c-Jun N-terminal kinase-3 (JNK3)/stress-activated protein kinase-β (SAPKβ) binds and phosphorylates the neuronal microtubule regulator SCG10

Sybille Neidhart[a], Bruno Antonsson[b], Corine Gilliéron[b], Francis Vilbois[b], Gabriele Grenningloh[a,1,*], Steve Arkinstall[c,1]

[a] *Institut de Biologie Cellulaire et de Morphologie, Université de Lausanne, rue du Bugnon 9, 1005 Lausanne, Switzerland*
[b] *Serono Pharmaceutical Research Institute, chemin des Aulx 14, 1228 Plan-les-Ouates, Geneva, Switzerland*
[c] *Serono Reproductive Biology Institute, Randolph, MA 02368, USA*

Received 5 September 2001; revised 17 October 2001; accepted 17 October 2001

First published online 2 November 2001

Edited by Richard Marais

Abstract The neuronal growth-associated protein SCG10 is enriched in the growth cones of neurons where it destabilizes microtubules and thus contributes to the dynamic assembly and disassembly of microtubules. Since its microtubule-destabilizing activity is regulated by phosphorylation, SCG10 may link extracellular signals to rearrangements of the neuronal cytoskeleton. To identify signal transduction pathways that may lead to SCG10 phosphorylation, we tested a series of serine–threonine-directed protein kinases that phosphorylate SCG10 in vitro. We demonstrate that purified SCG10 can be phosphorylated by two subclasses of mitogen-activated protein (MAP) kinases, c-Jun N-terminal/stress-activated protein kinase (JNK/SAPK) and p38 MAP kinase. Moreover, SCG10 was found to bind tightly and specifically to JNK3/SAPKβ. JNK3/SAPKβ phosphorylation occurs at Ser-62 and Ser-73, residues that result in reduced microtubule-destabilizing activity for SCG10. Endogenous SCG10 also undergoes increased phosphorylation in sympathetic neurons at times of JNK3/SAPKβ activation following deprivation from nerve growth factor. Together these observations indicate that activation of JNK/SAPKs provides a pathway for phosphorylation of SCG10 and control of growth cone microtubule formation following neuronal exposure to cellular stresses.

Key words: SCG10; Phosphorylation; Stress-activated protein kinase; c-Jun N-terminal kinase; Mitogen-activated protein; Scintillation proximity assay

1. Introduction

Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK3/SAPKβ) and p38/RK/CSBP (p38α) represent three subclasses of mitogen-activated protein (MAP) kinase that play an essential role within a number of intracellular signal transduction pathways [1–3]. Many MAP kinases are highly expressed within the central nervous system, particularly neurons, suggesting an important role in brain function. Recent observations suggest involvement in at least three distinct processes. Firstly, a role regulating neurotransmission and synaptic plasticity appears likely as ERKs become activated during synaptic activity [4,5], ERKs phosphorylate synapsin I at key regulatory sites [6,7], and this MAP kinase subclass has also been shown to play an important role in models of learning and memory [8–10]. A second function involving MAP kinases is neuronal differentiation. While blockade of ERK activation prevents nerve growth factor (NGF)-induced differentiation of PC12 cells [11], the effects of this neurotrophic factor can be mimicked by expression of mutant upstream components leading to constitutive activation of ERK MAP kinases [12]. Thirdly, neuronal apoptosis is also under tight control by MAP kinases, as blocking JNK3/SAPKβ, and p38 activation prevents death in NGF-deprived PC12 cells [13,14], in sympathetic neurons [15] and in glutamate-treated cerebellar granule cells [16], while excitotoxic death of hippocampal neurons is blocked in mice devoid of the JNK3 gene [17]. Powerful and rapid induction of selected MAP kinase phosphatases during neuronal differentiation [18] or following excitotoxic injury [19,20] is also consistent with an important role for MAP kinases controlling brain functions under both normal and pathological conditions. One major question relating to mechanisms by which MAP kinases may act to control different neuronal functions is the molecular identity of target proteins undergoing phosphorylation and which, as a consequence, exhibit altered function.

Currently, a number of proteins are known to undergo phosphorylation by different MAP kinases including phospholipase A2, additional kinases, nuclear receptors and transcription factors, as well as regulators of cell death and cytoskeletal function [21–25]. SCG10 is a neuron-specific membrane-associated protein highly concentrated in growth cones that has been shown to regulate microtubule dynamics [26]. SCG10 is found phosphorylated in brain and in vitro studies demonstrate that this modification regulates its microtubule-destabilizing activity and may be a mechanism linking cell surface signals to altered microtubule dynamics within the growth cone [27]. Although ERK MAP kinase p44 has been shown to phosphorylate SCG10 [27], a comparison with other

*Corresponding author. Fax: (41)-21-692 5105.
E-mail address: gabriele.grenningloh@ibcm.unil.ch (G. Grenningloh).

[1] These authors contributed equally.

Abbreviations: JNK/SAPK, c-Jun N-terminal/stress-activated protein kinase; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MBP, myelin basic protein; MS, mass spectrometry; SPA, scintillation proximity assay; NGF, nerve growth factor

0014-5793/01/$20.00
PII: S0014-5793(01)03090-3

MAP kinases has not yet been performed. This is a critical question as JNK3/SAPKβ and p38 MAP kinases undergo powerful activation by a number of stimuli distinct from those that activate ERKs, including a number of inflammatory cytokines and cellular stresses. In this report, we show that purified SCG10 can be phosphorylated not only by ERK, but also by all three major MAP kinase classes and, moreover, SCG10 binds tightly and specifically to JNK3/SAPKβ. Together these observations could indicate a new pathway for controlling neuronal growth cone function during inflammation or conditions of local cellular stress.

2. Materials and methods

2.1. Materials

Constitutively activated MAP kinase kinase (MEK1, JNKK2, MKK6) and inactive MAP kinase (ERK2, JNK3/SAPKβ, p38α) were generated as GST fusion proteins in *Escherichia coli* and purified using glutathione-Sepharose and nickel-agarose columns as described [28–30]. Recombinant SCG10 was purified as described earlier and stored in 20 mM Tris–HCl, 0.2 mM dithiothreitol (DTT), pH 7.5 at −80°C [31]. Myelin basic protein (MBP) was from Sigma (Division of Fluka Chemie, Buchs, Switzerland).

The following plasmids were generous gifts obtained as follows: pcDNA1-HA-p44 ERK1 from J. Pouyssegur (CNRS, Nice, France), pMT2T-HA-p54-SAPKβ from J.R. Woodgett (Ontario Cancer Institute, Toronto, ON, Canada), pcDNA3-HA-p38 from J.S. Gutkind (NIDR, National Institutes of Health, Bethesda, MD, USA). The expression vectors were transfected into COS-7 cells using FuGENE6 (Roche Diagnostics AG, Basel, Switzerland). [γ-^{33}P]ATP (1000 Ci/mmol) and streptavidin-coated scintillation proximity assay (SPA) beads were from Amersham Pharmacia Biotech, [γ-^{32}P]ATP (5000 Ci/mmol) was from DuPont de Nemours International S.A. (Regensdorf, Switzerland). The magnetic beads, BioMag-Streptavidin were from PerSeptive Diagnostic (Cambridge, UK), the Sulfo-NHS-LC-Biotin from Pierce (USA). Dulbecco's modified Eagle's cell culture medium and cell culture products were purchased from Life Technologies, Inc. (Basel, Switzerland), the 96-well plates (PET) were from Wallac (Finland), and murine EGF from Promega (Madison, WI, USA). All other chemicals were obtained from Sigma (Buchs, Switzerland). Sprague–Dawley rats and rat serum were supplied by Serono Pharmaceutical Research (Geneva, Switzerland). The anti-HA monoclonal antibody HA.11 was from Rowag Diagnostics (Zürich, Switzerland), the anti-NGF antibody from Roche Diagnostics, rabbit anti-Gqα is described in [32] and rabbit anti-SCG10 in [27]. Horseradish peroxidase conjugates with avidin, goat anti-mouse IgG, and goat anti-rabbit IgG were from Bio-Rad Laboratories (Glattbrugg, Switzerland).

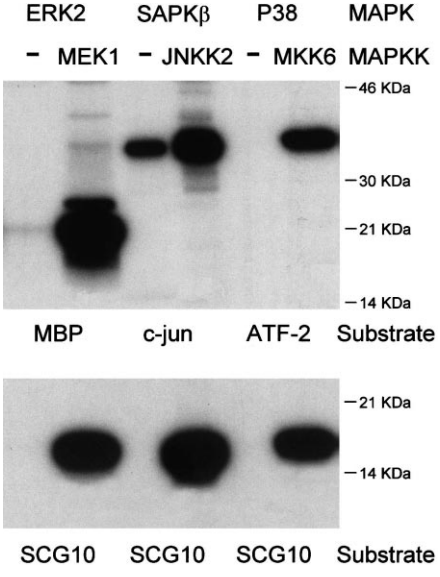

Fig. 1. In vitro phosphorylation of SCG10 by activated MAP kinases. Autoradiograph of phosphorylated SCG10 and control substrates (MBP, c-Jun, ATF-2) following separation on a 15% SDS–polyacrylamide gel. Substrates were incubated with purified recombinant MAP kinases (ERK2, JNK3/SAPKβ or p38α) and [γ-^{32}P]ATP in the presence or absence of the specific upstream MAP kinase kinase (MEK1, JNKK2 or MKK6). All three MAP kinases phosphorylate SCG10.

2.2. In vitro phosphorylation assays

Phosphorylation assays were performed in 60 μl at 30°C for 4 h. Reaction mixtures contained 20 μl kinase buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 0.2 mM sodium vanadate, 10 mM β-glycerophosphate, 2 mM DTT), 10 μl kinase substrate (15 μg MBP, 10 μg c-Jun, 10 μg ATF-2 or 10 μg SCG10), 10 μl MAP kinase (0.6 μg ERK2, 0.5 μg JNK3/SAPKβ, 0.5 μg p38α) with or without 10 μl MAP kinase kinase (0.1 μg MEK1, 0.2 μg JNKK2, 0.1 μg MKK6) and 10 μl of [γ-^{32}P]ATP mix (containing 6 μCi and 56 μM unlabeled ATP in 10 mM Tris–HCl pH 7.4). Reactions were initiated by addition of the ATP mix and incorporation of ^{32}P radioactivity was analyzed by electrophoresis on a 15% SDS–polyacrylamide gel and autoradiography.

2.3. Cell culture, transfection and stimulation

COS-7 cells were grown as previously described [28] and transfected using FuGENE6 (Roche Diagnostics) according to the manufacturer's instructions. Cells were transfected with 1 μg of the following plasmids: pcDNA1-HA-p44 ERK1, pMT2T-HA-p54-SAPKβ, pcDNA3-HA-p38. Cells were grown for 40 h before starvation and stimulation. For acute stimulation of MAP kinase activation, cells were starved by incubation in serum-free medium for 2 h followed by exposure for 10 min to EGF (10 nM), for 30 min to anisomycin (10 μg/ml), or for 30 min to H_2O_2 (0.5 mM) at 37°C.

2.4. Activation of MAP kinases in COS-7-stimulated cells and interactions with SCG10

2.4.1. Protein biotinylation. The proteins SCG10 and c-Jun were biotinylated according to the procedure provided by the manufacturer (Pierce, USA). To the samples in phosphate-buffered saline (PBS) was added 3-fold molar excess of Sulfo-NHS-LC-Biotin (Pierce, USA) and the samples were incubated at 4°C for 2 h in roller bottles protected from light. At the end of the incubation the samples were dialyzed against 50% glycerol with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT, 5 mM benzamidine. The samples were stored in aliquots at −80°C. The incorporation of biotin was determined with the assay kit from Pierce (ImmunoPure HABA). We incorporated 1.5 mol of biotin per mol of SCG10 and 5 mol of biotin per mol of c-Jun. For the precipitation assay (see below), the biotinylated proteins (c-Jun-Biotin and SCG10-Biotin) were coupled overnight by rotator mixing at 4°C to BioMag-Streptavidin magnetic beads as recommended by the manufacturer (PerSeptive Diagnostics, Cambridge, UK). BioMag c-Jun and BioMag SCG10 refer to the protein-bound magnetic beads.

2.4.2. Cell lysis. The transfected and stimulated cells (as described above) were washed twice in 2 ml of ice-cold PBS and scraped into Eppendorf tubes with 300 μl of Hibi lysis buffer (50 mM HEPES pH 7.4, containing 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 μg/ml leupeptin, 2 μl/ml aprotinin, 1 mM pefabloc). Cells were then homogenized using a syringe and the lysates were stored at −80°C until precipitation.

2.4.3. Precipitation. Aliquots (200 μl) of the COS-7 cell lysates were mixed with 800 μl of Hibi dilution buffer (18.3 mM HEPES pH 7.4, containing 14.2 mM NaCl, 0.07 mM EDTA, 0.03% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM pefabloc) and rotary-mixed for 1 h at 4°C, after which time they were centrifuged at 10 000 × *g* for 10 min at 4°C. For precipitation, 900 μl of supernatant was mixed by rotary mixing overnight at 4°C with 100 μl preformed BioMag c-Jun and BioMag SCG10. The following day, the magnetic particles were washed four times in 1 ml of ice-cold Hibi binding buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% Triton X-100) followed by final resuspension in 40 μl of kinase buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$,

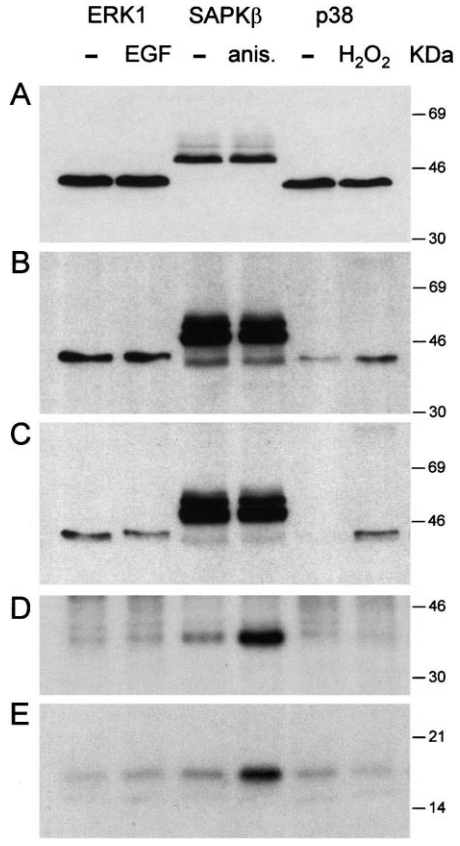

Fig. 2. JNK3/SAPKβ binds and phosphorylates SCG10. COS-7 cells were transfected with HA-tagged MAP kinases. After 40 h of growth and 2 h of serum starvation, cells were incubated in the absence (–) or the presence of 10 nM EGF (HA-ERK1), 10 μg/ml anisomycin (HA-SAPKβ), or 0.5 mM H_2O_2 (HA-p38). Cells were lysed and subjected to precipitation using biotinylated c-Jun or biotinylated SCG10 pre-bound to BioMag-Streptavidin magnetic beads. Immobilized c-Jun (B) and SCG10 (C) complexes were analyzed by Western blot using anti-HA epitope-specific monoclonal antibody. To control for expression of MAP kinases the cell lysates used for precipitation were subjected to Western analysis using anti-HA epitope monoclonal antibody (A). Data shown are representative of three separate experiments. B, C: While all three MAP kinases are expressed at similar amounts (A), only SAPKβ becomes highly enriched by the precipitation indicating that SAPKβ forms a complex with c-Jun or SCG10. The anti-HA immunoreactivity seen for ERK1 and p38 might represent non-specific binding to the beads. Quantification of the intensity of the bands on autoradiograms of three independent experiments by optic densitometry revealed that the gray values in lanes 3 and 4 (SAPKβ) were between 2000 and 3000 times higher than the values in lanes 1 and 2 (ERK1) or lanes 5 and 6 (p38). D, E: Autoradiograph of protein complex kinase assays following separation on a 15% SDS–polyacrylamide gel. The precipitated c-Jun- or SCG10-bound kinases were incubated in the presence of [γ-^{32}P]ATP for 30 min. Radioactivity is incorporated into c-Jun (D) and SCG10 (E) when bound to activated JNK3/SAPKβ. Control assays (immunoprecipitation of the MAP kinases using anti-HA epitope monoclonal antibody pre-bound to protein Sepharose beads followed by immune complex assays using MBP, or ATF-2 as substrates) have been performed to confirm activation of ERK1 and p38 (not shown; for experimental procedures see [29]).

20 mM β-glycerophosphate, 20 mM 4-nitrophenylphosphate, 0.1 mM sodium vanadate, 2 mM DTT). These samples were analyzed on SDS–PAGE and Western blotting using the ECL method from Amersham Pharmacia Biotech.

2.4.4. Protein complex kinase assays. Assays were performed by mixing 20 μl of bead suspensions with 10 μl of [γ-^{32}P]ATP (containing 2.5 μCi and 6 μM unlabeled ATP in 10 mM Tris–HCl pH 7.4) and 30 μl of kinase buffer followed by incubation for 30 min at 30°C. Reactions were terminated by adding 15 μl of 10× Laemmli sample buffer [33] and heated for 5 min at 95°C. Following centrifugation at 10 000×*g* for 5 min, supernatants were analyzed by SDS–PAGE (15% gels) and autoradiography.

2.5. SPA

SPA was used to measure phosphorylation of c-Jun and SCG10 as previously described [34]. The protein kinases and the biotinylated proteins used in this assay were as described above. To each well (96-well plates) was added 40 μl of assay solution containing 5 μl of biotinylated substrate (c-Jun-Biotin or SCG10-Biotin), 10 μl of MAP kinase (0.2 μg GST-JNK3/SAPKβ), 10 μl of MAP kinase kinase (GST-JNKK2), 10 μl of distilled water and the reactions were initiated by adding 5 μl of kinase buffer (160 mM HEPES pH 7.5, 80 mM $MgCl_2$, 8 mM DTT, 200 mM β-glycerophosphate, 0.8 mM sodium vanadate, 8 μM ATP, 900 nCi [γ-^{33}P]ATP).

2.6. Analysis of SCG10 phosphopeptides and tandem mass spectrometry (MS/MS) analysis

Purified SCG10 (100 μg) was phosphorylated by incubation with activated MAP kinase kinase (0.2 μg JNKK2), inactive MAP kinase (0.2 μg JNK3/SAPKβ), and 600 μM ATP in a total volume of 240 μl of kinase buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM 4-nitrophenylphosphate, 0.1 mM sodium vanadate, 2 mM DTT) containing 100 μM [γ-^{33}P]ATP (3000 dpm/pmol). Phosphorylated SCG10 was purified by high-performance liquid chromatography (HPLC), lyophilized, and digested by overnight incubation at 37°C in 90 μl of 100 mM Tris–HCl pH 8.5 containing 1 M urea, 20 mM methylamine, 1 mM DTT, and 5 μg of trypsin (sequencing grade). Peptides were separated by reverse-phase (RP) HPLC (Hewlett Packard HP1090) with a Brownlee C18 column (220×2.1 mm). Peptides were eluted with an acetonitrile gradient (in 0.1% trifluoroacetic acid) from 0 to 55% over 60 min, followed by 55–70% over 5 min. Fractions were collected, and radioactive ^{33}P-labeled phosphopeptides identified by scintillation spectrometry were sequenced by MS/MS. All MS experiments were carried out on a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nano-electrospray ion source [35].

2.7. NGF deprivation of sympathetic neurons

Sympathetic neurons from the superior cervical ganglia (SCG) of 1-day-old Sprague–Dawley rats (supplied by Serono Pharmaceutical Research, Geneva, Switzerland) were isolated, cultured and NGF-deprived as described previously [36]. The neurons were maintained for 7 days in the presence of NGF before being used for NGF with-

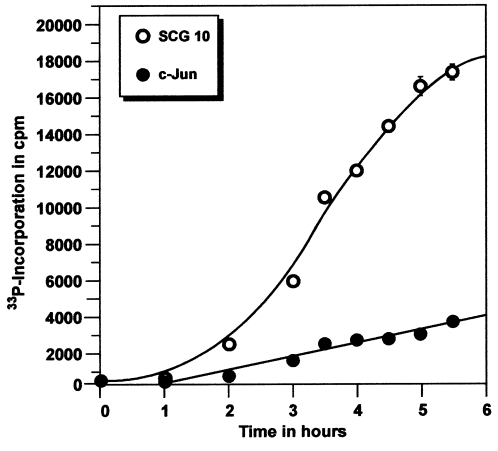

Fig. 3. Time dependence of the in vitro phosphorylation of biotinylated c-Jun and SCG10 proteins. Phosphorylation of biotinylated SCG10 and c-Jun by activated GST-JNK3/SAPKβ was measured using a 96-well plate-based SPA ($n=4$), by incubating JNK3/SAPKβ with biotinylated substrate (SCG10-Biotin or c-Jun-Biotin) in the presence of its MAP kinase kinase. The reaction was initiated with [γ-^{33}P]ATP and stopped at various time points by addition of 0.2 ml SPA beads solution.

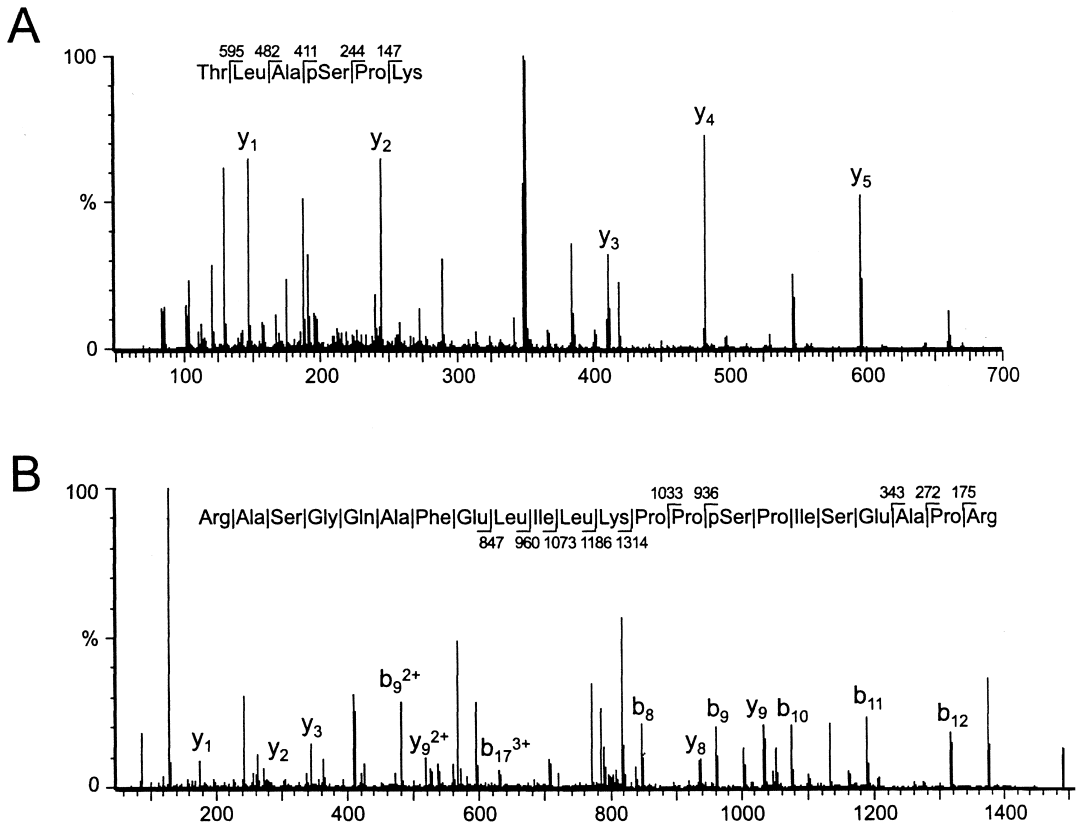

Fig. 4. MAP kinase-phosphorylated SCG10. To determine in vitro phosphorylation sites of the recombinant SCG10 after phosphorylation by JNK3/SAPKβ, the ^{33}P-phosphorylated SCG10 was digested with trypsin and the resulting peptides purified by RP-HPLC on a C18 column. Two peptides (A, B) eluted with the acetonitrile gradient (0–55%) in 0.1% trifluoroacetic acid were found to be ^{33}P-labeled. These peptides were submitted to MS/MS analysis on a Q-TOF mass spectrometer. The y_ns correspond to the ions generated by cleavage of the peptide bound after the *n*th amino acid from the COOH-terminal. The b_ns correspond to the ions generated from the NH_2-terminus. The charge, when higher than one, is indicated as superscript. The molecular weights of the identified peptide fragments are indicated in the peptide sequences. The sequences identified corresponded to TLASPK, residues 70–75, and RASGQAFELILKPPSPISEAPR, residues 48–69. The serine 73 and serine 62 (phosphoserine underlined) are found to be the sites of phosphorylation.

drawal. For Western blot, the growth medium from neuronal cultures was removed after various times of NGF deprivation and the remaining cells attached to the dish were harvested by scraping in a small volume of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, 50 mM glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 1 mM PMSF, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin), frozen in liquid nitrogen and stored at 80°C. The samples were heated at 90°C in Laemmli sample buffer [33], and the reaction products were separated on a 12% SDS–polyacrylamide gel, then transferred to nitrocellulose, and immunoblotted (ECL method of Amersham Pharmacia Biotech) using the rabbit anti-SCG10-BR antibody [27]. This antibody which was generated against in vitro phosphorylated (P-Ser-50, -62, -73 and -97) recombinant SCG10 recognizes both phosphorylated and non-phosphorylated SCG10.

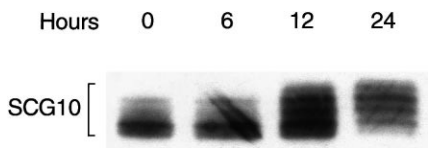

Fig. 5. Retardation of electrophoretic mobility of SCG10 expressed in rat sympathetic neurons following NGF deprivation. The sympathetic neurons were isolated and cultured as described in Section 2. After 7 days in culture, NGF-containing medium was removed, cultures were washed in NGF-free medium and incubated in medium containing anti-NGF antibody (100 ng/ml). The protein extracts were prepared at 0, 6, 12 and 24 h after NGF withdrawal and separated on 12% SDS–polyacrylamide gels. Western blot analysis was performed using the anti-SCG10-BR antiserum. Data are representative of three identical experiments.

3. Results and discussion

ERK1 has been reported previously to phosphorylate SCG10 [27]. To test the activity of other MAP kinase family members, we first incubated purified SCG10 with ERK2, JNK3/SAPKβ or p38α. Each MAP kinase was activated by a specific upstream MAP kinase kinase, MEK-1 for ERK2, JNKK2 for JNK3/SAPKβ and MKK6 for p38α. Fig. 1 shows that all three MAP kinases undergo powerful activation by the MAP kinase kinase and that ERK2, JNK3/SAPKβ or p38α all phosphorylate SCG10 to an extent similar to the control substrates. The use of purified recombinant proteins for these assays suggests that SCG10 phosphorylation results from a direct interaction with each MAP kinase. Similar observations were made using immunopurified HA epitope-tagged ERK1, JNK3/SAPKβ and p38α following expression and activation in COS-7 cells (data not shown).

Recently, several MAP kinases and their substrates have been shown to form tightly bound complexes, an interaction likely to facilitate specific and rapid phosphorylation of physiological target proteins within cells. This is exemplified by tight binding between JNK/SAPK family members and the target transcription factor c-Jun [29]. To test whether

SCG10 is able to bind any of the major mammalian MAP kinases, we transfected COS-7 cells with HA-ERK1, HA-JNK3/SAPKβ or HA-p38α and incubated cell lysates with biotinylated SCG10 pre-bound to streptavidin beads. Cell lysates were analyzed by Western blotting (Fig. 2A). Immobilized biotinylated c-Jun was used as a control and, as anticipated, was found to bind and precipitate HA-JNK3/SAPKβ from cell lysates (Fig. 2B). Interestingly, immobilized SCG10 also appeared to bind strongly and selectively to HA-JNK3/SAPKβ (Fig. 2C). To test whether JNK3/SAPKβ was active when bound to SCG10, washed beads were re-suspended in kinase buffer and incubated in the presence of [γ-^{32}P]ATP. Under these conditions, c-Jun is known to undergo phosphorylation by associated JNK/SAPK enzyme [37], this is clearly shown in Fig. 2D. In a similar fashion, SCG10 was also phosphorylated by bound JNK3/SAPKβ derived from cells stimulated by the JNK3/SAPKβ activator anisomycin (Fig. 2E). Phosphorylation of biotinylated SCG10 and c-Jun by activated JNK3/SAPKβ can also be measured using a 96-well plate-based SPA. Based on experiments using this assay, activated JNK3/SAPKβ phosphorylates SCG10 approximately 5-fold more rapidly than c-Jun (Fig. 3). Together these experiments demonstrate that although all three major MAP kinase subclasses can phosphorylate SCG10, only JNK3/SAPKβ was able to bind tightly to this substrate protein. Moreover, compared with the established JNK3/SAPKβ substrate c-Jun, SCG10 represents an excellent substrate for phosphorylation by activated JNK3/SAPKβ.

Previously SCG10 residues Ser-62 and Ser-73 were shown to be target sites for phosphorylation by the p44 ERK1 MAP kinase [27]. To test whether JNK3/SAPKβ phosphorylation occurs at identical sites, ^{33}P-phosphorylated SCG10 was digested with trypsin and the resulting peptides purified using RP-HPLC. Two peptides were found to be ^{33}P-labeled. Sequencing by MS/MS (Fig. 4) identified the phosphopeptides as TLASPK residues (70–75) and RASGQAFELILKPPSPISEAPR residues (48–69), with phosphorylation at Ser-73 and Ser-62, respectively (phosphoserine underlined). These sites are identical to those phosphorylated by p44 ERK1 suggesting that JNK3/SAPKβ, as demonstrated for p44 ERK1 [31], may also be effective at controlling microtubule-destabilizing activity of SCG10 within neuronal growth cones.

To assess whether SCG10 may be phosphorylated by JNK3/SAPKβ under physiological conditions, we next examined endogenous SCG10 expressed within SCG sympathetic neurons following deprivation from NGF. Neurotrophic factor withdrawal from this cell system has been reported by several groups to increase endogenous JNK3/SAPKβ activity and to lead to increased phosphorylation of c-Jun [38,39]. As assessed by Western blotting, immunoreactive SCG10 bands migrating with reduced electrophoretic mobility appear after 6–12 h of NGF removal. Such a profile of reduced SCG10 migration persists for at least 24 h (Fig. 5) and is consistent with the timing of JNK3/SAPKβ activation upon trophic factor deprivation [38,39]. A similar difference in the mobility of SCG10 protein in SDS–polyacrylamide gels has previously been observed in protein extracts of COS-7 cells transfected with wild-type SCG10 as compared with the mutant in which the serine phosphorylation sites have been mutated to alanines (data not shown). Furthermore, alkaline phosphatase treatment of brain extracts resulted in the disappearance of the slower migrating bands [27]. Thus, the observed gel shift in SCG neurons following NGF deprivation is most likely the result of increased phosphorylation. The tight correlation with activation of JNK3/SAPKβ family members strongly suggests that endogenous SCG10 becomes phosphorylated by this MAP kinase under conditions of cellular stress.

In summary, this report demonstrates that all three major classes of mammalian MAP kinase can phosphorylate the neuronal growth cone protein SCG10, although JNK3/SAPKβ appears specific insofar that it also binds tightly to this protein substrate. This could reflect kinase–substrate complex formation important for ensuring specific SCG10 phosphorylation by JNK3/SAPKβ under physiological conditions. JNK3/SAPKβ phosphorylation occurs at Ser-62 and Ser-73, residues which are also phosphorylated by p44 ERK1 and which result in reduced microtubule-destabilizing activity for SCG10 [27]. JNK3/SAPKβ is highly enriched in neurons and activation of this MAP kinase subclass may provide a pathway for phosphorylating SCG10 and thereby for controlling growth cone microtubule formation following neuronal exposure to inflammatory cytokines and/or a range of cellular stresses. Systems activating JNK3/SAPKβ may regulate microtubule dynamics in concert with pathways activating other kinases now known to phosphorylate SCG10 including p38 MAP kinase (this report), ERK, protein kinase A or p34 cdc2 kinase [27].

Acknowledgements: We thank Gilbert Di Paolo for this help and Stefan Catsicas for encouragement and advice. We are also grateful to Isabelle Martinou for providing the cultures of sympathetic neurons and Eric Bernardi for photographic work. This work was supported by Grant 31-50948.97 of the National Science Foundation of Switzerland to G.G.

References

[1] Widmann, C., Gibson, S., Jarpe, M.B. and Johnson, G.L. (1999) Physiol. Rev. 79, 143–180.
[2] Cobb, M.H. (1999) Prog. Biophys. Mol. Biol. 71, 479–500.
[3] Ono, K. and Han, J. (2000) Cell Signal. 12, 1–13.
[4] English, J.D. and Sweatt, J.D. (1996) J. Biol. Chem. 271, 24329–24332.
[5] Fukunaga, K. and Miyamoto, E. (1998) Mol. Neurobiol. 16, 79–95.
[6] Jovanovic, J.N., Benfenati, F., Siow, Y.L., Sihra, T.S., Sanghera, J.S., Pelech, S.L., Greengard, P. and Czernik, A.J. (1996) Proc. Natl. Acad. Sci. USA 93, 3679–3683.
[7] Matsubara, M., Kusubata, M., Ishiguro, K., Uchida, T., Titani, K. and Taniguchi, H. (1996) J. Biol. Chem. 271, 21108–21113.
[8] Kornhauser, J.M. and Greenberg, M.E. (1997) Neuron 18, 839–842.
[9] Blum, S., Moore, A.N., Adams, F. and Dash, P.K. (1999) J. Neurosci. 19, 3535–3544.
[10] Sweatt, J.D. (2001) J. Neurochem. 76, 1–10.
[11] Pang, L., Sawada, T., Decker, S.J. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 13585–13588.
[12] Cowley, S., Paterson, H., Kemp, P. and Marshall, C.J. (1994) Cell 77, 841–852.
[13] Park, D.S., Stefanis, L., Yan, C.Y.I., Farinelli, S.E. and Green, L.A. (1996) J. Biol. Chem. 271, 21898–21905.
[14] Horstmann, S., Kahle, P.J. and Borasio, G.D. (1998) J. Neurosci. Res. 52, 483–490.
[15] Harding, T.C., Xue, L., Bienemann, A., Haywood, D., Dickens, M., Tolkovsky, A.M. and Uney, J.B. (2001) J. Biol. Chem. 276, 4531–4534.
[16] Kawasaki, H., Morooka, T., Shimohama, S., Kimura, J., Hirano, T., Gotoh, Y. and Nishida, E. (1997) J. Biol. Chem. 272, 18518–18521.
[17] Yang, D.D., Kuan, C.Y., Whitmarsh, A.J., Rincon, M., Zheng,

T.S., Davis, R.J., Rakic, P. and Flavell, R.A. (1997) Nature 389, 865–870.
[18] Camps, M., Chabert, C., Muda, M., Boschert, U., Gilliéron, C. and Arkinstall, S. (1998) FEBS Lett. 425, 271–276.
[19] Boschert, U., Muda, M., Camps, M., Dickinson, R. and Arkinstall, S. (1997) NeuroReport 8, 3077–3080.
[20] Boschert, U., Dickinson, R., Muda, M., Camps, M. and Arkinstall, S. (1998) NeuroReport 9, 4081–4086.
[21] Waterman, W.H., Molski, T.F., Huang, C.K., Adams, J.L. and Sha'afi, R.I. (1996) Biochem. J. 319, 17–20.
[22] Geijsen, N., Dijkers, P.F., Lammers, J.J., Koenderman, L. and Coffer, P.J. (2000) FEBS Lett. 471, 83–88.
[23] Boulikas, T. (1995) Crit. Rev. Eukaryot. Gene Expr. 5, 1–77.
[24] Reynolds, C.H., Utton, M.A., Gibb, G.M., Yates, A. and Anderton, B.H. (1997) J. Neurochem. 68, 1736–1744.
[25] Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P. (1997) FEBS Lett. 409, 57–62.
[26] Riederer, B.M., Pellier, V., Antonsson, B., Di Paolo, G., Stimpson, S.A., Lutjens, R., Catsicas, S. and Grenningloh, G. (1997) Proc. Natl. Acad. Sci. USA 94, 741–745.
[27] Antonsson, B., Kassel, D.B., DiPaolo, G., Lutjens, R., Riederer, B.M. and Grenningloh, G. (1998) J. Biol. Chem. 273, 8439–8446.
[28] Muda, M., Boschert, U., Smith, A., Antonsson, B., Gilliéron, C., Chabert, C., Camps, M., Martinou, I., Ashwoth, A. and Arkinstall, S. (1997) J. Biol. Chem. 272, 5141–5151.
[29] Muda, M., Theodosiou, A., Gilliéron, C., Smith, A., Chabert, C., Camps, M., Boschert, U., Rodrigues, N., Davies, K., Ashworth, A. and Arkinstall, S. (1998) J. Biol. Chem. 273, 9323–9329.
[30] Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C. and Cohen, P. (1992) EMBO J. 11, 3985–3994.
[31] Antonsson, B., Montessuit, S., DiPaolo, G., Lutjens, R. and Grenningloh, G. (1997) Protein Expr. Purif. 9, 295–300.
[32] Edgerton, M.D., Chabert, C., Chollet, A. and Arkinstall, S. (1994) FEBS Lett. 354, 195–199.
[33] Laemmli, U.K. (1970) Nature 227, 680–685.
[34] Antonsson, B., Marshall, C.J., Montessuit, S. and Arkinstall, S. (1999) Anal. Biochem. 267, 294–299.
[35] Broekhuis, C.H., Neubauer, G., van der Heijden, A., Mann, M., Proud, C.G., van Venrooij, W.J. and Pruijn, G.J. (2000) Biochemistry 39, 3023–3033.
[36] Martinou, I., Fernandez, P.A., Missotten, M., White, E., Allet, B., Sadoul, R. and Martinou, J.-C. (1995) J. Cell. Biol. 128, 201–208.
[37] Camps, M., Nichols, A., Gilliéron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Science 280, 1262–1265.
[38] Virdee, K., Bannister, A.J., Hunt, S.P. and Tolkovsky, A.M. (1997) J. Neurochem. 69, 550–561.
[39] Eilers, A., Whitfield, J., Babij, C., Rubin, L.L. and Ham, J. (1998) J. Neurosci. 18, 1713–1724.