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Localization of Neuronal Growth–Associated, Microtubule-Destabilizing Factor SCG10 in Brain-Derived Raft Membrane Microdomains[1]

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Raft is a mobile membrane subdomain enriched in sphingolipid and cholesterol and also various signaling molecules. Previous observation suggested that brain-derived rafts contain tubulin but that rafts of non-neural origin do not. We hypothesized that SCG10, one of the neuronal growth-associated proteins (nGAPs), might be a neuron-specific molecule that anchors tubulin to neuronal rafts, and we explored biochemically its subcellular localization, interaction with tubulin, and effects on microtubule dynamics. In postnatal rat brain extracts, SCG10 was recovered mostly in membrane-associated fractions, and at least half was included in the raft fraction that was also enriched in GAP-43 and NAP-22. SCG10-enriched brain rafts also contained tubulin, and chemical cross-linking experiments revealed that SCG10 was closely associated with tubulin. In addition, SCG10 was able to inhibit polymerization of tubulin. These results indicate that SCG10 is a component of neuronal rafts as are other nGAPs, and suggest that SCG10 may be involved in signaling events in membranes for cytoskeletal reorganization around neuronal rafts.

Key words: caveolae, neuron, signaling, stathmin, tubulin.

A membranous microdomain called "raft" has received much attention because this membrane organelle region is rich in many signal transduction molecules (*1–4*), and thus seems to be a major activation site of cells for extracellular to intracellular signaling (for review, see (*5–7*)). Raft is involved in membrane trafficking toward endocytosis that occurs in most cells, and is also related to receptor internalization in neurons (*8–10*). Interestingly, the raft of brain origin contains tubulin as a major constituent, and is distinct from other nonneural cell- and/or tissue-derived rafts (*11*). Given that tubulin is a ubiquitous component of cells and that its expression occurs in all cell types, this fact suggests the possible existence of a neuron-specific protein that is abundant in and anchors tubulin to the neuronal raft. Raft is thought to be assembled in the Golgi region and thereafter transported to the distal plasma membrane (*5*). Since SCG10 is known to be a tubulin-binding protein (*12*), and also to be localized in the Golgi fraction (*13*, *14*) and/or the growth cones of developing neurons (*15*), we speculated that SCG10 might be a component of brain-derived raft microdomains. Moreover, SCG10 could be an anchoring molecule of membrane organelles, because it is palmitoylated on cysteine clusters in its N-terminally located hydrophobic domain (*13*). Thus, SCG10 fulfills multiple requirements as a potential neuronal anchoring protein of brain rafts.

One principal function of neuronal raft microdomains is considered to be transmission of signals from membrane receptors and/or cell adhesion molecules to effect reorganization of the intracellular cytoskeleton (*5*). In neurons, the control of both actin and microtubule networks is crucial for precise regulation of neuronal structural and functional plasticity (*16–19*). Accumulated evidence suggests that various Rho-related molecules regulate the actin cytoskeleton (*20–22*). On the other hand, there is little evidence as to what signals regulate microtubule dynamics, although recent results suggest that assembly and disassembly of microtubules are under the control of several factors including SCG10 and its related homologues (*23*). Among the SCG10 family members (*24–27*), stathmin is the most well-characterized microtubule regulatory molecule (*28–33*), and was shown to form a T2S complex consisting of one tubulin dimer and one stathmin molecule (*34*, *35*). Interestingly, this association is inhibited by phosphorylation of several serine residues on stathmin (*36*, *37*). SCG10 is a neuron-specific isoform of stathmin (*24*, *38*), is expressed predominantly in developing neurons (*15*), and, like stathmin, also binds tubulin dimers (*12*, *39*). SCG10 also bears multiple serine residues that can be phosphorylated by various protein kinases, and the SCG10–tubulin interaction is inhib-

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ited by phosphorylation (*40*) (Morii *et al.*, unpublished observations). SCG10 expression is developmentally regulated: high expression of SCG10 occurs in the neonatal stages, and its expression is reduced sharply during postnatal maturation (*15*, *41*, *42*). On the contrary, the expression of more recently identified SCG10-related molecules, such as RB3 and SCLIP (*i.e.*, SCG10-like-protein), is increased even in mature animals (*25–27*). Within the neuron, SCG10 is localized in the Golgi region as well as in the growth cone, and its membrane localization has been ascribed to its palmitoylation (*13*).

In the inner leaflet of neuronal rafts, many lipid-anchored proteins such as Src family protein kinases and trimeric G proteins are known to accumulate. In addition, brain-derived rafts contain at least two neuron-specific, lipid-anchored, calmodulin-binding proteins, *i.e.*, GAP-43 (also called B50, F1, and neuromodulin) and NAP-22, as major protein components (*11*, *43–45*). These results suggest that neuronal rafts are endowed with many neuronal growth–associated proteins (nGAPs) (*38*, *46*) and serve as a major site of signal transduction within neurons, which transduction may be tightly associated with neuronal growth as well as stimulation. Therefore, identification and characterization of raft components of neurons are important not only to dissect out the molecular components but also to understand the mechanism of information processing by the neuronal rafts.

In this study, we provide evidence that another nGAP, *i.e.*, SCG10, is a major component of neuronal rafts, forms a complex with other nGAPs, and is associated with tubulin in the brain-derived raft fraction. We also show that SCG10 can depolymerize microtubules with a similar efficiency to stathmin.

MATERIALS AND METHODS

Materials—Wistar rats (SLC, Shizuoka) were used for these experiments. The anti-tubulin monoclonal antibody (11-8C7) was produced using a microtubule fraction of rat brain as the antigen, and was screened by Western blotting for reactivity against the tubulin dimer. Chemical crosslinkers, *e.g.*, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and glutaraldehyde, were purchased from Pierce; PVDF membrane was from Millipore; and other chemical reagents including aprotinin, antipain, pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl (p-ABSF), phenylmethylsulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from either Nacalai Tesque or Wako.

Screening for SCG10 Monoclonal Antibody—Human SCG10 lacking its N-terminal 28 amino acids was expressed as a GST fusion protein (pGST-hSCG10) and purified by using a glutathione-Sepharose 4B column (Pharmacia). Immunization, cell fusion, and hybridoma selection were performed as described previously (*43*). Rat brain crude fraction (1,000 ×*g* supernatant of P3 brain homogenate) was used for the screening, and one clone (SCG10-4B4) was selected.

Construction of GST-SCG10 and SCG10-GFP and Cellular Transfection—The coding sequences of rat stathmin, SCLIP, and RB3 were generated by RT-PCR as described previously (*42*), and that of SCG10 was taken from pSCG10-8 (*38*). The deletion mutants were prepared by PCR using appropriate sets of primers. Their amplified DNA fragments were subcloned into pGEX-series expression vectors (Pharmacia) for generating glutathione-S-transferase (GST)–fused proteins or into pEGFP-N1 vectors (Clontech) to make GFP-fused proteins. For GST-SCG10 and related proteins, the expression of recombinant proteins in BL21 was induced by incubation with 0.1 mM IPTG at 18°C overnight. The cells were collected by centrifugation and stored at –80°C until used. The frozen cells were thawed, suspended in PBS containing 1 mM EDTA, 1 mM pABSF, 10 μg/ml leupeptin, and 1 μg/ml aprotinin, and sonicated. The lysate was centrifuged at 20,000 ×*g* for 1 h. The recombinant proteins were purified from the supernatant by passage through a column of glutathione-Sepharose 4B, and concentrated with an Ultrafree-15 centrifugal device (Millipore). The protein concentrations were determined by CBB staining on SDS-PAGE with BSA as a standard. The transfection of COS cells was performed by using Lipofectamine-PLUS reagents (Gibco-BRL). Cells were collected 24 h later, and cellular lysates were used for Western blot analysis. Details of plasmid construction and expression procedures are available upon request.

Subcellular Fractionation of Triton-Soluble and -Insoluble Fractions—Brain from rats at postnatal day 7 (P7) was homogenized in TME solution (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA; pH 7.5) in the presence of a protease inhibitor cocktail (1 mM PMSF, 1 μg/ml aprotinin, 0.01 mg/ml of pepstatin A, and antipain) and centrifuged at 100,000 ×*g* for 60 min. The cytoplasmic supernatant (CS) and the pellet fraction were obtained. The pellet fraction was further homogenized in TME containing 2% Triton X-100 plus protease inhibitors, and centrifuged at 100,000 ×*g* for 90 min. The supernatant was recovered as the Triton-soluble (TS) fraction. The remaining Triton-insoluble (TIS) fraction, *i.e.*, pellet fraction (P1) was separated into two layers, a loosely packed white layer (upper) and a tightly packed brown layer (lower). The upper white layer was suspended with gentle pipetting after the addition of small amount of TME solution (P2). The lower brown layer that remained attached to the centrifuge tubes was then suspended in TME with a Teflon-glass homogenizer (P3). To further fractionate the P2 fraction, P2 suspension was placed on the top of a continuous sucrose gradient (0.4–1.4 M) in a centrifuge tube and centrifuged at 35,000 rpm for 5 h (Hitachi, RPS40Ti). After centrifugation, samples were aspirated from the bottom and fractionated. Each fraction was diluted with TME, then centrifuged at 100,000 ×*g* for 60 min. The precipitates were suspended in TME and subjected to Western blotting using 4B4 and 8D5 (*43*) antibodies against SCG10 and NAP-22, respectively.

Chemical Crosslinking—EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; final conc. of 12 mM) was added to the raft fraction (P2), which was then incubated for 5 min at 37°C. Proteins were resolved by 8% SDS-PAGE followed by Western blotting using antibodies against tubulin and SCG10.

Microtubule Sedimentation Assay—Tubulin was prepared from bovine brains by two cycles of assembly and disassembly. The resulting crude tubulin, the purity of which was estimated to be about 75%, was referred as to C2S-tubulin. Tubulin assembly was carried out for 20 min at 37°C in a reaction mixture containing 20 μM C2S-tubulin, 1 mM GTP, RB buffer (0.5 mM $MgCl_2$, 1 mM EGTA, and

0.1 M MES-KOH; pH 6.8), and the mixture was separated by centrifugation at 100,000 ×*g* for 20 min at 35°C. The resulting supernatant (S) was collected, and the pellet (P) was dissolved in RB buffer. The S and P samples were separated by SDS-PAGE and stained with CBB. Quantification of the band density of tubulin was performed by using a densitometer (ATTO). The extent of microtubule formation was expressed as a ratio of the band intensity in lane P to the sum of those in lanes S and P (*i.e.*, total amount of tubulin), and the relative inhibition of polymerization was calculated from the extent of microtubule formation in the absence of SCG10 or stathmin as 0% inhibition.

RESULTS

Generation and Characterization of SCG10-Specific Monoclonal Antibody 4B4—We generated a monoclonal antibody using a stathmin-like domain of SCG10 that lacked the N-terminally located hydrophobic region (including 28 amino acids). On screening 325 hybridomas, we isolated a single clone (SCG10-4B4). This antibody reacted with the GST-SCG10 fusion protein and showed no reaction with the GST protein itself (data not shown). To further test the specificity of the 4B4 antibody, we expressed SCG10 family proteins fused with GFP in COS cells and examined blots of them with the 4B4 antibody. The antibody recognized a band corresponding to the GFP-SCG10 fusion protein, which had a molecular size of about 50 kDa, and did not cross-react with other family members (Fig. 1), thus indicating the 4B4 antibody to be specific for SCG10. The antibody was also used to detect the expression of native SCG10 in developing rat brain (Fig. 2). The Western blot showed apparently at least four bands distributed around 22–30 kDa, which are designated as bands "a–d" in Fig. 2. Band "a" should be non-phosphorylated SCG10, and "b–d" could correspond to phosphorylated SCG10, since alkaline phosphatase treatment abolished these upper bands (Morii *et al.*, data not shown). The temporal expression of SCG10, with sharp reduction at postnatal day 21, is consistent with the previous observation made by using a polyclonal SCG10 antibody (*41*). Heterogeneity in the mobility on SDS-PAGE and the expression pattern during development coincide well with the previous reports on SCG10 (*2, 6, 16, 20*). These results confirmed the specificity of the 4B4 antibody and further clarified that the antibody did not cross-react even with the closely related SCLIP and RB3. The 4B4 antibody was also used to stain SCG10 in dissociated neurons in culture, and intensive staining at the growth cones was noted (Morii *et al.*, data not shown).

Fractionation and Subcellular Localization of SCG10—We determined the subcellular distribution of SCG10 by biochemical fractionation followed by Western blotting using the aforementioned 4B4 antibody. We used P7 brain, since the brain was enriched in SCG10 at this stage (see Fig. 2). The brain homogenate was fractionated by a series of centrifugations (see "MATERIALS AND METHODS") into several fractions: CS, cytoplasmic supernatant; TS, Triton-soluble fraction; P1, P2, and P3 as TIS, Triton-insoluble fractions. P2 was further fractionated on a linear sucrose density gradient. Western blotting of these fractions showed that after the first homogenization, almost all of SCG10 was recovered in the insoluble fraction, and some part of this SCG10 is solubilized with Triton X-100 (Fig. 3A). About 40% of the SCG10 was solubilized with Triton X-100 and about half of the total amount of SCG10 was recovered in the P2 fraction. The P2 fraction was further fractionated on a sucrose gradient, and Western blotting was performed using the 4B4 antibody (Fig. 3B). SCG10 was recovered in low-density fractions that peaked around fractions 16 and 17. NAP-22, a marker protein of brain rafts, was also recovered in these fractions; but NAP-22 peaked at the slightly denser fraction 14. Another raft-enriched protein, tubulin, was quantified by densitometry of a CBB-stained gel. The tubulin distribution apparently paralleled that of SCG10 (Fig. 3C). Other proteins including Thy-1 and trimeric G proteins were also recovered in

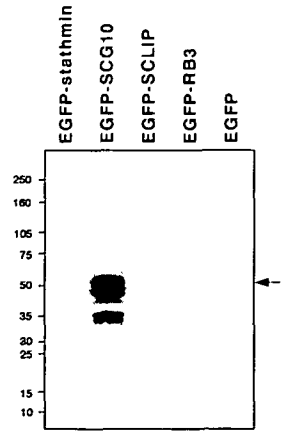

Fig. 1. **Specificity of anti-SCG10 monoclonal antibody 4B4.** The coding region of each of the SCG10 family molecules was expressed in COS cells as C-terminally tagged GFP-fusion proteins, and 5 μg of total protein was separated by 10% SDS-PAGE, followed by Western blotting using the 4B4 monoclonal antibody. The size of a major band around 50 kD corresponds closely to the estimated molecular mass of the SCG10-GFP fusion protein. Note that no cross-reactivity is observed with other members of the SCG10 family proteins.

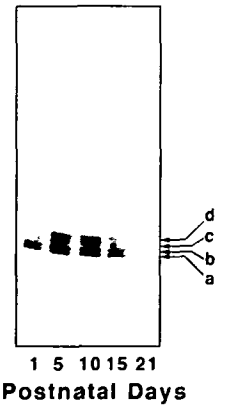

Fig. 2. **Specific detection by 4B4 antibody of endogenous SCG10 in rat brain extracts.** Whole brain extracts were prepared from the brains of Wister rats at postnatal days 1, 5, 10, 15, and 21, and 5 μg of protein of each extract was separated on a 13% SDS–polyacryl amide gel and blotted with anti-SCG10 antibody 4B4. Note that at least four bands (a–d) are apparently visible, and each band corresponds to phosphorylated and non-phosphorylated forms of SCG10 (see text for details).

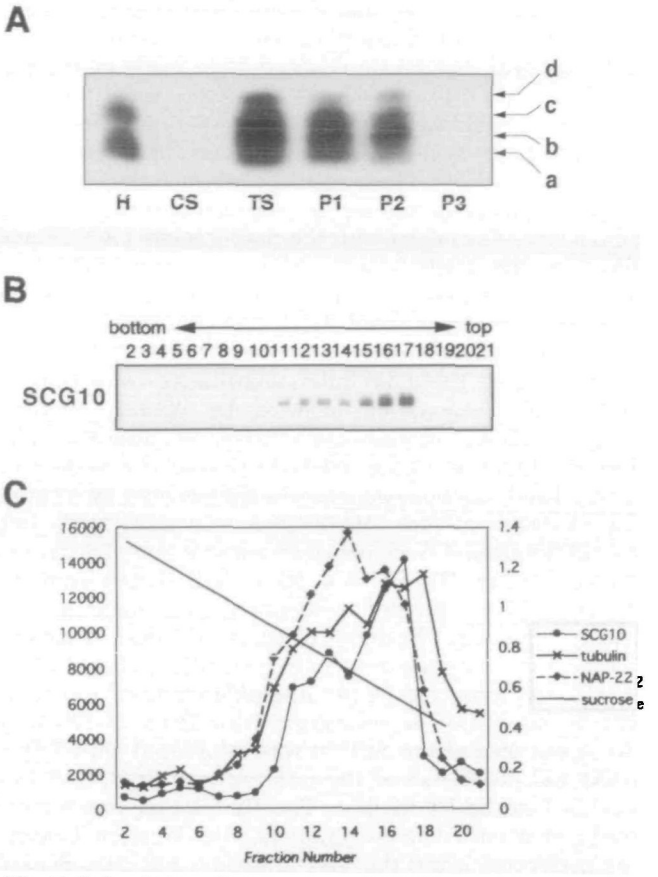

Fig. 3. **Subcellular distribution of SCG10 and its colocalization with other nGAPs and tubulin in the brain-derived raft fraction.** (A) Western blot analysis of brain fractions. Five micrograms of protein of each fraction was analyzed by Western blotting as in Fig. 2. H, homogenate; CS, crude supernatant; TS, Triton supernatant; P1, Triton pellet; P2, Triton loose upper layer; and P3, Triton tight lower layer. (B) Fractionation of SCG10 in the P2 fraction by sucrose density gradient centrifugation. SCG10 was visualized on a Western blot by using the ECL system. (C) Summary of fractionation of SCG10, NAP-22, and tubulin through a sucrose density gradient centrifugation. Each fraction was subjected to SDS-PAGE and Western blotting using 4B4 and 8D5 antibodies against SCG10 and NAP-22, respectively. The amount of each protein was quantified by densitometry of each immunoreactive band and the amounts of tubulin in a CBB-stained gel.

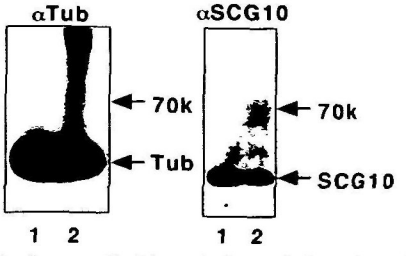

Fig. 4. **Chemical cross-linking of the raft fraction.** A mixture of fractions (#16 and #17) derived from rat brain (*i.e.*, the raft fraction) was treated (lane 2) or not (lane 1) with 12 mM EDC, and separated on an 8% SDS-PAGE gel. Shown are the results of Western blot performed by using anti-tubulin antibody 11-8C6 (αTub) and anti-SCG10 antibody 4B4 (αSCG10). In the 8% SDS-PAGE gel, SCG10 migrated to the gel front and therefore phosphorylated subforms of SCG10 were unresolved.

the raft fraction; however, the distribution of neither of these proteins paralleled that of SCG10 or tubulin (data not shown). The overall fractionation data support the idea that SCG10 is colocalized with tubulin in the Triton-insoluble fraction.

Direct Association of SCG10 with Tubulin—To further determine whether SCG10 directly binds tubulin or not, we performed a chemical cross-linking experiment. The addition of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), a zero-length cross-linker, to the brain-derived raft fraction, *i.e.*, P2 fraction (#16 and #17 in Fig. 3B), caused the appearance of a new band of about 70 kDa that reacted with both antibodies to tubulin and to SCG10 (see Fig. 4).

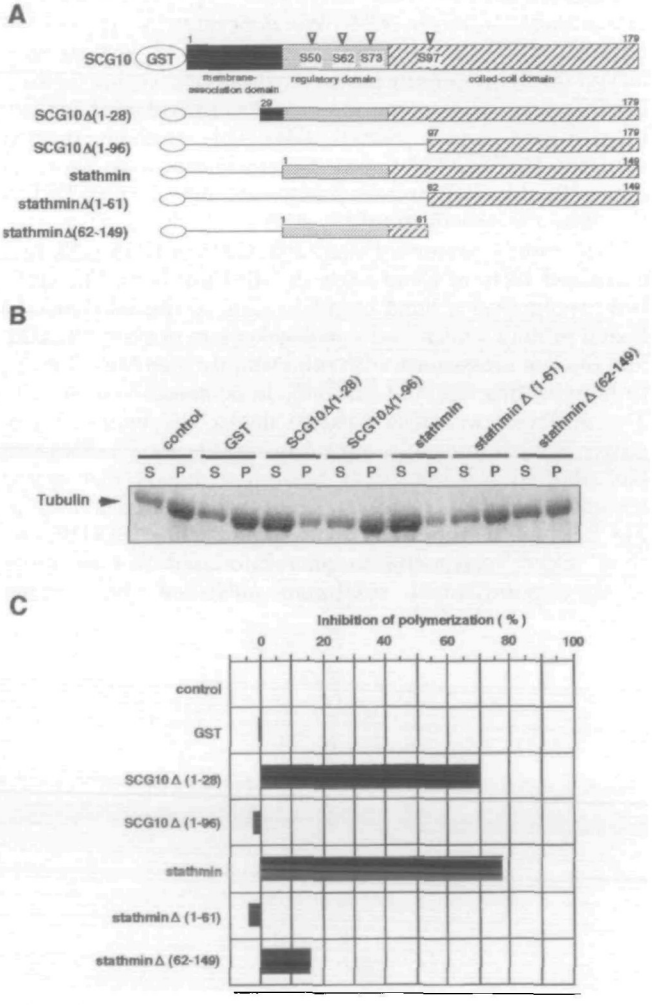

Fig. 5. **Inhibitory effects of SCG10 on microtubule polymerization.** (A) Schematic representation of the deletion mutants of GST-SCG10 and GST-stathmin used in this study. (B) Microtubule polymerization was measured in the presence of GST-SCG10 or GST-stathmin by using a sedimentation assay. After the incubation of tubulin (*i.e.*, C2S) with GST-SCG10, microtubules, *i.e.*, polymerized tubulin, were separated by ultracentrifugation; and tubulin in the supernatant (S) and pellet (P) fractions was detected on a 10% SDS-PAGE gel by CBB staining. (C) Effects of SCG10, stathmin, and their partial deletion constructs on microtubule polymerization. The band density of tubulin, which was detected in lanes S and P in (B), was measured, and relative inhibition of polymerization was determined. The amount of tubulin in lane P of the control was defined as 0% inhibition of microtubule formation.

Since tubulin is a dimeric protein, chemical cross-linking experiments result in the formation of higher (over 100 kDa) molecular mass complexes. Furthermore, because the cross-linking occurs at random, the cross-linked proteins usually show a heterogeneous mobility in SDS-PAGE. The broad, high-molecular-mass reactive band observed in Fig. 4 (anti-tub, lane 2, the lower part of this band is shown at the top) corresponds to the tubulin dimers and/or oligomers. To detect the binding of tubulin to SCG10, therefore, short-term cross-linking experiments using low doses of the cross-linkers were attempted. This resulted in the appearance of a 70-kDa band reactive both to anti-tubulin and to anti-SCG10 antibodies (Fig. 4). A similar result was obtained when glutaraldehyde was used as a cross-linker (data not shown). These data provide direct evidence of SCG10-tubulin association in the brain-derived raft microdomain, and suggests that tubulin and SCG10 reside, at least in part, in close proximity on rafts.

SCG10 Inhibits Polymerization of Tubulin to Form Microtubules—The effect of SCG10 on microtubule polymerization was previously examined by using a partial construct that lacked the N-terminally located hydrophobic segment (*39*). By using a centrifugation-mediated precipitation assay, we confirmed the effect of such a construct (SCG10Δ1-28) on the inhibition of microtubule formation, and compared its activity with that of stathmin and other partial deletion constructs (Fig. 5). The addition of the GST-fused SCG10 (SCG10Δ1-28) to the microtubule protein fraction (the so-called C2S fraction) inhibited microtubule formation as much as 70–80%, as in the case of stathmin. In contrast, constructs containing only a portion of the C-terminal domain (SCG10Δ1-96 and stathminΔ1-61) had little effect on the microtubule formation, although the stathmin construct that retained the central domain and a small portion of its C-terminal domain (stathminΔ62-149) showed a small but significant effect (15–20%). These results confirmed the previous observation (*38*), but further suggested that the amino acid sequences around the central regulatory domain (rich in potential phosphorylation sites) and a portion of the C-terminal domain are essential for the inhibition of microtubule formation *in vitro*.

DISCUSSION

In this study, we demonstrated that SCG10, a tubulin-binding membrane protein, is a component of the brain-derived raft. SCG10 was previously shown to localize in the growth cone and the Golgi region in cultured neurons and in PC12 cells (*13–15*). The N-terminal region of SCG10 is known to be necessary for membrane targetting and Golgi localization, and two cysteine residues (Cys^{22} and Cys^{24}) in this region are sites of palmitoylation (*13*). This palmitoylation facilitates the direct membrane interaction of SCG10 through the N-terminal region, similarly to the case of caveolin (*47*). Raft is assembled at the Golgi region and then transported to the cell membrane (*5*, *6*). The intracellular localization of SCG10, thus, coincides well with the currently understood scheme of assembly and transport of the raft microdomains.

Colocalization of SCG10 and Tubulin in Brain-Derived Raft Microdomains—In a previous study, we showed that tubulin is a major protein in the brain-derived raft (*11*). In the rafts prepared from non-neuronal cells and tissues, tubulin has never been reported to be present (*5*, *6*, *47*). This suggests that the mechanism of localization of tubulin in brain-derived rafts involves interaction with some neuron-specific protein in raft, rather than the direct interaction with lipids in raft. Our current result showed that at least a certain proportion of SCG10 in raft is localized in close proximity to tubulin monomers. Detection of a 70-kDa complex suggests that the complex is T1S (one tubulin monomer and one SCG10), although the chemical cross-linking experiments also detected additional bands, though less efficiently, at high molecular weight regions including 120 kDa. Therefore, the SCG10 in the raft microdomain seems not to be related to tubulin depolymerization, but rather may be involved in membrane-tubulin interaction. Thus, SCG10 may be a tubulin anchor in the neuronal rafts, although further quantitative analyses on the amounts and affinity of the interaction between tubulin and SCG10 in the raft membrane microdomains are needed to clarify this expectation. Expression of SCG10 was relatively strong in neonatal stages, as was shown in Fig. 2, whereas tubulin localization to the raft membranes was also observed in the adult brain (*11*). This may further suggest that other recently identified SCG10 family proteins (RB3 and SCLIP) may function in tubulin anchoring in the rafts of mature neurons.

We showed here that the presence of SCG10 has negative effects, as observed with stathmin, on microtubule formation from tubulin. However, a remarkable fact about the interaction between tubulin and SCG10 and/or stathmin is that the tubulin-depolymerization activity is strongly inhibited by phosphorylation of stathmin (*37*) and also of SCG10 ((*40*); Morii *et al.*, unpublished data). Interestingly, multiple bands were detected by the anti-SCG10 4B4 antibody on the Western blot of the brain-derived rafts, and the presence of the slower migrating species suggested that most of SCG10 is phosphorylated (see Fig. 3A). Heterogeneity of the bands may be ascribed to differential phosphorylation by different protein kinases, such as cAMP-dependent protein kinase, MAP kinase, and cell-cycle dependent kinase (or its related homologues) (*25*, *26*, *40*). However, similar phosphorylated SCG10 was also present in the TS fraction (lacking membrane rafts), and thus the biological meaning of phosphorylation of SCG10 in the neuronal rafts is unclear at present. Future studies on the regulatory mechanism of SCG10 with respect to microtubule polymerization and the effects of various levels of phosphorylation on its activity will help to elucidate further the functional roles of SCG10 in the neuronal rafts.

Roles of nGAPs as Components of Neuronal Raft—The neuronal raft is a major site of signaling over synapse. Why are so many nGAPs present in the neuronal raft? A potential reason for the presence of various nGAPs including SCG10, GAP-43, and NAP-22 may be that the raft is a transient structure (*5*, *6*, *47*), and such a structure may further be transformed into caveola, spines, presynaptic and/or postsynaptic membrane subdomains in neurons. At postsynaptic sites, a variety of signaling molecules including phosphoserine and phosphotyrosine mediators are involved in transmitting signals secreted by receptor and channel activation (*48*, *49*), and these signals should lead to micro-structural changes at the postsynapse, which should be orchestrated by cytoskeletal reorganizations of actin fibers and microtubules (*18*, *19*). The facts that GAP-43 reg-

ulates actin polymerization (*20*, *50–55*) and SCG10 regulates microtubule depolymerization (*39*), and that both are further regulated by phosphorylation suggest that these nGAPs may coordinate neuronal activation signals that lead to neuronal structural changes, which provide a basis for neuronal structural plasticity (*46*, *56*, *57*).

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