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Role of Ser50 Phosphorylation in SCG10 Regulation of Microtubule Depolymerization

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Members of the stathmin-like protein family depolymerize microtubules (MTs), probably due to the ability of each stathmin monomer to bind two tubulin heterodimers in a complex (T_2S complex). SCG10, a member of this family, is localized in the growth cone of neurons. It has four identified sites of serine phosphorylation (S50, S63, S73, and S97). Of these, S50 and S97 are phosphorylated by cAMP-dependent protein kinase, an enzyme involved in growth cone guidance. When the equivalent sites in stathmins are phosphorylated, they lose their ability to depolymerize MTs. We investigated the specific role of the two cAMP-dependent protein kinase (PKA) phosphorylation sites in SCG10. A mutant of SCG10 phosphorylated only on S50 retained the ability to depolymerize MTs, but SCG10 phosphorylated on S97 or on both S50 and S97 lost MT-depolymerizing activity. Surface plasmon resonance studies revealed that the phosphorylation of SCG10 at these sites reduced the tubulin heterodimer binding, mainly due to a reduced rate of association. In particular, compared to the two other phosphorylated forms, SCG10 phosphorylated at S50 had a significantly smaller dissociation constant for the binding of the first tubulin heterodimer and larger association and dissociation rate constants for the binding of the second heterodimer. This indicates that the phosphorylation of S50 compensates for the effect of phosphorylation at other sites by modulating T_2S complex formation. Furthermore, these results suggest that S50-P maintains MT-depolymerizing activity, which indicates that the biological functions of phosphorylation at S50 and S97 are different.

Key words: growth cone; microtubules; surface plasmon resonance; phosphorylation; cAMP-dependent protein kinase

SCG10 is a developmentally expressed, neuron-specific protein (Curmi et al., 1997; Mori and Morii, 2002) that is concentrated in growth cones (Lutjens et al., 2000; Mori and Morii, 2002). The N-terminus of SCG10 includes sites for palmitoylation, which enables SCG10 to be attached to growth cone vesicles, likely mediating its transport to the growth cone area (Lutjens et al., 2000; Grenningloh et al., 2004). SCG10 is a member of the stathmin-like protein family, proteins that are known to depolymerize microtubules (MTs; Riederer et al., 1997; Gavat et al., 1998; Curmi et al., 1999; Grenningloh et al., 2004). Phosphorylation of SCG10 and other stathmins inhibits their MT-depolymerizing activity. Like the other stathmin family members, SCG10 has four sites of serine phosphorylation: S50, S63, S73, and S97 (Antonsson et al., 1998). These sites are phosphorylated by distinct protein kinases (Antonsson et al., 1998) both in vitro and in vivo. For example, S50 and S97 are catalyzed by cAMP-dependent protein kinase (PKA). Interestingly, in some cases the PKA pathway controls axonal guidance, a process that involves the regulation of MT polymerization.

To examine the significance of the PKA-dependent phosphorylation of SCG10 at S50 or S97, we examined the effect of a truncated SCG10 corresponding to amino acids 35–179 (ΔSCG10) and two site-directed mutants of ΔSCG10, S97A, and S50A. We succeeded in producing

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Fig. 1. Recombinant SCG10, its mutants, and their phosphorylation. As other stathmin-like proteins, SCG10 has four putative sites of serine phosphorylation (S50, S62, S73, and S97). The recombinant form of SCG10 used in the current study, which consisted of residues 35–179, lacked the N-terminal hydrophobic region containing the palmitoylation sites. The S50A and S97A mutants used in this study were site-directed mutants of ΔSCG10.

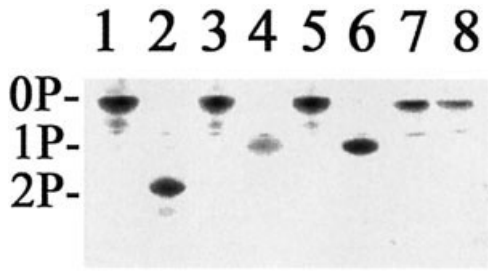

Fig. 2. Analysis of PKA-dependent phosphorylation of SCG10 and its mutants by urea-PAGE gel shift assay. ΔSCG10 and its mutants were phosphorylated by PKA catalytic subunit in a buffer containing Mg^{2+} and ATP and then separated by 8% urea-PAGE. Doubly phosphorylated proteins migrate faster than do singly phosphorylated proteins. Lane 1, wild-type; lane 2, wild-type phosphorylated by PKA (S50/S97-P); lane 3, S97A; lane 4, S97A phosphorylated by PKA (S50-P); lane 5, S50A; lane 6, S50A phosphorylated by PKA (S97-P); lane 7, S50A/S97A; lane 8, S50A/S97A reacted with PKA, which was not phosphorylated by PKA. The positions corresponding to two, one, and zero phosphorylated sites are indicated as 2P, 1P, and 0P, respectively.

SCG10 that was phosphorylated at only at S50 (S50-P) or S97 (S97-P). We also produced SCG10 phosphorylated at both sites (S50/S97-P). We examined the ability of these phosphorylated forms of SCG10 to affect MT polymerization. We also directly analyzed the interaction between the phosphorylated SCG10 and tubulin using surface plasmon resonance (SPR). We succeeded in measuring tubulin-binding kinetics using an on-chip PKA-phosphorylation method, and we confirmed that PKA-dependent phosphorylation of SCG10 reduced the binding of tubulin heterodimers. In addition, we found that the association rate constant for the binding of the first tubulin heterodimer was reduced by phosphorylation and that compared to the other two phosphorylated forms, S50-P had a smaller dissociation constant for the binding of the first heterodimer and larger rate constants for the binding of the second tubulin heterodimer. Our present results suggest that the phosphorylation of S50 helps maintain the ability of SCG10 to depolymerize MTs, indicating that it plays a different role than S97 phosphorylation in regulating growth cone behavior.

MATERIALS AND METHODS

Recombinant SCG10

Because full-length SCG10 has low solubility due to the hydrophobic N-terminal region, we prepared an N-terminal-deleted SCG10 (ΔSCG10; Fig.1) to generate a glutathione-S-transferase (GST)-SCG10 fusion protein (Antonsson et al., 1997). N-terminal-deleted SCG10 (ΔSCG10; Fig.1) was generated by inserting the region encoding amino acids 35–179 into pGEX 6P-1 (Amersham Biosciences). *Escherichia coli* (BL21) was transformed with the construct using standard methods (Ohyama et al., 2002) to express the ΔSCG10 protein. The ΔSCG10-expressing transformants were frozen and then suspended and sonicated in a buffer consisting of 20 mM HEPES (pH 7.2), 50 mM NaCl, 1 mM EDTA, and 1% Triton X-100. The fusion protein was purified using Glutathione 4B beads (Amersham Biosciences). After 2 hr of mixing at 4°C, the beads were washed with three times with phosphate-buffered saline (PBS), equilibrated with protease buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol), and cleaved overnight at 4°C with PreScission protease (Amersham Biosciences). The purified proteins were analyzed by SDS-PAGE to confirm their purity and homogeneity.

Tubulin Preparation

MT proteins containing tubulin and microtubule-associated proteins were purified from rat brain by two cycles of reversible temperature-dependent polymerization in PEM buffer (80 mM PIPES, pH 6.9, 1 mM EGTA, and 1 mM $MgCl_2$) according to the procedure of Shelanski et al (1973). Tubulin was purified using a phosphocellulose column (Shelanski et al., 1973). Purified tubulin was stored at −80°C in PEM buffer containing 1 mM GTP.

Analysis of MT Polymerization

MT assembly and disassembly were measured using a light-scattering assay. MT proteins were diluted to 2 mg/ml in PEM buffer containing 1 mM GTP. To examine the effect of the recombinant SCG10 proteins, MTs were polymerized for 20 min at 36°C and then ΔSCG10 protein (5 μM final concentration, 1:40 vol/vol) was added. The samples were then cooled to 4°C to depolymerize the MTs, and another cycle of MT assembly was initiated by raising the temperature to 36°C. The absorbance at 350 nm was measured in using a U-2000 spectrophotometer (Hitachi).

MT assembly was measured quantitatively using a sedimentation assay (Riederer et al., 1997). After the light-scattering assay, the MTs were mixed with SCG10, collected, and cooled for over 1 hr at 4°C to depolymerize MTs. These samples were then repolymerized for 30 min at 36°C, overlaid on a cushion of 60% glycerol in PEM buffer, and centrifuged for 30 min at 28,000 × *g* and 35°C. The supernatants (containing soluble tubulin) and the pel-

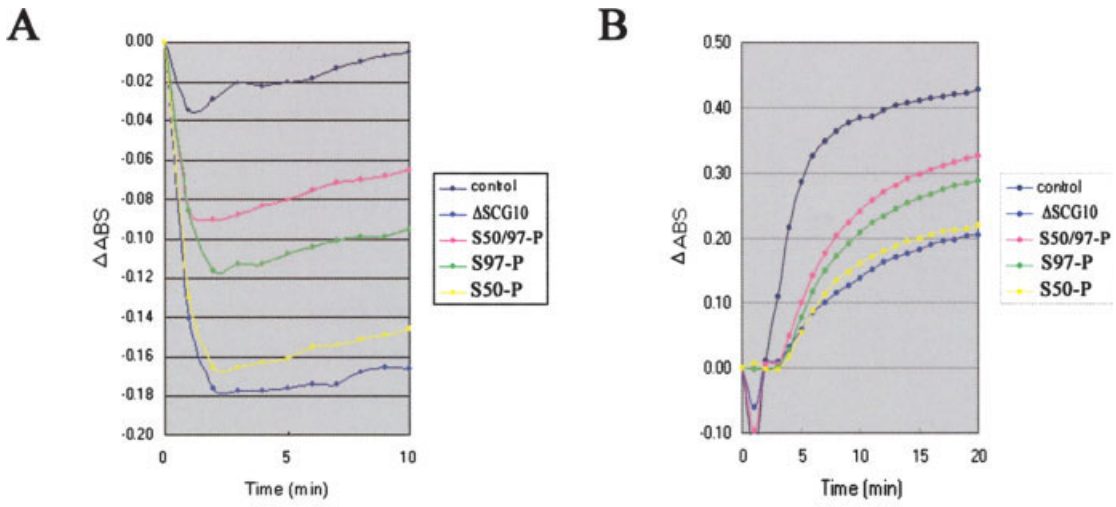

Fig. 3. The disassembly (**A**) and the assembly (**B**) of MTs in presence of ΔSCG10, its mutants, and their phosphorylated forms. MT dynamics were measured using light scattering at 350 nm. MT assembly was analyzed quantitatively. MTs were mixed with SCG10 derivatives, collected, cooled for 1 hr at 4°C, repolymerized for 30 min at 36°C, overlaid on a cushion of 60% glycerol in PEM buffer, and then centrifuged for 30 min at 28,000 × *g* and 35°C. S50-P had MT-depolymerizing activity equivalent to the unphosphorylated ΔSCG10. The results are representative of four separate experiments.

lets (containing MTs) were collected separately and analyzed by SDS-PAGE. The gel was analyzed using NIH Image software.

In Vitro Phosphorylation

GST-ΔSCG10 and mutants were phosphorylated using the PKA catalytic subunit (Sigma) in phosphorylation buffer (20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP) at 25°C for 4 hr, and then purified by cleavage using PreScission protease. The phosphorylation was confirmed by gel shift on 8% urea-PAGE (pH 8.3; Asakawa and Azuma, 1990), which showed that the ΔSCG10 band was shifted toward the cathode according to the number of the phosphorylated residues. Both the phosphorylated and nonphosphorylated ΔSCG10s were concentrated in PEM buffer.

SPR

SPR studies of the effects of on-chip phosphorylation were monitored using a BIAcore 3000 system (BIAcore, Inc.). ΔSCG10 proteins (1,000 resonance units) were immobilized on a CM5 sensor chip using an Amine Coupling Kit (BIAcore, Inc.). Three of four flow cells were used for the wild-type, S50A, and S97A proteins, and the fourth was used as a blank control to subtract the bulk effect. The measurements were carried out at 25°C with 1, 2, 3, 5, and 10 μM of purified tubulin α/β heterodimer in PEM buffer containing 1 mM GTP at a flow rate of 10 μl/min. The immobilized ΔSCG10 proteins were phosphorylated by the PKA catalytic subunit (0.1 U/μl) diluted in phosphorylation buffer, and injected at a low flow rate of 1 μl/min for 5, 15, 40, 60, and 120 min. After each cycle, 3 μM tubulin was injected to evaluate the affinity for SCG10. BIAevaluation v4.1 (BIAcore, Inc.) was used for kinetic analyses. Because the binding ratio between stathmin family proteins and tubulin dimer is believed to be 1:2 (Curmi et al., 1997), we used a bivalent binding model for fitting the data.

RESULTS

Phosphorylation of ΔSCG10 and its Mutants

To confirm the specificity of the protein kinases and the corresponding phosphorylation sites of ΔSCG10, we phosphorylated the truncated SCG10 and its mutants and analyzed the phosphorylation using a urea gel shift assay (Fig. 2). In this assay, phosphorylation enhances the ability of the protein to migrate through the gel. We confirmed that the mobility of S50A/S97A SCG10 did not change after incubation with PKA, indicating that this mutant is not phosphorylated by PKA (Fig. 2). PKA thus phosphorylates only S50 and S97. S50D or S97D, mutations that are expected to mimic the phosphorylations, had lower mobilities than did the singly phosphorylated proteins (data not shown).

Effect of PKA-Mediated Phosphorylation on MT Assembly and Disassembly

MT polymerization and depolymerization were monitored using the change in turbidity at 350 nm. MT disassembly was inhibited rapidly by the nonphosphorylated ΔSCG10 (Fig. 3A). Each nonphosphorylated form of ΔSCG10, S50A, and S97A had a similar MT-depolymerizing activity to one another (data not shown). S50/S97-P and S97-P had considerably reduced abilities to inhibit MT disassembly. In contrast, the MT-depolymerizing activity of S50-P was similar to the nonphosphorylated form (Fig. 3A). Also, S50-P inhibited MT assembly as effectively as the nonphosphorylated form did (Fig. 3B), whereas S50/S97-P and S97-P had reduced abilities to inhibit MT assembly (Fig. 3B).

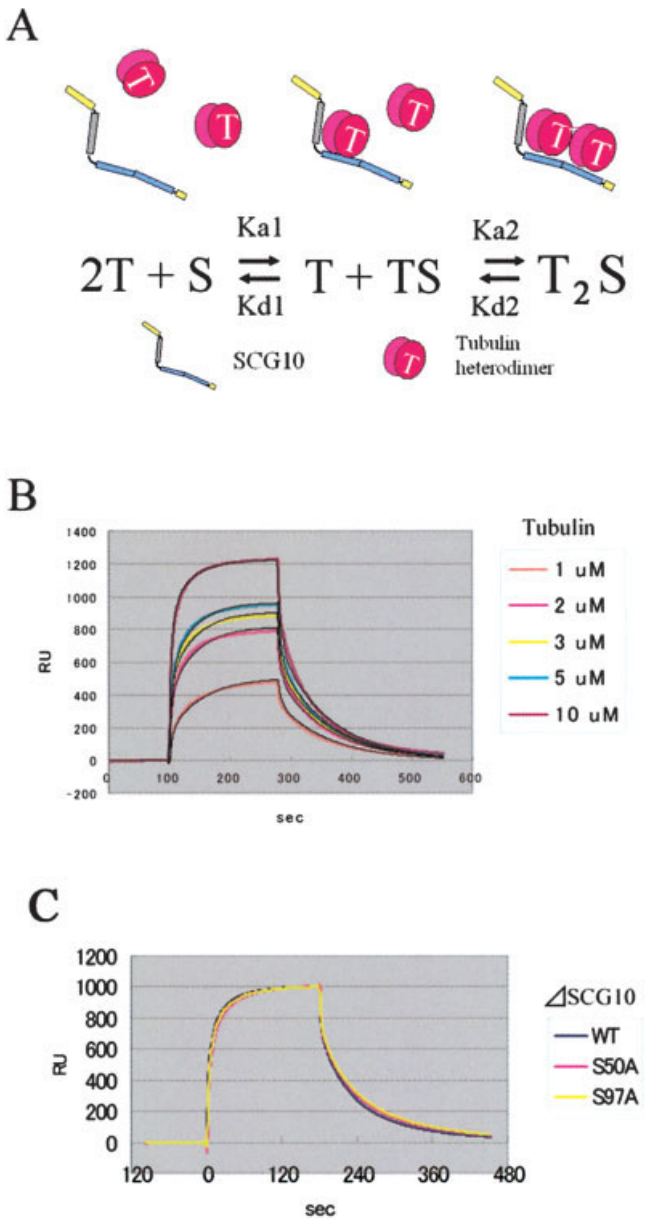

Fig. 4. Analysis of tubulin binding by ΔSCG10 and its mutants by SPR. **A:** The bivalent model for the T_2S complex. Stathmin family proteins are thought to form complexes with tubulin heterodimers in a 1:2 ratio, which is known as the T_2S complex. **B:** The net sensorgram from tubulin binding to ΔSCG10 derivatives at different tubulin concentrations was analyzed using a bivalent model, providing a good fit to the data. **C:** The sensorgrams from the nonphosphorylated forms of ΔSCG10, S50A, and S97A were similar. Tubulin concentration used was 3 μM each.

SPR Analysis of the Effect of On-Chip Phosphorylation Reveals That S50-P Retains Tubulin-Binding Activity

Analysis of tubulin binding by SPR revealed that the reaction is quantitatively dependent on the tubulin concentration flowing through the reaction cell. Using this method, we were able to measure the rate constants for the binding reaction and to analyze the dynamics of assembly and disassembly. Because SCG10 is believed to form a complex of two tubulin heterodimers with each stathmin monomer, which is referred to as the T_2S complex (Fig. 4A), curve fitting of the data was carried out using a bivalent model. The bivalent model provided good fits for the SCG10-tubulin association data (Fig. 4B). The nonphosphorylated forms of ΔSCG10, S50A, and S97A had similar tubulin-binding profiles (Fig. 4C), indicating that these three forms of SCG10 have similar tubulin-binding activities (see also Table I).

We next directly assessed the effect of on-chip phosphorylation on the kinetics of tubulin binding (Fig. 5). Phosphorylation of ΔSCG10 by PKA clearly reduced the maximal binding (R_{max}) of tubulin binding. Between 5 and 60 min, the decrease in tubulin binding was dependent on the length of the phosphorylation reaction. After this time, R_{max} was constant, which indicated that the phosphorylation level reached a maximum at 60 min. The R_{max} was lower for the doubly phosphorylated SGC10 (S50/S97-P) than it was for singly phosphorylated proteins (S50-P or S97-P).

The association rate constants for the binding of the first tubulin heterodimer (k_{a1}) to S50/S97-P and S97-P were significantly lower than they were for the corresponding nonphosphorylated forms (Table I). In addition, S50-P had a smaller dissociation constant for the binding of the first heterodimer (K_{D1}) than did the other two phosphorylated forms (Table I). The dissociation constant for the binding of the second tubulin dimer (K_{D2}) itself did not significantly differ, but both the association (k_{a2}) and the dissociation (k_{d2}) rate constants of S50-P were significantly larger than were those of S97-P and S50/S97-P (Table I). These results suggest that S50-P has higher affinity for the binding of the first tubulin heterodimer and a more rapid turnover for the binding of the second heterodimer than it does for the other two phosphorylated forms.

DISCUSSION

SCG10, a stathmin-like protein family member that promotes MT disassembly, is found in growth cones and is known to be phosphorylated by PKA on S50 and S97 (Mori and Morii, 2002; Grenningloh et al., 2004). To determine the effect of these two phosphorylation sites, we examined both tubulin binding and MT dynamics. We demonstrated that, unlike S97-P and S50/S97-P, S50-P retained MT-regulating activity. To study the specific effects of the phosphorylations on MT binding by SCG10, we devised an on-chip phosphorylation method. Our present results are the first report of a direct kinetic analysis of a phosphorylated stathmin protein. Specifically, we found that S50-P had a lower k_{d1} and K_{D1} than did the other two phosphorylated forms, indicating that S50-P forms a more stable complex with tubulin heterodimers and that it results in lower tubulin turnover than other phosphorylated forms.

TABLE I. Kinetic Parameters of Tubulin Heterodimer Binding to SCG10 and its Mutants as Determined by Surface Plasmon Resonance[†]

SCG10 mutant	k_{a1}[a] (10^3/Ms)	k_{d1}[b] (10^{-2}/s)	k_{a2}[a] (10^{-5}/RU)	k_{d2}[b] (10^{-3}/s)	K_{D1}[c] (10^{-5}M)	K_{D2}[c] (10^2RU/s)
ΔSCG10	3.51 ± 2.02	10.8 ± 3.56	2.23 ± 1.49	9.60 ± 9.35	3.59 ± 1.44	3.84 ± 0.88
S50A	3.47 ± 0.97	9.6 ± 2.70	1.73 ± 0.57	5.95 ± 0.76	2.90 ± 2.07	3.69 ± 1.07
S97A	4.78 ± 1.35	13.7 ± 3.17	1.13 ± 0.52	5.54 ± 0.45	2.99 ± 0.46	5.74 ± 2.59
S50/97-P	1.12 ± 0.88*	5.47 ± 3.89	3.12 ± 2.41	4.39 ± 1.81	5.83 ± 2.96	1.90 ± 1.06
S97-P	1.82 ± 0.36*	6.06 ± 3.75	1.90 ± 1.36	5.15 ± 2.83	3.43 ± 2.07	3.13 ± 0.87
S50-P	2.34 ± 0.77*	0.956 ± 0.06**	22.6 ± 9.93***	56.9 ± 19.2***	0.46 ± 0.18**	2.59 ± 0.53

[†]Results represent means ± standard deviation ($n = 6$).

[a]Values of k_{a1} and k_{a2} are the association rate constants for the binding of the first and second tubulin heterodimers, respectively.

[b]Values of k_{d1} and k_{d2} are the dissociation rate constants for the binding of the first and second tubulin heterodimers, respectively.

[c]Values of K_{D1} and K_{D2} are the dissociation constants for the binding of the first and second tubulin heterodimers, respectively.

*$P < 0.05$ for S50/97-P *vs.* ΔSCG10, S97-P *vs.* S50A, and S50-P *vs.* S97A; ** $P < 0.05$, *** $P < 0.01$ for S50-P *vs.* S97-P and S50/97-P (by Student's *t*-test). Also see Fig. 4A and its legend.

MT Dynamics and Tubulin-Binding Kinetics in the Presence of Phosphorylated SCG10

The urea-PAGE gel shift assay demonstrated that S50A/S97A SCG10 was not phosphorylated by PKA, confirming that PKA phosphorylates SCG10 at only S50 and S97. Using S50A and S97A, we succeeded in producing and distinguishing S97-P from S50-P. The doubly phosphorylated form, S50/S97-P had a lower ability than S97-P did to promote depolymerization and inhibit polymerization of MT; however, both of these phosphorylated forms decreased MT depolymerization. These results are consistent with the fact that the tubulin-binding activity of S97-P and S50/S97-P is reduced (Fig. 5). SPR analysis revealed that phosphorylation did not change the K_{D1} or K_{D2} for tubulin binding, indicating that phosphorylation by PKA did not alter the affinity for tubulin. Because SPR analysis allows for real-time monitoring and measurement of the reaction rate, we were able to show that phosphorylation at S50 or S97 reduced the k_{a1}, suggesting a slower association of phospho-SCG10 and tubulin. This reduces the ability of SCG10 to sequester tubulin, thereby attenuating MT disassembly.

The parameters determined by SPR revealed that S50-P had a smaller K_{D1} and larger k_{a2} and k_{d2} than did the other phosphorylated species that we examined. This indicates that S50-P induces a more stable complex with the first tubulin heterodimer and promotes a faster turnover of the second tubulin heterodimer. It is likely that these properties of S50-P compensate for the effect of other phosphorylations on MT dynamics.

In COS cells, overexpression of S50/S97D or S97D but not S50D enhances MT assembly (Antonsson et al., 1998). Our present results are consistent with the idea that the aspartates mimicked phosphoserine. Also in agreement with our results, structural analysis reveals that the phosphorylation of serine 63 in stathmin, which corresponds to S97-P in SCG10, destroys the α-helix within the tubulin-binding site (Steinmetz et al., 2001). Because S50-P is located within a regulatory domain, it is likely that the effects of phosphorylation at S50 and S97 are different.

In the SPR study, we adopted a bivalent model for the SCG10-tubulin complex because one SCG10 molecule is thought to bind two tubulin heterodimers, also referred to as the T_2S complex model (Curmi et al., 1997). Although our K_{D1} values are generally higher than those described in other reports using a univalent model (Table I), we confirmed that the bivalent model fits better to our data than the univalent model (data not shown).

Significance of S50 Phosphorylation in SCG10

Activation of PKA by cAMP is a potent regulator of growth cone guidance (Ming et al., 2001; Kao et al., 2002; Nishiyama et al., 2003). We suspect that SCG10 is a physiologically important substrate for PKA and a target of cAMP in the growth cone. The control of MT assembly is believed to be one of the most important aspects for the regulation of growth cone behavior (Dent and Gertler, 2003; Gordon-Weeks, 2004). There are many components that act as MT regulators, including microtubule-associated proteins and Tau, which promote MT polymerization. These two proteins are polymerization factors, and like depolymerization factors such as stathmin/Op18 proteins, they are known to be modulated by phosphorylation (Gordon-Weeks, 2004).

S50 is phosphorylated in the growth cone (M.I., unpublished results). This is the also the major site of phosphorylation in the brain (G.G., personal communication). As in stathmins, S50 in SCG10 may be phosphorylated by kinases in addition to PKA (Wittmann et al., 2003); however, of these kinases, PKA is the most abundant in isolated growth cones (T.T. and M.I., in preparation). The phosphorylation of S50-P by PKA thus is expected to be an important regulator of growth cone behavior.

The phosphorylation of SCG10 on S50 and other sites had been believed to reduce the ability to inhibit the disassembly of MT assembly, but our present results clearly reveal that the effect of S50-P on MT dynamics is very similar to that of the nonphosphorylated form of SCG10. S50-P and S97-P thus should have different effects on MT dynamics.

Because SCG10 interacts with other molecules besides tubulin (Nixon et al., 2002), S50-P of SCG10

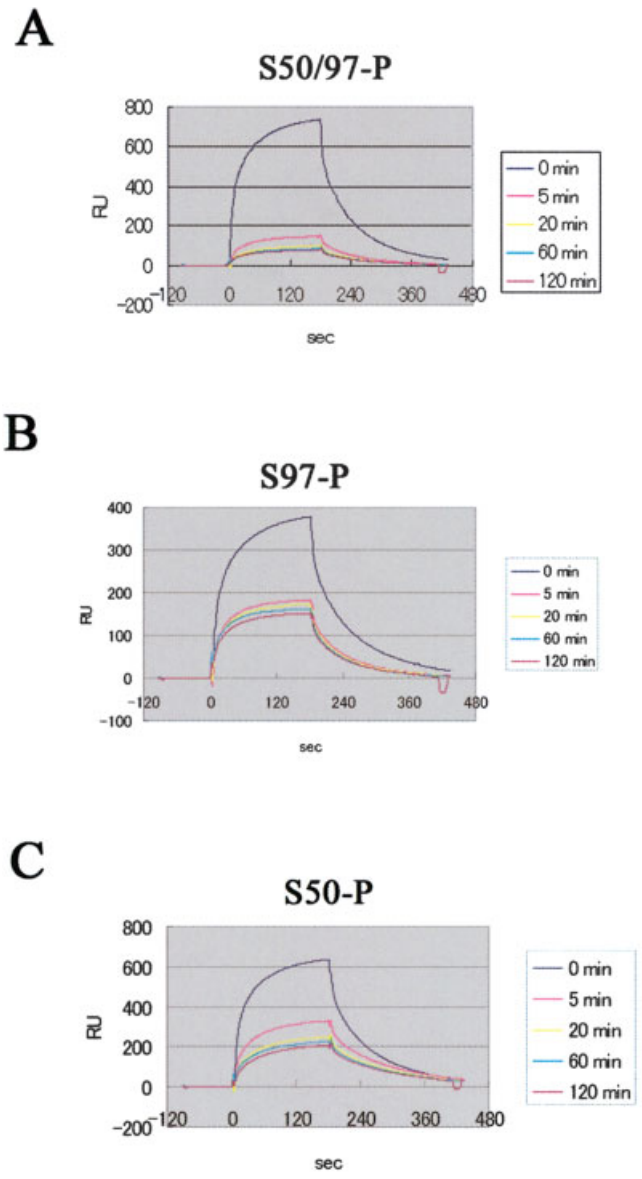

Fig. 5. Sensorgrams for tubulin binding to the phosphorylated forms of SCG10 after on-chip phosphorylation by PKA. The sensorgrams for S50/S97-P (**A**), S97-P (**B**), and S50-P (**C**) are shown after different times of phosphorylation by PKA. A plateau was reached for the binding of 3 μM tubulin after 60 min of phosphorylation.

may be involved in growth cone functions independent of effects on MT dynamics. To determine whether this occurs, we are now using real-time video analysis to investigate the localization and movements of green fluorescent protein (GFP)-SCG10 and its S50A mutant.

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