Biochemical and Biophysical Research Communications 462 (2015) 346–351

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

N-glycosylation modulates filopodia-like protrusions induced by sez-6 through regulating the distribution of this protein on the cell surface

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ARTICLE INFO

Article history:
Received 23 April 2015
Available online 7 May 2015

Keywords:
Dendrite
Extracellular matrix
Motopsin
Neuro2a
Neurogenesis

ABSTRACT

Seizure-related gene 6 (sez-6) is a trans-membrane protein expressed by neuronal cells that modulates dendritic branching. It has three clusters of eleven possible *N*-glycosylation sites in the extracellular domain region: sugar chain (SC)1-3, SC4-7, and SC8-11. Recent reports suggest that *N*-glycosylation modulates the membrane trafficking and function of trans-membrane proteins. Here, we studied the role of *N*-glycosylation in sez-6 function. We transfected mutants lacking one, two, or all *N*-glycosylation clusters into neuro2a cells. A mutant lacking all *N*-glycosylation was transported to the cell membrane. Mutants lacking one cluster (sez-6 ΔSC1-3, ΔSC4-7, ΔSC8-11) were evenly distributed on the cell membrane and secreted into the conditioned medium, as in wild-type sez-6; in contrast, the unglycosylated mutant, sez-6 ΔSC1-11, and mutants having only one cluster (sez-6 SC1-3, SC8-11) were localized in some portions on the cell membrane. Despite sez-6 SC4-7 having only one cluster, it was transported like the wild type. Among mutants behaving like the wild type, sez-6 ΔSC1-3 and ΔSC4-7 reduced neurite formation. Interestingly, mutants lacking SC4-7 (sez-6 ΔSC4-7) did not affect the formation of filopodia-like protrusions. In contrast, other mutants as well as the wild type induced it, suggesting that SC4-7 is crucial for filopodia-like protrusions. Our results indicate that *N*-glycosylation regulates cell morphology through modulating the cell surface distribution of sez-6 protein.

1. Introduction

Seizure-related gene 6 (sez-6) is a trans-membrane protein that is expressed by neuronal cells in an activity-dependent manner [1]. The expression of sez-6 starts around embryonic day 11.5 and continues in the cerebral and piriform cortices, amygdala, hippocampus, and striatum [2,3]. This expression pattern suggests the possibility that sez-6 modulates the construction of neuronal circuits and plasticity. Actually, mice lacking sez-6 show abnormal behaviors such as impaired spatial memory and attenuation of ambulation. The loss of sez-6 increases dendritic branching with a decrease in mean length and reduces postsynaptic excitability [4]. However, it is still unknown how sez-6 modulates dendritic arborization and synaptic excitability. Sez-6 is composed of a threonine-rich domain, two complement C1r/C1s, Uegf, Bmp1 (CUB) domains, five short consensus repeat (SCR) domains, a transmembrane domain, and a short cytoplasmic domain [1]. Recently, we have reported that sez-6 interacts with motopsin, an extracellular serine protease [5], the loss of the function of which causes severe mental retardation in humans and abnormal social behavior in mice [6,7]. The SCR/CUB domains of sez-6 bind to the proline-rich/kringle domain located at the N-terminal of motopsin. The interaction of these proteins prevents neurite protrusion enhanced by motopsin overexpression.

Sez-6 contains eleven possible *N*-glycosylation sites [1]. *N*-glycosylation is believed to contribute to the folding and stability of target proteins and to modulate the interaction of proteins with one another [8]. Recent reports indicate that impaired *N*-glycosylation causes various disorders in the central nervous system. Abnormal *N*-glycosylation of neurotransmitter-related proteins, such as

Abbreviations: CUB, complement C1r/C1s Uegf Bmp1; DMEM-H, Dulbecco's Modified Eagle Medium (high glucose); Endo, endoglycosidase; FBS, fetal bovine serum; GFP, green fluorescent protein; GFP-f, farnesylated green fluorescent protein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PNGase, peptide N glycosidase; RIPA, radio-immunoprecipitation assay; PRG, plasticity-related gene; RT, room temperature; SC, sugar chain; SCR, short consensus repeat; Tris, tris-(hydroxymethyl)-aminomethane; TBS-Tween, tris-buffered saline containing 0.05% Tween20.

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http://dx.doi.org/10.1016/j.bbrc.2015.04.139
0006-291X/

glutamate receptor 6 kainate receptor subunit, γ-aminobutyric type A receptor, and excitatory amino acid transporters 1 and 2, has been reported in patients with schizophrenia [9–11]. Altered glycosylation of apolipoprotein E was also shown to be associated with the accumulation of amyloid β42 in an animal model of Niemann-Pick type C disease [12]. These reports suggest the possibility that *N*-glycosylation contributes to sez-6 function.

In this study, we investigated the role of *N*-glycosylation in the distribution and function of sez-6 protein. We found that *N*-glycosylation is necessary for the cell surface distribution of sez-6 and the induction of filopodia-like protrusions.

2. Materials and methods

2.1. Plasmid

Eleven possible *N*-glycosylation sites exist in the amino acid sequence of mouse sez-6 and they form three clusters: sugar chain (SC)1-3 around the threonine-rich domain, SC4-7 in the 1st SCR/CUB domain, and SC8-11 around the 2nd SCR/CUB domain (Fig. 2A). Here, we term sez-6 lacking all *N*-glycosylation sites sez-6 ΔSC1-11. Sez-6 with an *N*-glycosylation cluster and without one are sez-6 SC (the number of *N*-glycosylation sites) and sez-6 ΔSC (the number of deleted *N*-glycosylation sites), for example, sez-6 SC1-3 or sez-6 ΔSC1-3. An 1800-bp fragment of sez-6 cDNA (nucleotides 491-2350; GenBank Accession No.BC053011) was chemically synthesized with the introduction of mutations (Genscript USA Inc., Piscataway, NJ) so that all possible glycosylated Asn were converted to Gln: N244Q, N286Q, N310Q, N396Q, N419Q, N433Q, N437Q, N538Q, N580Q, N704Q, and N719Q (Supplemental Fig. 1). To construct various missense mutants of sez-6 lacking a cluster of glycosylation sites, parts of mutant cDNA were amplified and inserted into pXY/mouse sez-6. Then, open reading frames were subcloned into an expression vector, pcDNA3.1 Myc-His. The details of this are described in the supplementary material.

A plasmid vector, pAcGFP1-F, for the expression of farnesylated green fluorescent protein (GFP-f) was purchased from Takara Bio Inc. (Ohtsu, Japan).

2.2. Transfection

Neuro2a cells (CCL-131) were cultured in Dulbecco's Modified Eagle Medium (high glucose) (DMEM-H; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 7% fetal bovine serum (FBS) at 37 °C in air-5% CO_2. Cells (7.0×10^3 cells/hole) were plated on glass slides with 4 holes printed with a highly water-repellant mark (Matsunami Glass Ind. Ltd., Osaka, Japan) for immunocytochemistry. For western blot analyses, cells (1.8×10^5 cells/well) were plated on a 12-well tissue culture plate (NIPPON Genetics Co. Ltd., Tokyo, Japan). The next day, the medium was exchanged for fresh DMEM-H supplemented with 2% FBS. Plasmid DNA (0.35 μg/hole in a 4-hole glass slide) was mixed with Metafectene Pro reagent (μg plasmid DNA:μl reagent = 1:4; Biontex Laboratories GmbH, Munich, Germany) in 30 μl of serum-free medium and incubated for 20 min at room temperature (RT). For cells on 12-well plates, plasmid DNA (0.5 μg/well) was mixed with the reagent in 50 μl of serum-free medium at a ratio of 1:5. The mixture was applied to cells with 100 μl of medium containing 2% FBS. Cells were cultured for a further 48 h at 37 °C.

To investigate the intracellular localization of mutant proteins, an expression vector for a mutant was co-transfected with an equal amount of pAcGFP1-F. For morphological analysis, plasmid for the expression of a mutant protein or pAcGFP1-F was transfected.

2.3. Western blots

Two days after transfection, cells were washed with ice-cold phosphate-buffered saline (PBS) and rinsed in ice-cold sonication buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.9, 150 mM NaCl, 1 mM $CaCl_2$, 5 mM ethylenediaminetetraacetic acid) containing protease and phosphatase inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan) on ice for 40 min. Cells were collected in a tube and sonicated for 20 s, and then centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was further centrifuged at 20,000 × *g* for 1 h at 4 °C. The resulting supernatant was collected as the soluble fraction. Pellets were suspended in ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktail on ice for 40 min and sonicated as described above. The extract was centrifuged at 20,000 × *g* for 1 h at 4 °C and the supernatant was recovered as the membrane fraction.

Proteins were separated in 8.5% or 10% SDS polyacrylamide gel under reducing conditions and transferred to Immobilon-P membrane (EMD Millipore, Billerica, MA). This membrane was incubated in blocking buffer (5% skim milk in tris-(hydroxymethyl)-aminomethane (Tris)-buffered saline containing 0.05% Tween20 (TBS-Tween) for 10 min at RT, and then reacted with anti-sez-6 antibody (1:1000 dilution) in TBS-Tween overnight at RT. Anti-sez-6 antibody was prepared as previously described [3]. The following day, the membrane was washed with TBS-Tween four times and incubated with anti-rabbit IgG (1:5000 dilution) conjugated with horseradish peroxidase (HRP) (Cell Signal Technology, Inc., Dnvers, MA) in TBS-Tween for 2 h at RT. After washing as described above, immunoreactivities were detected using an Image Quant LAS-4000 (GE Healthcare, Little Chalfont, U.K.) and Luminata Crescendo Western HRP Substrate (EMD Millipore).

2.4. Deglycosylation of sez-6 by enzyme treatment

Neuro2a cells transfected with pcDNA3.1/mouse sez-6 or pcDNA3.1/mouse sez-6 ΔSC1-11 were lysed in ice-cold RIPA buffer for 10 min on ice. Cell lysate was recovered and centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was added to 1/10 volume of 10 × Glycoprotein denaturing buffer (New England Biolabs Inc., Ipswich, MA) and heated at 100 °C for 10 min. An aliquot of denatured cell lysate was reacted with 500 units of endoglycosidase (Endo) H in G5 reaction buffer (New England Biolabs Inc.) or 500 units of peptide N glycosidase (PNGase) F in G7 reaction buffer (New England Biolabs Inc.) at 37 °C for 1 h. The mixture was analyzed by western blot analysis as described above.

2.5. Immunocytochemistry

Neuro2a cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4 °C. We did not permeabilize cells to analyze the intracellular distribution of sez-6 protein, since anti-sez-6 antibody recognizes the extracellular domain of this protein. After washing with PBS three times, cells were incubated with rabbit anti-sez-6 antibody (1:1000 dilution) in PBS containing 1% normal goat serum overnight at 4 °C. When cell morphology was analyzed, cells were faintly permeabilized with 0.006% Triton X-100 and 0.004% saponin in PBS. In this case, GFP-f was detected using rat anti-GPF antibody (1:1500 dilution; Nacalai Tesque, Inc.). After washing three times, immunoreactions were visualized using anti-rabbit IgG and/or anti-rat IgG labeled with Alexa Fluor 488 or 594 (1:1000 dilution; Thermo Fisher Scientific Inc., Waltham, MA). Specimens were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA). Immunocytochemical images were obtained with an all-in-