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Journal: Experimental neurology

Year: 2006 Nov

Volume: 202

Issue: 1

Pages: 152-60

DOI: 10.1016/j.expneurol.2006.05.026

Published in final edited form as:
Exp Neurol. 2006 November ; 202(1): 152–160. doi:10.1016/j.expneurol.2006.05.026.

Taxol and tau overexpression induced calpain-dependent degradation of the microtubule-destabilizing protein SCG10

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Abstract

Microtubule-stabilizing and -destabilizing proteins play a crucial role in regulating the dynamic instability of microtubules during neuronal development and synaptic transmission. The microtubule-destabilizing protein SCG10 is a neuron-specific protein implicated in neurite outgrowth. The SCG10 protein is significantly reduced in mature neurons, suggesting that its expression is developmentally regulated. In contrast, the microtubule-stabilizing protein tau is expressed in mature neurons and its function is essential for the maintenance of neuronal polarity and neuronal survival. Thus, the establishment and maintenance of neuronal polarity may down-regulate the protein level/function of SCG10. In this report, we show that treatment of PC12 cells and neuroblastoma cells with the microtubule-stabilizing drug Taxol induced a rapid degradation of the SCG10 protein. Consistently, overexpression of tau protein in neuroblastoma cells also induced a reduction in SCG10 protein levels. Calpain inhibitor MDL-28170, but not caspase inhibitors, blocked a significant decrease in SCG10 protein levels. Collectively, these results indicate that tau overexpression and Taxol treatment induced a calpain-dependent degradation of the microtubule-destabilizing protein SCG10. The results provide evidence for the existence of an intracellular mechanism involved in the regulation of SCG10 upon microtubule stabilization.

Keywords

SCG10; Tau; Taxol; Microtubules; Degradation; Calpain

Introduction

The elongation and rapid shortening of microtubules, a process referred to as dynamic instability, is crucial during neuronal differentiation (Mitchison and Kirschner, 1984; Dent and Gertler, 2003). This process responds to external cues that control and guide molecular changes required for the establishment of domains necessary for neuronal function (Dent and Gertler, 2003). Several proteins that affect microtubule's dynamic instability during neuronal differentiation have been identified (Dent and Gertler, 2003). Some of these proteins, such as SCG10 and stathmin, destabilize microtubules whereas others like the micro-tubule-associated protein tau promote microtubule stabilization (Riederer et al., 1997; Buèe et al., 2000; Grenningloh et al., 2004). Several studies indicated that both microtubule-

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stabilizing and -destabilizing proteins are essential for neurite outgrowth and neuronal function (Buèe et al., 2000; Grenningloh et al., 2004). However, it is still unknown whether these functional diverse proteins can affect or regulate each other's functions to coordinate microtubule's dynamic instability.

SCG10 is a neuronal-specific protein of the same gene family as stathmin (Grenningloh et al., 2004). The level of SCG10 protein is elevated during development and reduced in adults, suggesting that its expression is developmentally regulated. This protein is found to associate with membrane rafts and Golgi (Lutjens et al., 2000; Maekawa et al., 2001). Additionally, SCG10 is enriched in growth cones during neurite outgrowth (Lutjens et al., 2000; Grenningloh et al., 2004). Analysis of purified SCG10 protein from brain protein extract detected the presence of higher molecular weight SCG10 species (Antonsson et al., 1998). Further studies demonstrated that this shift in molecular weight is due to phosphorylation. In vitro studies have shown that protein kinases A (PKA) phosphorylates SCG10 at Ser50 and Ser97, whereas mitogen-activated protein kinase (MAPK) does it at Ser62 and Ser73 (Antonsson et al., 1998; Grenningloh et al., 2004). Furthermore, cyclin-dependent kinases, such as CDK5, have been shown to phosphorylate SCG10 at Ser73 (Grenningloh et al., 2004). These phosphorylation events adversely affect the microtubule-destabilizing activity of SCG10 protein (Antonsson et al., 1998; Grenningloh et al., 2004).

The microtubule-associated tau proteins are preferentially expressed in neurons (Buèe et al., 2000). However, tau expression has been also detected in other cell types (Buèe et al., 2000). Similarly to SCG10, the biological function of tau proteins is adversely affected by phosphorylation (Buèe et al., 2000; Lee et al., 2001). Hyperphosphorylation of tau proteins and its aberrant intracellular aggregation have been identified as a hallmark in a family of neurodegenerative disorders, collectively known as tauopathies (Lee et al., 2001). The best known and studied tauopathy is Alzheimer's disease (AD). The pathological role of tau in these disorders is underscored by the identification of mutations in the tau gene of kindred afflicted by neurodegeneration (Lee et al., 2001). Most of the identified mutations interfered with the ability of tau proteins to bind tubulin and stabilize micro-tubules (Buèe et al., 2000; Lee et al., 2001). Based on these findings, a loss-of-function model has been suggested to explain tau-induced neurodegeneration (Feinstein and Wilson, 2005). Consistently, a tauopathy mouse model treated with low dosages of the microtubule-stabilizing drug Taxol exhibited a restoration of fast axonal transport, increase axonal microtubules, and improved motor impairments (Zhang et al., 2005). Additionally, Taxol protected primary neurons from the toxic effect induced by β-amyloid peptides ($A\beta_{1–42}$) and mitigated tau phosphorylation (Michaelis et al., 2004). These results suggest that microtubule depolymerization due to disruption of the biological function of tau may play a fundamental role in the molecular mechanism underlying tau-induced neurodegeneration.

Regulation of the biological function of microtubule-stabilizing and -destabilizing proteins may be inherently interconnected. Here, we showed that both Taxol treatment and tau overexpression induced a drastic reduction in SCG10 protein level. Calpain inhibitor but not proteasomal or caspase inhibitors blocked this reduction in SCG10 protein. Collectively, these results suggest that microtubule stabilization triggers a signaling cascade that leads to the activation of calpains and degradation of microtubule-destabilizing proteins.

Materials and methods

Materials

Antibodies used in this project are polyclonal antibodies WK S44 specific to human tau (aa 162–178; 1:500; generated by Dr. Yen's laboratory; DeTure et al., 2002), SCG10-BR (Antonsson et al., 1998), SCG10-GL (DiPaolo et al., 1997), anti-actin (C-11; Santa Cruz

Biotechnology), and anti-cleaved caspase-3 (Asp175; Cell Signaling Technology). Monoclonal antibodies used are anti-α-tubulin and anti-ubiquitin (P4D1) from Santa Cruz Biotechnology. Secondary antibodies used in western blot analysis are peroxidase-conjugated goat anti-rabbit (1:4000) or goat anti-mouse (1:2000) Ig antibodies (Chemicon, Temecula, CA, USA).

Pharmacological agents used are described below. Taxol (Paclitaxel) and Nocodazole were purchased from Sigma. All other drugs, including epoxomicin, MG132, calpain inhibitor III (MDL-28170), caspase inhibitor I (Z-VAD-FMK), lactacystin, H-89 dihydrochloride, roscovitine, MEK inhibitor (PD 98059), and JNK inhibitor II (SP600125) were purchased from EMD Biosciences. The specific concentrations used are described in the text.

Culture and drug treatment of PC12 cells

Pheochromocytoma cells (PC12) were maintained in growth media [DMEM supplemented with 7.5% fetal calf serum (FCS), 7.5% horse serum (HS), 3.5 g/L glucose, 3.7 g/L sodium bicarbonate, pH 7.3, 10 μg/mL streptomycin, and 10 U/mL penicillin] at 37°C/5%CO_2. Cultured undifferentiated PC12 (5×10^6 cells/plate; five plates per treatment) were treated with different concentrations of Taxol (dissolved in ethanol) for 24 h. As control, equal volume of ethanol was added to culture PC12 cells for the same interval of time. When indicated, PC12 cells were pre-treated with specific inhibitors, at the concentration specified in the text, for 3 h before the addition of 10 μM Taxol to the medium. To induce differentiation, 100 ng/mL of nerve growth factor (NGF) was added directly to PC12 cells cultured in minimal medium [DMEM supplemented with 3.5 g/L glucose, 3.7 g/L sodium bicarbonate, pH 7.3, 1% FCS, 1% HS, 10 μg/mL streptomycin, and 10 U/mL penicillin] at 37°C/5%CO_2. PC12 cells were incubated in NGF containing media for three days prior to Taxol (10 μM) treatment for 24 h NGF-differentiated PC12 cells (for 3 days) were pre-treated with calpain inhibitor (MDL28170) for 3 h prior to the addition of Taxol (10 μM) to the medium.

Neuroblastoma cells and tau overexpression

The Tet-off inducible system was used to generate stable transfectant (M1C) expressing wild-type human tau (4R0N). M1C cells were derived from human neuroblastoma BE(2)-M17D cell line and maintained in growth media [DMEM supplemented with 10% fetal bovine serum (FBS), 4.5 g/L of glucose, 3.7 g/L sodium bicarbonate, pH 7.3, 0.3 g L-glutamine, 400 μg/mL G418, 1 μg/mL puromycin, 2 μg/mL tetracycline (Tet), 100 μg/mL zeocin, and 100 μg/mL hygromycin] at 37°C/5% CO2. The cells were seeded in 10 cm dish at 2×10^6 cells per plate (60–70% confluence) a day before Tet-off induction. Tau expression was induced by thoroughly washing the cells with DMEM (without Tet) and adding fresh medium with different concentrations (1 ng/ml, 10 ng/ml, or 100 ng/ml) of Tet. Cells were cultured in the indicated medium for seven days. Fresh medium was supplemented on the third and fifth day. When indicated, M1C cells were pre-treated with calpain inhibitor MDL28170 (10 μM) or proteasome inhibitor MG132 (1.0 μM or 10 μM) for 3 h before incubation in reduced concentration of Tet (1 ng/mL) for 48 h.

Western blot analysis

After the indicated incubation time, PC12 and M1C cells were collected and centrifuged to remove culture medium. The cell pellet was resuspended in 1 mL of lysis buffer [20 mM Tris base, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM PMSF; 5 mM sodium pyrophosphate; 30 mM β-glycerophosphate; 30 mM sodium fluoride]. The resuspended cells were passed ten times through a syringe with a 27G1/2″ needle. The cell lysate was spun down at 18,000×*g* for 2 min. The supernatant was removed and the pellet resuspended in lysis buffer (500 μL). Total protein concentration was estimated using the colorimetric

Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Recombinant BSA was used as a standard.

For western blot analysis, samples of equal protein concentration (10 μg) were loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE). Proteins resolved by gel electrophoresis were transferred to nitrocellulose membrane (BioRad, Hercules, CA, USA), which were then incubated in blocking solution [5% dry milk; 0.1% Tween 20 in 1× TBS] for 1 h prior to overnight incubation with the indicated primary antibody. After washing with 1× TBS containing 0.1% Tween 20, the membranes were incubated at room temperature with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies and washed again. Immunoreacted proteins were visualized with enhanced chemiluminescence system (ECL plus; Amersham Biosciences, Piscataway, NJ, USA) and exposure to X-ray film. When indicated, membranes were re-probed after extensive washing (3 h) and incubation in blocking solution. Quantitative analyses were carried out using MCID Imaging analysis software. Films of two different exposure times (5 s and 20 s) per antibody (i.e., anti-SCG10 and anti-tubulin) were scanned. Then, the scanned images were use to determine the relative intensity of the signal detected creating squares of the same size enclosing the protein bands. The relative intensity detected in both films was corrected using the signal detected from anti-tubulin from the respective film. The ratio was the same in both films. The numbers reported represent the mean of three independent experiments and the error bar shows the standard error [$\sigma/(\sqrt{n})$] per treatment.

Results

SCG10 protein level is reduced upon Taxol treatment

Treatment of PC12 cells with increasing concentrations of the microtubule-stabilizing drug Taxol (0.1–10 μM) induced a steady decline in the level of SCG10 protein (Fig. 1A; SCG10) after 24 h of incubation. Upon Taxol treatment, an SCG10 protein band of higher molecular weight than those observed in control samples was detected, suggesting that post-translational modification of SCG10 proteins may be induced upon Taxol treatment (Figs. 1A and C, arrow). Additionally, there is a protein band detected at approximately ~17 kDa (Fig. 1A, asterisk). Because the SCG10 antibody SCG10-BR has been shown to cross-react with stathmin, the specific SCG10 antibody SCG10-GL was also used (Fig. 2). The results showed that this protein band is likely to be derived from SCG10 (Fig. 2). The quantification of three representative samples from each treatment condition demonstrated that, in 24 h, the total level of SCG10 protein was significantly ($p<0.0001$) reduced upon Taxol treatment (Fig. 1B). These results suggest that Taxol-mediated microtubule stabilization induces rapid degradation of the microtubule-destabilizing SCG10 protein in PC12 cells.

The observed reduction in SCG10 protein level could be related to the process of cell death after Taxol treatment and not an intrinsic cellular response to microtubule stabilization. In order to gain information that helps to discriminate between these two possibilities, undifferentiated PC12 cells were treated with 10 μM Taxol and samples collected at different intervals (Fig. 1C). Taxol treatment of PC12 cells induced a reduction in SCG10 protein level after 4 h of incubation (Fig. 1C, compare lanes 1–2 with lanes 5–6; SCG10). Further reduction on SCG10 protein level was observed after 12 h (Fig. 1C, lanes 3 and 7; SCG10) and 24 h (Fig. 1C, lanes 4 and 8; SCG10). Previous report indicated that SCG10 is associated with plasma membrane and Golgi (Lutjens et al., 2000; Maekawa et al., 2001). Therefore, both supernatant (Fig. 1C, lanes 1–4) and pellet (Fig. 1C, lanes 5–8) fractions were analyzed after Taxol treatment. In both fractions, we observed similar reduction on SCG10 protein level (Fig. 1C; SCG10). However, western blot analysis using anti-stathmin antibodies showed that Taxol does not affect stathmin protein levels (Fig. 1C; Stath). These

results indicate that Taxol treatment of PC12 cells induced a decrease in the overall amount of SCG10 proteins, but not related proteins such as stathmin.

Previous studies have demonstrated that microtubule-disrupting drugs, such as nocodazole and colchicines, also induced cell death (Kim et al., 2002). Thus, PC12 cells were treated with nocodazole [either 8.5 mM (Fig. 1D, lane 2) or 17 mM (Fig. 1D, lane 3)] for 4 h. After treatment, cells were harvested and western blot analysis was performed. In contrast to what was observed in Taxol-treated cells, acute nocodazole treatment did not induce a decrease in SCG10 protein levels (Fig. 1D, lanes 2–3), despite the induction of apoptosis as illustrated by the appearance of cleaved caspase-3 (Fig. 1D, compare lanes 1–3; Clev-Caspase3). Importantly, these results indicate that the reduction in SCG10 protein level is not due to the reported activation of apoptosis associated with both Taxol and nocodazole treatments (Wang et al., 2000; Kim et al., 2002). Thus, Taxol treatment triggers a molecular mechanism that leads to the degradation of the microtubule-destabilizing SCG10 protein in PC12 cells.

Proteasomal inhibition induced the expression of smaller SCG10 protein species

The ubiquitin–proteasome system (UPS) has been linked to rapid degradation of proteins in response to environmental cues and insults (Kornitzer and Ciechanover, 2000). Pharmacological studies were conducted in order to rule out a potential role of the UPS in Taxol-induced SCG10 degradation. Undifferentiated PC12 cells were pre-treated for 3 h with different concentrations of the proteasome inhibitor MG132 (Fig. 2A, lanes 3–5). After pre-treatment, PC12 cells were incubated in presence of Taxol (10 μM; Fig. 2A, lanes 2–4) for 24 h. Western blot analyses utilizing anti-ubiquitin antibody indicated the accumulation of ubiquitinated proteins, confirming that the proteasome was efficiently inhibited (Figs. 2A and 3B; Ub). The protein level of SCG10 protein was restored by all different MG132 concentrations. Interestingly, however, SCG10 proteins recovered were of smaller molecular weight (~17 kDa) than the most prominent band detected in control samples (Fig. 2A, compare lanes 1, 3, and 4; SCG10). The accumulation of this shorter SCG10 protein species was also observed in samples from cells pre-treated with a different and more specific proteasomal inhibitor, epoxomicin (Fig. 2B, lanes 3–4; SCG10). These results suggest that proteasomal inhibition may block Taxol-induced SCG10 degradation. However, it is also possible that this phenomenon is independent of the effect exerted by Taxol on PC12 cells.

In order to determine if the MG132- and epoxomicin-induced accumulation of the small SCG10 protein species depends on Taxol treatment, we treated PC12 cells with MG132 alone for 24 h. As shown in Fig. 2A (lane 5), the smaller SCG10 protein species was also detected in PC12 cells treated with MG132 alone. These results suggest that the small SCG10 species induced by exposure of PC12 cells to both Taxol and proteasome inhibitor was derived most likely from the major 17-kDa SCG10 species induced by proteasomal inhibition. The 17-kDa species is about 3.6 kDa smaller than that deduced from the amino acid sequences of intact SCG10 protein, raising the possibility that it may be a novel SCG10 isoform. Alternatively, it may represent a truncated SCG10 cleaved by activation of proteases as a result of proteasomal inhibition. Regardless, it is questionable whether Taxol-induced SCG10 degradation involves proteasomes. Therefore, these results indicate that inhibition of the UPS may affect a different molecular mechanism than the one induced by Taxol treatment, leading to the accumulation of an apparent lower molecular weight SCG10 protein.

Calpain-dependent SCG10 protein reduction upon Taxol treatment

It has been demonstrated that Taxol treatment induced the activation of calpain in proliferating cells (Wang et al., 2004). Therefore, it is plausible that Taxol-induced activation of calpain may mediate the degradation of SCG10 protein in PC12 cells. In order

to determine the role that calpains may play in Taxol-induced SCG10 degradation, undifferentiated PC12 cells were pre-treated with either inhibitor to caspase (Fig. 3B, lane 3) or calpain (Fig. 3B, lane 4) for 3 h, followed by Taxol (10 μM) treatment for 24 h (Fig. 3B, lanes 2–4). PC12 cells treated with either caspase inhibitor (Fig. 3A, lane 2) or calpain inhibitor (Fig. 3A, lane 3) alone for 20 h were used as control. In contrast to that observed in cells treated with either MG132 or epoxomicin (Fig. 2), no changes in SCG10 protein levels were detected in cells treated with either vehicle control (Fig. 3A, lane 1) or in cells treated with inhibitors either to caspase or calpain (Fig. 3A, compare lanes 1–3).

As expected, Taxol induced the activation of apoptosis as illustrated by the detection of caspase-3 cleaved product (Fig. 3B, lane 2, Clev-Caspase3). This activation was efficiently blocked by the caspase inhibitor Z-VAD-FMK (Fig. 3B, Clev-Caspase3, compare lanes 2 and 3). In spite of the inhibition of Taxol-induced caspase activation, SCG10 protein levels were not restored (Fig. 3B, lane 3, SCG10). On the contrary, the calpain inhibitor MDL-28170 did block Taxol-induced decrease in SCG10 protein level (Fig. 3B, lane 4, SCG10). The accumulated SCG10 protein, however, has an apparent higher molecular weight than SCG10 proteins found in control samples (Fig. 3B, compare lanes 1 and 4).

SCG10 plays a crucial role in neuronal development (Grenningloh et al., 2004). In order to determine the effect of Taxol on SCG10 protein levels in differentiating cells, PC12 cells were cultured in NGF-containing medium for three days. NGF-induced morphological changes were monitored by light microscopy. Approximately 80% of PC12 cells showed neurite extension after three days of incubation in NGF-containing medium (data not shown), indicating that most cells were committed to differentiation. At this point, NGF-differentiated PC12 cells were treated with different concentrations of Taxol (Fig. 3C, lane 2=1.0 μM and lane 3=10 μM) for 24 h. Consistently, SCG10 protein levels were drastically reduced in cells cultured in presence of Taxol and not vehicle control (Fig. 3C, compare lanes 1–3). Pre-treatment of NGF-differentiated PC12 cells with calpain inhibitor, followed by treatment with Taxol (10 μM), blocked the observed reduction in SCG10 protein level (Fig. 3C, lane 4, Cl). These results suggest that Taxol-induced SCG10 degradation, in both undifferentiated and NGF-differentiating PC12 cells, is calpain dependent. Previous reports indicated that MG132 could inhibit calpain protease activity. However, the accumulation of two different SCG10 protein species after treatment with calpain (Figs. 3B and C) and proteasome (Fig. 2) inhibitors indicates that there is not cross-inhibition due to lack of specificity. Furthermore, MG132 provoked the accumulation of a lower molecular weight SCG10 protein band in the absence of Taxol treatment (Fig. 2A, lane 5), whereas calpain inhibitor MDL-28170 did not exert any effect on SCG10 in the absence of this treatment (Fig. 3A, lane 3). Therefore, these results suggest that two distinct mechanisms may mediate SCG10 protein degradation in PC12 cells.

Inhibition of known SCG10 kinases did not block Taxol-induced SCG10 degradation

Immunoblotting analysis of control samples, utilizing anti-SCG10 antibodies, showed three major protein bands (Fig. 4A, lane 1, SCG10). Previous studies demonstrated that the level of phosphorylation affects the gel electrophoresis mobility of SCG10 proteins (Antonsson et al., 1998). Therefore, it is reasonable to suggest that the SCG10 protein-banding pattern observed upon Taxol treatment could represent different phosphorylation states (Fig. 4A, lane 1; SCG10). The level of this form of SCG10 increased substantially upon calpain inhibitor treatment (Figs. 3B and C). Consequently, these results suggest that hyperphosphorylation of SCG10 protein may precede its degradation and kinases known to phosphorylate SCG10, such as protein kinases A (PKA), mitogen-activated protein kinase (MAPK), or CDK5, may be involved in Taxol-induced SCG10 degradation. This possibility was tested by pre-treatment of undifferentiated PC12 cells with either PKA (H89), MAPK (PD98059), or CDK5 (Roscovitine) inhibitor prior to Taxol treatment for 24 h (Fig. 4A,

lanes 3–5). In addition, these inhibitors were used in different combinations to rule out any concurrent contribution of these kinases (Fig. 4A, lanes 6–9). Western blot analyses of samples collected from different treatments indicated that none of the kinase inhibitors restore the SCG10 protein level after Taxol treatment (Fig. 3D, compare lane 1 with lanes 3–9). Interestingly, however, an anti-SCG10 antibody immunoreactive band corresponding to the second SCG10 species (Fig. 4A, lanes 5 and 7–9, asterisk) was observed when CDK5 inhibitor was used. However, CDK5 inhibitor did not restore SCG10 protein to control levels (Fig. 4A, compare lanes 1 and 9). These results indicate that kinases implicate in SCG10 phosphorylation are not involved in Taxol-induced SCG10 degradation. Alternatively, SCG10 phosphorylation may not be a regulatory factor in the molecular mechanism leading to its degradation.

Overexpression of Tau protein induced calpain-dependent SCG10 degradation

Recently, it has been shown that suppression of tau expression makes cells more sensitive to Taxol treatment (Rouzier et al., 2005). Furthermore, previous reports suggested that tau and Taxol compete for the same binding site on microtubules to induce their stabilization (Kar et al., 2003; Samsonov et al., 2004). Thus, the observed Taxol-induced SCG10 degradation could be also triggered by microtubule stabilization due to overexpression of tau proteins. This possibility was tested by using neuroblastoma cells expressing human tau (4R0N) protein under the control of the Tet-Off system (Ko et al., 2005; see Materials and methods). As previously reported (Ko et al., 2005), the level of tau expression corresponded inversely to the concentration of tetracycline in the culturing medium (Fig. 5A, lanes 1–4; Tau) and tau overexpression led to microtubule bundling. The highest tau expression was detected in protein extract from cells cultured in medium containing 1 ng/mL of tetracycline after seven days (Fig. 5A, lane 4; Tau). Moreover, very little exogenous tau were detected in cells maintained in media containing 100 ng/mL or higher concentrations (Fig. 5A, lane 3; Tau). Interestingly, the overexpression of tau proteins induced a reduction in SCG10 protein level comparable to that observed in Taxol-treated cells (Fig. 5A, lanes 1–4; SCG10). The induction of tau expression, with 10 ng/mL or 1 ng/mL, induced SCG10 degradation (Fig. 5A, compare lanes 3–4). The result indicates that increase tau expression lead to induction of SCG10 protein degradation.

In PC12 cells, Taxol-induced decrease in SCG10 protein level is calpain-dependent (Fig. 3). Therefore, in order to establish a mechanistic correlation between Taxol treatment and tau overexpression, M1C neuroblastoma cells were pre-treated with either MG132 (Fig. 5B, lanes 3–4) or calpain (Fig. 5B, lane 5) inhibitor followed by induction of tau overexpression (Tet= 1 ng/mL) for 2 days. Consistently, overexpression of tau proteins induced the elimination of SCG10 proteins (Fig. 5B, compare lanes 1 and 2; SCG10). This effect was not blocked by pre-treatment with different concentrations of the proteasomal inhibitor MG132 (Fig. 5B, lanes 3–4; SCG10), despite the fact of an increase accumulation of ubiquitinated proteins (Fig. 5B, lanes 3–4; Ub). Interestingly, however, MG132 did also increase the accumulation of tau proteins in comparison to untreated cells (Fig. 5B, compare lanes 2–4; Tau). This MG132-induced accumulation of tau proteins is under investigation (to be published elsewhere). On the other hand, pre-treatment of neuroblastoma cells with calpain inhibitor blocked the reduction of SCG10 induced by tau overexpression (Fig. 5B, compare lanes 2 and 5; SCG10). This finding indicates that, in response to tau overexpression, SCG10 is degraded in a cal-pain-dependent manner. Consistently with what was observed in PC12 cells, Taxol treatment of M1C cells without tau induction (Fig. 5C, lane 2; SCG10 and Tau) also resulted in SCG10 degradation (Fig. 5C, compare lanes 1and 2). As control, non-tau transfected M1C neuroblastoma cells (Fig. 5C, lanes 3–4) were treated with Taxol (Fig. 5C, lane 4) to corroborate that the observed reduction in SCG10 protein was not due to overexpression of tau proteins. As expected, Taxol treatment induced

SCG10 protein degradation in these founder M1C neuroblastoma cells (Fig. 5C, compare lanes 3 and 4). Collectively, these results indicate that both Taxol treatment and tau overexpression may activate equivalent signaling cascades that lead to calpain activation and SCG10 protein degradation.

Discussion

Microtubule dynamic instability plays a crucial role during neuronal differentiation. This process requires the biological function of microtubule-stabilizing and -destabilizing proteins. The expression of the microtubule-destabilizing protein SCG10 increases during neuronal differentiation (Grenningloh et al., 2004). In mature neurons and adult brains, the amount of SCG10 protein is reduced (Grenningloh et al., 2004). However, previous reports demonstrated a transient increase in the expression of SCG10 after induction of long-term potentiation in the rat hippocampal Schaffer–collateral CA1 pathway and during axonal regeneration (Peng et al., 2003). In contrast, the expression of the microtubule-stabilizing protein tau is constant during development and adulthood (Buèe et al., 2000). Collectively, these findings indicate that there may be a molecular mechanism that regulates the function and expression of these functional diverse proteins.

Here we describe the degradation of the microtubule-destabilizing SCG10 protein triggered by overexpression of the microtubule-stabilizing protein tau or treatment with the anti-mitotic drug Taxol, which is blocked by the calpain inhibitor MDL-28170. In undifferentiated and differentiated PC12 cells, Taxol treatment induced a rapid reduction in the SCG10 protein level that could be blocked by calpain inhibitors (Figs. 1 and 2). Calpain inhibition induced the formation of higher molecular weight SCG10 species (Fig. 3). This SCG10 higher molecular weight species may represent hyperphosphorylated SCG10, suggesting that phosphorylation may precede its degradation. However, pharmacological inhibition of kinases, known to phosphorylate SCG10, did not block the reduction of SCG10 exerted by Taxol treatment (Fig. 4). Importantly, both over-expression of tau proteins and Taxol treatment in neuroblastoma cells induced reduction of SCG10 protein level (Fig. 5). This effect was also blocked by pre-treatment with calpain inhibitor, indicating that comparable molecular mechanisms mediate Taxol-induced SCG10 degradation in both rat PC12 and human neuroblastoma cells. Taken together, these results suggest that microtubule stabilization may activate a cellular response to down-regulate the function of the microtubule-destabilizing SCG10 protein that is blocked by the calpain inhibitor MDL-28170.

Treatment of PC12 cells with proteasome inhibitors induced neurite outgrowth and the accumulation of a small SCG10 species. Pre-treatment of PC12 cells with both calpain and proteasomal inhibitors followed by Taxol treatment promoted the accumulation of this SCG10 species (data not shown). This result indicates that the effect exerted by proteasomal inhibition on the accumulation of the SCG10 shorter form is independent of the Taxol-activated pathway that leads to calpain-dependent degradation of SCG10 proteins. The expression of this novel SCG10 species could be linked to the process of neurite outgrowth induced by proteasomal inhibition. However, further experiments are needed in order to understand the molecular properties of this novel SCG10 protein species and its role in MG132-induced neurite outgrowth.

The biological function of calpains has been shown to play an important role in cell development, cell motility, growth cone motility and guidance, learning, and memory, among other cellular processes (Croall and DeMartino, 1991; Sato and Kawashima, 2001). Additionally, abnormal activation of calpains has been associated with the development of different diseases, including AD and Type-II diabetes mellitus (Zatz and Startling, 2005).

Therefore, appropriate activation and function of calpains are essential for cellular survival and, consequently, prevention of diseases. Interestingly, Wang et al. (2004) recently reported that inhibition of calpain protects against peripheral neuropathy commonly observed in patients treated with the antiancer drug Taxol (paclitaxel). In this study, inhibition of calpain activity was shown to prevent axonal degeneration and neuropathy in mice treated with Taxol (Wang et al., 2004). Therefore, it is reasonable to predict that unregulated calpain-dependent degradation of SCG10 may affect neuronal polarity and transmission.

We hypothesize that the stabilization of microtubules, by means of either Taxol treatment or increase expression of tau proteins, activates a signaling cascade to down-regulate the function of the microtubule disrupting SCG10 protein. Alternatively, overexpressed tau proteins and Taxol molecules may prevent the binding of SCG10 protein to tubulin molecules, rendering it susceptible to degradation. This mechanism may also coordinate the activity of microtubule-stabilizing and -disrupting proteins during neuronal development and function. In mature neurons, particularly in those involved in learning and memory, this putative pathway may become inactivated during induction of long-term potentiation to allow the expression of SCG10 and transient regulation of microtubule dynamics required for neuronal plasticity. Consequently, this novel molecular mechanism may play a central role in coordinating cytoskeleton changes required during neuronal development and function.

Acknowledgments

This study was supported, in part, by NIH/NINDS-Ruth L. Kirschstein National Research Service Award F32NS047930 (to I.E.V.), National Institutes of Health Grant AG17216 (to S-H.Y), and Mayo Clinic Foundation.

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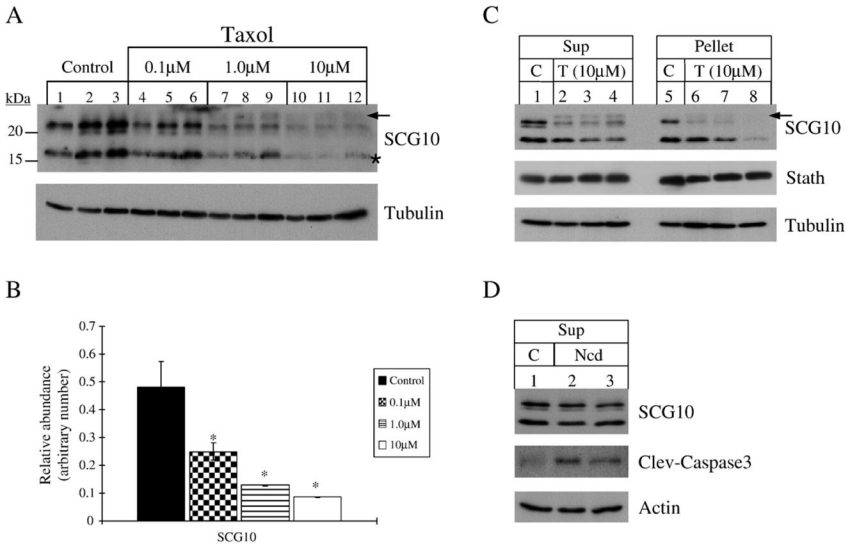

Fig. 1.

Taxol treatment of undifferentiated PC12 cells induced reduction in SCG10 protein level. (A) Undifferentiated PC12 cells were treated with the indicated Taxol concentrations for 24 h. Control samples (C; lanes 1–3) were treated with vehicle control (40 μL ethanol) for the same period of time. The cells were harvested and equal protein concentration (10 μg) was loaded on a 12.5% SDS–PAGE. Western blot analysis was performed utilizing anti-SCG10-BL antibody. As loading control, the membrane was re-probed with anti-tubulin antibody. (B) Quantification of SCG10, in each treatment condition, indicated a reduction of up to 90% in 10 μM Taxol (see Materials and methods). Bar per sample set represent the mean of three independent experiments and the error bar shows the standard error [$\sigma/(\sqrt{n})$] (*p value<0.0001). (C) Time course experiment using 10 μM Taxol was carried out (lanes 2 and 6=4 h, lanes 3 and 7=12 h, and lanes 4 and 8=24 h). Reduction in SCG10 protein level was observed as early as 4 h in both supernatant (Sup-lanes 2–4) and Pellet (lanes 6–8) fractions. In contrast, the protein level of stathmin (Stath) was unchanged. Tubulin was used as a loading control. (D) Nocodazole does not affect SCG10 protein level. Undifferentiated PC12 cells were treated with the microtubule-destabilizing drug nocodazole (Ncd; lanes 2=8.5 mM and lane 3=17 mM) for 4 h. As control (C; lane 1), cells were treated with DMSO (25 μL). Western blot analysis demonstrated that Ncd treatment of PC12 cells does not affect SCG10 protein levels, despite the activation of caspases (Clev-Caspase3). Actin was used as a loading control. The arrow in panels A and B indicates slow migrating SCG10 protein induced after Taxol treatment. The asterisk in panel B indicates the ~17-kDa SCG10 species.

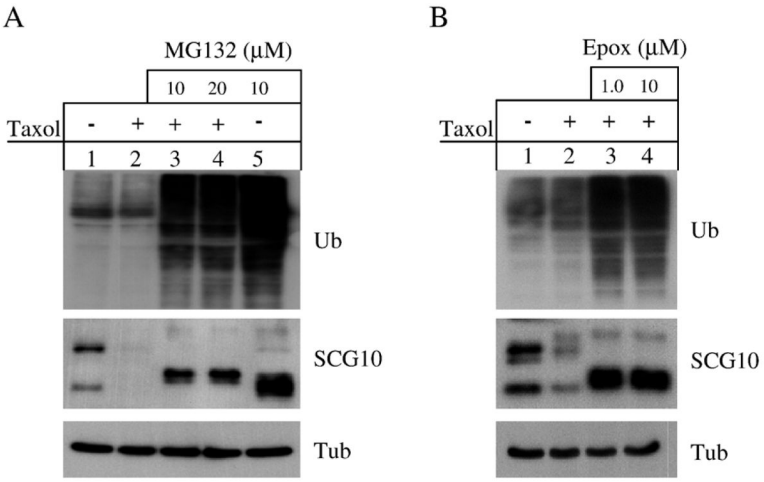

Fig. 2.

Proteasome inhibition induced the expression of shorter SCG10 protein species. (A) Undifferentiated PC12 cells were treated with Taxol (10 μM) in the absence (lane 2) or presence (lanes 2–4) of the indicated concentrations of the proteasome inhibitor MG132. As control (lane 1), cells were treated with vehicle control [ethanol (40 μL) and DMSO (20 μL)]. Undifferentiated PC12 cells were also treated with MG132 alone (lane 5). (B) PC12 cells were pre-treated with a different proteasomal inhibitor, epoxomicin (lanes 3–4), at the indicated concentrations followed by Taxol treatment. As control, PC12 cells were treated with vehicle control (lane 1) or Taxol alone (lane 2). In panels A and B, the antibodies anti-SCG10-GL (SCG10), anti-ubiquitin P4D1 (Ub), and anti-tubulin (Tub) were used for western blot analyses. The results indicated that proteasome inhibition affects SCG10 expression independent of the effect exerted by Taxol treatment.

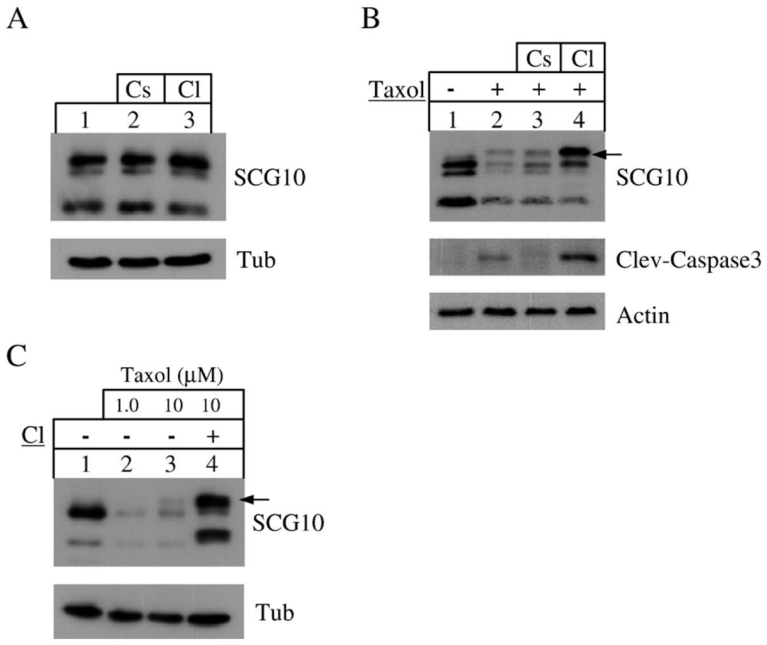

Fig. 3.

Taxol-induced and calpain-dependent SCG10 degradation. In order to determine the role of caspases and calpain activation on the reduction in SCG10 protein level upon Taxol treatment (10 μM), undifferentiated PC12 cells were pre-treated with either caspase (Cs; Z-VAD-FMK; 40 μM) or calpain (Cl; MDL-28170; 10 μM) inhibitor for 3 h. (A) Undifferentiated PC12 cells were treated with Cs (lane 2) or Cl (lane 3) for 24 h to determine the effect that caspases and calpain exerts on SCG10 independently of Taxol treatment. As control, PC12 cells were treated with DMSO (lane 1). Western blot analysis of the samples collected was performed utilizing SCG10-BL antibodies. Tubulin immunoblotting was performed as loading control. The result indicates that treatment with Cs or Cl did not affect SCG10 protein levels. (B) Undifferentiated PC12 cells were pre-treated with either Cs (40 μM, lane 3) or Cl (10 μM, lane 4) for 3 h. After pre-treatment, Taxol (10 μM; lanes 2–4) was added to the medium for 24 h. In lane 1, we loaded protein lysate from PC12 cells treated with vehicle control (ethanol and DMSO). Western blot analyses were performed, employing the indicated antibodies. The result indicates that SCG10 protein is accumulated after Taxol treatment only if undifferentiated PC12 cells were pre-treated with Cl. Actin was used as a loading control. (C) NGF-differentiated PC12 cells were treated for 24 h with either 1.0 μM (lane 2) or 10 μM (lanes 3–4) Taxol. As control, differentiated PC12 cells were treated with ethanol and DMSO (lane 1). Consistently, Taxol treatment induced SCG10 protein degradation. Pre-treatment of differentiated PC12 cells with Cl (10 μM, lane 4) for 3 h, followed by Taxol (10 μM) treatment for 24 h, blocked the observed Taxol-induced SCG10 degradation (lane 4). Tubulin immunoblotting was used as loading control. The arrow in panels A and B indicates the slow migrating SCG10 protein species recovered after Cl treatment.

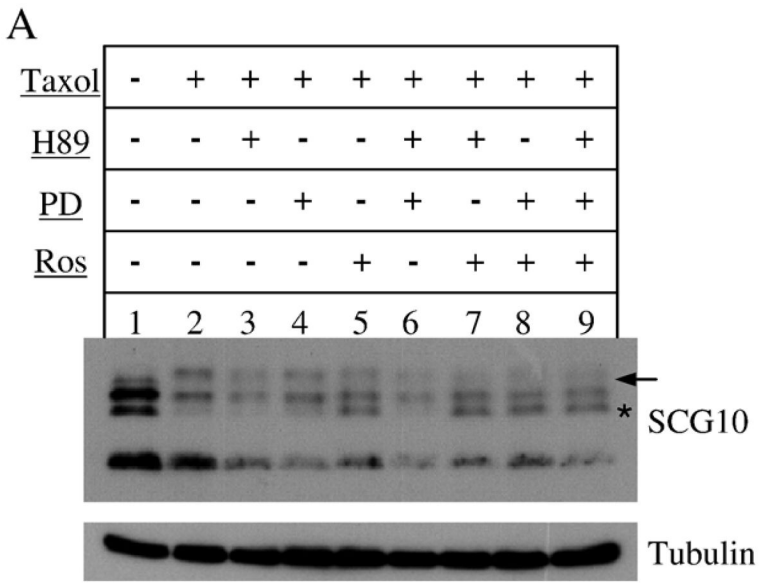

Fig. 4.

Inhibition of known SCG10 kinases did not blocked Taxol-induced SCG10 degradation. (A) Undifferentiated PC12 cells were pre-treated with PKA (20 μM; H89; lanes 3, 6–7 and 9), MEK (30 μM; PD; PD98059, lanes 4, 6, and 8–9), and CDK5 (5 μM; Ros; Roscovitine, lanes 5 and 7–9) inhibitors for 3 h. Then, Taxol (10 μM) was added to the medium. Cells were harvested after 24 h of incubation with the indicated drugs. The SCG10 protein levels were accessed by western blot analysis utilizing anti-SCG10-BR antibodies (SCG10). Tubulin was used as a loading control (Tub). The arrow marks the higher molecular weight SCG10 species induced upon Taxol treatment. The asterisk indicates the location of the SCG10 protein shift upon Ros treatment.

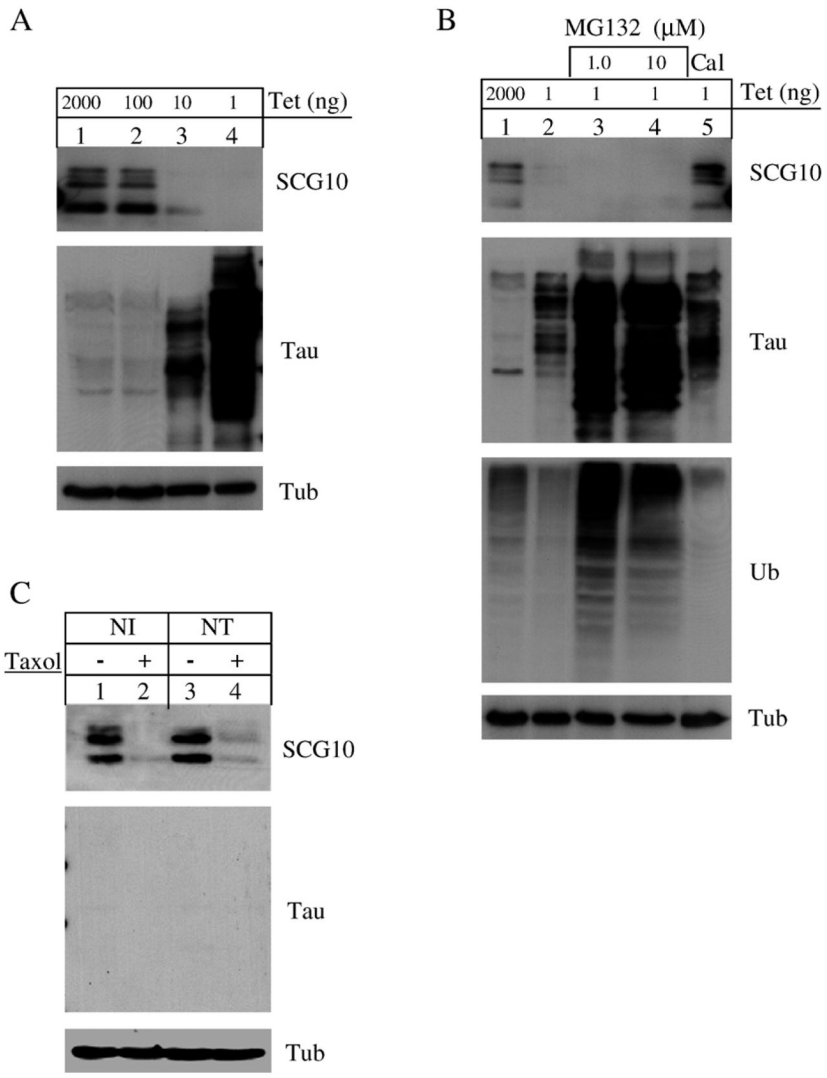

Fig. 5.

Overexpression of Tau protein induced calpain-dependent SCG10 degradation. (A) The expression of human tau in M1C cells was induced by decreasing concentration of tetracycline (Tet, lanes 2–4). Protein extract from neuroblastoma cells under uninducible conditions was used as control (lane 1). The cells were harvested after 7 days of incubation. The steady-state level of SCG10 and tau proteins was accessed by western blot analysis, employing the antibodies anti-SCG10-BR (SCG10) and human-specific anti-tau (WKS44). (B) M1C cells were pre-treated with either MG132 (lanes 3–4, at the indicated concentration) or calpain (10 μM; Cal; lane 5) inhibitor for 3 h prior to the induction of tau expression for 2 days. The protein extracts from neuroblastoma cells without (lane 1) or with (lane 2) induction of tau overexpression were used as control. Western blot analysis was conducted utilizing the indicated antibodies. Anti-ubiquitin (Ub) immunoblotting was used to determine the accumulation of ubiquitinated proteins after MG132 treatment. (C) M1C cells without tau induction (NI, lanes 1–2) or non-tau transfected neuroblastoma cells (NT, lanes 3–4) were treated with Taxol (10 μM; lanes 2 and 4) for 24 h. Western blot analysis utilizing anti-SCG10BR (SCG10) or anti-Tau (Tau) antibodies was used to determine the level of these proteins. In panels A,–C, anti-tubulin (Tub) immunoblotting was used as a loading control.