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

Chiharu Hidaka, Shinichi Mitsui*

Department of Rehabilitation Sciences, Gunma University Graduate School of Health Sciences, 3-39-22 Showa-machi, Maebashi, Gunma 371-8514, Japan

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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.

* Corresponding author. Fax: +81 27 220 8950.
E-mail address: smitsui@gunma-u.ac.jp (S. Mitsui).

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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-

one fluorescence microscope, BZ-8100 (Keyence Co., Ltd., Osaka, Japan).

2.6. Image analysis and statistics

The numbers of processes of which the length from the edge of the cell body was over 1 μm were counted using Image J software (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/). Processes were defined as neurites and filopodia-like structures according to their width: neurite, >1.5 μm; filopodia-like protrusions, ≤1.5 μm. Data were analyzed using the statistical package SPSS version 21 (IBM Co., Ltd., New York, NY). Statistical comparisons were performed by ANOVA followed by Tukey's *post hoc* test for pairwise comparisons. *P* values of less than 0.05 were considered significant. Data are presented as mean ± standard error.

3. Results

3.1. Confirmation of N-glycosylation on sez-6 and mutants

As we previously reported [3], western blot analysis detected two major bands at 190 kDa and 160 kDa in the membrane fraction of cells expressing wild-type sez-6, while only a 190-kDa band was detected in the conditioned medium (Fig. 1A). Treatment of the membrane fraction with PNGase F reduced the molecular mass of the two bands to 110 kDa, whereas only the 160-kDa band was sensitive to Endo H treatment, suggesting that the 190-kDa and 160-kDa bands represented mature and immature *N*-glycosylation of sez-6, respectively (Fig. 1B). In contrast, sez-6 ΔSC1-11 was detected as a single band of 110 kDa and treatment with neither PNGase F nor Endo H reduced its molecular mass, indicating that sez-6 ΔSC1-11 was not glycosylated.

Western blot analysis confirmed that the mutants lacked *N*-glycosylation as predicted. In the membrane fraction, sez-6 lacking a cluster of *N*-glycosylation (sez-6 ΔSC1-3, sez-6 ΔSC4-7, sez-6 ΔSC8-11) showed higher mobility than wild-type sez-6 (Fig. 2B). Sez-6 SC4-7 showed delayed mobility compared with mutants having another *N*-glycosylation cluster (sez-6 SC1-3 and sez-6 SC8-11). This suggests that the region of SC4-7 contained the largest amount of *N*-glycan among the *N*-glycosylation clusters. All mutants lacking only one cluster of *N*-glycosylation (sez-6 ΔSC1-3, sez-6 ΔSC4-7, sez-6 ΔSC8-11) were detected in the conditioned medium, like wild-type sez-6 (Fig. 2C). In contrast, among mutants lacking two clusters of *N*-glycosylation, only sez-6 SC4-7 was detected in the conditioned medium. A mutant lacking all *N*-glycosylation sites (sez-6 ΔSC1-11) was also undetectable in conditioned medium.

3.2. Intracellular distribution of sez-6 mutants

To clarify the effect of *N*-glycosylation on the intracellular distribution of sez-6, neuro2a cells expressing GFP-f and each sez-6 mutant were analyzed (Fig. 3). Wild-type sez-6 was well distributed on the cell surface and co-localized with GFP-f, even in fine protrusions. In contrast, sez-6 ΔSC1-11 lacking all *N*-glycosylation sites was localized in some places, but not detected at protrusions. The distribution of other mutants was similar to that of either wild-type sez-6 or sez-6 ΔSC1-11. Mutants lacking one cluster of *N*-glycosylation: sez-6 ΔSC1-3, sez-6 ΔSC4-7, and sez-6 ΔSC8-11, showed distributions similar to that of wild-type sez-6. Among mutants having only one cluster of *N*-glycosylation, the distribution of sez-6 SC4-7 was similar to that of wild-type sez-6, whereas sez-6 SC1-3 and sez-6 SC8-11 were localized on the cell surface like sez-6 ΔSC1-11. Hence, the intracellular distribution of sez-6 mutants appears to be related to the secretion of these proteins. Mutants secreted into the conditioned medium were well distributed all over the cell surface, whereas mutants undetected in the conditioned medium were clustered on the cell membrane. It should be noted that all mutants were transported to the cell surface, since sez-6 was detected under non-permeabilized conditions.

3.3. Effects of N-glycosylation of sez-6 on filopodia-like protrusions on neuro2a cells

To clarify the effect of *N*-glycosylation on cell morphology, we analyzed five mutants showing a distribution similar to that of wild-type sez-6 (Fig. 4). We analyzed cell morphology by measuring the immunoreactivity of sez-6 mutants, since they were evenly distributed on the cell surface including in filopodia-like fine protrusions. Furthermore, transfection of only a plasmid alleviated the stress to express exogenous genes in comparison with co-expression with GFP-f.

Overexpression of wild-type sez-6 induced filopodia-like protrusions, but did not affect the number of neurites. In terms of neurite number, ANOVA (the main effect of transfectant, $F(5, 299) = 4.879$, $P < 0.001$) and *post hoc* analysis indicated that the

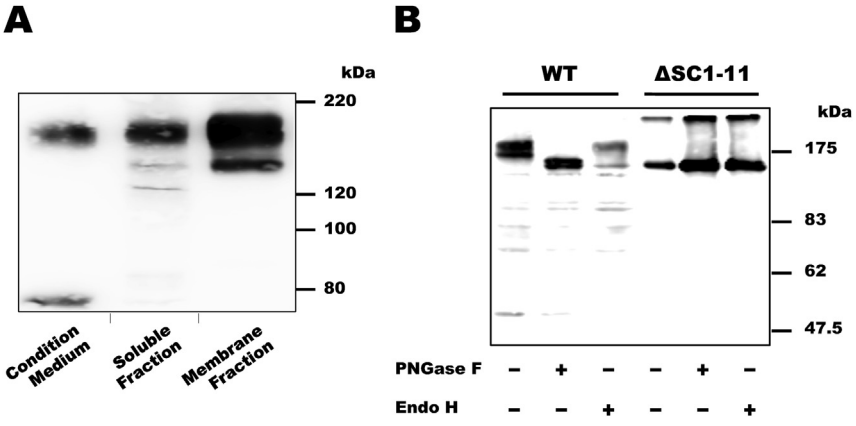

Fig. 1. Confirmation of *N*-glycosylation sites on sez-6. (A) Neuro2a cells transiently expressed wild-type sez-6. The cell lysates were collected 48 h after transfection and analyzed by western blotting using anti-sez-6 antibody. Only a 190-kDa band was detected in the conditioned medium, whereas both 190-kDa and 160-kDa bands were detected in the soluble and membrane fractions. (B) The membrane fractions of cells expressing wild-type sez-6 (WT) and sez-6 ΔSC1-11 were treated with PNGase F and Endo H. Both 190-kDa and 160-kDa bands in wild-type sez-6 were sensitive to PNGase F treatment, whereas only the 160-kDa band was sensitive to Endo-H treatment. In contrast, PNGase F- and Endo-H-treated sez-6 ΔSC1-11 appeared at the same position as the untreated one. Twenty micrograms of protein was applied in each lane.

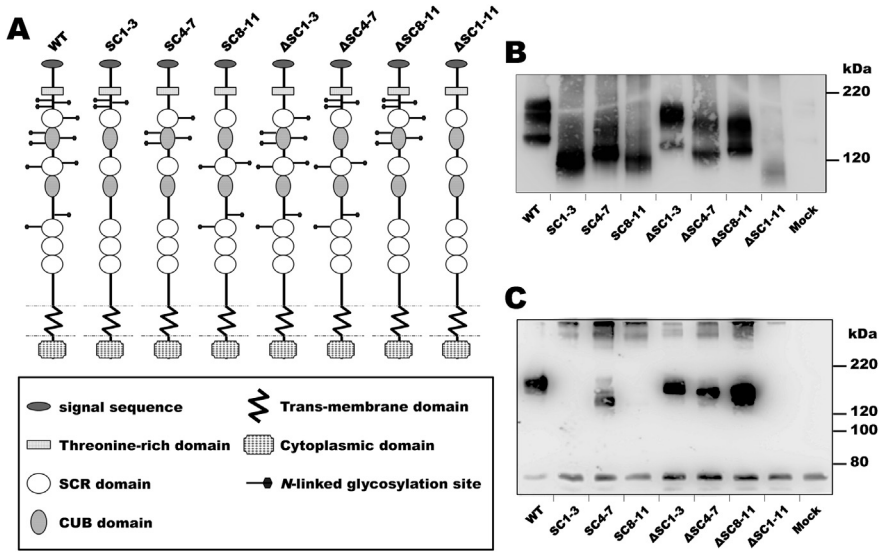

Fig. 2. Effect of *N*-glycosylation on the processing of sez-6 protein. Neuro2a cells were transfected with a plasmid for wild-type sez-6 (WT), sez-6 mutants, which lacked one, two or all *N*-glycosylation clusters, or mock vector. The conditioned medium and the membrane fraction were analyzed. (A) Schematic structures of wild-type sez-6 and mutants. (B) Membrane fractions. Mutants secreted into the conditioned medium showed both low-mobility and high-mobility bands, although mutants that were not detected in the conditioned medium indicated only a high-mobility band. Twenty micrograms of protein was applied in each lane. (C) Conditioned medium. Wild-type sez-6 and some mutants were detected in the conditioned medium, whereas others were not.

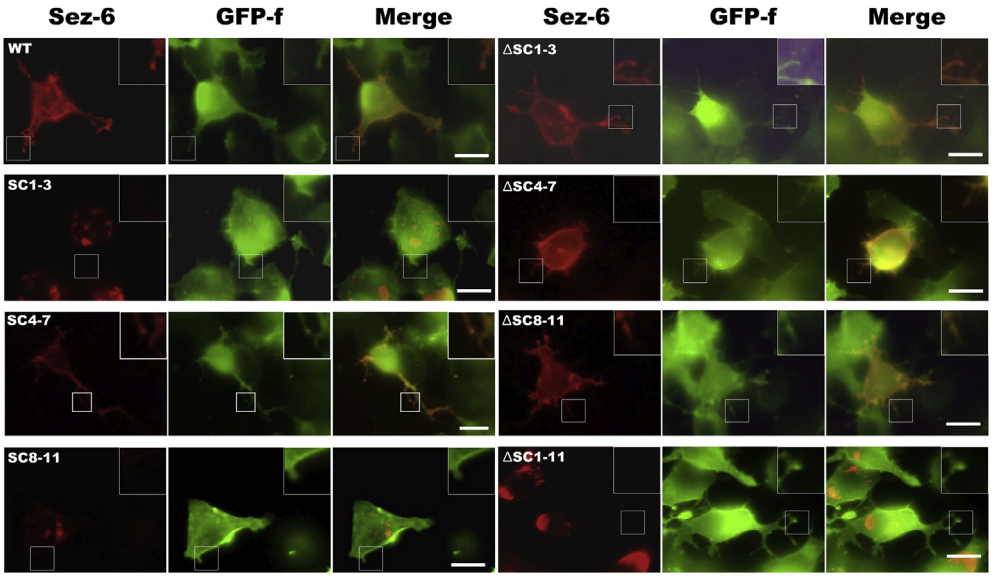

Fig. 3. Effect of *N*-glycosylation on the intracellular distribution of sez-6 protein. Neuro2a cells were transfected with an expression vector for wild-type sez-6 (WT) or mutant sez-6 as well as pAcGFP1-F. Sez-6 protein was detected with anti-sez-6 antibody under non-permeabilized conditions. Green fluorescence was derived from the expressed GPF-f proteins. Some mutants were evenly distributed on the cell membrane like wild-type sez-6, whereas others were clustered. Left, sez-6; middle, GFP-f; right, merged image. A magnified image of fine protrusions is shown in the upper right of each image. Scale bar, 20 μm.

expression of sez-6 ΔSC1-3 or sez-6 ΔSC4-7 significantly decreased the number of neurites (Fig. 4B). Other mutants (sez-6 SC4-7 and sez-6 ΔSC8-11) also appeared to decrease the number of neurites; however, this was not statistically significant. Interestingly, mutants excluding sez-6 ΔSC1-3 significantly decreased the number of filopodia-like protrusions in comparison with wild-type sez-6 (the main effect of transfectant, $F(5, 298) = 47.044$, $P < 0.001$). Mutants except for sez-6 ΔSC4-7 had a significantly increased number of filopodia-like protrusion compared with mock transfectant, whereas sez-6 ΔSC4-7 reduced the number to that of mock transfectant (Fig. 4C). *N*-glycosylation at SC4-7 appears to be the most effective in terms of filopodia-like protrusions.

4. Discussion

Western blot analysis indicated that sez-6 SC4-7 was secreted into the conditioned medium, whereas neither sez-6 SC1-3 nor SC8-11 was secreted (Fig. 2). All mutants lacking only one cluster of *N*-glycosylation were secreted, suggesting that at least two clusters are necessary for correct trafficking of sez-6. SC4-7 may

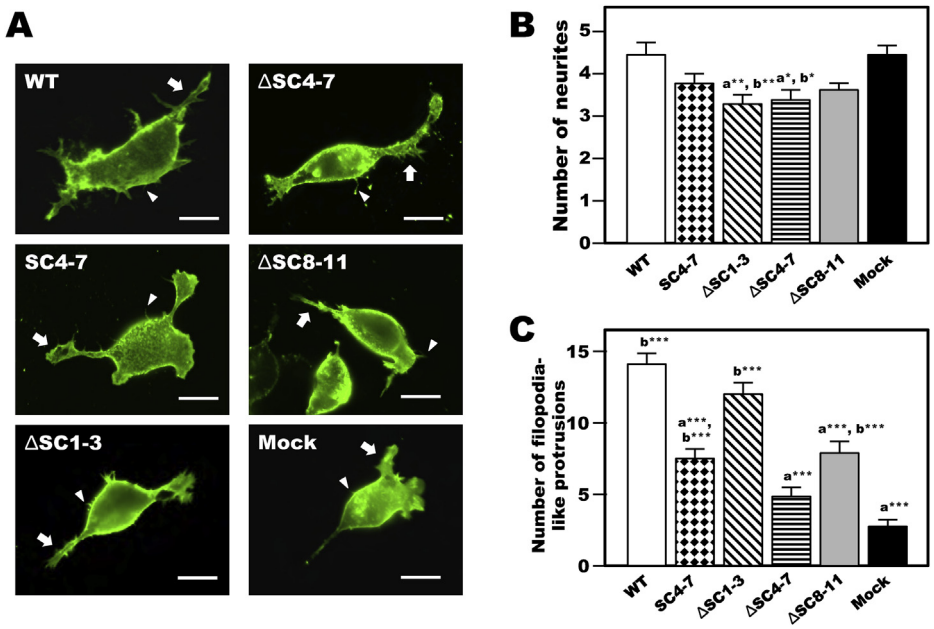

Fig. 4. Effects of *N*-glycosylation of sez-6 on neurites and filopodia-like protrusions. Cell morphology of neuro2a cells expressing wild-type sez-6 (WT), a mutant, or GFP-f was analyzed at 48 h after transfection. (A) Typical images of transfected cells are shown. Arrows and arrowheads indicate neurites and filopodia-like protrusions, respectively. Scale bar, 20 μm. (B) Number of neurites. Sez-6 ΔSC1-3 and sez-6 ΔSC4-7 significantly decreased the number of neurites compared with wild-type sez-6 and mock transfectant. WT, 4.5 ± 0.3, n = 51; SC4-7, 3.8 ± 0.2, n = 53; ΔSC1-3, 3.3 ± 0.2, n = 55; ΔSC4-7, 3.4 ± 0.2, n = 38; ΔSC8-11, 3.6 ± 0.2, n = 53; mock, 4.4 ± 0.3, n = 55. (C) Number of filopodia-like protrusions. Most mutants decreased the number of filopodia-like protrusions compared with WT. In particular, for sez-6 ΔSC4-7, it declined to the basal level. a, Significant difference compared with wild-type sez-6. b, Significant difference compared with mock transfectant. WT, 14.1 ± 0.8, n = 51; SC4-7, 7.5 ± 0.6, n = 54; ΔSC1-3, 12.1 ± 0.7, n = 55; ΔSC4-7, 5.0 ± 0.5, n = 35; ΔSC8-11, 8.0 ± 0.7, n = 54; mock, 2.9 ± 0.3, n = 55. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

compensate for another cluster of *N*-glycosylation, since it contains the largest *N*-glycans among the *N*-glycosylation clusters. Glycosidase treatments revealed that the cellular fraction (the soluble and membrane fractions) contained a mature form and an immature form of wild-type sez-6 showing resistance and sensitivity to Endo H treatment, respectively. Only SC4-7 appears to be sufficient but not essential for the correct processing of sez-6 protein, since both sez-6 SC4-7 and sez-6 ΔSC4-7 showed a mature form in the cellular fraction and were secreted into the conditioned medium. In contrast, the immature form of sez-6 SC1-3 and SC8-11 was mainly detected in the cellular fraction, while the mature form was rarely detected. Recent reports indicate that the loss of *N*-glycosylation prevents the maturation of residual *N*-glycans and the correct trafficking of tyrosinase and acid sensing ion channel 1a (ASIC1a) [13,14]. Similarly, some *N*-glycosylation may be required for the maturation of *N*-glycan and the correct processing of sez-6.

Mutant proteins that were detected in the conditioned medium, like wild-type sez-6, were well distributed on the cell membrane, including filopodia-like fine protrusions (Fig. 3). In contrast, sez-6 SC1-3, SC8-11, and ΔSC1-11 were localized at some portions. Since we did not permeabilize cells with any detergent, these mutant proteins were detected on the cell surface. Loss of *N*-glycosylation was shown to reduce the surface presentation of ASIC1a markedly, but did not completely inhibit it [14]. In addition, mutated plasticity-related gene (PRG) 3 lacking *N*-glycosylation and glycan-depleted acetylcholinesterase were found to fail to localize at the cell membrane [15,16]. Together with these reports, our results suggest that the maturation of *N*-glycosylation of sez-6 may promote the correct distribution on the cell surface, but is not crucial for membrane trafficking.

We analyzed the effect of sez-6 mutants on the cell morphology, since sez-6 modulates the dendritic arborization pattern [4]. Overexpression of wild-type sez-6 did not affect neurite protrusion, as we previously reported [5] (Fig. 4B). However, sez-6 ΔSC1-3 and ΔSC4-7 significantly decreased the number of neurites, suggesting that these mutants disturbed neurite formation. It was unexpected that the overexpression of sez-6 increased the number of filopodia-like protrusions, since our previous study did not detect such an effect [5]. This discrepancy may be attributable to the difference of the site where filopodia-like protrusions are located. In the previous study, filopodia-like structures on neurites were counted, whereas those on somatic bodies were analyzed here. Sez-6 ΔSC4-7 restrained filopodia-like protrusions to the basal level (Fig. 4C). Other mutants including sez-6 SC4-7 also significantly decreased the number of filopodia-like protrusions, but still enhanced them compared with the mock transfectant. These results suggest that SC4-7 is crucial for filopodia-like protrusions and others may improve the efficiency of SC4-7 function.

There are some reports indicating that *N*-glycosylation modulates filopodia-like protrusions. Overexpression of deglycosylated mutant of the sodium channel β4 subunit increased the number of filopodia-like protrusions and accelerated neurite extension, when compared with cells expressing the wild type [17]. ASIC1a has two *N*-glycosylation sites at Asn^{366} and Asn^{393}. A mutation of N366Q enhances expression on the cell surface and induces dendritic spines of hippocampal neurons [14]. This mutation showed increased amplitude, density, and pH sensitivity of pH-activated current. Furthermore, N366Q mutant contributes to acidosis-dependent spine loss, like wild-type ASIC1a. In contrast, N393Q mutation showed the opposite effects on acidosis-induced spine loss. Overexpression of PRG3 induces filopodia formation in several cell lines, whereas deglycosylation invalidates this effect, like in sez-6 mutants [16].

Although it is conceivable that ion channels such as ASIC1a and a sodium channel modulate the formation of filopodia and spines by evoking membrane depolarization, the mechanism underlying the induction of filopodia-like protrusions by sez-6 is currently obscure. Our finding that SC4-7 is critical for membrane trafficking,

Table 1
Phenotypes of cells expressing sez-6 mutants are summarized.

Mutant	Sez-6 ΔSC1-11	Sez-6 SC1-3	Sez-6 SC4-7	Sez-6 SC8-11	Sez-6 ΔSC1-3	Sez-6 ΔSC4-7	Sez-6 ΔSC8-11
Membrane trafficking	○	○	○	○	○	○	○
Secretion	×	×	○	×	○	○	○
Distribution on the cell surface	×	×	○	×	○	○	○
Neurite formation	N. D.	N. D.	○	N. D.	×*	×*	○
Filopodia formation	N. D.	N. D.	Δ	N. D.	○	×	Δ

○, similar to wild-type phenotype.
Δ, intermediate phenotype between wild-type and mock-transfectant.
×, similar to mock-transfectant.
×*, means decrease of neurite formation.
N. D., not determined.

distribution on the cell surface, and the protrusion of filopodia-like structures is suggestive (summarized in Table 1). SC4-7 exists on the first set of SCR and CUB domains located at the N-terminal side [1]. Interestingly, these domains preferentially interact with motopsin, which induces neurite extension, and such interaction restores motopsin function [5]. Some proteins containing CUB domain or SCR domain are known to modulate neurotransmitter receptors through the interaction with extracellular proteins [18]. Furthermore, recent reports indicate that an extracellular CUB domain induces filopodia protrusions through stimulation of an intracellular phosphorylation cascade [19,20]. The interaction of SCR/CUB domains of sez-6 with other molecules may contribute to filopodia-like protrusions. Therefore, we should focus on the molecules interacting with SCR/CUB domains of sez-6 and the molecular mechanism, including signal cascades, by which sez-6 enhances filopodia-like protrusions in future work.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.139.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.139.

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