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이학석사 학위논문

**SGIP1 α induces membrane
tubulation and forms homodimer
via its unique additional
hydrophobic region in MP domain**

SGIP1 α -MP 도메인의 소수성
부위를 통한 membrane tubule 및
homodimer 형성에 관한 연구

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ABSTRACT

SGIP1 α induces membrane tubulation and forms homodimer via its unique additional hydrophobic region in MP domain

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The early steps of clathrin-mediated endocytosis (CME) require membrane deformation and invaginations, and BAR domains or FCH domain proteins such as endophilin or PACSIN have been implicated in these processes. SGIP1 (SH3-domain GRB2-like endophilin interacting protein 1) is an endophilin interacting protein and is composed of N-terminal membrane phospholipid binding (MP) domain, proline rich domain (PRD) in the middle, and C-terminal μ -homology domain (μ HD). It is known to be specifically expressed in the brain and to function in the regulation of energy homeostasis although its role in the nervous system is not fully understood. Recent study showed that it's the longest splicing variant, SGIP1 α , plays a role in CME by interacting with

phospholipids and Eps15 and that MP domain of SGIP1 α binds to phospholipid and deforms the plasma membrane. The MP domain, however, doesn't show any sequence homology to well-known tubule-forming BAR or EFC domain, therefore how MP domain of SGIP1 α induces membrane tubulation has been in question. Here, I found that unlike SGIP1, SGIP1 α has additional 28 amino acids in MP domain. I also found that the additional 28 amino acids in MP domain are essential for SGIP1 α to induce membrane tubule while SGIP1 which lacks this additional amino acids failed to induce membrane tubule. The sequence analysis of additional 28 amino acids in MP domain identified a highly hydrophobic region, flanked by two positively charged regions. Using various point mutants of MP domain, I found that the upstream of hydrophobic region of SGIP1 α is crucial for tubulation. I also found that SGIP1 α forms homodimer via MP domain and the region between the hydrophobic region and the subsequent positively charged region is responsible for homodimerization. Evidently, SGIP1 α mutants lacking hydrophobic region forms neither membrane tubule nor homodimer. Taken together, my results suggest that the hydrophobic region in additional 28 amino acids of SGIP1 α MP domain plays an essential role in membrane tubulation and homodimerization. Considering that SGIP1 is a brain-specific protein, the unique feature in SGIP1 α MP domain may confer its special role in nervous system although it requires further study.

Keywords : SGIP1, SGIP1 α , CME, membrane tubulation, homodimerization

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Figure 6. SGIP1 α can form a homodimer via MP domain

Introduction

Endocytic process internalizes membrane receptors and extracellular components such as membrane-bound and soluble cargo into the cells. In addition, endocytosis is crucial for regulating signaling pathways that could decide which pathway is to be activated or terminated. Although there are several different mechanism for endocytosis, clathrin-mediated endocytosis (CME) is the best characterized so far.

The CME can be subdivided into several steps: 1) the nucleation of clathrin coated pit (CCP); 2) cargo selection; 3) maturity of clathrin coat and membrane curvature induction; 4) vesicle scission and clathrin uncoating [1].

The first step of CME is to define the sites to which clathrin will be recruited, and where membrane invagination is induced. FCHO proteins that are involved in the nucleation of CCPs arrive to the site of newly forming endocytic vesicles. Once FCHO proteins bind to the plasma membrane where PtdIns(4,5)P₂ is enriched, membrane invagination is induced before AP-2 and clathrin recruitment. This curvature can be a marker for the assembly of other components such as Eps15 and intersectin which recruit AP-2 to the plasma membrane [2, 3].

The next step is cargo selection. This step is mediated by AP-2 and other cargo specific adaptor proteins [4]. Once cargo binds to the transmembrane receptor on the plasma membrane, nucleation module of FCHO/Intersectin

complex recruits AP-2 to the receptor. Then, AP-2 binds to the cytoplasmic tail of receptors and forms a cargo-AP-2 complex. This complex acts as a trigger to increase the local concentration of PIP₂, leading to extend the coated membrane [5]. After cargo is selected by AP-2, clathrin assembly is initiated and, clathrin coats start to form a curved spherical structure. This process provides the force to emerge vesicle budding from membrane and formulates the neck region [6].

Highly invaginated clathrin coated membrane undergoes dynamin mediated fission. Once dynamin-GTP is assembled around the neck of nascent vesicles, dynamin twists narrow tubules in response to GTP hydrolysis, leading to pinch off the vesicles from the membrane [7]. Finally, after clathrin coated vesicles are pinched off from the plasma membrane, uncoating protein such as Rab5, auxillin and HSc70 are recruited to release clathrin coats from the endocytic vesicles [8].

SGIP1, Src homology 3-domain growth factor receptor-bound 2-like endophilin interacting protein 1, is specifically expressed in the brain and to function in the regulation of energy homeostasis [9]. SGIP1 consists of an N-terminal membrane phospholipid-binding (MP) domain, followed by prolin rich domain (PRD) and a C-terminal μ -homology domain (μ HD). Although it is not clear how SGIP1 acts as a regulator of energy balance, increased hypothalamic SGIP1 gene expression is a common physiological feature of obesity and diabetes.

Recent study shows that, the longest splicing variant of SGIP1, SGIP1 α

interacts with Eps15 and adaptor protein 2, AP-2. Eps15 is an adaptor protein of CME, and organizes a complex necessary for coated vesicle formation. AP-2 is a key component of the CCP, and plays a crucial role in both the organization and the function of plasma membrane coated pits [10]. In addition, AP-2 is constitutively associated with Eps15. Accordingly, SGIP1 α was expected to play a role in CME by interacting with Eps15 and AP-2 [11].

SGIP1 α MP domain is known as phospholipid binding domain, and binds not only to PS which is rich in brain lipids, but also PI 3-phosphate, PI 4-phosphate, PI 3,4-bisphosphate, PI3,5-bisphosphate, or PI(4,5) P₂ [11]. Along with membrane binding property, the MP domain deforms plasma membrane into narrow tubular structure, indicating that MP domain might be involved in vesicle formation during endocytosis although MP domain doesn't show any sequence homology with other membrane tubulating domains such as BAR domain in FBP17, ENTH domain in epsin and PH domain in dynamin [12].

While I was searching for the previously published articles regarding SGIP1 and SGIP1 α , I found many studies have misused SGIP1 as SGIP1 α , and there is no comparative study of SGIP1 and SGIP1 α .

Here, I found that compared to SGIP1, SGIP1 α has two additional regions; 28 amino acids in MP domain and 20 amino acids in a C-terminal region [11]. Using sequence analysis, additional 28 amino acids in MP domain identified as a highly hydrophobic region, flanked by two positively charged regions. I found that SGIP1 α MP domain plays an essential role in membrane tubulation and

homodimerization. Along with this hydrophobic region, the upstream of hydrophobic region is essential for MP domain-mediated membrane tubulation. I further found that the hydrophobic region followed by a positively charged region is crucial for homodimerization.

Taken together, my results suggest that the hydrophobic region in the additional 28 amino acids of SGIP1 α MP domain plays an essential role in membrane tubulation and homodimerization. Since SGIP1 lacks these additional region, my results also raise a possibility that despite they are isoforms of each other, SGIP1 and SGIP1 α may have distinct functions in cells. Considering that SGIP1 α is a brain-specific protein, the unique feature in SGIP1 α MP domain may confer its special role in nervous system although it requires further study.

Material and Method

A. DNA constructs and antibody

SGIP1 α construct originated from mouse was provided by Marek Michalak (University of Alberta, Edmonton, Alberta, Canada). Each domains of SGIP1 α were amplified by polymerase chain reaction and sub cloned into the expression vectors which are GFP, HA and FLAG. All constructs were verified by sequencing. The following antibodies were used: Anti-GFP rabbit polyclonal antibody was raised against GST-GFP. The antiserum was affinity-purified with the fusion proteins covalently bound to AminoLink Resin (Thermo scientific); Tubulin- β (Abcam); SGIP1 (Abcam, ab102839); Anti-HA (Covance, Princeton, NJ); FLAG (sigma, F3165). HRP-conjugated secondary antibodies were obtained from Jackson ImmunoReserch (WestGrove, PA)

B. Mutagenesis

The MP domain mutant constructs of SGIP1 α were made by two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. After finishing PCR reaction using SGIP1 MP domain as a template, add Dpn I restriction enzyme (NEB) to each PCR product. Those products incubated in 37 $^{\circ}$ C water bath for 1hr to digest the parental plasmid. Subsequently, 1 λ of those products were transfer to the competent cell (DH5 α) and incubated on ice for 30min. After heat shock for

1min 15s at 42°C and then place the products on ice 10minuites. Lastly, each products plate on agar plates containing antibiotic for plasmid vector and incubate at 37°C for more than 16hrs.

C. Cell culture and Transfection

Experiments were carried out following the guideline set forth by Seoul National University Council Directive for the proper care and use of laboratory animals. HEK293T cells (ATCC, Manassas, VA) and COS-7(Korean Cell Line Bank, Seoul, South Korea) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C and 5% CO₂. COS-7 cells (1.0×10^5) were plated on coverslips one day before transfection in only Dulbecco's modified Eagle's medium (Invitrogen). Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) reagent according to the manufacturer's protocol, and cells were grown for 18hours – 24hours.

D. Co-immunoprecipitation and Immunoblotting

For identifying that SGIP1 α form a homodimer *in vivo*, HEK293T cells were transfected with Lipofectamine 2000(Invitrogen). Transfected cells were washed twice with cold PBS and extracted at 4 °C for 1 h in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM aprotinin). Then, the samples were clarified by centrifugation at 15,000 \times g for 15 min, and protein concentrations were determined with a Bradford protein assay reagent kit (Bio-Rad). Samples containing 1 mg of total protein were immunoprecipitated for 4 h with anti-GFP antibody, followed by an additional 2 hours of incubation at 4 °C with protein A-Sepharose beads (GE Healthcare). The beads were extensively washed with lysis buffer and subjected to SDS-PAGE and transferred to a PVDF (Bio-Rad) membrane which is previously activated by 100% methanol. The membrane was blocked with 5% skim milk/TBS-T for 1 h, washed for 5min, and probed with primary antibody for 2 hours at room temperature. After extensive washing in TBS-T, the membrane was incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch). Proteins were visualized with ECL reagent (GE Healthcare).

E. Cell imaging and Microscopy

To detect short tubule of SGIP1 α , COS-7 cells were fixed in 4% formaldehyde, 4% sucrose in phosphate-buffered saline (PBS) for 15min. After fixation, cells were mounted on the slide glass, using fluorescence mounting medium (DAKO). Fluorescence images were acquired on a Nikon eclipse Ti-U inverted microscope equipped with a plan Fluor 40x 1.30 NA oil-immersion objective (Nikon, Tokyo, Japan), a GFP optimized filter set (Omega Optical, Brattleboro, VT), and an ORCA-R2 CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Confocal images were acquired on an Olympus FV-1000 confocal microscope with a 60 \times 1.35 NA oil lens driven by FluoView 1000. Cells which overexpressed the desired protein were excited with 488 nm (from an EGFP laser).

F. Sequence analysis

To detect the hydrophobic character and charge distribution of SGIP1 α , Hydrophobicity plot of MP domain was created by means of ExpASy Service ProtScale (<http://web.expasy.org/protscale>), and charge distribution is demonstrated by EMBOSS explorer, a graphical user interface to the EMBOSS suite of bioinformatics tools (<http://emboss.bioinformatics.nl/cgi-bin/emboss>).

Results

1. SGIP1 is expressed in brain and in cell line of neuronal lineage.

To examine the cellular distribution of SGIP1, various cell lysates and brain lysates from different species were analyzed by immunoblotting with anti-SGIP1 antibody. FLAG tagged SGIP1 α (Flag-SGIP1 α) over expressed in HEK293T cells was used a positive control and endogenous SGIP1 α is supposed to be detected around 130 kD. SGIP1 α was strongly expressed in brain lysates, neuron lysate and neuroblast cell line, N1E-115, whereas SGIP1 α was not detected in glia lysate, HEK293T cell, or COS-7 cells. This result indicates that SGIP1 α is specifically expressed in brain and in cell line of neuronal lineage (Fig. 1).

2. SGIP1 α induces membrane tubulation via its MP domain.

SGIP1 is an endophilin interacting protein and is composed of an N-terminal membrane phospholipid-binding (MP) domain, followed by proline rich domain (PRD) and a C-terminal μ -homology domain (μ HD). Unlike SGIP1, its longest splicing variant SGIP1 α has additional 28 amino acids in MP domain (aa 34-61) and 20 amino acids in C-terminal region (aa 550-569) (Fig. 2A). SGIP1 α is known to play prominent roles in CME by binding to membrane phospholipids and deforming the plasma membrane via its MP domain [11]. I first tested the expression patterns of each domain of SGIP1 α and whether

which domain(s) is required for membrane tubulation. COS-7 cells were transfected either with GFP-tagged full length or each domains or deletion mutants of SGIP1 α and cells were observed with confocal microscope. Consistent with the results from the previous study, MP domain containing constructs, GFP-SGIP1 α , GFP-SGIP1 α MP, and GFP-SGIP1 α $\Delta\mu$ HD formed membrane tubules whereas mutants lacking MP domain did not (Fig. 2B). MP domain, however, doesn't show any sequence homology to the well-known tubule forming BAR or EFC domain. Therefore how MP domain of SGIP1 α induces membrane tubulation has been in question. Previous study suggested that amino acids 1 to 97 of SGIP1 α are responsible for the formation of tubular structure [11]. In this study, I constructed amino acids 1 to 115 and named it as GFP-SGIP1 α MP115. Next, I characterized the sequence properties of MP115 domain using hydropathy plot and charge distribution plot. (Fig. 2C). Hydrophobicity of SGIP1 α MP domain was analyzed using the Kyte-Doolittle hydropathy plots and charge distribution was assayed by EMBOSS explorer, a graphical user interface to the EMBOSS suite of bioinformatics tools. The hydropathic index revealed the hydrophobic central region of MP115 domain around amino acid 43 to 55. Charge distribution plot showed two flanking positive charged regions (amino acid 33 to 43 and amino acid 72 to 87) to the hydrophobic region. I have subdivided MP domain into five regions. The N-terminal region (MP₁), the first positively charged region (P₁), the hydrophobic region (H₁), the second positively charged region (P₂) and the rest of the region (MP₂) (Fig. 2D).

3. Point mutation experiments reveal the essential residues for membrane tubulation by MP domain.

Considering the sequence analysis of MP115, hydrophobic region (H₁) region and two positively charged regions (P₁ and P₂) could have a role in membrane tubulation. To identify the essential amino acid residues of MP115 domain for membrane tubulating activity, candidate amino acids were point-mutated. The positively charged amino acids (Lysine, K) in P₁ and P₂ region of GFP tagged SGIP1 α MP 115 domain were changed to neutral amino acid (Glutamine, Q); K36/37/38/41Q, K82/83/86/87Q respectively. In addition to positively charged regions, hydrophobic amino acids (Phenylalanine, F; Tryptophan, W) in H₁ region were changed to negatively charged amino acid (Glutamate, E); F50E, W51E (Fig. 3A). GFP tagged point mutants were transfected to HEK293T cells, and cell lysates were immunoblotted with anti-GFP antibody (Fig. 3B) to check their expressions on correct sizes. Then, COS-7 cells were transfected with various GFP tagged MP domain point mutants and were examined with a fluorescence microscope. In the control which expressed GFP-SGIP1 α MP115 domain (MP115), short and long tubular structures were observed. In contrast, no long tubular structures were observed in point mutants of P₁ region; K36/37/38Q, K41E, K36/37/38/41Q. Point mutants in P₂ region; K86/87Q, K82/83/86/87Q had no effect on tubule formation. Compared to the point mutation results of positively charged regions (P₁ and P₂), point mutants in H₁ region showed different effects on tubulation. F50E was

expressed cytoplasmically and induced both short and long tubular structures, but the expression of W51E was confined in a nucleus (Fig. 3C). My results suggest that H₁ region and P₁ region are both involved in the processes of MP domain-induced membrane tubulation.

4. The upstream of hydrophobic region is essential for MP domain-mediated membrane tubulation.

To test which region of MP domain could induce membrane tubules in cells, I constructed various deletion mutants and performed tubulation analysis by expressing following deletion mutants in COS-7 cells. SGIP1 α MP115 Nt29: N-terminal MP₁ region up to 29th amino acid representing positive value in a charge distribution plot; SGIP1 α MP115 Δ Nt22: SGIP1 α MP115 construct lacking the N-terminal MP₁ region up to 22th amino acid; SGIP1 α Full length Δ Nt 34-61 and SGIP1 α MP115 Δ Nt 34-61: SGIP1 α and SGIP1 α MP115 lacking additional 28 amino acids in MP domain (Fig. 4A). COS-7 cells were transfected with GFP tagged deletion mutants and were examined with a fluorescence microscope. I found that SGIP1 α MP115 Δ Nt22 failed to induce membrane tubule and neither did SGIP1 α MP115 Nt29, indicating that MP₁ region and other regions in MP domain are required for membrane tubulation. I also found that both SGIP1 α Full length Δ Nt 34-61 and SGIP1 α MP115 Δ Nt 34-61 constructs showed no tubulation. The above results indicate that and P₁ regions in addition to MP₁ region are necessary for membrane tubulation (Table

1). Since SGIP1 α Full length Δ Nt 34-61 is indeed SGIP1 which has an MP₁ region but neither a H₁ nor a P₁ region in its MP domain, this explains why SGIP1 fails to induce membrane tubule.

5. SGIP1 α can form a homodimer via MP domain.

BAR domain proteins form a homodimer and make a concave-up structure of the positively charged surface and allow the surface to interact with the anionic phospholipid membrane. Self-association is important for inducing and stabilizing membrane curvatures [13-15]. To determine whether SGIP1 α can be self-assembled or not, HEK293T cells were co-transfected with GFP, GFP-SGIP1 α , GFP-SGIP1 α MP, GFP-SGIP1 α PRD, GFP-SGIP1 α μ HD and FLAG-SGIP1 α and subsequently immunoprecipitated with specific antibodies. The result showed that FLAG-SGIP1 α interacted with GFP-SGIP1 α and GFP-SGIP1 α MP. This indicates that SGIP1 α can be self-assembled via its MP domain (Fig. 5A). To test further, HEK293T cells were co-transfected with GFP, GFP-SGIP1 α , GFP-SGIP1 α MP, GFP-SGIP1 α PRD, GFP-SGIP1 α μ HD and HA-SGIP1 α MP domain and subsequently immunoprecipitated with specific antibodies. As expected, the blotting data clearly showed that GFP-SGIP1 α and GFP-SGIP1 α MP strongly interact with HA-SGIP1 α MP, indicating that SGIP1 α forms a homodimer through its MP domain (Fig. 5B).

6. Point mutation experiments reveal the essential residues for homodimerization of SGIP1 α .

To further identify critical regions for homodimerization of SGIP1 α MP domains, various point mutations and deletion constructs of SGIP1 α MP115 were co-transfected with HA-SGIP1 α MP domain in HEK293T cells. Since SGIP1 α homodimerizes via its MP domain, mutation or deletion of critical residues in MP domain could result in defective homodimerization. HEK293T cells were co-transfected with GFP, GFP-SGIP1 α , GFP-SGIP1 α MP 115, various point mutants of MP 115, deletion mutants and HA-SGIP1 α MP and subsequently immunoprecipitated with specific antibodies. In H₁ region, F50E point mutant showed defects in homodimerization whereas W51E point mutant did not. K82/83/86/87Q mutant can form a homodimer although to a less extent compared to that of control, implicating that H₁ and P₂ regions are partially responsible for SGIP1 α to form a homodimer (Fig. 6). Consistently, SGIP1 α MP Nt29 and SGIP1 α MP115 Δ Nt 34-61 failed to induce homodimerization since in both cases H₁ and P₂ regions are deficient (Table 2). This result showed that SGIP1 α MP forms a homodimer via its H₁ and P₂ regions, and SGIP1 which has neither H₁ nor P₁ region in its MP domain could not form a homodimer.

Figure 1. SGIP1 is expressed in brain and in cell line of neuronal lineage.

Immunoblotting analysis of different cell lysates and brain lysates from different species using anti-SGIP1 antibody. Thirty microgram of protein was loaded in each lane. HEK293T cell; Human embryonic kidney cell, COS-7 cell; African green monkey kidney cell, SV40 transformed, Brain lysate; E18 rat whole brain tissue lysate, Neuron lysate: E18 rat primary cortical neuron culture lysate, Glia: E18 rat primary astroglia culture lysate, and N1E-115 cell: Adrenergic clone derived from the mouse neuroblastoma.

Figure 1.

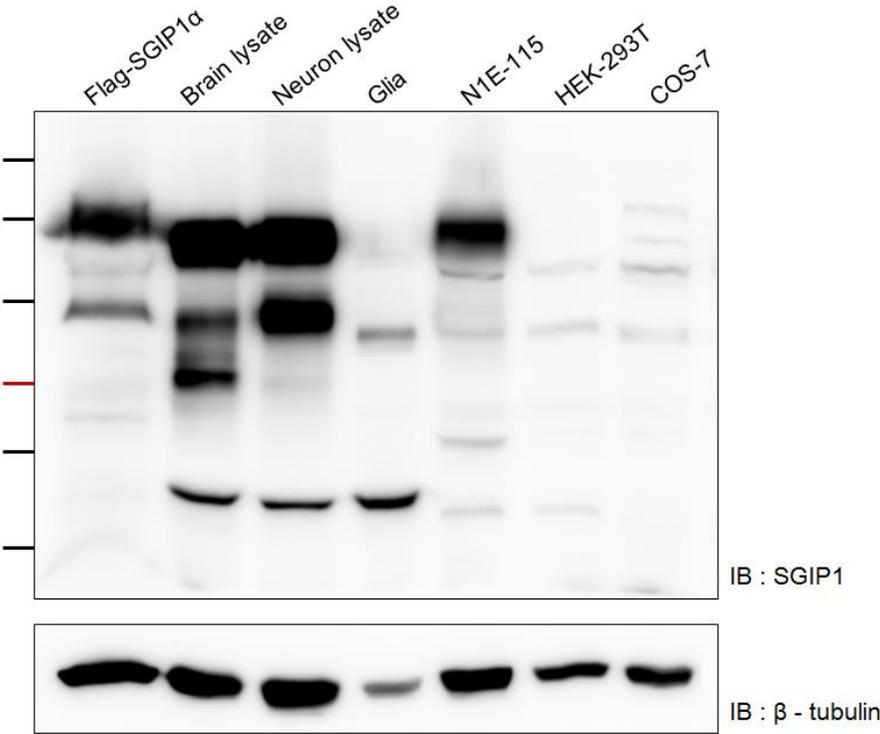
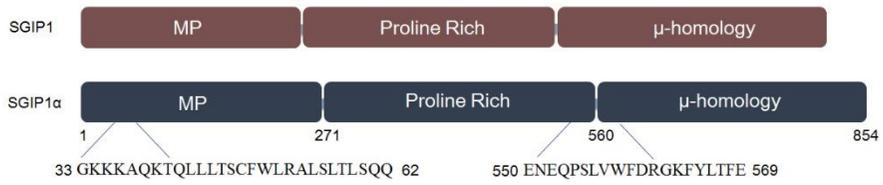


Figure 2. SGIP1 α induces membrane tubulation via its MP domain.

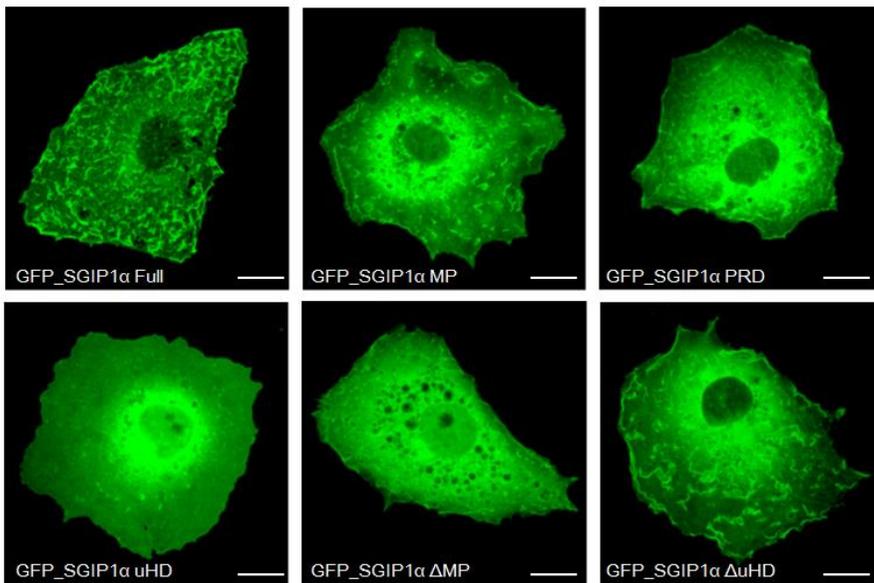
(A) Schematic diagram of SGIP1 α and its domain structure. (B) COS-7 cells were transfected with pEGFP- SGIP1 α full length (aa 1-854), pEGFP- SGIP1 α MP (aa 1-271), pEGFP- SGIP1 α PRD (aa 265-560), pEGFP- SGIP1 α μ HD (aa 556-854), pEGFP- SGIP1 α Δ MP (aa 265-854), or pEGFP- SGIP1 α Δ μ HD (aa 1-560). Eighteen hours after transfection, cells were observed with a confocal microscope. Scale bars, 20 μ m. (C) Schematic representation of the SGIP1 α MP domain, which is further subdivided into five regions. The N-terminal region (MP₁), the first positively charged region (P₁), the hydrophobic region (H₁), the second positively charged region (P₂) and the rest of the region (MP₂). (D) Analysis of hydrophobicity and charge distribution of MP domain amino acid sequence. Hydrophobicity plot of MP domain was created by means of ExPASy Service ProtScale. The larger positive hydropathic index indicates the more hydrophobic region. Charge distribution plot was obtained by the EMBOSS suite of bioinformatics tools. The electric charges were represented as positive and negative value along with its amino acid position.

Figure 2.

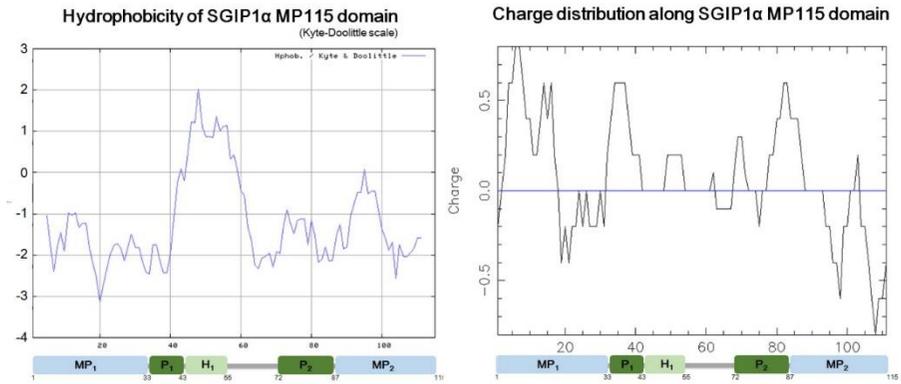
A.



B.



C.



D.

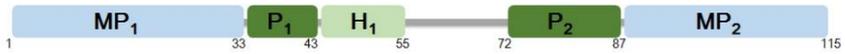
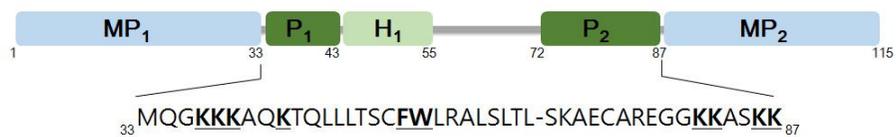


Figure 3. Point mutation experiments reveal the essential residues for membrane tubulation by MP domain.

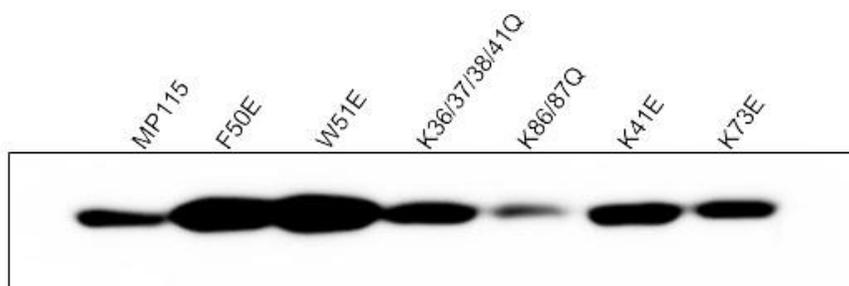
(A) All candidate amino acids were point-mutated (bold and underlined letters). The positively charged amino acids in P₁ region and P₂ region were changed to neutral amino acid (glutamine) and hydrophobic amino acids in H₁ region were changed to negatively charged amino acid (glutamate). (B) GFP tagged point mutants were transfected to HEK 293T cells, and cell lysates were immunoblotted with anti-GFP antibody. All constructs were expressed well on correct sizes. (C) COS-7 cells were transfected with various GFP tagged MP domain point mutants. Eighteen hours after transfection, cells were examined with a fluorescence microscope. Membrane tubules are subdivided into either short or long tubule, and the effects of each point mutants on either tubule structure are indicated as “+” or “-”. Scale bar, 20 μm.

Figure 3.

A.



B.



C.

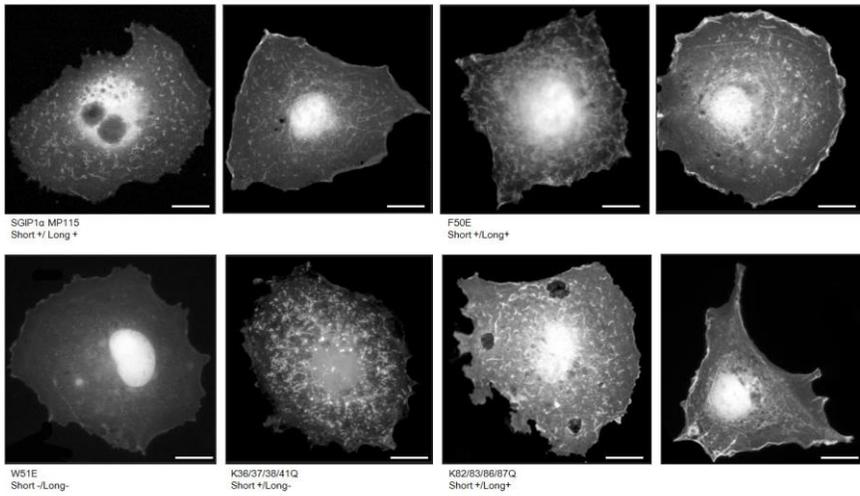
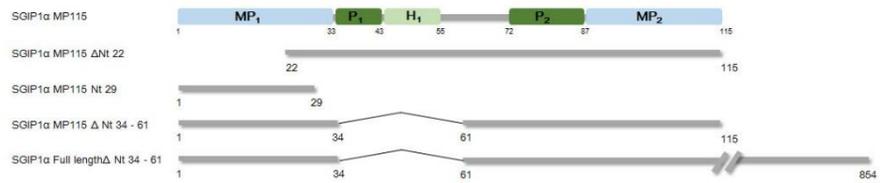


Figure 4. The upstream of hydrophobic region is essential for MP domain-mediated membrane tubulation.

(A) Schematic representation of various deletion mutants of SGIP1 α MP domain. (B) Epifluorescence micrographs of COS-7 cells transfected with GFP tagged deletion mutants of SGIP1 α . Eighteen hours after transfection, cells were imaged with a fluorescence microscope. The effect of each point mutants on either long or short tubule structure are indicated as “+” or “-“. Scale bar, 20 μ m

Figure 4.

A.



B.

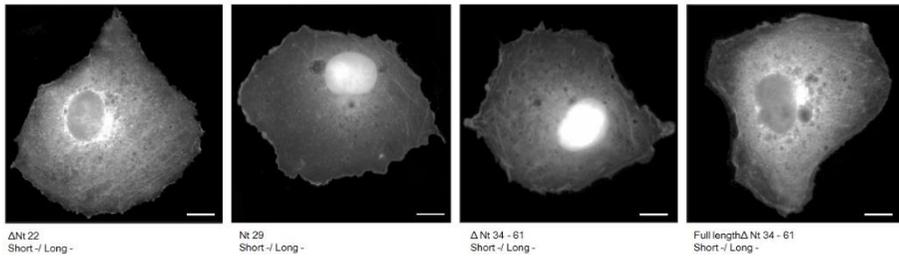


Table 1. The upstream of hydrophobic region in MP domain is the essential region for tubulation.

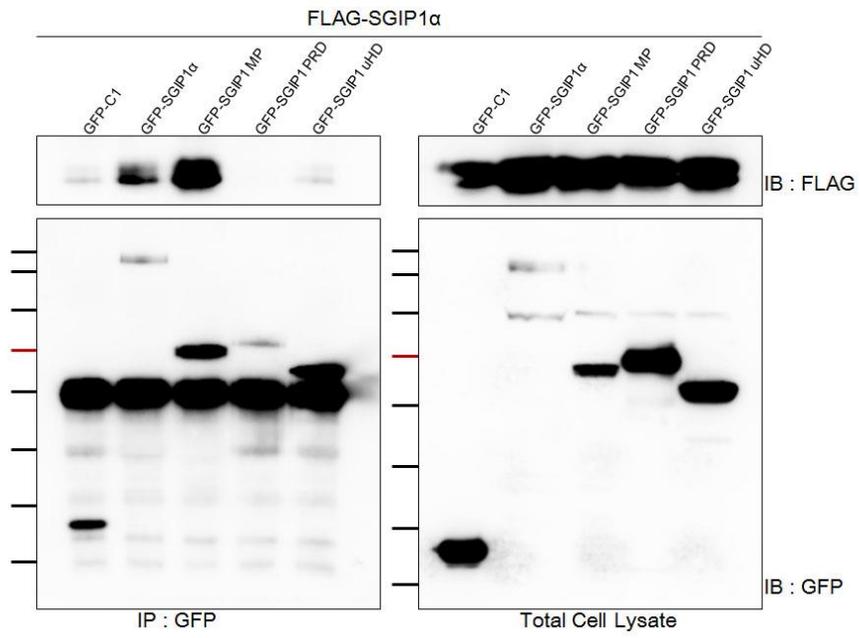
Construct	Short tubule	Long tubule
Positively charged region (P₁)		
K36/37/38Q	+	-
K41Q	+	-
K36/37/38/41Q	+	-
Hydrophobic region (H₁)		
F50E	+	+
W51E	-	-
Positively charged region (P₂)		
K73E	+	+
K82/83Q	+	+
K82/83/86/87Q	+	+
MP domain length		
ΔNt 22	-	-
Nt 29	-	-
MP Δ Nt 34 – 61	-	-
Full length Δ Nt 34 – 61	-	-

Figure 5. SGIP1 α can form a homodimer via MP domain

(A,B) HEK 293T cells were co-transfected with GFP, GFP-SGIP1 α , or various GFP-tagged domains and FLAG-SGIP1 α (A) or HA-SGIP1 α MP domain (B). Forty eight hours after transfection, the cells were lysed, immunoprecipitated with anti-GFP antibody, and immunoblotted with anti-FLAG antibody or anti-HA antibody, respectively. IP, immunoprecipitation; IB, immunoblot

Figure 5.

A.



B.

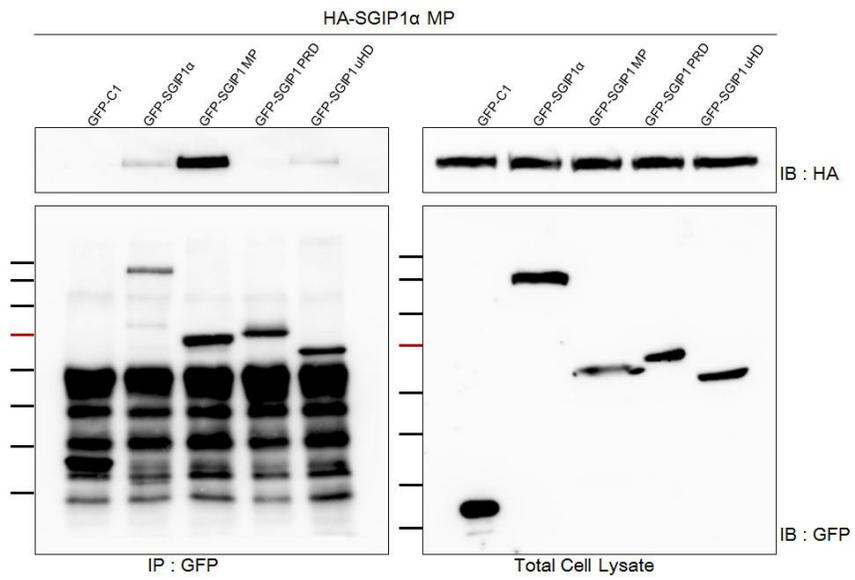


Figure 6. Point mutation experiments reveal the essential residues for homodimerization of SGIP1 α .

HEK 293T cells were co-transfected with HA-SGIP1 α MP domain and GFP, GFP-SGIP1 α MP115, various GFP-tagged point mutants or GFP-tagged deletion mutants, lysed, and immunoprecipitated with anti-GFP antibody followed by immunoblotting with anti-HA antibody.

Figure 6.

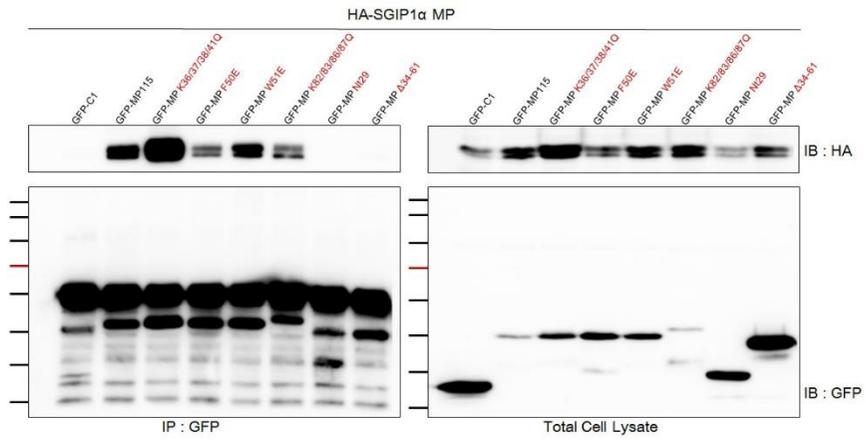


Table 2. The hydrophobic region and the following positively charged region is responsible for homodimerization.

Construct	Dimerization
Full length	+
MP 115	+
Positively charged region (P₁)	
K36/37/38/41Q	+
Hydrophobic region (H₁)	
F50E	+/-
W51E	+
Positively charged region (P₂)	
K82/83/86/87Q	+
MP domain length	
Nt 29	-
MP Δ Nt 34 – 61	-

Table 3. Tubule formation and homodimerization of various SGIP1 α **MP domain mutants**

Construct	Short tubule	Long tubule	Dimerization
Full length	+	+	+
MP	+	+	+
MP 115	+	+	+
Positively charged region (P ₁)			
K36/37/38Q	+	-	+
K36/37/38/41Q	+	-	+
K41E	+	-	+
Hydrophobic region (H ₁)			
F50E	+	+	+/-
W51E	-	-	+
Positively charged region (P ₂)			
K73E	+	+	+
K86/87Q	+	+	+
K82/83/86/87Q	+	+	+/-
MP domain length			
Δ Nt 22	-	-	+
Nt 29	-	-	-
MP Δ Nt 34 – 61	-	-	-
Full length Δ Nt 34 – 61	-	-	-

Discussion

Recent studies reveal that the MP domain of SGIP1 α functions as a membrane tubulator and is involved in clathrin-mediated endocytosis (CME) [11, 16]. The MP domain, however, exhibits no significant homology to other tubulating domains such as BAR domain and EFC domain, it remains elusive how the MP domain induces membrane tubules [2, 11].

To identify the essential regions for SGIP1 α -MP domain's tubulating activity, I characterized the sequence properties of the MP domain using both hydropathy analysis and charge distribution. Based on these results, I subdivided the MP domain into five regions; the N-terminal region representing positive charge (MP₁), the hydrophobic region (H₁) flanked by two positively charged regions which are the first positively charged region (P₁) and the second positively charged region (P₂), and the rest of the region (MP₂). Although SGIP1 and its longest splicing variant SGIP1 α both contain the MP domain, unlike SGIP1, SGIP1 α has 28 additional amino acids in the MP domain (a.a 34-61) which are composed of the positively charged region (P₁) and subsequent hydrophobic region (H₁).

To determine the crucial region(s) for membrane tubulation, various deletion mutants of SGIP1 α -MP115 were tested. I found that either MP₁ region only or the region without MP₁ failed to form membrane tubules. In addition, deletion mutant which lacks the additional 28 amino acids did not form membrane

tubules. These results indicate that MP₁ and additional 28 amino acids are involved in membrane tubule formation. Although I haven't investigated the exact mechanism in a current study, based on previous findings regarding membrane tubulating proteins, it is possible that positively charged N terminal region of MP domain binds phospholipids in the plasma membrane and then following hydrophobic region is partially embedded into lipid bilayer, leading to deformation of the lipid bilayer into narrow tubules [17-19].

To further pinpoint the critical amino acid residue(s) for membrane tubulation, I converted the positively charged amino acids into neutral amino acids in P₁ and P₂ regions, which might inhibit electrical charge interaction between phospholipids in the plasma membrane and the MP domain. I found that P₂ region mutant induced both short and long tubules like SGIP1 α full-length did while P₁ region mutant induced only short tubules without long tubules. These results suggest that SGIP1 α -MP domain forms membrane tubules by forming interactions between the negatively charged phospholipids in the membrane and positively charged P₁ region of the MP domain.

Endophilin, an N-BAR domain protein, is known to generate high curvature membrane by interacting with phospholipids via its hydrophilic face and by embedding into the bilayer through its hydrophobic region [17]. I tested whether hydrophobic region (H₁) of MP domain has any effect on membrane tubulation. At first, I hypothesized that F50 and W51 would be key residues in the hydrophobic region, therefore I converted these amino acids to glutamate to reduce hydrophobicity, expecting that these mutant constructs could inhibit

membrane tubulation. The results, however, showed that F50E mutant formed membrane tubules like SGIP1 α full-length did while W51E mutant was confined in nucleus which might be from the loss of its targeting to the cytosol. Accordingly, I found that either single point mutant (F50E or W51E) or double point mutant (F50E /W51E) does not really change the hydrophobicity of the MP domain. Evidently, to get better understanding of the role of hydrophobic region in membrane tubulation, it is necessary to reduce hydrophobicity of MP domain. According to the analysis of hydropathy plot, I selected a few candidate amino acids. Using *in silico* hydropathy simulation, I found that converting these amino acids to either hydrophilic residues or arginine that has the lowest hydrophobicity among amino acids caused a dramatic reduction of hydrophobicity of the MP domain. Further study will be focused on these candidate residues.

The tubulating domains such as BAR and F-BAR proteins form a dimer or an oligomer by self-association which is essential for their activity for CME [13, 14, 20]. SGIP1 α -MP domain could be self-dimerized. As expected, SGIP1 α forms a homodimer via the MP domain. I found that the region without MP₁ didn't affect its homodimerization. However, either only MP₁ region or mutant without the additional 28 amino acids in MP domain failed to form a homodimer, indicating that additional 28 amino acids region in the MP domain is essential for homodimerization. Interestingly, the degree of homodimerization was reduced in cells expressing P₂ region mutant and F50E

mutant in H₁ region.

My results showed that additional 28 amino acids region in MP domain deforms the plasma membrane into narrow tubules and is self-assembled to make a homodimer. The regions responsible for inducing membrane tubulation and forming a homodimer in SGIP α largely overlap with each other within the hydrophobic region (H₁). However, even in H₁ region, I found that membrane tubulation and homodimerization are mediated by different amino acid residues, implying that two activities are distinctly regulated by different residues.

Rynditch's group found that SGIP1 forms a complex with intersectin-1 in clathrin-coated pits. Intersectin-1 is an endocytic scaffolding protein and forms an interacting network of endocytic proteins during CME. They found that SH3A and SH3E domains of intersectin-1 interact with proline rich domain (PRD) of SGIP1 *in vitro* [16]. In addition, SGIP1 α directly binds to AP2 through APA region, the AP2 activator motif. Adaptor protein-2 (AP2) is a key component of CME and mediates the constitutive endocytosis of cargo proteins [21]. AP2 thereby binds target proteins and assembles the machinery for internalization of cargo. Thus, as SGIP1 α binds to AP2 via APA region, it can stabilize nascent endocytic pits by exposing membrane and cargo binding site of AP2 [22].

This is the first study to identify the role(s) of additional region in MP domain and I believe that my findings would have a big impact on future studies regarding the roles of SGIP1 and SGIP1 α in the nervous system.

There are three proteins that are closely related to SGIP1 α : F-BAR proteins FCHo1 and 2 and the yeast endocytic protein, Syp1p [12, 23]. These proteins have a similar domain arrangement even though the sequence homology among them is relatively low. All four proteins including SGIP1 α have N-terminal lipid-binding domain; FCHo1/2 have F-BAR domains, SGIP1 α has an MP domain and Syp1p has an FCH domain. They also share proline rich domains in the center and partially conserved μ -homology domain in their C-terminal [12, 24]. FCH domain of Syp1p is quite similar to F-BAR domain of FCHo1/2 but MP domain of SGIP1 α does not show any sequence homology to these two domains, suggesting that MP domain of SGIP1 α may bind to phospholipids by different mechanism. Based on the localization of FCHo1, Syp1p and SGIP1 α at the endocytic site, they are supposed to play roles in endocytosis. According to recent study from Drubin's group Syp1p, septin-associated protein, is a component of the CME in budding yeast. Syp1p arrives early at endocytic site and forms a mark to recruit the coat module such as clathrin. Syp1p interacts with Ede1p, a EH-domain protein that is well-known for an endocytic site formation [12, 23]. Previous study showed that SGIP1 α interacts with Eps15 during clathrin coated pit formation [11]. Interestingly, Eps15 is homolog of Ede1p [25]. This suggests that SGIP1 α /Eps15 play the roles as an early endocytic module like Syp1p/Ede1p.

Although early arriving endocytic proteins regulate and facilitate the early steps of CME initiation, the mechanism governing CCP site selection and

subsequent nucleation in mammal have remained elusive. Recent study from McMahon's group first shed a light on this ambiguity. They revealed the role of FCHo1/2 in CCV budding and marking site of CCV formation [24, 26]. According to this study, FCHo1/2 bind to the plasma membrane and sculpt the initial bud site. FCHo1/2 bind to Eps15 and intersectin which subsequently recruit clathrin machinery for CCV formation [4, 21, 27]. Thus, they concluded that FCHo1/2 play a role as a nucleator for CME [24, 26]. Although MP domain of SGIP1 α and the EFC/F-BAR domain of FCHo1/2 show no significant amino acid sequence homology, they have functional similarity. Indeed, in their paper, McMahon's group tested the idea whether SGIP1 could replace the function of FCHo1/2 in the condition of FCHo1/2 knock-down. SGIP1, however, failed to rescue the CCP formation when endogenous FCHo1/2 were depleted [24]. Although they concluded that unlike FCHo1/2, SGIP1 is not a CCP nucleator, my current results bring their conclusion regarding the function of SGIP1 into a question. I found here that SGIP1 has neither H₁ nor P₁ regions in its MP domain and thus failed to form a homodimer and membrane tubules. SGIP1 α , however, forms a homodimer and induces membrane tubules via its additional amino acids in MP domain. Therefore, it is highly likely that if SGIP1 α is used instead of SGIP1, it could act as a nucleator and rescue CCP formation in FCHo1/2 depleted cells.

I showed that SGIP1 α , but not SGIP1, the activities of membrane tubulation and homodimerization via its MP domain. These membrane deforming

properties as well as homodimerization may act in vesicle formation and contribute to the localization of other endocytic proteins for clathrin coat assembly during endocytosis. Furthermore, since SGIP1 α has similar domain arrangement with FCHo1/2, known as a nucleator of CME, it is possible that SGIP1 α has a role in clathrin-coated vesicle formation, and further studies are necessary to better understand the actual role of SGIP1 α as a nucleator of CME.

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Abstract in Korean (국문 초록)

Clathrin mediated endocytosis (CME) 과정은 세포막 변형과 함입 과정을 통하여 시작되며, 이러한 초기과정은 BAR나 FCH 도메인을 포함하는 단백질에 의해 조절된다. SGIP1 (SH3-domain GRB2-like (endophilin) interacting protein 1)은 endophilin과 상호작용을 하는 단백질로써, N말단의 Membrane phospholipid binding (MP) 도메인, 중간 부분의 Proline rich 도메인 (PRD) 그리고 C 말단의 μ -homology 도메인 (μ HD)로 이루어져 있다. 처음 SGIP1은 뇌에서 특이적으로 발현되고 energy homeostasis 조절에 관여하는 단백질로 보고되었으나, 실제 nervous system에서의 역할에 대해서는 아직 연구된 바가 없다. 최근 SGIP1의 가장 긴 splicing variant인 SGIP1 α 가 phospholipid에 결합하고 세포막 변형을 일으킴을 보고하였고, Eps15과의 상호작용을 통하여 CME 과정에 작용하는 것을 밝혔다. SGIP1 α -MP도메인은 membrane tubule 형성에 관여하지만, 기존에 알려진 BAR나 EFC 도메인과는 아미노산 서열에서 어떠한 유사성도 보이지 않는다. 그러므로, SGIP1 α -MP 도메인이 어떠한 메커니즘에 의해 membrane tubule을 형성하는 지는 불확실하다. SGIP1 α -MP 도메인은 SGIP1-MP 도메인에 없는 28개의 아미노산을 추가적으로 갖고 있는데, 아미노산 서열을 분석한 결과

추가된 아미노산이 양전하를 띠는 부위에 둘러싸여 있는 소수성 부위를 확인하였다. 위 사실을 바탕으로 다양한 SGIP1 α -MP 도메인의 point 및 deletion mutant를 제작하여 실험한 결과, MP도메인은 N 말단부터 소수성 부위까지를 통하여 membrane tubule을 형성하고, 소수성 부위와 뒤따르는 양전하를 띠는 부분이 homodimer 형성에 중요함을 확인하였다. 위 결과를 토대로, SGIP1 α -MP 도메인의 소수성 부위가 membrane tubule과 homodimer 형성에 중요한 역할을 하는 것을 증명하였다. SGIP1이 뇌에서 특이적으로 발현되는 단백질을 고려해볼 때, SGIP1 α -MP도메인이 nervous system에서 특별한 역할을 담당할 것으로 사료된다.

주요어 : SGIP1, SGIP1 α , 클라트린 매개 세포 내 이입, 세포막 미세소관 형성, 동질 이량체

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