SCG10 is a neuron-specific, membrane-associated protein that is highly concentrated in growth cones of developing neurons. Previous studies have suggested that it is a regulator of microtubule dynamics and that it may influence microtubule polymerization in growth cones. Here, we demonstrate that in vivo, SCG10 exists in both phosphorylated and unphosphorylated forms. By two-dimensional gel electrophoresis, two phosphoisoforms were detected in neonatal rat brain. Using in vitro phosphorylated recombinant protein, four phosphorylation sites were identified in the SCG10 sequence. Ser-50 and Ser-97 were the target sites for protein kinase A, Ser-62 and Ser-73 for mitogen-activated protein kinase and Ser-73 for cyclin-dependent kinase. We also show that overexpression of SCG10 induces a disruption of the microtubule network in COS-7 cells. By expressing different phosphorylation site mutants, we have dissected the roles of the individual phosphorylation sites in regulating its microtubule-destabilizing activity. We show that nonphosphorylatable mutants have increased activity, whereas mutants in which phosphorylation is mimicked by serine-to-aspartate substitutions have decreased activity. These data suggest that the microtubule-destabilizing activity of SCG10 is regulated by phosphorylation, and that SCG10 may link signal transduction of growth or guidance cues involving serine/threonine protein kinases to alterations of microtubule dynamics in the growth cone.

SCG10 is a growth-associated protein abundant in the growth cones of developing neurons (1–3). The gene encoding SCG10 is a member of the stathmin gene family (4). Both stathmin and SCG10 are microtubule (MT) 1 -destabilizing factors (5–7). In in vitro assays of MT assembly, these molecules inhibit microtubule polymerization and induce depolymerization. Unlike stathmin, which is a cytosolic protein and expressed in most tissues (8), SCG10 is a membrane-associated and neuron-specific protein (1, 9). The expression of SCG10 is developmentally regulated, with high levels in embryonic and postnatal nervous system (1, 2). In the adult, its expression persists in several brain regions that are associated with synaptic plasticity (10), and up-regulation of SCG10 has been found following lesion experiments (11). Overexpression of SCG10 in a neuronal cell line was found to enhance neurite outgrowth (7). Moreover, SCG10 is highly concentrated in the central domain of growth cones (3) where the distal ends of MTs are in a dynamic state of growth and shrinkage (12–14). Thus, SCG10 may be a regulator of MT dynamic instability during neurite outgrowth and structural plasticity. While the role of MTs in growth cone motility and neurite elongation is well established (15–18), little is known about their regulation in response to the environment and the signaling pathways involved. An understanding of phosphorylation and dephosphorylation events regulating the activity of SCG10 may lead to important insights into the intracellular mechanisms that modulate growth cone motility.

We have previously shown that recombinant SCG10 is an in vitro target for the serine/threonine protein kinases PKA, MAP kinase, and cyclin-dependent kinase (CDK) p34cdc2 (19). Both MAP kinase and PKA are present in growth cones and associated with microtubules (20). The CDK p34cdc2 is not expressed in neurons, but another member of the CDK family, CDK5/p25, which is highly homologous to p34cdc2, has been identified in neurons (21, 22) and is localized in growth cones (23).

Here, we demonstrate that SCG10 is phosphorylated in vivo and we have identified four phosphorylation sites in the recombinant protein using liquid chromatography/electrospray ionization mass spectrometry. To further elucidate the molecular mechanism of SCG10 function, we have analyzed the effect of phosphorylation on the activity of the protein in intact cells. Our findings suggest that SCG10 is a phosphoprotein in developing brain and that its MT-destabilizing activity is regulated by phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Antibodies—** A rabbit antibody directed against SCG10 (anti-SCG10-BR) was generated by injecting 80 μg of recombinant, NH2-terminal truncated SCG10 that had been phosphorylated with PKA, MAP kinase, and p34cdc2 (19) in complete Freund’s adjuvant. One month after

**Mass spectrometry**—MS, mass spectrometry; MS/MS tandem mass; LC, liquid chromatography; PBS, phosphate-buffered saline; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
the first injection, the animal was boosted with 2 weeks every 40 μg of antigen in incomplete Freund’s adjuvant. Five days after the third boost, the animal was deeply anesthetized with nembutal and bled by cardiac puncture. The anti-SCG10 serum was tested for specificity in Western blots. For immunofluorescence experiments, the previously described mouse monoclonal antibody for α-tubulin (clone B-5-1-2, Sigma) was used.

Tissue Preparation, Immunoblots, and Dephosphorylation—Wistar rats of different ages (birth, postnatal days (P) 0, 5, 10, 15, and 20) and adult rats (3 months) were prepared for biochemistry as described earlier and cultured in serum-free (15 mM MOPS, pH 7.0, 10 mM MgCl2, 0.5 mM EGTA) and digested on-column (total on-column digestion time of 2 min). Peptide fragments were trapped onto a reversed phase C18 column and eluted into the ion source of the mass spectrometer as described previously (29). Tryptic peptides were separated on a 15 cm × 300-μm capillary C18 column (LC Packings) operated at 5 μl/min using a linear gradient of 1 to 21% buffer B in 5 min and 21 to 41% buffer B in 15 min. Electrospray mass spectra were acquired by scanning the mass spectrometer from 300–1800 Da in a 3-s using a 0.5-Da step and a 2.0-ms dwell time each.

Enzymatic Digestion and LC/MS Characterization—Aliquots of protein samples, corresponding to 5–25 pmol, were injected onto an immunoaffinity column (PerSeptive Biosystems, Framingham, MA) and digested on-column (total on-column digestion time of 2 min). Twenty peptides were described earlier and stored in 20 mM Tris-HCl, 0.2 mM DTT, pH 7.5 at –80 °C (19, 28). The purified protein was over 98% pure on reverse phase-HPLC and showed apparent homogeneity on SDS-PAGE. For the in vitro phosphorylation, cells were transfected with an inactive SCG10 construct where the first 99–100 amino acids were deleted. In both cases 100% of the interphase cells were scored, and the percentage of cells containing polymerized MTs was considered as cells containing polymerized MT if they contained more than 50 intact MTs. In three independent experiments, 50 cells were scored, and the percentage of cells containing polymerized MTs was calculated. As controls, we analyzed untransfected cells and cells that were transfected with an inactive SCG10 construct where the first 99 amino acids were deleted. In both cases 100% of the interphase cells were scored.
Mapping of SCG10 Phosphorylation Sites

RESULTS

SCG10 Phosphoisoforms in Rat Brain—An anti-SCG10 serum, raised against in vitro phosphorylated recombinant SCG10 recognized two major bands in Western blots of postnatal brain extracts, one at 22 kDa and one at 25 kDa (Fig. 1A). As expected, due to the developmental change of SCG10 protein in rat brain (2), the levels of expression decreased after postnatal day 5 and became undetectable in the adult (Fig. 1A).

Following alkaline phosphatase treatment of P5 brain extract, the band at 25 kDa was no longer detectable, indicating that it corresponds to one or several phosphorylated forms of SCG10 (Fig. 1B). Two-dimensional gel electrophoresis revealed three isoforms of SCG10 in postnatal brain extract (Fig. 1C). The electrophoretic mobility of these isoforms was compared with that of in vitro phosphorylated, NH2-terminal truncated SCG10 (28) (as described under “Experimental Procedures”) (Fig. 1, D–F). A mixture of nonphosphorylated SCG10 and SCG10 that was phosphorylated by CDK or by a combination of CDK, MAP kinase, and PKA generated a SCG10 pattern similar to that found in brain extracts (Fig. 1G). These results indicate that postnatal rat brain contains unphosphorylated as well as two different phosphoisoforms of SCG10.

Phosphorylation Sites in SCG10—For determination of the phosphorylation sites, recombinant SCG10 was in vitro phosphorylated by PKA, MAP kinase, and CDK as described under “Experimental Procedures.” The phosphorylated samples were first analyzed by ion-spray mass spectrometry to determine the number of phosphorylation sites. Ion-spray mass spectrometry gave a mass of 20,624 ± 1.4 for unphosphorylated SCG10, which is in agreement with the calculated molecular mass (20,624 Da). After phosphorylation with PKA, the main molecular mass was found to be 20,803, which corresponds to diphosphorylated SCG10. MAP kinase phosphorylation gave both mono- and diphosphorylated SCG10 with molecular masses of 20,715 and 20,803, respectively. However, after 120 min of incubation with the kinase, the protein was entirely diphosphorylated (Fig. 2A). Samples phosphorylated with CDK showed mainly monophosphorylated protein with a molecular mass of 20,715. To map the sites of phosphorylation, the samples were digested with either trypsin or Glu-C, and the peptides were separated on reverse phase-HPLC and sequenced with tandem mass spectrometry (MS/MS).

After trypsin digestion of the sample phosphorylated by PKA, three peptides were identified by the stepped orifice voltage technique and with a shift in the molecular masses on LC/MS. One peptide gave a (M + 3H)+ signal at m/z 815.5 which corresponds to M, 2,447 (Fig. 3A). The calculated M, for the monophosphorylated peptide 48–69 is 2,445. When the peptide was sequenced, serine 50 was identified as the site of phosphorylation. The second peak corresponded to the peptide 49–69 and confirmed the phosphorylation site as serine 50. The third shifted peak was at a (M + 2H)2+ signal of m/z 632.8 corresponding to a molecular mass of 1,266 (Fig. 3B). The monophosphorylated peptide 95–104 has a calculated M, of 1,266. MS/MS sequencing of the peptide revealed that the phosphorylation site was serine 97. The phosphorylation sites were confirmed by sequencing of two phosphorylated peptides from a Glu-C digestion (amino acid 42–55 and 93–99).

In the samples phosphorylated with MAP kinase and digested with trypsin two phosphorylated peptides were identified and found to be shifted in the LC/MS spectrum (Table I). When the first peptide showing a (M + H)+ ion at m/z 696 was sequenced by MS/MS, the phosphorylation site was identified as serine 73. The second peptide gave a (M + 2H)2+ signal of m/z 1145, which corresponds to a molecular mass of 2,290. In the SCG10 sequence this corresponds to a monophosphorylated peptide generated through cleavage at amino acid 49 and 69 (M, 2,289). Sequencing of the peptide revealed that serine 62 was the site of phosphorylation (Fig. 2B). Digestion with Glu-C also generated two phosphorylated peptides (amino acid 67–83 and 56–66). Sequencing of the peptides confirmed the phosphorylation sites.

Phosphorylation with CDK resulted in one phosphorylated peptide generated through cleavage at amino acid 49 and 69 (M, 2,291). Using the anti-SCG10-BR antibody or Coomassie Blue staining, protein extracts prepared from different developmental stages (P0–P20 and adult) of rat brain were analyzed by SDS-PAGE. One- and two-dimensional SDS-polyacrylamide gel electrophoresis, and the percentage of cells containing polymerized MTs was determined as described above.

showed a dense MT network. To compare the activity of SCG10 in transfected cells with that of a soluble form of SCG10 missing the membrane-binding domain (9), 100 transfected cells from three independent experiments were counted regardless of their level of expression, and the percentage of cells containing polymerized MTs was determined as described above.

FIG. 1. Detection of SCG10 phosphoisoforms in rat brain by one- and two-dimensional SDS-polyacrylamide gel electrophoresis. A, protein extracts prepared from different developmental stages (P0–P20 and adult) of rat brain were analyzed by SDS-PAGE (5–20% gels, 50 μg of protein per lane) and Western blot. Immunostaining of the membranes for SCG10 using the anti-SCG10-BR antibody revealed two major bands (at 22 and 25 kDa) and two minor bands (at 21 and 23 kDa). The band at 21 kDa corresponds to a degradation product (3). B, brain extracts from P5 rat brain were incubated in the absence (−) or the presence (+) of alkaline phosphatase. Note the disappearance of the upper bands upon dephosphorylation. C–G, NEPHGE-SDS gel electrophoresis of P5 brain extract and recombinant NH2-terminal truncated SCG10 followed by Western blot analysis (C and G) using the anti-SCG10-BR antibody or Coomassie Blue staining (D–F). C, P5 brain extract; D, nonphosphorylated SCG10; E, CDK phosphorylated SCG10; F, SCG10 multiphosphorylated by MAP kinase, CDK, and PKA; G, P5 brain extract mixed with nonphosphorylated, CDK phosphorylated, and multiphosphorylated SCG10. Protein migration is from acidic (left) to basic (right).
peptide with a \((M + H)^+\) signal at \(m/z\) 695.5. MS/MS sequencing showed that the peptide corresponded to amino acids 49–69.

The results are summarized in Table I and Fig. 4, A and B, and compared with the phosphorylation sites of stathmin.

Regulation of SCG10 Activity—SCG10 inhibits the assembly of microtubules and induces their disassembly in vitro (7). To study the effect of SCG10 on microtubules in intact cells and to identify the role of phosphorylation in its activity, we have transiently transfected COS-7 cells, which do not express endogenous SCG10. We assessed the effects of expression of wild-type SCG10 on the MT array by immunofluorescence staining using an anti-\(\alpha\)-tubulin antibody. Then, we tested mutants in which the serines, individually or in combination, were mutated to alanine or aspartate to prevent or mimic phosphorylation, respectively (Fig. 4 C). In cells expressing low levels of wild-type SCG10 in which SCG10 staining was observed in the area of the Golgi apparatus, as previously shown (9), no obvious abnormalities could be observed. These cells were similar to the controls where 100% of the interphase cells showed a dense MT network. However, cells expressing high levels of SCG10, where the protein was also abundant in the cytoplasm (Fig. 5B), showed a dramatic, sometimes complete, disappearance of the microtubule network (Fig. 5, A and B). When we compared the activity of wild-type SCG10 with that of a construct that is missing the NH\(_2\)-terminal membrane-binding domain and thus expressed as a cytosolic protein (9), we found that SCG10 is less active in the membrane-bound form. While 43.7 ± 1.2% of cells transfected with full-length SCG10, only 20.3 ± 0.9% of cells transfected with soluble SCG10 contained polymerized MTs (\(p < 0.001\)).

We compared the effects of high level expression of wild-type SCG10 with the effects of SCG10 in which serine-to-alanine substitutions were made at each individual site (S50A, S62A, S73A, and S97A) or at dual sites (S50A,S97A, S62A,S73A). Analysis showed no statistically significant difference in depolymerizing activity between these proteins (Fig. 6). However, the nonphosphorylatable mutant (S50A,S62A,S73A,S97A) was significantly more active in depolymerizing the microtubule network than the wild-type protein (Figs. 5, C and D, and 6). The percentage of cells containing polymerized microtubules decreased from 13.3 ± 0.9% for wild-type to 3.0 ± 1.5% for the nonphosphorylatable mutant (\(p < 0.001\)) (Fig. 6).

To further test the role of phosphorylation in regulating the MT destabilizing activity of SCG10, mutants in which serine residues were substituted with aspartate residues at one, two, or all four sites were analyzed (Fig. 4C). While introducing acidic charges to mimic phosphorylation at either Ser-50 or Ser-62 had no significant effect on the activity of SCG10, single mutations at Ser-73 (S73D) and Ser-97 (S97D) significantly
decreased SCG10 activity (Fig. 6). The percentage of cells containing polymerized microtubules increased from 13.3 ± 0.9% for wild-type to 24.0 ± 0.6% and 27.0 ± 1.5%, respectively, for these mutants (p < 0.001). Double phosphorylation at Ser-50 and Ser-97 (S50D, S97D) and at Ser-62 and Ser-73 (S62D, S73D) showed a further increase in cells containing microtubules to 31.7 ± 2.8% and 28.7 ± 1.3%, respectively (p < 0.001). The greatest reduction in SCG10 activity was observed when all four phosphorylation sites were substituted by aspartate (S50D, S62D, S73D, and S97D) resulting in an increase of cells containing MTs to 46.7 ± 0.9% (p < 0.001) (Fig. 6). To assess whether endogenous phosphorylation of SCG10 in
COS-7 cells contributes to reducing its activity, the double aspartate mutant S62D,S73D was further mutated on Ser-50 and Ser-97 with alanine substitutions to prevent phosphorylation on these two residues (S50A,S97A,S62D,S73D). The percentage of cells transfected with S50A,S97A,S62D,S73D which contained polymerized MTs (25.3 ± 1.8%) was similar to that of cells transfected with S62D,S73D (28.7 ± 1.3%), indicating that the endogenous phosphorylation on Ser-50 and Ser-97 in this mutant does not contribute significantly to decreasing SCG10 activity (Fig. 6). Altogether, our results suggest that phosphorylation negatively regulates the microtubule-depolymerizing activity of SCG10 and that all four sites participate in this regulation, although the relative importance of each site varies.

**DISCUSSION**

SCG10, a growth cone-enriched MT-destabilizing protein, has been recently characterized as an in vitro substrate for various serine/threonine kinases including PKA, MAP kinase, and CDK (19). We have found that SCG10 is phosphorylated in vivo in developing rat brain. The in vivo isoforms correspond to unphosphorylated, monophosphorylated, and multiphosphorylated forms. More detailed studies will be required to reveal the specific phosphorylation states of this protein in cells and tissues under a variety of physiological conditions.

In this work, the sites of SCG10 phosphorylated by PKA, MAP kinase, and CDK have been identified. Our results, based upon tryptic peptide mapping followed by LC/MS and MS/MS sequencing, are summarized in Fig. 4A, where they are compared with the sites that have been reported for the related protein stathmin (33–35). The PKA phosphorylation sites present in SCG10 are conserved to the in vitro and in vivo phosphorylation sites known for stathmin. The sites for MAP kinase phosphorylation were identified as Ser-62 and Ser-73 of SCG10. Both sites contain a proline residue C-terminal to the serine consistent with the serine (threonine)-proline specificity of this kinase (Fig. 4B). In contrast to stathmin, where the major site for MAP kinase was Ser-25, no preferred phosphorylation site was found for SCG10. Two sites (Ser-25 and Ser-38) are phosphorylated by CDK in stathmin, whereas we found only one major site in SCG10 that was phosphorylated by this kinase (Ser-73). Since the kinase used for these experiments is not expressed in neurons, the neuronal cdc2-like kinase CDK5/p25, which was also found to efficiently phosphorylate SCG10 (data not shown), may be the physiologically relevant kinase. Our results suggest that the function of SCG10 is regulated by multiple protein kinases and that the kinases, PKA, MAP kinase, and CDK/p25, all three of which are present in growth cones (20, 23), are good candidates to phosphorylate SCG10 in vivo.

To reveal physiological functions of SCG10 phosphorylation, we assessed whether phosphorylation at the identified sites had an effect on the MT-destabilizing activity of the protein. We found that overexpression of wild-type SCG10 in COS-7 cells caused disruption of the MT network consistent with its microtubule-depolymerizing effect in vitro (7). A similar activity was recently reported for the cytosolic protein stathmin upon transfection (6) or microinjection of recombinant protein (36) into cells. However, SCG10 was significantly less active than a truncated cytosolic form of the protein, and its effect was observed mainly in highly overexpressing cells, where SCG10 localization was not restricted to the area of the Golgi complex (9) but also found in the cytoplasm. This may indicate that Golgi-associated SCG10 is either not very active or, more likely, not in a subcellular compartment where it can induce depolymerization of interphase microtubules. It is not known yet whether SCG10 functions while it is bound to organelles in neuronal growth cones (3) or whether it has to be released from membranes.

By expressing a series of phosphorylation site mutants, we showed that the MT-destabilizing effect of SCG10 could be modulated. While the nonphosphorylatable mutant showed higher activity than the wild-type protein, the activity of the mutant in which phosphorylation on all four sites was mimicked by an aspartate residue was greatly reduced. These data suggest that the nonphosphorylated state of SCG10 represents the most active form of the protein. Observations of stathmin in transfected or microinjected cells (6, 36) as well as in an in vitro assay of MT assembly (37, 38) suggest a similar mechanism of regulation of the activity of the two proteins. However, for stathmin it has been reported that alanine substitution on only two of the four serines (Ser-25 and Ser-38) increased its MT-destabilizing activity to nearly the same extent as mutating all four sites. This was not the case for SCG10, where the S50A,S62A,S73A,S97A mutant was significantly more active than any single or double alanine substitutions. Interestingly, these two serine residues in stathmin (Ser-25 and Ser-38) are not precisely conserved in the SCG10 sequence (Fig. 4B) and exhibit differences between stathmin and SCG10 in their phosphorylation by MAP kinase and CDK (4A).

We also tested the effect of mutation of phosphorylation sites in SCG10 by introducing aspartate residues to replace each of the four phosphorylatable serines, both individually and in

**FIG. 4. Schematic representation of the phosphorylation sites of stathmin and SCG10 and of SCG10 phosphorylation site mutants.** A, comparison of NH2-terminal regulatory regions of stathmin and SCG10 indicating the sites phosphorylated by PKA, MAP kinase, and CDK. The serine residues phosphorylated by PKA are conserved between the stathmin and SCG10 sequence: Ser-16 and Ser-63 in stathmin correspond to Ser-50 and Ser-97 in SCG10, respectively. B, amino acid alignment of stathmin and SCG10 encompassing the sites phosphorylated by MAP kinase and CDK. C, site-directed mutagenesis of SCG10 phosphorylation sites. The four serine residues were mutated either to alanine or to aspartate, in various combinations. These mutants were expressed in COS-7 cells.

The two most phosphorylated serine residues of stathmin are Ser-25 and Ser-38, which are phosphorylated by PKA and CDK, respectively. In stathmin it has been reported that alanine substitution on only one of these sites, Ser-38, decreased its activity, whereas the double alanine mutant was more effectively inhibited (39). To determine whether similar results could be obtained with SCG10, we introduced Ser-25A,S38A and Ser-25A,S38D mutants, in which phosphorylation on all four sites was mimicked by a mutation to an aspartate residue. Interestingly, these mutants showed much higher activity than the wild-type protein, the activity of the double aspartate mutant being greater than that of the double alanine mutants (Fig. 6A). These data suggest that the nonphosphorylated state of SCG10 represents the most active form of the protein. On the other hand, these results also suggest that differential phosphorylation in vivo may contribute to the regulation of SCG10 activity.

To determine whether differential phosphorylation of SCG10 occurs in vivo, we performed immunoprecipitation on COS-7 cultures transiently transfected with S50A,S97A,S62D,S73D, a double mutant in which phosphorylation on all four sites was mimicked by alanine substitution. The amount of SCG10 co-immunoprecipitated with a specific antibody was determined by Western blotting. The results show that the MT-destabilizing activity of SCG10 could be modulated. While the nonphosphorylatable mutant showed higher activity than the wild-type protein, the activity of the mutant in which phosphorylation on all four sites was mimicked by an aspartate residue was greatly reduced. This data suggest that the nonphosphorylated state of SCG10 represents the most active form of the protein. The endogenous phosphorylation of SCG10 on Ser-50 and Ser-97 in the double aspartate mutant was greatly reduced. These data suggest that the nonphosphorylated state of SCG10 represents the most active form of the protein. However, when stathmin it has been reported that alanine substitution on only two of the four serines (Ser-25 and Ser-38) increased its MT-destabilizing activity to nearly the same extent as mutating all four sites. This was not the case for SCG10, where the S50A,S62A,S73A,S97A mutant was significantly more active than any single or double alanine substitutions. Interestingly, these two serine residues in stathmin (Ser-25 and Ser-38) are not precisely conserved in the SCG10 sequence (Fig. 4B) and exhibit differences between stathmin and SCG10 in their phosphorylation by MAP kinase and CDK (4A).

We also tested the effect of mutation of phosphorylation sites in SCG10 by introducing aspartate residues to replace each of the four phosphorylatable serines, both individually and in
with a mouse monoclonal antibody to S50D, S62D, S73D, and S97D (E and F). Cells were double-immunostained with a mouse monoclonal antibody to α-tubulin (A, C, and E) and a rabbit antiserum to SCG10 (B, D, and F). In cells expressing high levels of SCG10 (arrowheads), both wild-type and alanine mutants induced a nearly complete disruption of microtubule arrays (A–D). In contrast, the aspartate mutant showed much less disruptive activity (E and F). Scale bar, 25 μm.

In summary, our results strongly suggest that the activity of SCG10 is controlled by phosphorylation and that its activity can be down-regulated to different extents by multiple phosphorylations. Therefore, SCG10 may be a key factor that links growth or guidance cues to the local control of MT assembly in growth cones. Fine tuning of its activity, possibly by several signal transduction pathways that act in concert, may be involved in regulation of the dynamics of MTs required for growth cone advance and turning.

Acknowledgments—We thank all members of the neurobiology group at Geneva Biomedical Research Institute for their helpful discussion. We are grateful to R. Golsteyn for experiments on SCG10 phosphorylation with neuronal CDK. We also thank A. Bernard, H. Blasey, J. Y. Bonnefoy, N. Gullu, C. Hebert, S. Herren, P. Gruber, S. Montessuit, R. Porchet, L. Potier, and E. Sebille for their help at various stages of this work. We thank S. Catsicas and J.K. Staple for many helpful comments and critical reading the manuscript.

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FIG. 5. Double immunofluorescence analysis of MTs in COS-7 cells transfected with SCG10 phosphorylation site mutants. Cells were transfected with constructs encoding wild-type SCG10 (A and B), the nonphosphorylatable alanine mutant S50A, S62A, S73A, S97A (C and D) or the phosphorylation-mimicking aspartate mutant S50D, S62D, S73D, and S97D (E and F). Cells were double-immunostained with a mouse monoclonal antibody to α-tubulin (A, C, and E) and a rabbit antiserum to SCG10 (B, D, and F). In cells expressing high levels of SCG10 (arrowheads), both wild-type and alanine mutants induced a nearly complete disruption of microtubule arrays (A–D). In contrast, the aspartate mutant showed much less disruptive activity (E and F). Scale bar, 25 μm.

FIG. 6. Quantitative analysis of SCG10 MT-destabilizing activity in COS-7 cells transfected with phosphorylation site mutants. Cells were transfected with wild-type (WT) and mutant SCG10 cDNA constructs (see Fig. 4C for description) and double-immunostained for α-tubulin and SCG10 48 h after transfection. Cells with cytosolic SCG10 staining were assessed for their MT content and scored when they contained more than 50 individual MTs. Results are expressed as percentages of cells containing MTs and were obtained from 3 individual experiments (n = 50; **p < 0.001).

various combinations. Of the single mutants, only S73D and S97D were statistically different from wild-type, but not from each other. Also the double mutants were not statistically different from each other, though they were different from the S50D, S62D, S73D, and S97D mutant. Whether the decreased activity of aspartate mutants is caused by a reduced binding to tubulin as is the case for in vitro phosphorylated stathmin (37, 38) needs to be determined.
Mapping of SCG10 Phosphorylation Sites

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J. Biol. Chem. 1998, 273:8439-8446.
doi: 10.1074/jbc.273.14.8439

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