SGIP1α Is an Endocytic Protein That Directly Interacts with Phospholipids and Eps15

SGIP1 has been shown to be an endophilin-interacting protein that regulates energy balance, but its function is not fully understood. Here, we identified its splicing variant of SGIP1 and named it SGIP1α. SGIP1α bound to phosphatidylinositol and phosphoinositides and deformed the plasma membrane and liposomes into narrow tubules, suggesting the involvement in vesicle formation during endocytosis. SGIP1α furthermore bound to Eps15, an important adaptor protein of clathrin-mediated endocytic machinery. SGIP1α was colocalized with Eps15 and the AP-2 complex. Upon epidermal growth factor (EGF) stimulation, SGIP1α was colocalized with EGF at the plasma membrane, indicating the localization of SGIP1α at clathrin-coated pits/vesicles. SGIP1α overexpression reduced transferrin and EGF endocytosis. SGIP1α knockdown reduced transferrin endocytosis but not EGF endocytosis; this difference may be due to the presence of redundant pathways in EGF endocytosis. These results suggest that SGIP1α plays an essential role in clathrin-mediated endocytosis by interacting with phospholipids and Eps15.

Clathrin-mediated endocytosis governs not only the routine uptake of membranes and nutrient receptors but also the internalization of several ligand-stimulated receptors, channels, and transporters (1). Clathrin triskelions assemble into polyhedral lattices on the cytoplasmic surface of the plasma membrane. Binding of clathrin to the plasma membrane is mediated by adaptor proteins that interact with clathrin and with specialized cytoplasmic motifs of transmembrane proteins and/or phospholipids (2–6). The major adaptor protein is the AP-2 complex that consists of α-, β2-, γ2-, and μ2-adaptons. The AP-2 complex recruits other adaptor proteins, such as amphiphysin, β-arrestin, epsin, and Eps15. These adaptor proteins can also interact with each other and with other components, such as dynamin, of clathrin-mediated endocytosis machinery.

Accompanied by the binding of clathrin and its adaptor proteins to the plasma membrane, membrane curvature is generated to form a coated pit (1). The process of membrane curvature leads to the formation of a deeply invaginated membrane followed by the fission of a nascent coated vesicle. Adaptor proteins, such as epsin and amphiphysin, directly bind to and deform liposomes into tubules in vitro (3, 7–10). These proteins, together with dynamin, play crucial roles in membrane curvature and fission for the formation of clathrin-coated vesicles (1). They directly interact with membrane phosphoinositides through phospholipid-binding domains such as the ENTH domain in epsin, the BAR domain in amphiphysin, the EFC/FCH and BAR domain in FBP17, and the pleckstrin homology domain in dynamin (11–13). These domains deform the plasma membrane into narrow tubules.

We attempted to isolate a novel tubulin- and/or MT-binding protein and purified a protein with a molecular mass of about 100 kDa (p100). During the study, p100 was found to be highly homologous to a recently reported protein named Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1), the function of which is not fully understood (14). It is likely that p100 is a longer splicing variant of SGIP1; therefore, it was named SGIP1α. It remains

5 The abbreviations used are: ENTH, epsin N-terminal homology; FBP17, formin-binding protein 17; SGIP1, Src homology 3-domain growth factor receptor-bound 2-like interacting protein 1; aa, amino acid(s); Abs, antibodies; mAb; monoclonal Ab; pAb, polyclonal Ab; BAR, Bin-Amphiphysin-Rvs; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; EFC, extended FCH; FCH, Fes-CIP4 homology; EGF, enhanced green fluorescent protein; GST, glutathione S-transferase; MBP, maltose-binding protein; MP, membrane phospholipid-binding; MT, microtubule; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI(4,5)P2, PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; RNAi, RNA interference; sIRNA, small interfering RNA; Tfn, transferrin; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

‡ This study was supported by grants-in-aids for Cancer Research and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to GenBank™ /EBI Data Bank with the accession number(s) AB262963 and AB262964.

5 The abbreviations used are: ENTH, epsin N-terminal homology; FBP17, formin-binding protein 17; SGIP1, Src homology 3-domain growth factor receptor-bound 2-like interacting protein 1; aa, amino acid(s); Abs, antibodies; mAb; monoclonal Ab; pAb, polyclonal Ab; BAR, Bin-Amphiphysin-Rvs; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; EFC, extended FCH; FCH, Fes-CIP4 homology; EGF, enhanced green fluorescent protein; GST, glutathione S-transferase; MBP, maltose-binding protein; MP, membrane phospholipid-binding; MT, microtubule; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI(4,5)P2, PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; RNAi, RNA interference; sIRNA, small interfering RNA; Tfn, transferrin; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
Role of SGIP1α in Endocytosis

eclusive whether its tubulin binding is significant, but we have found that SGIP1α is an endocytic protein, which directly interacts with phosphoinositides and Eps15.

EXPERIMENTAL PROCEDURES

Tubulin Blot Overlay—Tubulin blot overlay was performed as described (15, 16) with some modifications. Samples to be tested were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 5% fat-free powder milk overnight at 4 °C and then equilibrated with PEM buffer (100 mM PIPES/NaOH, pH 6.8, 1 mM EGTA, 1 mM MgCl₂, and 1 mM DTT) for 10 min at room temperature. The membrane was then incubated with 30 µg/ml tubulin in PEM buffer containing 1% bovine serum albumin for 1 h at 4 °C. After washing in PBS, the membrane was subjected to immunoblot analysis using anti-tubulin α mAb (DM1A, Sigma-Aldrich).

Purification of p100 and Mass Spectrometry—All the purification procedures were carried out at 0–4 °C. Twenty rat brains were homogenized in 200 ml of buffer A (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT) in a ceramic grinder. The homogenate was subject to immunoblot analysis using anti-tubulin α mAb (DM1A, Sigma-Aldrich).

FIGURE 1. Identification of p100 as a tubulin-binding protein. A, tubulin blot overlay on various rat tissues. Various rat tissue homogenates (30 µg of proteins each) were subjected to SDS-PAGE (8% polyacrylamide gel) followed by tubulin blot overlay. β tubulin blot overlay on recombinant SGIP1α. COS7 cells were transfected with pcMV-Myc-SGIP1α (full-length), and the cell lysate (20 µg of protein) was subjected to SDS-PAGE (8% polyacrylamide gel) followed by tubulin blot overlay or immunoblot analysis using anti-Myc mAb. B, immunoblot.

were collected. The active fractions (fractions 16–19) were subjected to SDS-PAGE followed by silver staining (18). After the protein band corresponding to p100 was excised and subjected to in-gel trypsin digestion, the resultant peptides were desalted by Zip tips C18 (Millipore) and subjected to infusion nanoESI-TOF mass spectrometry (QSTAR Pulsar i Applied Biosystems/MDS SCIEX). Partial aa sequences were obtained with peptide ion fragmentation due to high collision energy. The sequences obtained were subjected to a search for sequence similarity against the current NCBI data base using the Mascot Search Program (Matrix Science Ltd.).

Construction of Expression Vectors—Oligonucleotide primers, 5′-CGG GAT CCA TGA TGG AAG GAC TGA AAA AAC TCA ATC TCG AGT TAG TTA TCT GCC AAG TAC TTT CCT GC-3′ and 5′-ATC TCG AGT TAG TTA TCT GCC AAG TAC TTT CCT GC-3′, were designed, and mouse SGIP1α cDNA was obtained by PCR using mouse cDNA as a template. Rat Eps15 cDNA was obtained by PCR using mouse cDNA as a template. (DBD/EMBL/GenBank™ accession number AB262963). Expression vectors were constructed in pGex5X-3 (GE Healthcare Bio-Science Corp.), pMal C2 (New England Biolabs), pEGFP-C1 (Clontech), and pCMV-Myc (19). SGIP1α-1 (aa 1–280), SGIP1α-2 (aa 261–580), SGIP1α-3 (aa 561–854), SGIP1α-4 (aa 1–97), SGIP1α-5 (aa 98–280), SGIP1α-6 (aa 251–390), SGIP1α-7 (aa 428–854), and rat Eps15 (aa 593–834) were obtained by PCR. SGIP1α mutant (T2001C, C2004G, C2007A, A2010G, and C2013T) was generated using the site-directed mutagenesis kit (Stratagene) without changing the aa sequence. GST fusion and MBP fusion proteins were purified using glutathione-Sepharose beads (GE Healthcare Bio-Science Corp.) and amylose resin beads (New England Biolabs Inc.), respectively.

Abs—A rabbit pAb against SGIP1α was raised against GST-SGIP1α-6 (aa 251–390). The antiserum was affinity-purified with the fusion protein covalently coupled to N-hydroxysuccinimydyl-activated Sepharose (GE Healthcare Bio-Science Corp.). The following Abs were purchased from commercial sources: mouse anti-Myc mAb (9E10) (American Type Culture Collection); mouse anti-tubulin α mAb (clone DM1A) (Sigma-Al-
Role of SGIP1α in Endocytosis

SEPTEMBER 7, 2007 • VOLUME 282 • NUMBER 36 • JOURNAL OF BIOLOGICAL CHEMISTRY 26483

15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and immunostained with various Abs. DilC16(3) (Invitrogen) staining was performed as described (20). Images were taken with a fluorescence microscope (BX51, Olympus) or a confocal microscopy system (BX50 and Fluoview FV300; Olympus) at room temperature. The fluorochromes used included Alexa Fluor 488 and 594, Texas-red conjugated EGF, and DilC16(3) (Invitrogen). A ×60 oil immersion objective, NA 1.40 (Olympus) was used. Images were assembled with PhotoShop (Adobe). In each plate, photographs were cropped, and each fluorochrome was adjusted for identical brightness and contrast to represent the observed images.

The quantification of colocalization was performed using MetaMorph imaging system software (21). Briefly, background was subtracted from unprocessed images, and the percentage of SGIP1α pixels overlapping Eps15, α-adaptin, or EGF pixels was measured. For the colocalization in the plasma membrane region, images were acquired to display the entire cell surface adhering to culture dishes (22). The colocalization percentage was determined in a restricted region of the images extending 15 pixels from the cell edge toward the cytoplasm. For the colocalization in the intracellular region, images were acquired at upper planes from the ventral surface. The colocalization percentage was determined in a region of the images excluding 15 pixels from the cell edge. Data were shown as the means ± S.E. of three independent experiments.

Liposome Binding and Liposome Tubulation Assays—Liposome binding assay was performed as described (12). PE/PC liposomes consisted of 70% PE, 20% PC, and 10% PI or various phosphoinositides. Where indicated, liposomes consisted of 80% PE, 20% PC, and various percentages of PS (with a corresponding reduction in PE). MBP-SGIP1α-4 (50 μg/ml) was incubated with 1 mg/ml liposomes for 15 min at room temperature followed by centrifugation. To calculate the Kd value, various doses of GST-SGIP1α-4 were incubated with 0.2 mg/ml liposomes (60% PE, 20% PC, and 20% PI(4,5)P2) for 30 min at 4 °C followed by centrifugation. Comparable amounts of the supernatant and pellet

drich); rabbit anti-EGFP pAb and mouse anti-EGFP mAb (MBL Co.); mouse anti-α adaptin mAb, mouse anti-γ adaptin mAb, and mouse anti-Eps15 mAb (BD Biosciences); rabbit anti-Eps15 pAb (Covance); and secondary Abs conjugated with Alexa Fluor 488 and 594 (Invitrogen).

Cell Culture, Transfection, and Immunofluorescence Microscopy—COS7 cells were transfected with pEGFP-SGIP1α (full-length), pEGFP-SGIP1α-1 (aa 1–280), pEGFP-SGIP1α-2 (aa 261–580), pEGFP-SGIP1α-3 (aa 561–854), pEGFP-SGIP1α-4 (aa 1–97, MP domain), or pEGFP-SGIP1α-5 (aa 98–280). Cells were examined with a fluorescence microscope. Scale bars, 10 μm. B, origination of tubular structures from the plasma membrane. COS7 cells transfected with pEGFP-SGIP1α-4 (MP domain) were fixed without permeabilization and then stained with DilC16(3). Samples were examined with a fluorescence microscope. Insets, enlarged images of dashed boxes. Scale bar, 10 μm.

FIGURE 2. Membrane tubulation by SGIP1α overexpression. A, the N-terminal region (aa 1–97, MP domain) responsible for the formation of tubular structures. Upper panel, structures of full-length SGIP1α and various fragments of SGIP1α. Lower panel, COS7 cells were transfected with pEGFP-SGIP1α (full-length), pEGFP-SGIP1α-1 (aa 1–280), pEGFP-SGIP1α-2 (aa 261–580), pEGFP-SGIP1α-3 (aa 561–854), pEGFP-SGIP1α-4 (aa 1–97, MP domain), or pEGFP-SGIP1α-5 (aa 98–280). Cells were examined with a fluorescence microscope. Scale bars, 10 μm. B, origination of tubular structures from the plasma membrane. COS7 cells transfected with pEGFP-SGIP1α-4 (MP domain) were fixed without permeabilization and then stained with DilC16(3). Samples were examined with a fluorescence microscope. Insets, enlarged images of dashed boxes. Scale bar, 10 μm.
fractions were subjected to SDS-PAGE followed by CBB staining. The protein amount was quantified by scanning using NIH image (version 1.61). Liposome tubulation assay was carried out as described (12).

**In Vitro Binding Assays and Immunoprecipitation**—To examine the in vitro binding of SGIP1α to Eps15, MBP-Eps15 or MBP alone (200 pmol each) was incubated with GST-SGIP1α-3 or GST alone (200 pmol each) immobilized on beads in PBS containing 0.1% Triton X-100. After washing, bound proteins were subjected to SDS-PAGE followed by CBB staining.

Immunoprecipitation was performed as follows. Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM EGTA, 5 mM EDTA, 150 mM NaCl, 15 mM NaF, 1.5 mM Na3VO4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) followed by centrifugation. The supernatant (1 mg of protein) was incubated for 3 h at 4 °C with anti-SGIP1α pAb or control rabbit IgG. Protein G-Sepharose beads were added to the sample, which was further incubated for 3 h at 4 °C. After the beads were thoroughly washed with the same buffer, bound proteins were subjected to SDS-PAGE followed by immunoblot analysis.

**RNAi**—Stealth double-stranded RNAs were purchased from Invitrogen. The sequence of siRNA specific to SGIP1α was 5’-UUU ACC CAG AAA UCC UUG GUA UUG G-3’ (corresponding nucleotide 1997–2021 relative to the start codon) and a double-stranded RNA targeting luciferase (5’-CGU ACG CGG AAU ACU UCG AAA UGU C-3’) was used as a control. N1E115 cells were transfected with 20 nM siRNA using Lipofectamine 2000 reagent (Invitrogen). After 24 h, a second transfection was performed, and the cells were cultured for 72 h and subjected to various experiments.

**FIGURE 3. Binding of SGIP1α to phospholipids.** A, binding specificity and dose dependence. Upper panel, phospholipid specificity. MBP-SGIP1α-4 (MP domain) was incubated with synthetic PE/PC liposomes supplemented with 10% of the indicated phospholipid followed by ultracentrifugation. Comparable amounts of the supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE (10% polyacrylamide gel) followed by CBB staining. Lower panel, PS dose dependence. Synthetic liposomes supplemented with various percentages of P5 or brain lipid (Folch lipid) were also analyzed. Asterisks indicate proteolytic product of MBP-SGIP1α-4. PI(3,4)P2, PI 3,4-bisphosphate; PI(3,5)P2, PI 3,5-bisphosphate; PI(3,4,5)P3, PI 3,4,5-trisphosphate. B, quantitative analysis. Various amounts of MBP-SGIP1α-4 (MP domain) were mixed with PE/PC liposomes supplemented with 20% PI(4,5)P2 followed by ultracentrifugation. Amounts of free and bound MP domain were calculated by determining the protein amounts from the supernatant and pellet fractions with a densitometer. Inset, Scatchard analysis. C, liposome tubulation by the MP domain. Brain lipid liposomes containing 5% rhodamine-conjugated PE were incubated with MBP alone or MBP-SGIP1α-4 (MP domain) followed by confocal microscopy. Scale bar, 5 μm.

**FIGURE 4. Binding of SGIP1α to Eps15 and the AP-2 complex.** A, structures of Eps15 and pPrey clones. B, in vitro direct binding of SGIP1α to Eps15. MBP alone or MBP-Eps15 (aa 593–834) was incubated with GST alone or GST-SGIP1α-3 (aa 561–854) immobilized on glutathione-Sepharose beads. Each original sample and eluate were subjected to SDS-PAGE (12% polyacrylamide gel) followed by CBB staining. C, in vivo binding of SGIP1α to Eps15 and the AP-2 complex. N1E115 cell lysate was subjected to immunoprecipitation (IP) with anti-SGIP1α pAb. The immunoprecipitate was then subjected to SDS-PAGE (8% polyacrylamide gel) followed by immunoblot analysis using anti-SGIP1α pAb, anti-Eps15 mAb, anti-α-adaptin mAb, and anti-γ-adaptin mAb. IB, immunoblot.
Endocytosis Assays—Endocytosis in COS7 cells was assayed using fluorescent ligands as described (12, 20). Endocytosis assays in N1E115 cells using fluorescent ligands were performed as described (12, 20) with slight modifications. Briefly, cells were starved with serum-free Dulbecco’s modified Eagle’s medium for 2 h and incubated with 25 μg/ml Alexa Fluor 594-conjugated Tfn (Invitrogen) or 20 ng/ml Texas red-conjugated, biotinylated EGF (Invitrogen) for 10 min at 37 °C. After acid stripping, cells were fixed with formaldehyde. The average intensity of internalized ligands per cell area was determined using MetaMorph imaging system software (23). Endocytosis assays in N1E115 cells using radioactive ligands were performed as described (24, 25) with slight modifications. Briefly, cells were starved for 2 h and incubated with 10 nM 125I-labeled Tfn (PerkinElmer) or 1.5 ng/ml 125I-labeled EGF (PerkinElmer) for the indicated periods of time at 37 °C in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin. Internalized and surface radioactivity was quantified by a gamma counter. Nonspecific binding was measured for each time point in the presence of 100-fold molar excess of the same unlabeled ligand.

Other Procedures—Tubulin was prepared from fresh porcine brains as described (26, 27). The yeast two-hybrid library constructed from adult rat brain cDNA was screened using pBTM116HA-SGIP1α-7 (aa 428–854) as bait as described (28, 29). Protein concentrations were performed with bovine serum albumin as a reference protein (30). SDS-PAGE was performed as described (31).

RESULTS

Identification of p100 as a Tubulin-binding Protein—To detect tubulin- and/or MT-binding proteins, we performed tubulin blot overlay. When various rat tissue homogenates were subjected to blot overlay, several bands with various molecular masses were detected (Fig. 1A). Of these protein bands, a thick band of about 100 kDa (p100) was detected only in the brain. By successive column chromatographies, p100 was copurified with another band of about 110 kDa (p110) (supplemental Fig. 1). These protein bands were separately excised out from the gels, digested with trypsin, and subjected to mass spectrometry. From the p100 protein band, four peptide sequences were obtained (supplemental Fig. 2A). A data base search revealed that the peptide sequences of p100 were identical to those of a mouse hypothetical protein (GenBank accession number BC017596). p110 was found to be CLIP-115, a brain-specific MT-binding protein (32). We performed PCR to amplify cDNA of the hypothetical protein using mouse brain cDNA as a template. Our cloned cDNA encoded a protein that consisted of 854 aa and showed a calculated molecular weight of 91,719 (DDBJ/EMBL/GenBank accession number AB262964) (supplemental Fig. 2A). This protein contained a proline-rich domain in the middle region, whereas it did not possess any conserved domains in the N-terminal or C-terminal region (see Fig. 2A).

During the study, the mouse hypothetical protein was found to be a recently reported neuronal protein, named SGIP1 (14). When the aa sequences of our clone and SGIP1 were aligned, they showed 94% identity (supplemental Fig. 2A). Our clone had two inserted peptide sequences at its N-terminal and middle regions. It is likely that our clone is a longer splicing variant of SGIP1; therefore, the protein encoded by our clone was named SGIP1α. To confirm whether SGIP1α is detected by tubulin blot overlay, we expressed a Myc-tagged protein in COS7 cells. Myc-tagged SGIP1α was detected by blot overlay (Fig. 1B).

![FIGURE 5. Colocalization of SGIP1α with Eps15 and the AP-2 complex. A, N1E115 cells. B, COS7 cells. Cells were transfected with pEGFP-SGIP1α (full-length) and double-stained with anti-EGFP mAb and anti-Eps15 pAb or with anti-EGFP pAb and anti-α-adaptin mAb. Samples were examined with a confocal microscope. Insets, enlarged images of dashed boxes. Scale bars, 10 μm.](image-url)
Membrane Tubulation by SGIP1α Overexpression—To examine the binding of SGIP1α to tubulin and/or MTs in intact cells, we examined the subcellular distribution of SGIP1α. When transiently expressed at a high level in COS7 cells, EGFP-SGIP1α (full-length) showed short tubular structures (Fig. 2A). EGFP-SGIP1α-1 (aa 1–280) and -4 (aa 1–97) also showed tubular structures, whereas other fragments, including EGFP-SGIP1α-2 (aa 261–580), -3 (aa 561–854), and -5 (aa 98–280), did not. These results indicate that the N-terminal region (aa 1–97) of SGIP1α is responsible for the formation of tubular structures. These structures appeared to be different from those of MTs but were reminiscent of those recently reported to be formed by phospholipid-binding BAR and EFC domains (11, 12, 20). To examine the possibility that the N-terminal region (aa 1–97) binds to the plasma membrane and deforms it into tubules, COS7 cells overexpressing EGFP-SGIP1α-4 (aa 1–97) were stained with DiIC16(3). DiIC16(3) is a lipophilic fluorescent probe used for plasma membrane staining (33). The tubular structures of EGFP-SGIP1α-4 were completely overlapped with DiIC16(3) staining (Fig. 2B). This result indicates that tubular structures originate from the plasma membrane. It remains elusive whether SGIP1α binds to tubulin and/or MTs in intact cells. Based on the observation that the N-terminal region (aa 1–97) of SGIP1α binds to membrane phospholipids as described below, we named this region the MP domain, which shows no significant homology to any proteins in the current protein data base.

Direct Binding of SGIP1α to Phospholipids—To examine whether the MP domain directly interacts with membrane phospholipids in vitro, we performed liposome cosedimentation assay. Although MBP-SGIP1α-4 (MP domain) did not bind to synthetic liposomes composed of PE and PC, it strongly bound to liposomes containing PS, PI 3-phosphate, PI 4-phosphate, PI 3,4-bisphosphate, PI 3,5-bisphosphate, or PI(4,5)P2 (Fig. 3A). The MP domain faintly bound to liposomes containing PI, PI 5-phosphate, or PI 3,4,5-trisphosphate. The percentage of PS in liposomes for maximal binding was about 10%. The MP domain bound to brain lipids ( Folch fraction) rich in PS (about 50% of total lipids). This binding specificity of the MP domain is similar to that of the FBP17 EFC domain (11, 12). The Kd value of the MP domain for PI(4,5)P2 was calculated to be about 5 × 10⁻⁷ m (Fig. 3B). This value is comparable with those of the PLCβ1 pleckstrin homology domain, the epsin ENTH domain, and the FBP17 EFC domain, which are known to bind PI(4,5)P2 with high affinity (8, 9, 12). We next examined using synthetic liposomes containing rhodamine-conjugated PE whether the MP domain deforms liposomes into tubes. MBP-SGIP1α-4 induced tubulation, whereas MBP alone did not (Fig. 3C). Taken together, these results indicate that SGIP1α binds to membrane phospholipids and deforms membranes into tubes through the MP domain.

Interaction of SGIP1α with Eps15 and the AP-2 Complex—We then attempted to identify an SGIP1α-interacting protein(s) using the yeast two-hybrid method. We screened 2.5 × 10⁶ clones of a prey cDNA library from rat brain with a bait construct, pBTM116HA-SGIP1α-7 (aa 428–854), and 14 independent clones were obtained. Of these clones, pPrey clones 1–6 encoded C-terminal regions of Eps15 (Fig. 4A). Eps15 is an essential adaptor protein of clathrin-mediated endocytic machinery (34–37). It directly interacts with the AP-2 complex and is involved in the formation of clathrin-coated pits. Eps15 consists of three Eps15 homology (EH) domains, a coiled–coil region, an Asp-Pro-Phe (DPF) repeat region, and two ubiquitin-interacting motifs (UIMs) (34). To examine the in vitro
direct binding of SGIP1α and Eps15, we prepared MBP-Eps15 (aa 593–834). It bound to GST-SGIP1α-3 (aa 561–854) immobilized on glutathione-Sepharose beads, whereas MBP alone did not (Fig. 4B). Neither MBP alone nor MBP-Eps15 bound to GST immobilized on beads. These results indicate that the C-terminal region of SGIP1α directly binds to the DPF repeat region of Eps15 in vivo.

We next examined the in vivo binding of SGIP1α to Eps15 by immunoprecipitation. For this purpose, we used N1E115 neuroblastoma cells because Northern and Western blot analyses showed that SGIP1α was predominantly expressed in neural tissues (supplemental Fig. 2B). When endogenous SGIP1α was immunoprecipitated from N1E115 cells with anti-SGIP1α pAb, endogenous Eps15 was co-precipitated (Fig. 4C). α-Adaptin, a component of the AP-2 complex, was also co-precipitated with SGIP1α and Eps15; however, γ-adaptin, a component of the AP-1 complex, was not. We further examined the localization of SGIP1α, Eps15, and the AP-2 complex by immunofluorescence microscopy. When transiently expressed at a low level in N1E115 cells, EGFP-SGIP1α showed a punctuate staining pattern and was colocalized with endogenous Eps15 and α-adaptin (Fig. 5A). The percentage of SGIP1α-Eps15 colocalization in the plasma membrane region was similar to that in the intracellular region (38.6 ± 3.2 versus 41.1 ± 0.9%, respectively), whereas the percentage of SGIP1α-α adaptin colocalization in the plasma membrane region was higher than that in the intracellular region (27.7 ± 2.5 versus 14.7 ± 1.8%, respectively). When EGFP-SGIP1α overexpression induced membrane tubulation in COS7 cells, Eps15 was recruited to membrane tubules (Fig. 5B). These results indicate that SGIP1α interacts with Eps15 and the AP-2 complex in intact cells.

Localization of SGIP1α at Clathrin-coated Pits/Vesicles—We examined the subcellular localization of SGIP1α when cells were incubated with EGF. EGFP-SGIP1α-expressing N1E115 cells were incubated with EGF for 1 h at 4 °C and moved to a 37 °C incubator. At 0 and 1 min, SGIP1α was colocalized with EGF at the plasma membrane (Fig. 6A). The percentages of SGIP1α-EGF colocalization were as follows: 0 min, 19.4 ± 1.9% (plasma membrane region) and 3.5 ± 0.8% (intracellular region); and 1 min, 21.5 ± 1.7% (plasma membrane region) and 6.7 ± 0.5% (intracellular region). When EGFP-SGIP1α-expressing COS7 cells were incubated with EGF at 4 °C, SGIP1α was colocalized with EGF in tubules at the plasma membrane (Fig. 6B). Taken together with the above observation that SGIP1α is colocalized with Eps15 and the AP-2 complex, these results indicate that SGIP1α is localized at clathrin-coated pits/vesicles.

Involvement of SGIP1α in Clathrin-mediated Endocytosis—We examined the involvement of SGIP1α in endocytosis by overexpression. When EGFP-SGIP1α was transiently expressed in COS7 cells, the uptake of Alexa Fluor 594-conjugated Tfn and Texas red-conjugated EGF was remarkably decreased (Fig. 7A). Within control cells, Tfn and EGF were internalized and observed as dots. Only about 25 and 30% of SGIP1α-overexpressing cells showed Tfn and EGF uptake, respectively, whereas about 90% of control cells did.

We next examined by knockdown using siRNA whether SGIP1α is involved in endocytosis. Western blot analysis revealed that SGIP1α siRNA reduced the expression level of SGIP1α in N1E115 cells (Fig. 8A). In cells with reduced SGIP1α expression, the uptake of Alexa Fluor 594-conjugated Tfn was remarkably reduced when compared with that in cells treated with control siRNA by microscopic analysis (Fig. 8B). In contrast, the uptake of Texas red-conjugated EGF in cells treated with SGIP1α siRNA was hardly reduced when compared with that in cells treated with control siRNA. Similar results were obtained with a quantitative assay that measures the internalization of [125I]-labeled Tfn and EGF (Fig. 8C). When EGFP-SGIP1α mutant, in which siRNA target sequence was silently mutated, was transiently expressed in SGIP1α knockdown cells, Tfn uptake was restored (Fig. 9A and B). Taken together, these findings indicate that SGIP1α is involved in clathrin-mediated endocytosis and required for Tfn endocytosis but not for EGF endocytosis in N1E115 cells.

DISCUSSION

In this study, we identified SGIP1α as a tubulin-binding protein by tubulin blot overlay. It remains elusive whether its tubulin binding is significant. However, the phosphoinositide-binding ENTH domain in epsin has been shown to bind to tubulin and MTs (38). Moreover, the FCH domain, which constitutes the phospholipid-binding EFC domain together with the coiled-coil domain (11, 12), was originally identified as an MT-binding domain (39). It is generally thought that MTs are not required for endocytosis, but the tubulin- and/or MT binding
Role of SGIP1α in Endocytosis

The MP domain binds to phospholipids but shows no significant homology to other phospholipid-binding domains. However, the MP domain exhibits similar properties to BAR and EFC domains (7, 10–13, 20). First, these domains do not show high specificity for phosphoinositides; MP, BAR, and EFC domains show relatively non-specific binding to negatively charged phospholipids. Second, the SGIP1/H9251 MP domain as well as BAR and EFC domains forms an oligomer. The oligomerization of BAR and EFC domains is thought to be important for membrane tubulation (7, 11, 20). The BAR domain is proposed to sense and generate membrane curvature for vesicle formation during endocytosis (10, 13). Therefore, the MP domain, via its membrane-deforming property, may also act in vesicle formation during endocytosis.

We have shown, by overexpression, knockdown, and localization studies, that SGIP1α is involved in clathrin-mediated endocytosis. SGIP1α overexpression as well as knockdown inhibits endocytosis, and this effect may be due to sequestration of its binding protein(s) from functional clathrin-mediated endocytosis machinery. This idea is consistent with the observation that Eps15 is recruited to membrane tubules formed by SGIP1α overexpression. Eps15 is an important adaptor protein of clathrin-mediated endocytic machinery (34–37). Several lines of evidence suggest that Eps15 regulates clathrin coat assembly by interacting with its binding partners including the AP-2 complex. This role of Eps15 in clathrin coat assembly is likely to be closely related to its unique localization at the growing edges of clathrin-coated pits (40). SGIP1α may contribute to the unique localization of Eps15 for clathrin coat assembly during endocytosis. It has been shown that Eps15 and the AP-2 complex are required for Tfn endocytosis but not for EGF endocytosis (35, 37, 41, 42). Consistently, we have shown by knockdown that SGIP1α is required for Tfn endocytosis but not for EGF endocytosis. This different requirement of SGIP1α in clathrin-mediated endocytosis may be due to the presence of redundant pathways, such as the Cbl-CIN85-endophilin pathway, in EGF endocytosis (43). It is most likely that SGIP1α plays an essential role in clathrin-mediated endocytosis not only by deforming the plasma membrane but also by interacting with Eps15 and the AP-2 complex. Further studies are necessary for our better understanding of the role of SGIP1α in endocytosis.
Acknowledgments—We thank Dr. Y. Takai (Osaka University, Osaka, Japan) for pBTM116-HA vector and Dr. K. Kaibuchi (Nagoya University, Nagoya, Japan) for N1E115 cells.

REFERENCES

1. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 37–44
2. Kirchhausen, T. (2000) Nat. Rev. Mol. Cell Biol. 1, 187–198
3. Slepnev, V. I., and De Camilli, P. (2000) Nat. Rev. Neurosci. 1, 161–172
4. Owen, D. J., Collins, B. M., and Evans, P. R. (2004) Annu. Rev. Cell Dev. Biol. 20, 153–191
5. Sorkin, A. (2004) Curr. Opin. Cell Biol. 16, 392–399
6. Traub, L. M. (2005) Biochim. Biophys. Acta 1744, 415–437
7. Takei, K., Slepnev, V. I., Haucke, V., and De Camilli, P. (1999) Nat. Cell Biol. 1, 33–39
8. Itoh, T., Koshiba, S., Kigawa, T., Yokoyama, S., and Takei, K. (1999) Mol. Biol. Cell 10, 2489–2499
9. Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A., and Takai, Y. (1999) J. Cell Biol. 145, 539–549
10. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
11. De Zeeuw, C. I., Hoogenraad, C. C., Goedknegt, E., Hertzberg, E., Neubauer, A., Gais, M., and Galljart, N. (1997) J. Cell Biol. 139, 951–961
12. Kashiba, S., Kigawa, T., Yokoyama, S., and Takei, K. (1999) Mol. Biol. Cell 10, 2489–2499
13. Conner, S. D., and Schmid, S. L. (2003) J. Cell Biol. 162, 773–779
14. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1999) J. Biol. Chem. 274, 28059–28066
15. Hess, P. H., Waheed, S., and Schmid, S. L. (2001) J. Cell Biol. 153, 657–666
16. Conner, S. D., and Schmid, S. L. (2003) Trends Biochem. Sci. 28, 598–603