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Site-specific phosphorylation of SCG10 in neuronal plasticity: Role of Ser73 phosphorylation by *N*-methyl D-aspartic acid receptor activation in rat hippocampus

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Abstract

Accumulated evidence suggests that actin and microtubule regulating proteins contribute to neuronal structural dynamics, which subsequently affect neuronal plasticity. SCG10 is a neuronal-specific stathmin protein with microtubule destabilizing activity that is affected by multiple phosphorylation, at least in vitro. SCG10 has four major phosphorylation sites: Ser50 and Ser97 targeted by protein kinase A (PKA), and Ser62 and Ser73 targeted by mitogen-activated protein kinase (MAPK). To explore the potential roles of site-specific phosphorylation in physiological models, we developed phosphorylation site-specific antibodies and examined the SCG10 status in primary cultured hippocampal neurons and tissues. Although SCG10 is concentrated in growth cones and the Golgi apparatus in primary cultured neurons, the phosphorylated form was also detected in both regions, suggesting that MT dynamics within the growth cone may be regulated by protein phosphorylation. In the adult hippocampus, an intense stimulus such as kainate treatment induced a rapid phosphorylation of Ser73 within 15 min that was sustained for at least 60 min. This response was mediated through the *N*-methyl D-aspartic acid (NMDA) receptor and was ablated by the antagonist MK-801. The MAPK enzyme Erk2 was simultaneously activated along a similar time course to SCG10, suggesting that Erk2 may directly phosphorylate Ser73. These results demonstrate that changes in the phosphorylation status of SCG10 in vivo, dependent upon neural activity and/or plasticity, could affect the microtubule dynamics in neuronal dendrites.

Keywords: Stathmin; Microtubule dynamics; Cytoskeleton; Seizure

SCG10, which belongs to a small family that includes stathmin, SCLIP and RB3, is one of abundant protein in growth cones during brain development [10,13,19]. All family members act as microtubule (MT)-destabilizing factors to suppress MT polymerization and to induce MT depolymerization [5,20] and, with the exception of stathmin have a palmitoylated N-terminal domain through which they anchor to the Golgi apparatus and vesicles [8]. While stathmin is ubiquitously expressed, the expression of other members is mostly limited to neurons and is developmentally regulated [22,25]. SCG10 is the dominant species in immature neurons, while SCLIP predominates in mature neurons. In contrast, RB3 expression levels are low in both the immature and mature brain, but appear to be induced during seizure [4]. In the adult brain, SCG10 gene expression is up-regulated in lesion-based experiments and long-term potentiation (LTP) [17,21,26] and down-regulated following the monocular deprivation of visual input [12]. Thus, SCG10 may serve as a regulator of MT dynamics in structural plasticity.

MT-destabilizing activity of SCG10 is regulated by multiple phosphorylation. SCG10 has been characterized as a substrate of serine/threonine protein kinases such as PKA and MAPK in vitro [3]. Four phosphorylation sites were identified: Ser50 and Ser97 are targeted by PKA, while Ser62 and Ser73 are phosphorylated by MAPK [2]. The MT-destabilizing activity showed an inverse correlation with the number of phosphorylated sites, and

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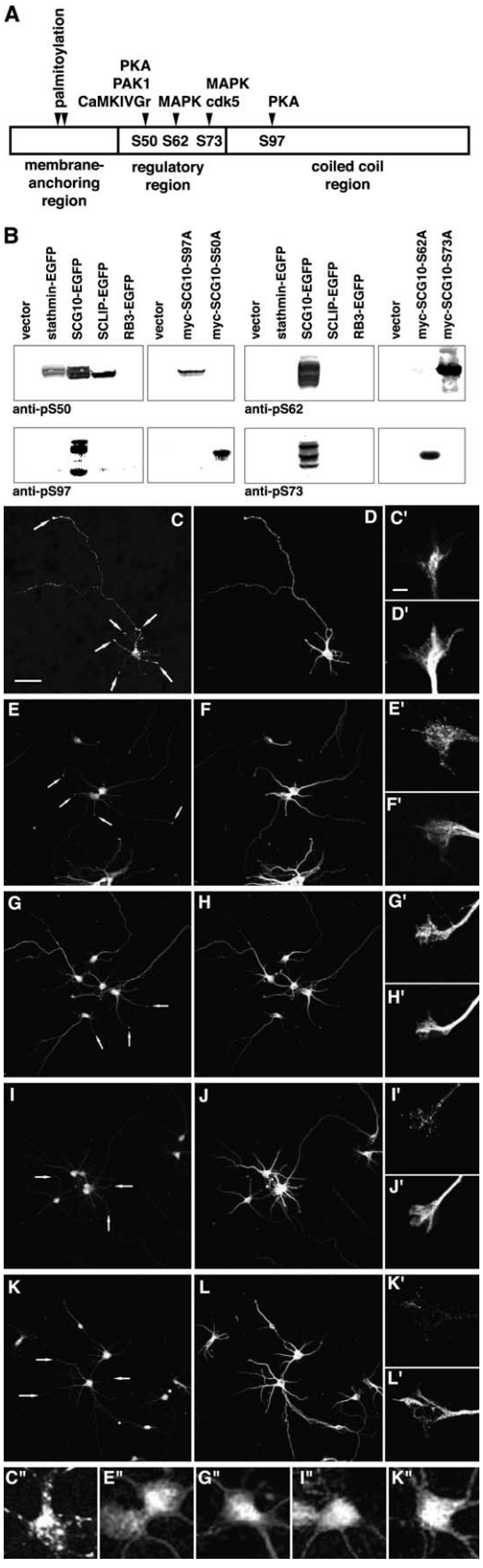

phosphorylation at all four sites caused an almost total loss of activity. This fine-tuning of SCG10 activity by several signal transduction pathways may be part of a regulatory mechanism for MT dynamics.

In this study, we developed anti-phospho specific antibodies to demonstrate SCG10 phosphorylation status and measured the levels of phosphorylation in rat hippocampus after an intense stimulation such as kainate or pentylenetetrazole (PTZ) treatment.

All experiments were approved by the Animal Research Committee of National Institute for Longevity Sciences and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Rats used in the present study were obtained from Japan SLC (Shizuoka, Japan).

Rabbit antiserum against SCG10 (SG-C) was developed using a C-terminal synthetic peptide (RNKELQVELSG) conjugated to KLH. Rabbit anti-phosphoserine SCG10 antibodies, anti-pS50, anti-pS62, anti-pS73, and anti-pS97, respectively, were raised against the phosphorylated synthetic KLH-conjugated peptides NKRA[pS]GQAF, CILKPP[pS]PISEAPRT, RTLA[pS]PKKK, and GRRK[pS]QEAQV. The specific antibodies were purified by peptide affinity column chromatography. Anti-SCG10 monoclonal antibody (clone 4B4) was prepared as reported by Maekawa et al. [16].

The coding regions of the SCG10 family (stathmin, SCG10, SCLIP, and RB3) were subcloned into a pEGFP-N1 vector or a pCIneo-myc vector. Nonphosphorylatable mutants (SA mutants) of SCG10 were generated by PCR using oligonucleotide mutagenesis primers, to replace the codon for the target serine with an alanine. All constructs were verified by sequencing.

Acute chemical seizure was induced in male Wistar rats (8–10 w) by intraperitoneal (i.p.) injection of kainate (Wako, Japan) at 30 mg/kg or PTZ (Sigma, USA) at 50 mg/kg in 0.9% saline. Hippocampi were immediately dissected on ice and homogenized in a buffer containing 50 mM Tris–Cl (pH 7.5), 1 mM EDTA, protease inhibitors (1 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml E-64, and 1 mM PMSF), and phosphatase inhibitors (5 mM NaF, 25 mM β-glycerophosphate, and phos-

Fig. 1. The subcellular localization of SCG10 phospho-isoforms in primary cultured hippocampal neurons. (A) SCG10 consists of three functional regions: a membrane anchoring region, a regulatory region, and a coiled coil region. Arrowheads indicate sites of phosphorylation by PKA (Ser50 and Ser97) and MAPK (Ser62 and Ser73). By analogy with stathmin, Ser50 and Ser73 may also be phosphorylated by PAK1/CaMKIVGr and cdk5, respectively. (B) The phospho-specific antibodies were validated in COS cells, transiently overexpressing stathmin family proteins or SCG10 SA mutants using Lipofectamine PLUS (Invitrogen). One day post-transfection, cells were stimulated with forskolin for anti-pS50 and anti-pS97 detection, or by the replacement of fetal bovine serum following serum starvation for anti-pS62 and anti-pS73 detection. The cell extracts were analyzed by Western blotting. (C–L) Primary cultured hippocampal neurons were stained using anti-SCG10 (C), anti-pS50 (E), anti-pS62 (G), anti-pS73 (I), and anti-pS97 (K) antibodies. Cells were co-stained with anti-α-tubulin (D, F, H, J, and L). Arrows indicate the nerites tips. Panels C′–L′ and panels C″–K″ show stained images of a growth cone and a cell body from the main panel at a high magnification, respectively.