Targeting of SCG10 to the Area of the Golgi Complex Is Mediated by Its NH₂-terminal Region*

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SCG10 is a neuronal growth-associated protein that is concentrated in the growth cones of developing neurons. SCG10 shows a high degree of sequence homology to the ubiquitous phosphoprotein stathmin, which has been recently identified as a factor that destabilizes microtubules by increasing their catastrophe rate. Whereas stathmin is a soluble cytosolic protein, SCG10 is membrane-associated, indicating that the protein acts in a distinct subcellular compartment. Identifying the precise intracellular distribution of SCG10 as well as the mechanisms responsible for its specific targeting will contribute to elucidating its function. The main structural feature distinguishing the two proteins is that SCG10 contains an NH₂-terminal extension of 34 amino acids. In this study, we have examined the intracellular distribution of SCG10 in PC12 cells and in transfected COS-7 cells and the role of the NH₂-terminal domain in membrane-binding and intracellular targeting. SCG10 was found to be localized to the Golgi complex region. We show that the NH₂-terminal region (residues 1–34) was necessary for membrane targeting and Golgi localization. Fusion proteins consisting of the NH₂-terminal 34 amino acids of SCG10 and the related protein stathmin or the unrelated protein, β-galactosidase, accumulated in the Golgi, demonstrating that this sequence was sufficient for Golgi localization. Biosynthetic labeling of transfected COS-7 cells with [³H]palmitic acid revealed that two cysteine residues contained within the NH₂-terminal domain were sites of palmitoylation.

The neuronal growth-associated protein SCG10 belongs to the same gene family as stathmin, a ubiquitous phosphoprotein. In contrast to stathmin, which is a highly soluble cytosolic protein (1), SCG10 is associated with cellular membranes (2). SCG10 was first identified as a gene expressed in sympathetic neurons but not in chromaffin cells (3). The expression of SCG10 is high in the developing nervous system (2), and its mRNA is nerve growth factor-inducible in PC12 cells (4). The SCG10 protein is localized to the perinuclear cytoplasm and enriched in growth cones of developing neurons in culture (2). SCG10 shares 74% amino acid identity with stathmin (5), which has been implicated in signal transduction mechanisms due to its phosphorylation in response to extracellular stimuli regulating proliferation and differentiation in different cell types (Ref. 1; for review see Ref. 6). In cells of the immune system, a role for the control of cell cycle has been proposed (7–9). We have previously found that in PC12 cells, stathmin is required for neuronal differentiation in response to nerve growth factor (NGF) (10). More recently, it has been reported that stathmin increases the catastrophe rate of microtubules, suggesting that it is a regulator of microtubule dynamics (11). We have observed a similar function for SCG10 in regulating the dynamic instability of microtubules during neurite outgrowth (12). However, SCG10 may act in specific subcellular compartments to which it is targeted via its membrane association.

In this study, we have defined the intracellular localization of SCG10 in PC12 cells and transfected COS-7 cells as the area of the Golgi complex. In addition, we have studied the mechanism responsible for the membrane binding and targeting of SCG10 to this area. Because the structural difference between stathmin and SCG10 lies in the NH₂-terminal region of SCG10, we generated several constructs encoding truncated and chimeric proteins for the transfection of COS-7 cells. We demonstrated that the NH₂-terminal region of SCG10 contains a signal required for its targeting to the area of the Golgi complex and sufficient to target stathmin as well as the unrelated protein, β-galactosidase, to this subcellular region. Furthermore, the presence of two cysteines within the NH₂ terminus of SCG10, a potential locus for palmitoylation, prompted us to investigate thio-acylation as a possible lipid modification for the membrane localization of SCG10.

MATERIALS AND METHODS

Antibodies and Cytochemical Probes—The rabbit antisera raised against the NH₂-terminal amino acids 15–27 of stathmin has been previously described (13) (gift of Dr. A. Sobel, Paris, France). For SCG10, a rabbit antiserum and mouse mAbs were raised against a fusion protein containing the entire rat coding sequence fused to the bacterial TrpE protein (14). The rabbit antisera was further purified by sequential absorption on a protein A-Sepharose column followed by affinity chromatography on a glutathione S-transferase-stathmin-Affi-Gel-10 column to eliminate antibodies cross-reacting with stathmin. The mouse mAbs were purified on a superagger Fast IgG column (Spectrun) and by gel filtration on Superdex200 (Pharmacia, Uppsal, Sweden). The following antibodies were generous gifts: mouse mAbs against the KT3 tag (15) and the Glu-Glu tag (16) (both from Dr. G. Walter, San Diego, CA). The following reagents were obtained from commercial sources: mouse mAbs directed against β-galactosidase (Promega, Madison, WI), γ-adaptin, FITC-conjugated wheat germ agglutinin (Sigma, Buchs, Switzerland), and mouse mAbs against α-mannosidase II

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‡ The abbreviations used are: NGF, nerve growth factor; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; WGA, wheat germ agglutinin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SCG-GAL, chimeric construct where the NH₂ terminus of SCG10 has been fused to the β-galactosidase moiety; SCG-STAT, chimeric construct where the NH₂ terminus of SCG10 has been fused to stathmin.
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(BaBCo, Berkeley, CA).

Generation of Stathmin and SCG10 Constructs—Standard DNA procedures were performed according to Sambrook et al. (17). The human stathmin cDNA and the rat SCG10 cDNA were generous gift of Dr. A. Sobel (Paris, France) and Dr. N. Mori (Kyoto, Japan), respectively. The cDNA fragments of algae stathmin and stathmin II were amplified by PCR using primers containing BanHI and XbaI extensions and then cloned into the pcDNA3 vector (Invitrogen) at the corresponding sites for expression in COS-7 cells. A NH2-terminal truncated SCG10 construct ΔNSCG10 was generated by PCR starting from amino acid 35. A chimeric construct GAL-SCG containing the NH2-terminal region of SCG10 (amino acid 1–34) fused to the β-galactosidase polypeptide (amino acid 3–1001) was generated by PCR and in-frame ligation. A chimeric construct STAT containing the same NH2-terminal domain of SCG10 (amino acid 1–34) fused to the stathmin polypeptide (amino acid 3–150) was generated similarly. For experiments where exogenously expressed expression had to be distinguished from endogenous expression in COS-7 cells, stathmin and SCG-STAT were epitope-tagged at the COOH-terminus with a RT5 tag (TPPPEPET; Ref. 16) or a Glu-Glu tag (EYMPME; Ref. 19) using PCR. Site-directed mutagenesis to replace cysteines with alanines at positions 22 and 24 of SCG10 sequence was performed according to PCR procedures previously described (20). All constructs were confirmed by DNA sequencing.

Cell Culture and Transfection—PC12 cells were cultured as described (17). Cultures were grown on coverslips coated with poly-L-lysine and laminin (Collaborative Biomedical Products). COS-7 cells were grown in a humidified 37°C incubator with 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transient transfections were performed with the Bio-Rad electroporation system using 200 V and 960 microfarad. 15 μg of DNA were used for each transfection in electrotransport buffer (150 mM NaCl, 20 mM HEPES, pH 7.4) at a cell density of 106/ml. After transfection, the cells were plated on glass coverslips precoated with 1 mg/ml polyethyleneimine (Sigma) in 0.03 M borate buffer, pH 8.3, for immunofluorescent studies or on 100-mm Petri dishes for biochemical procedures. In some experiments, cells were treated with 10 μM nocodazole (Sigma) for 2 h and processed for immunocytochemistry.

Immunofluorescence—Cultures were fixed in 4% formaldehyde in sulfate buffer (90 mM Na2SO4, 30 mM K2SO4, 5.8 mM MgCl2, 0.25 mM CaCl2, 1.0 mM NaH2PO4, pH 7.4) for 20 min and then rinsed with MTBS (66 mM NaCl, 100 mM Tris-HCl, pH 7.4). Cells were incubated with primary antibodies diluted in MTBS containing 10% rat serum, 0.3% Triton X-100, 2% bovine serum albumin overnight at 4°C. After rinsing in MTBS, cells were incubated with either fluorescein- or Texas Red-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Vector, Peterborough, UK) for 30 min at room temperature. Wheat germ agglutinin was used at 2.5 μg/ml concomitantly with secondary antibodies. Cultures were rinsed in MTBS and mounted using Vectashield mounting medium (Vector).

Metabolic Labeling with [3H]Palmitate and [3H]Myristate—COS-7 cells were transiently transfected with Glu-Glu-tagged wild-type and mutated SCG10 constructs where cysteines 22 and 24 were individually or concomitantly substituted with alanines. 48 h after electroporation, cells were collected from 100-mm dishes, rinsed twice with Dulbecco’s modified Eagle’s medium and labeled for 4 h at 37°C in 2 ml of serum-free Dulbecco’s modified Eagle’s medium containing 800 μCi of [3H]palmitate or [3H]myristate (DuPont NEN). Cells were then pelleted, rinsed twice in ice-cold PBS, and lysed in 400 μl of 6% perchloric acid containing 0.1% sodium deoxycholate, 10 mM NaF and 0.5% sodium deoxycholate, 10 mM NaF supplemented with a mixture of proteases inhibitors (Complete, Boehringer, Mannheim, Germany). SCG10 was then immunoprecipitated for 3 h at 4°C with constant rotation using 50 μg/ml anti-GluGlu mouse mAbs. 30 μl of 50% protein A-Sepharose/PBS (Pharmacia) were then added to the immunoprecipitation buffer, and samples were rotated for 1 h at 4°C. Beads were washed twice with immunoprecipitation buffer, and proteins were eluted for 5 min at 95°C in 50 μl of sample buffer. Proteins were then electrophoresed in duplicates on 8–16% SDS-PAGE. The gels were processed either for fluorography or for immunoblotting.

Subcellular Fractionation and Immunoblotting—Transiently transfected COS-7 cells were allowed to grow for 48 h after electroporation. For the continuous sucrose gradient, four 10-cm diameter Petri dishes were used. The confluent monolayers were washed twice with PBS at 4°C, scraped in 2.5 ml of PBS, and centrifuged for 5 min at 1000 rpm in a Heraeus 1.0 R megafuge at 4°C. After rinsing the cells in 2.5 ml of homogenization buffer (0.25 M sucrose, 3 mM imidazole, pH 7.4), the cell pellet was reconstituted for 10 min at 2000 rpm at 4°C. The cells were then resuspended in 0.4 ml of homogenization buffer and harvested by seven passages through a 22G 1/4 needle fitted on a 1-ml plastic syringe. The homogenate was centrifuged for 10 min at 2000 rpm at 4°C, and the post-nuclear supernatant was collected (1 mg of total protein). The supernatant was then centrifuged at 4°C with the same sucrose gradient poured from 45% (1.58 M) and 11.5% (0.35 M) sucrose solutions containing 3 mM imidazole, pH 7.4. The gradient was centrifuged for 17 h at 35’000 rpm at 4°C in a Beckman L8–80 ultracentrifuge using a SW60 rotor. Eight fractions of about 0.5 ml each were collected from the bottom using a peristaltic pump at low flow, and sucrose concentration was determined by refractometric means. Samples were precipitated in 15-μl aliquots according to Wolsey and Flugge (22) and resuspended in sample buffer. For the cytosol membranes preparation, transiently transfected COS-7 cells were collected 48 h after electroporation and pelleted at 2000 × g for 5 min. The cells were then harvested by sonication in ice-cold homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) and centrifuged at 4000 × g for 2 min to remove nuclei and unbroken cells. Cytosol and membranes were prepared by centrifugation of the supernatants at 58,000 rpm (150,000 × g) for 40 min at 4°C in a TL100.2 rotor (Beckman Instruments). The pellets were washed three times and then resuspended to the same volume of homogenization buffer as the cytosol fraction. The samples were analyzed by SDS 8–16% PAGE and immunoblotted with specific antibodies. Cultures were rinsed in MTBS and mounted using Vectashield mounting medium (Vector).

RESULTS

SCG10 Is Localized to the Area of the Golgi Complex in PC12 Cells and Transfected COS-7 Cells—We generated a specific antiserum and mAbs against SCG10 to compare the subcellular localization of stathmin and SCG10 in PC12 cells and in transfected COS-7 cells. For stathmin, we obtained a rabbit antiserum from A. Sobel (INSERM, Paris). The specificity of the stathmin and SCG10 antibodies was tested on immunoblots where the stathmin antisem specifically recognized bacterially produced rat stathmin and not SCG10 and the purified SCG10 antiserum as well as the mAb labeled specifically recombiant SCG10 and not stathmin (data not shown). When COS-7 cells were transfected by electroporation with a construct encoding stathmin, no staining was observed with the SCG10 antiserum in cells with both stathmin and SCG10 expression constructs (Fig. 1, A and B). Both, purified SCG10 antiserum and mAbs gave indistinguishable staining patterns in transfected COS-7 cells (Fig. 1, C and D) and in PC12 cells (Fig. 2). Double immunostaining of PC12 cells demonstrated that stathmin was homogenously distributed in the cytoplasm as expected for a soluble protein (Fig. 2A). In contrast, the SCG10 mAbs recognized punctate structures in the perinuclear region (Fig. 2B). To investigate the nature of the punctate structures, we performed double stainings of PC12 cells with two markers of the Golgi complex: α-mannosidase II, a glycoprotein enriched in the cis/medium Golgi region (23) and wheat germ agglutinin (WGA), a lectin that binds to N-acetylgalactosamine and N-acetylenuraminic acid residues found predominantly in the trans Golgi apparatus/network (24) (Fig. 2, C–F). SCG10 immunoreactivity colocalized with both markers, indicating that SCG10 is localized in the area of the Golgi complex.

To determine whether the subcellular localization of SCG10 in the Golgi complex region is mediated by an intrinsic feature of the structure of SCG10, we transiently transfected COS-7 cells with both stathmin and SCG10 expression constructs (Fig. 3). To distinguish transfected stathmin from stathmin that was endogenously expressed at low levels in COS-7 cells, at the 3′ end of the stathmin cDNA, we added a fragment encoding the 6-amino acid epitope “Glu-Glu” (EYMPME; Ref. 19) or the KT3 epitope (TPPPEPET; Ref. 18), which are recognized by specific
mAbs (15, 16). No SCG10 immunoreactivity was observed in wild-type COS-7 cells stained with the SCG10 antiserum (Fig. 1B). Immunofluorescence staining of transfected COS-7 cells revealed distinct distribution patterns of stathmin and SCG10 as observed in the PC12 cells. Unlike stathmin, which was diffusely distributed throughout the cytoplasm (Fig. 1A), SCG10 was highly concentrated in the region of the Golgi complex as shown by its colocalization with WGA and another marker of the trans Golgi compartment, γ-adaptin (25) (Fig. 4). SCG10 staining remained similar to that of WGA (Fig. 5, A and B) or γ-adaptin (Fig. 5, C and D) following disruption of the Golgi organization with the microtubule-depolymerizing drug nocodazole (26). Although most of the structures were labeled with both SCG10 and the Golgi markers, a significant proportion showed a staining for SCG10 but not for the Golgi markers and conversely (Fig. 5, A–D). In PC12 cells, SCG10 remained colocalized with α-mannosidase II following nocodazole treatment, whereas brefeldin A (20 μg/ml, 20 min), which induces a breakdown of the Golgi stacks and a redistribution of resident Golgi proteins into the endoplasmic reticulum (27, 28), caused SCG10 and α-mannosidase II stainings to disappear (data not shown).

SCG10 Cofractionates with the Golgi Marker γ-Adaptin in Transfected COS-7 Cells—In order to further characterize the association of SCG10 with the Golgi apparatus, we employed a cell fractionation procedure based on a sucrose density gradient. Post-nuclear supernatants from transfected COS-7 cells were centrifuged on a continuous sucrose gradient ranging linearly from 37 to 12% (w/w), and fractions were analyzed by immunoblotting. SCG10 was enriched in heavy fractions, particularly in fractions 3 and 4 (32–28%), but significant amounts of the protein were detected in the other fractions (Fig. 6). The Golgi marker γ-adaptin showed a distribution comparable with that of SCG10 and was enriched in fractions 3–5, consistent with our previous immunofluorescent studies (Fig. 6). Total cellular proteins measured in each fraction show a distinct distribution (Fig. 6). SCG10-enriched fractions were then treated with high salt, nonionic detergent, and alkali in order to determine the nature of the association with Golgi membranes. Consistent with previous results obtained with SCG10-enriched smooth microsomes (2), the protein was completely extracted with 1% Triton X-100 but only partially with carbonate pH 11.4 (40–50%). No significant extraction with high salts (1.5 M) was observed (data not shown).

The NH₂-terminal Region of SCG10 Is Necessary and Sufficient for Membrane Binding and Targeting to the Golgi Complex—SCG10 has a structural feature that is not present in stathmin (amino acid 1–34, Fig. 3). This NH₂-terminal domain contains a stretch of hydrophobic amino acids, which has been proposed to be responsible for membrane attachment of SCG10 (2). In order to address the question whether the differential localization of stathmin and SCG10 is mediated by this NH₂-terminal region, we generated several plasmid constructs expressing both deletion and fusion proteins (Fig. 3). These were transfected into COS-7 cells and analyzed by double immunofluorescence. When COS-7 cells were transfected with a construct in which the NH₂-terminal region of rat SCG10 (amino acids 1–34) was deleted, the truncated SCG10 molecule was found in the cytoplasm. The appearance was very similar to
stathmin expression (Fig. 7). Therefore, removal of this domain led to a complete solubilization of the remaining region. This indicates that the first 34 amino acids of SCG10 contain information required for association with the Golgi complex.

When COS-7 cells were transfected with a construct in which the NH₂-terminal domain of SCG10 was fused in frame to stathmin, the chimeric stathmin molecule, just like SCG10,

Fig. 3. Schematic representation of the NH₂-terminal regions of SCG10 and stathmin and the constructs expressed in COS-7 cells. Top panel, sequence alignment of the NH₂-terminal amino acids of SCG10 (amino acid 1–50) and stathmin (amino acid 1–16). Closed dots indicate the cysteines (22 and 24) contained in the NH₂ terminus of SCG10, which have been replaced by an alanine. Bottom panel, diagram showing all the constructs investigated in this report. STAT, stathmin; ΔNSCG10, SCG10 where the first 34 amino acid have been deleted; GAL, β-galactosidase.

Fig. 4. Intracellular distribution of SCG10 in transiently transfected COS-7 cells. Cells were stained with the rabbit serum anti-SCG10 (A and C) and counterstained with a mAb directed to the Golgi marker γ-adaptin (B) and with the FITC-coupled lectin WGA (D). SCG10 immunoreactivity colocalizes with the Golgi markers in transfected cells (arrows). Scale bar, 25 μm.

Fig. 5. Effect of nocodazole on the intracellular distribution of SCG10 in transfected COS-7 cells. Following 2 h of treatment with 10 μM nocodazole, cells were stained with the rabbit serum anti-SCG10 (A and C) and counterstained with the FITC-coupled lectin WGA (B) and with a mAb directed to the Golgi marker γ-adaptin (D). SCG10 immunoreactivity colocalizes to a large extent with the Golgi markers after disruption of Golgi apparatus organization with the drug (arrowheads). Scale bar, 25 μm.

Fig. 6. Cofractionation of SCG10 and γ-adaptin in a sucrose gradient. The post-nuclear supernatant (about 1 mg of total protein) was centrifuged on a 4-ml continuous sucrose gradient (37–12%), and 15-μl aliquots from each fraction were subjected to SDS-PAGE and Western blot analysis. SCG10 and γ-adaptin immunoblots show a comparable distribution of both proteins (A). The percentage of sucrose (w/w) content and the distribution of total protein in the corresponding fractions are indicated in the graph (B).

was concentrated in the region of the Golgi complex as revealed by colocalization with the Golgi marker WGA (Fig. 6). This finding suggests that the NH₂-terminal domain of SCG10 contains information sufficient to target the otherwise cytosolic stathmin to this intracellular compartment. To further define the mechanism responsible for intracellular targeting of SCG10, we transfected COS-7 cells with a construct in which the NH₂-terminal domain of SCG10 was fused to an unrelated protein, β-galactosidase. Although β-galactosidase had a diffuse cytosolic localization, the SCG10 (1–34)/β-galactosidase fusion protein was concentrated in the region of the Golgi complex (Fig. 9).

In addition to the immunofluorescence analysis, we studied
the membrane binding properties of the deletion and fusion proteins. The distribution of the proteins in the cytosolic and total membrane fractions from transiently transfected COS-7 cells was detected by Western blot analysis using anti-KT3 mAb (to detect stathmin expression), anti-SCG10, and anti-β-galactosidase antibodies. Stathmin and β-galactosidase were mainly found in the soluble fraction (Fig. 10, C), whereas SCG10 was located predominantly in the particulate fraction (Fig. 10, M). In contrast, ΔNSCG10 was predominantly in the soluble fraction and SCG-GAL and SCG-STAT, in which the NH$_2$-terminal domain of SCG10 was fused to β-galactosidase or stathmin, respectively, were in the particulate fraction (Fig. 10). This subfractionation analysis indicates that the NH$_2$-terminal 34 amino acids of SCG10 contain enough information for membrane association.

The NH$_2$-terminal Domain of SCG10 Contains Two Cysteines That Are Sites for Palmitoylation—Cysteines have been shown to be targets for post-translational modification, mainly palmitoylation, in other neuronal proteins concentrated in axons and nerve terminals, such as GAP-43 and SNAP-25 (29, 30). Also SCG10 contains two closely spaced cysteine residues within the NH$_2$-terminal region, at positions 22 and 24 (Fig. 3), which may represent sites for palmitoylation. To study whether SCG10 was palmitoylated, COS-7 cells were transfected with Glu-Glu epitope-tagged SCG10 and incubated with [3H]palmitic acid or [3H]myristic acid for 4 h. SCG10 was isolated by immunoprecipitation using anti-Glu-Glu mAbs and analyzed by SDS-PAGE and fluorography. To compare the expression levels of SCG10, the immunoprecipitates were analyzed by Western blotting using the SCG10 antiserum. As a control, nontransfected cells were also labeled, immunoprecipitated, and subjected to electrophoresis. The incorporation of tritium into SCG10 was detected from [3H]palmitic acid but not from [3H]myristic acid (Fig. 11, left panel). Then both cysteines (22 and 24) were mutated to alanines to determine if they were involved in the palmitoylation of SCG10. The single and double mutants and wild-type SCG10 were expressed in COS-7 cells, and the cells were labeled with [3H]palmitic acid. The absence of radioactivity in SCG10 in which both cysteines have been mutated suggests that the attachment of palmitic acid to SCG10 occurs through thioester linkage to cysteines (Fig. 11, right panel). In agreement with these data, hydroxylamine treatment, which is known to hydrolyze thioester at pH 7 (31), released the radioactivity associated with SCG10 (data not shown). We found that the Cys$^{22}$ mutant (Fig. 11, A22) incor-
To assess whether palmitoylation contributes to the membrane binding ability of SCG10, we transiently transfected COS-7 cells with constructs in which cysteine 22 and/or 24 have been replaced by alanines. Double immunofluorescence analysis revealed that the mutated proteins did not colocalize with the Golgi marker protein γ-adaptin but showed a more cytosolic distribution as compared with the wild type (Fig. 12). In some cells, however, localization to the Golgi complex appeared to be retained when Cys22 was mutated (Fig. 12C). As shown in Fig. 13, in all three cases, the mutations caused only partial solubilization of the protein, whereas removal of the entire NH₂-terminal domain led to nearly complete solubilization. In all subfractionation analysis by Western blot, we defined the cytosol and membrane contamination compared with the wild type.

DISCUSSION

In this study, we have investigated the molecular mechanism responsible for the membrane targeting of the neurospecific protein SCG10, which is closely related to the ubiquitous soluble protein stathmin. Analysis of the subcellular localization in PC12 cells and transfected COS-7 cells indicated that SCG10 colocalized with the region of the Golgi complex and that both specific structural elements and palmitoylation within the NH₂-terminal region were responsible for membrane association and localization in this area.

For these studies, we used existing antibodies for stathmin and generated polyclonal and mAbs for SCG10, all three of which were highly specific in Western blots and immunofluorescence. Because native PC12 cells contain only barely detectable levels of SCG10 (data not shown), for these experiments, PC12 cells were treated with NGF for 24 h. It was known from previous results that SCG10 is up-regulated in response to NGF in this cell line (4). NGF-stimulated PC12 cells showed strong immunoreactivity for both stathmin and SCG10, but the subcellular distribution of these two very similar proteins was strikingly different. Whereas stathmin immunoreactivity was cytosolic as expected for a soluble protein (1), SCG10 accumu-
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FIG. 13. Distribution of SCG10 mutant proteins in cytosolic and membrane fractions prepared from transiently transfected COS-7 cells. Cells were transfected with wild-type SCG10 (WT), Cys to Ala22 mutant (A22), Cys to Ala24 mutant (A24), or Cys to Ala22 and Cys to Ala24 mutant (A22A24) or SCG10 lacking the first 34 amino acids (ΔN). The graph shows the results of three independent experiments in which the protein levels were measured by optic densitometry of Western blot autoradiograms. Mutation of one or both cysteines into alanine results in a partial solubilization of the proteins into the cytosolic fraction. In contrast, deletion of the NH2-terminal domain leads to nearly complete solubilization into the cytosolic fraction.

related to the perinuclear region. This is in agreement with previous studies that have shown a punctate SCG10 staining of the perinuclear cytoplasm in neuronal cell bodies (2). Here, we have further defined the localization of SCG10 to the area of the Golgi complex.

Both endogenous protein in NGF-induced PC12 cells and exogenous protein in transfected COS-7 cells colocalized with markers for the Golgi compartment, including α-mannosidase II, γ-adaptin, and the lectin WGA (Figs. 2 and 4). SCG10 immunoreactivity was sensitive to the Golgi-disrupting agent brefeldin A (data not shown) and remained associated to a large extent, although not completely, with the Golgi markers following perturbation of Golgi organization with nocodazole (Fig. 5). Consistent with colocalization studies, SCG10 largely cofractionated with γ-adaptin following subcellular fractionation of transfected COS-7 cells on a sucrose density gradient. Altogether, these results strongly suggest that SCG10 is associated with the Golgi apparatus, although further electron microscope-based analysis will be required to determine the precise location in the Golgi compartment.

SCG10 was described as a membrane-associated but not an integral membrane protein, whose association with smooth microsomal membranes was efficiently disrupted with nonionic detergent but only partially with alkali (2). Structural features of the SCG10 protein sequence led to the speculation that a short stretch of hydrophobic amino acids within the NH2-terminal domain of SCG10 (and not conserved in stathmin) might mediate membrane interaction (2). We examined this possibility by separate expression of stathmin, SCG10, a NH2-terminal deleted SCG10, and SCG10-stathmin and SCG10-β-galactosidase fusion proteins in transfected cells. Our results indicate that specific signals within the NH2-terminal 34 amino-acids of SCG10 are required not only for membrane binding but also for its targeting to the area of the Golgi complex. Moreover, we showed that the NH2-terminal domain is sufficient to target heterogeneous proteins to the same region. The results of immunofluorescence were always consistent with results obtained from crude subcellular fractionation. Because these experiments were performed in transfected COS-7 cells, the mechanism through which the NH2-terminal region of SCG10 targets the protein to this subcellular region is not neuron-specific.

In the case of other nerve terminal proteins, GAP-43 and SNAP-25, fatty acylation (palmitoylation) on cysteine residues was shown to mediate membrane association (29, 30, 32). SCG10 also contains two cysteines within the NH2-terminal region. Therefore, we investigated whether SCG10 was palmitoylated on these cysteines and whether palmitoylation may be the mechanism responsible for the membrane targeting. Indeed, both cysteines 22 and 24 were found to be palmitoylated in transfected COS-7 cells, but Cys24 was palmitoylated to a higher extent. Interestingly, the Cys22 mutant was more labeled than the wild-type protein. Because the mutant SCG10 proteins showed less degradation products in the Western blots, this may indicate that the wild-type protein is more susceptible to proteolytic cleavage that may remove the radioactivity. Furthermore, replacement of Cys22 or Cys24 by alanine caused a cytosolic distribution of a large proportion of the protein. These data suggest an important role of cysteine residues in the membrane targeting of SCG10.

However, because mutation of the cysteines did not result in complete solubilization of SCG10, in addition to palmitoylation other signals contained within the NH2-terminal domain must be responsible for the membrane distribution and targeting of this protein. A similar finding was obtained for glutamic acid carboxylase, which exists in two major isoforms, one of which is palmitoylated and Golgi complex-associated. The targeting of this isoform does not depend on palmitoylation but on a signal within the NH2-terminal 27 amino acids of the protein (33). However, the NH2-terminal regions of SCG10 and glutamic acid carboxylase do not share sequence similarity. Thus, the precise mechanism by which the NH2-terminal domain of SCG10 targets the protein to the subcellular compartments remains to be established. Interestingly, basic amino acids in the NH2 terminus of Src, which is myristoylated, have been involved in membrane binding through electrostatic interaction with acidic phospholipids (34). The presence of five basic amino acids (four lysines and one arginine) in the first 34 NH2-terminal amino acids of SCG10 (Fig. 3) suggest that a similar mechanism may be involved. Future transfection experiments of SCG10 constructs in neurons may elucidate whether the same signals that target SCG10 to the Golgi complex region mediate the targeting to the growth cones or whether additional signals or an interaction with other proteins are required for growth cone targeting.

The SCG10-related protein stathmin was shown to destabilize microtubules by increasing their catastrophe rate (transition from a growing to a shrinking state) (11). Our recent studies have identified a similar role for SCG10 as a mediator of neurite outgrowth through regulation of microtubule dynamics (12). Owing to its membrane association, SCG10 is likely to function in specific subcellular compartments, including Golgi and neuronal growth cones. The dynamic interaction between intracellular organelles and microtubules may specifically require proteins, like SCG10, acting as microtubule-disrupting factors to regulate trafficking events. Alternatively, SCG10 might use the sorting machinery to access to neuronal growth cones, regulating neurite outgrowth-associated events via its action on microtubules dynamics.

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