1 day *in vitro* (DIV) or 3 DIV, the cells were transfected with 0.3 μg of an expression plasmid for mammalian cells by magnetofection using a NeuroMag kit (OZ Biosciences, Marseille cedex, France) according to the manufacturer's manual.

The mouse neuroblastoma cell line Neuro-2a (ATCC No. CCL-131) and COS1 (ATCC No. CRL-1650) cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies Co.) containing 7% fetal bovine serum at 37 °C in 5% CO_2.

2.5. Immunoprecipitation

COS1 cells were plated on a six-well plate and transfected with 1 μg of D-HA/motopsin and 2 μg of pcDNA3.1/mouse Sez-6-Myc using Fugene 6 (Roche Applied Sciences, Mannheim, Germany) according to the instruction manual. After 2 days, the cells were lysed in 0.5 mL of ice-cold RIPA buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate). The lysate was centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. The supernatant was incubated with 2 μg of rabbit IgG for 2 h at 4 °C, and then incubated with 10 μL of Protein A Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 3 h. The lysate was then centrifuged at $5000 \times g$ for 5 min at 4 °C. The supernatant was supplemented with 2 μg of rabbit anti-Myc tag IgG (Sigma–Aldrich Co., St. Louis, MO) and incubated at 4 °C overnight. Protein A Sepharose was added to the reaction mixture and incubated for 3 h. Protein A Sepharose was then recovered after centrifugation at $5000 \times g$ for 5 min at 4 °C, and washed with 1 mL of washing buffer (20 mM Tris–Cl, pH 8.0, 136 mM NaCl, 0.05% Tween-20) three times. The bound proteins were eluted in 40 μL of SDS-sample buffer.