**INTRODUCTION**

The BRIs are a new family of integral membrane proteins containing at least three members (BRI1-3). At present, there is some information available pertaining to the biological function(s) of BRIs in neurodegenerative disease. Vial et al. (1, 2) reported on two families of British and Danish origin, affected by a dementing illness similar to Alzheimer’s disease, who had mutations in the BRI2 gene. However, it is notable that the BRI2 gene has been expressed in all tissues tested so far, with high levels of expression in the heart, placenta, kidney, and pancreas; while BRI3 (Accession No. AF272043) is a brain-specific type II integral membrane protein. Northern blot analysis has indicated that BRI 3 mRNA is expressed principally in the brain (3).

Furthermore, Wickham et al. (4) found that BRI1 was a binding partner of β-amyloid protein converting enzyme (BACE) 1, and was also efficiently processed by furin. Greater knowledge on the function of BRI3, as a nerve-specific member of the BRI protein family, could lead us to discover its roles in neuronal development and/or in certain neural diseases. Therefore, we performed a two-hybrid screening of a human fetal brain cDNA library to identify putative downstream effectors of BRI3. Here, we report on a microtubule-destabilizing protein - SCG10 (superior cervical ganglia, neural specific 10) - that was found to associate with the N-terminal domain of BRI3.

SCG10 is a member of the stathmin family of proteins (5, 6). It is a neuron-specific and membrane-associated, microtubule-destabilizing protein of neuronal growth cones. Since the overexpression of SCG10 strongly enhances neurite outgrowth, SCG10 appears to be an important factor for the dynamic assembly and disassembly of growth cone microtubules during axonal elongation (7).

In a continuing effort to understand the role of BRI1 within the nervous system, we discovered a unique link between BRI1 and the cytoskeletal network that may play significant roles in neuronal development and differentiation.

**RESULTS**

**Identification of SCG10 as a BRI1-binding protein**

To identify proteins that associate with BRI1, a yeast two-hybrid screen was performed. The 59 residues of the BRI1 N-terminal [BRI1 (N)] were used as bait, since full-length BRI1 contains a transmembrane region, which is well known for its low efficiency in the yeast two-hybrid system. Since BRI1 is predominantly expressed in brain tissue, a human fetal brain cDNA library was used to screen its interacting proteins. Of the 29 positive clones screened, one encoded a protein called SCG10.

**BRI1 and SCG10 associate in vitro and in vivo**

Native SCG10 protein is difficult to purify, and even more difficult to obtain in high concentration (8). The N-terminal region of SCG10 (amino acids 1-34) was thought to be important for membrane association, and the removal of this region re-
Fig. 1. GST pull-down assay showing interactions of BRI3 and SCG10. (A) Polyclonal anti-BRI3 antibody recognized endogenous BRI3 protein from SH-SY5Y cells, but not from HeLa cells. (B) Coomassie Brilliant Blue staining of the GST fusion proteins used for the pull-down assay. The indicated recombinant GST fusion proteins, immobilized on Sepharose 4B beads, were incubated with SH-SY5Y cell lysate. (C) The pull-down assay showing BRI3 bound and eluted from immobilized GST-ΔSCG10 fusion proteins, but not from GST proteins. (D) Co-immunoprecipitation of BRI3 with SCG10. 293ET cells were co-transfected with pcDNA3.1/V5-BRI3 and pcDNA4/Myc-SCG10, and the cells were immunoprecipitated with anti-Myc or anti-Flag mAb and detected by Western blotting with anti-V5 mAb. (E) Co-localization of BRI3 and SCG10. EGFP-BRI3 and DsRED-SCG10 co-localized in neuroblastoma SH-SY5Y cells when co-expressed.

resulted in a soluble protein (ΔSCG10) that is closely homologous to conventional stathmin (9). Thus, an N-terminal truncated GST-tagged form of ΔSCG10 was constructed to facilitate the purification of SCG10.

To determine whether recombinant SCG10 could bind to endogenous BRI3 in vitro, GST-ΔSCG10 fusion protein immobilized on glutathione beads was used to pull down endogenous BRI3 from SH-SY5Y cells, in which the BRI3 gene is endogenously expressed, and was examined by RT-PCR (data not shown) and Western blot analysis (Fig. 1A). As shown in Fig. 1C, the GST-ΔSCG10 fusion protein pulled down endogenous BRI3. As a control, GST protein alone did not pull down BRI3. We also performed an immunoprecipitation assay in 293ET cells to confirm the BRI3-SCG10 association in vivo by using overexpressed full length BRI3 and SCG10 (Fig. 1D). BRI3 was found to co-immunoprecipitate with SCG10 from the extracts of 293ET cells (Fig. 1D, right lane), and no relative band was found in the control (left lane). These in vitro and in vivo results confirm the association of the two proteins detected in the yeast two-hybrid system.

Co-localization of BRI3 and SCG10 in SH-SY5Y cells

Since the expression of SCG10 is predominantly confined to neuronal tissue (10), we selected neuroblastoma SH-SY5Y cells to examine the subcellular localization patterns of human SCG10 and BRI3. SH-SY5Y cells were transfected with GFP-tagged BRI3 and RFP-tagged SCG10, and their subcellular localization patterns were assessed by the direct measurement of GFP or RFP signals. A mainly perinuclear co-localization of BRI3 and SCG10 was shown in the SH-SY5Y cells by the overlay signals (Fig. 1E, yellow), supporting their association described above.

Influence of BRI3 binding on the ability of SCG10 to modulate microtubule assembly and disassembly

SCG10 belongs to the stathmin protein family. A well-characterized activity of SCG10 is its ability to prevent microtubule assembly (7). To evaluate the effect of BRI3 binding on the microtubule disassembly activity of SCG10, an in vitro assay of microtubule assembly was employed. First, tubulin was purified from porcine brain, as shown by SDS-PAGE analysis in Fig. 2A. Then, the incubation of soluble tubulin at 37°C in the presence of GTP resulted in its polymerization into microtubules, and this process was followed by a turbidimetric assay (Fig. 2B). Consistent with previous results (8), the addition of ΔSCG10 (10 μM) resulted in decreased tubulin polymerization under the assay conditions (Fig. 2C). However, the addition of BRI3 (N) had no effect on tubulin polymerization (data not shown). To evaluate the effect of BRI3 on the ability of ΔSCG10 to modulate microtubule assembly, the assays were performed and repeated by adding ΔSCG10 (10 μM) and varying the concentrations of BRI3 (N). However, even at concentrations as high as 30 μM, BRI3 had no discernible effect on the ability of ΔSCG10 to attenuate microtubule assembly (Fig. 2D).

In addition to its ability to prevent microtubule assembly, SCG10 is also capable of initiating microtubule disassembly both in vitro (7) and in intact cells (11). Although the precise mechanism by which SCG10 triggers microtubule disassembly is not clear, this process can be monitored by the turbidimetric assay described above. Under conditions in which tubulin was polymerized and subsequently treated with ΔSCG10, ΔSCG10 promoted the disassembly of assembled microtubules (Fig. 3A). However, no obvious effect could be detected when the assembled microtubules were treated with...
BRI3 (N) (data not shown). Remarkably, the addition of BRI3 (N) blocked ΔSCG10-induced microtubule disassembly in a dose-dependent manner (Fig. 3B). In fact, the microtubule disassembly induced by 10 μM ΔSCG10 was almost completely abated, upon the addition of 30 μM BRI3 (N).

Effects of BRI3 and SCG10 Co-expression on microtubule organization

SCG10 is a microtubule-destabilizing protein that induces microtubule depolymerization in vitro and when overexpressed in cultured cells (9, 12).

The interaction and co-localization of BRI3 and SCG10 led us to examine the possible effects of this interaction on the microtubule-destabilizing activity of SCG10. When expressed alone in PC12 cells, RFP-SCG10 localized in the area of the Golgi complex, and the microtubule network was disrupted (Fig. 4C). In contrast, no obvious change in the microtubule network was observed in the cells expressing RFP-BRI3 alone (Fig. 4B), when compared to the untransfected cells (Fig. 4A). However, the overexpression of RFP-BRI3 reversed the disassembly of the microtubule network induced by GFP-SCG10 (Fig. 4D).

Effects of BRI3 on SCG10-mediated PC12 cell neurite outgrowth induced by NGF

PC12 cells are rat pheochromocytoma cells that undergo differentiation into a neuronal-like phenotype when stimulated with NGF, and have been used widely as a model of neuronal differentiation. It was reported that the overexpression of SCG10 enhanced NGF-induced neurite outgrowth in PC12 cells (7). The above evidence suggests that BRI3 may modulate neurite extension through interaction with SCG10. To examine the influence of SCG10 and BRI3 protein expression on the NGF-induced neurite outgrowth of PC12 cells, the cells were transfected with GFP- or RFP-tagged forms of SCG10, BRI3, or both SCG10 and BRI3 (Fig. 4E, F). Twenty-four hours after transfection, NGF was added to induce neuronal differentiation. As shown in Fig. 4E-F and summarized in Fig. 4G, the overexpression of SCG10 enhanced NGF-induced neurite outgrowth as expected, and the overexpression of BRI3 alone slightly attenuated neurite outgrowth as compared with untransfected cells (Fig. 4G); however, the overexpression of BRI3 together with SCG10 attenuated the outgrowth of neurites at all time points examined (Fig. 4F, G). These results reveal that BRI3 is able to attenuate neurite outgrowth through its interaction with SCG10.
**DISCUSSION**

In this paper, we describe a novel association between SCG10 and BR1, a neuron-specific member of the BRI protein family. The interaction of BR1 with SCG10 was identified in a yeast two-hybrid system and confirmed by in vitro and in vivo studies. The analysis of microtubule polymerization and depolymerization revealed a clear functional consequence from BR1 (N)-SCG10 interaction. Although BR1 had no effect on the ability of SCG10 to block microtubule assembly, there was a striking effect on SCG10-induced disassembly. The mechanism for SCG10-mediated destabilization of microtubules is not yet clear, and there has been considerable controversy in the field concerning this activity of stathmin proteins.

It is intriguing that BR1 blocked only microtubule disassembly, whereas microtubule assembly remained unaffected. Recently, Nixon et al. (13) reported on the identification of RGSZ1 as a binding partner of SCG10. An analysis of in vitro microtubule polymerization/depolymerization indicated that the binding of RGSZ1 to SCG10 is similar to the binding of BR1 to SCG10, which blocked the ability of SCG10 to induce microtubule disassembly. The authors hypothesized that perhaps the interaction of RGSZ1 with SCG10 alters the microenvironment of SCG10, such that it resembles more the high pH (7.5) condition than the low pH (6.8) condition, when SCG10 specifically induces microtubule catastrophes. However, this hypothesis is based on data obtained from stathmin, rather than SCG10. Although highly similar in sequence and closely related (14, 15), differences exist between the two, which may complicate these assumptions. Further work is needed to clarify whether SCG10 does indeed behave like stathmin with regard to the separation of its microtubule-regulating activities, as well as to clarify the mechanism by which the affinity of SCG10 for tubulin is influenced by BR1 or RGSZ1.

The present results raise intriguing questions concerning the physiological role of BR1 in the brain. Although neuron cells also contain MAPs that act to stabilize microtubules, the relative contribution of individual proteins controlling stabilization and destabilization in regions of neuronal outgrowth is a matter of debate. At present, it is not clear what prevents the depolymerization of microtubules when they need to be stabilized, e.g., in response to extrinsic guidance cues.

One of the questions surrounding the BR1-SCG10 interaction is its relevance to the functions of neurons, where distinct populations of both stable and labile microtubules coexist. The growing tips of extending processes contain particularly high levels of very labile polymers, and a number of studies have shown that axonal growth and guidance strongly depend on the highly dynamic behavior of microtubules (MTs) in these motile structures (15-17). The dynamic instability of MTs may be an important factor for their rapid growth response to various environmental signals. Neurons contain a dense array of MTs, suggesting a significant contribution of this cytoskeletal element to the architecture and function of these highly specialized cells.

It is well known that drugs decrease the dynamic behavior of MTs, inhibiting neurite extension (18, 19). Thus, growth cone advance and the rate of neurite elongation rely on the proper control of MT assembly and disassembly. Indeed, it is believed that SCG10 enhances neurite outgrowth by increasing the dynamic instability of microtubules in neuron cells. Our finding that the interaction of BR1 with SCG10 attenuates SCG10-induced microtubule disassembly and stabilizes the microtubule network to some extent, raises the possibility that
BRI3 associates with SCG10

BRI3 may inhibit neurite outgrowth by decreasing the dynamic instability of microtubules caused by SCG10.

The precise mechanism through which BRI3 inhibits the neurite outgrowth of PC12 cells was not determined in the present study. SCG10 is a brain-specific member of the stathmin protein family, and all members of this family share a common stathmin domain possessing microtubule-depolymerizing activity (20). The present evidence for the interaction of this domain with the N-terminal of BRI3 raises the possibility that BRI3 may affect the stathmin domain function. The idea that BRI3 could contribute to axonal outgrowth is an interesting hypothesis that may provide a link between a neuron-specific integral membrane protein and neuronal differentiation.

Okazaki et al. (21) reported that SCG10 compartmentalization and metabolism may be altered in AD (Alzheimer’s disease), possibly due to mechanisms related to tangle formation in this disease. Recent progress on Down’s syndrome showed that SCG10 is almost undetectable in neuronal precursor cells from Down’s syndrome patients. In cell culture, the Down’s syndrome cells showed a reduction of neurogenesis, as well as decreased neurite length and abnormal changes in neuron morphology (22). It is also intriguing that Wickham et al. (4) showed the N-terminal fragment of BRI3 interacting with BACE1; we presume BACE1 maybe a competency protein of SCG10 and also involved in the dynamic behavior of MTs. We anticipate that future work on BRI3 will enhance our understanding of neural development, plasticity, and degeneration.

**MATERIALS AND METHODS**

**Yeast two-hybrid screen**

The cDNA encoding the N-terminal of BRI3 [denoted BRI3 (N), comprising residues 1-59 of the BRI3 open reading frame] was ligated into pAS2-1 (CLONTECH). The two-hybrid screen was conducted in a human fetal brain cDNA library using the Matchmaker 2 System (CLONTECH) according to the manufacturer’s recommendations.

**Protein expression and purification**

Bacterial expression plasmids for the N-terminal truncation mutant of SCG10 (denoted ΔSCG10, comprising residues 35-179), and the N-terminal fragments of BRI3, were constructed in pGEX-6P-1 (Amersham Biosciences). ΔSCG10 was constructed in pET-28a+ (Novagen). The purification of soluble GST-ΔSCG10, GST-BRI3 (N), and His-tagged ΔSCG10 protein (His-ΔSCG10) was conducted according to standard procedures.

The eluted GST-BRI3 (N) and His-ΔSCG10 were dialyzed in PEM buffer [80 mM PIPES (pH 6.9), 1 mM EGTA, and 1 mM MgCl2].

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Fig. 4. Effect of BRI3 and SCG10 co-expression on microtubule organization and neuritis outgrowth of PC12 cells. (A) Microtubule organization in untransfected normal cells stained with β-tubulin antibody (green) and Hoechst 33342 (blue). (B) Microtubule organization in cells expressing DsRed1-BRI3 alone. Images of cells showing DsRed1-BRI3 (red) and tubulin (green) fluorescence. (C) Disrupted microtubule organization in cells expressing DsRed1-SCG10 alone. Images of cells showing SCG10 (red) and tubulin (green) fluorescence. (D) The co-expression of BRI3 and SCG10 stabilized the microtubule network. Images of cells showing DsRed1-BRI3 (red), EGFP-SCG10 (green), and tubulin (green). Tubulin was detected by indirect immunofluorescence. (E) The overexpression of SCG10 alone enhanced NGF-induced neurite outgrowth in PC12 cells; the overexpression of BRI3 alone had a negative effect on neurite formation. (F) Co-expression with SCG10 and BRI3 attenuated the outgrowth of neuritis. (G) Statistics of the effect on neurite outgrowth of SCG10 and BRI3. * P < 0.05
In vitro GST pull-down assay and antibody generation

GST or GST-tagged ∆SCG10 protein (5 μg) was incubated with cell lysate from SH-SY5Y cells (250 μg) in 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 1% NP40. The details of the pull-down protocol were described previously (25). Rabbit polyclonal anti-BRI3 antibody was generated using the N-terminal fragments of BRI3 as the antigen.

Co-immunoprecipitation (Co-IP)

293ET cells, growing in 10 cm plates with 90% confluence, were transfected with pcDNA3.1/V5-BRI3 and pcDNA4/Myc-SCG10 using LipofectAMINE®2000 (Invitrogen, Carlsbad, CA). After 24 h, cell extracts were prepared and subjected to immunoprecipitation via the standard procedure with mouse anti-V5 (1:3000, Invitrogen) or mouse anti-Flag Ab (as negative control Ab; 1:4000). The precipitates were run on SDS-PAGE and detected by subsequent Western blots with mouse anti-V5 (1:5000, Invitrogen).

Turbidimetric evaluation of microtubule assembly and disassembly

The assembly/disassembly of tubulin was measured using a light scattering assay as described previously (26). For the microtubule assembly assays, MAP-rich tubulin (2 mg/ml, final concentration) in PEM buffer containing 1 mM GTP at 4°C was mixed with ∆SCG10, BRI3 (N), or a combination of the two proteins. Microtubule assembly was initiated by raising the temperature to 37°C, and the absorbance at 350 nm was monitored over 10 min in an Ultrospect Model 4300 spectrophotometer. For the microtubule disassembly experiments, microtubules were prepared as described above, and the temperature was maintained at 37°C. ∆SCG10, BRI3 (N), or a combination of the two proteins were added, and the absorbance at 350 nm was monitored over 10 min. In the assembly/disassembly experiments, in which the effects of ∆SCG10 and BRI3 (N) together were tested, the two proteins were preincubated at 4°C for 1 h prior to use.

Cell culture, fluorescence, and immunofluorescence analysis

PC12 cells (rat pheochromocytoma cell line) were grown in DMEM supplemented with 5% fetal bovine serum and 10% equine serum. Neuroblastoma cells (SH-SY5Y) were maintained as described before (27).

Transient transfections of the PC12 cells were performed using LipofectAMINE®2000 (Invitrogen). The cells destined for fluorescence microscopy analyses were plated on glass coverslips coated with poly-D-lysine. The transfected cells were used for experiments 48 h after transfection. The fluorescence or immunofluorescence analyses were performed as described previously (28).

Effect of BRI3 on SCG10-mediated PC12 cell neurite outgrowth

PC12 cells were transfected with GFP or RFP-tagged forms of SCG10, BRI3, or with both SCG10 and BRI3. After 24 h, the cells were treated with 100 ng/ml of NGF. The cells were fixed after 24 and 48 h and stained with a mouse monoclonal anti-tubulin antibody. Cells bearing neurites, defined as processes of at least two cell body lengths, were scored (7). The percentage of neurite-bearing cells was determined by the average of three counts on at least 100 cells. The results shown represent the means ± S.D. of three separate experiments.

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