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Scribble controls NGF-mediated neurite outgrowth in PC12 cells

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ABSTRACT

Neurite outgrowth is mediated by dynamic changes of the cytoskeleton and is largely controlled by Rho GTPases and their regulators. Here, we show that the polarity protein Scribble controls PC12 cell neurite outgrowth in response to nerve growth factor. Scribble knockdown decreases neurite numbers and increases neurite length. This effect is linked to TrkA the cognate receptor for NGF as pharmacological inhibition of phosphorylated TrkA (pTrkA) reduces Scribble expression. Moreover, Scribble forms a complex with the MAPK components ERK1/2 in a growth factor dependent manner. In RNAi experiments where Scribble expression is efficiently depleted sustained ERK1/2 phosphorylation is reduced. Conversely, siRNA with intermediate Scribble silencing efficiency fails to match this effect indicating that ERK1/2 activation depends on basic Scribble protein levels. Finally, Scribble translocates to the plasma membrane in response to growth factor where it complexes with HRas and Rac1 suggesting that the phenotype activated by loss of Scribble may be a result of altered GTPase activity. Together, these results demonstrate a novel role for Scribble in neurite outgrowth of PC12 cells.

Introduction

Nerve growth factor (NGF) is a trophic growth factor that promotes diverse activities including signaling, differentiation and apoptosis in neuronal tissue. NGF is essential for cognitive function as disturbed signaling is associated with neurodegenerative disease (Siegel and Chauhan, 2000). In addition, reduced NGF expression correlates with the oncogenesis of neuroblastoma (Bogenmann et al., 1998). Another important role for NGF is in neuronal differentiation (Hall and Lalli, 2010). NGF signals through the TrkA receptor that activates various intracellular pathways including the Ras/ERK MAPK pathway which is associated with neurite and axon growth (Kaplan and Miller, 2000). While Ras drives signaling in this process, the Rho GTPase Rac1 regulates underlying actin changes. The activation of Rac1 is controlled by guanine nucleotide-exchange factors (GEFs) including βPIX, which binds to Rac1 and stimulates GDP/GTP exchange (Manser et al., 1998; Raftopoulou and Hall, 2004; Zhang et al., 2005). Various neurological disorders evoked by deficits in cytoskeleton dynamics have been linked to Rac1 and βPIX (Colomer et al., 1997; Fiala et al., 2002; Kutsche et al., 2000; Mendoza-Naranjo et al., 2007; Newey et al., 2005; Zhang et al., 2005), suggesting that alterations in function may cause abnormal signaling and loss of cytoskeleton structure.

Scribble is a peripheral membrane protein, which belongs to the LAP (leucine rich-repeats and PDZ) protein family including mammalian Erbin and Densin-180 (Zhang et al., 2006). The leucine-rich repeats (LRRs) of Scribble are important for its association to the plasma membrane (Legouis et al., 2003), while the 4 PDZ domains scaffold distinct proteins involved in cytoskeleton dynamics. For example, Scribble forms a complex with βPIX and GIT1 to control synaptic vesicle recycling in PC12 cells (Audebert et al., 2004). In cultured astrocytes Scribble binds βPIX to control Rho Cdc42 GTPase activity and directed migration (Osmani et al., 2006). Also, p-21 activated kinase activation by Rac1 is dependent on Scribble association and vital for control of epithelial cell migration (Nola et al., 2008). Thus, apart from an established role in apical-basal polarity, Scribble stabilizes transient polarized migration. This function is shared by the PAR complex members (Osmani et al., 2006; Pegtel et al., 2007; Sakakibara and Horwitz, 2006), which in other contexts antagonizes the activity of Scribble (McCaffrey and Macara, 2009; Nelson, 2009). The driving thrust for the motility in polarized cell migration is influenced by external cues. This shapes the cytoskeleton through the activity of Cdc42, which regulates the overall polarity, while Rac1 appears to control filopodia formation (Hengst et al., 2009). Yet, information of the external cues that activates Scribble and PAR proteins in polarized migration is lacking. Recently PAR3 was implicated in NGF-mediated axonal elongation (Hengst et al., 2009). It was demonstrated that up-regulation of

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PAR3 mRNA is NGF dependent and drives axonal growth. This may indicate that NGF is a common cue that activates polarity proteins in distinct settings.

In this study we have investigated the role of Scribble in NGF-induced differentiation of PC12 cells. Here, we show that RNAi mediated knock-down of Scribble in PC12 cells stimulated with NGF reduces neurite density but increases neurite length. In addition, expression of Scribble is induced by growth factor stimuli and corresponds to complex formation with Ras, Rac1, βPIX and ERK1/2. Together, these data reveal a novel and context-dependent role for Scribble in cytoskeleton alterations that underlies neurite outgrowth.

Materials and methods

Chemicals, antibodies and plasmids

Rat collagen type I from rat tail tendon was from Roche. Rabbit anti-GFP antibody, anti-ERK1/2 antibody, phospho anti-ERK1/2 antibody and phospho-pY490TrkA antibody was from Cell Signaling Technology. Goat anti-Scribble antibodies (C-20 and K-21), rabbit anti-HRas antibody and horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody was from Santa Cruz Biotechnology. Mouse anti-βActin antibody, rat-NGF (2.5S), k252a, Cycloheximide (CHX) and protease inhibitor cocktail were from SIGMA. Recombinant human EGF was from R&D systems. Cell culture medium DMEM and RPMI 1640, horse serum (HS), Fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), Opti-MEM buffer and transfection reagent (Lipofectamine 2000) were from Invitrogen. Protein G-Sepharose beads were from Millipore. Precast 4–20% Tris–HCl gradient SDS gels were from BIO-RAD. ECL chemiluminiscence kit, HRP-conjugated donkey anti-rabbit antibody, donkey anti-mouse antibody, Alexa594-conjugated donkey anti-goat and Alexa488 goat anti-rabbit and Alexa568 rabbit anti-goat were from GE Healthcare. Mounting media Fluoromount was from Southern Biotechnology. The pEYFP plasmid used as a control was from Clontech. Plasmids encoding CFP-tagged βPIX, GFP-tagged Rac1 and GFP-tagged HRas have been described previously (Mayhew et al., 2006; Subauste et al., 2000), and were obtained by Drs. Rick Horwitz, Gary Bokoch and Karel Svoboda (Addgene plasmids 15235, 12980 and 18662). The construct encoding GFP-RhoA was kindly provided by Dr. Michael Way.

Cell culture and transient transfections

PC12 and COS7 cells were maintained at 37 °C in 5% CO_2 in RPMI 1640 supplemented with 10% HS, 5% FBS and antibiotics (PC12) or in DMEM supplemented with 10% FBS and antibiotics (COS7). Transient plasmid transfection of COS7 cells was performed using Lipofectamine 2000 under serum free conditions according to the manufacturer's instructions. Where indicated, cells starved for at least 24 h were treated with 100 ng/ml NGF in serum free media supplemented with 1% HS or with 100 ng/ml EGF in media without serum or antibiotics. For pharmacological inhibition of TrkA in naïve cells, cultures were pre-treated with 200 nM of k252a followed by combined NGF stimulation for the indicated intervals. Protein synthesis inhibition was achieved by application of 50 μg/ml of CHX for 4 h following combined treatment with NGF for the indicated intervals. For evaluation of phenotypic differences by Scribble depletion, cells treated with siRNA for 48 h were stimulated with NGF in combination with siRNAs for 24 h. In co-precipitation and evaluation of protein levels, PC12 and COS7 cells were stimulated with NGF or EGF for 5 min or for intervals as indicated.

Western blotting and immunoprecipitations (IP)

Cells stimulated with growth factor for 5–60 min were washed with cold phosphate-buffered saline (PBS) and lysed by rocking for 45 min at 4 °C with 0.2 ml/6-well plate of RIPA buffer (50 mM TrisHCl, pH.7.5, 135 mM NaCl, 1% Triton-X100, 0.5% sodiumdeoxycholate, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_2VO_4 and protease inhibitor cocktail). Equal amounts of lysate eluted with 2× Laemmli sample buffer were subjected to SDS-PAGE gels and transferred to PDVF membrane for subsequent detection with primary antibody.

For co-precipitations in PC12 cells, confluent layers of starved cells were treated with NGF, washed with cold PBS and lysed by rocking for 45 min at 4 °C with 0.2 ml/6-well plate of RIPA. Lysates (approximately 300 μg of protein/0.2 ml) were incubated with 2 μg of anti-Scrib antibody (K-21) overnight at 4 °C, followed by incubation with protein G-Sepharose beads for 4 h. Beads were washed 4 times with ice-cold RIPA and bound proteins were eluted with 2× Laemmli sample buffer. Equal amounts of protein were subjected to SDS-PAGE and transferred to PDVF membrane for detection with primary antibody.

For co-precipitations in COS7, 80% confluent cells were transfected with 2 μg each of GFP-tagged constructs encoding HRas, Rac1, βPIX or GFP. Following treatment with EGF, cells were washed with cold PBS and lysed by rocking for 20 min at 4 °C with 0.2 ml/6-well plate of RIPA. Lysates were incubated with 2 μg of anti-Scrib antibody (C-20) overnight at 4 °C, followed by incubation with protein G-Sepharose beads for 4 h. Beads were washed 4 times with ice-cold RIPA and bound proteins were eluted with 2× Laemmli sample buffer. Equal amounts of protein were subjected to 4–20% gradient SDS-PAGE gels and transferred to PDVF membranes for subsequent treatment with primary antibody. Primary antibody dilutions were as follows: anti-ERK1/2 antibody (1:2000), phospho anti-ERK1/2 antibody (1:2000), phospho-pY490TrkA antibody (1:500), anti-Scrib antibody (C-20 and K-21; 1:300), anti-βActin antibody (1:2500), anti-HRas antibody (1:200) and anti-GFP antibody (1:1000). Following washing with PBS, membranes were incubated with corresponding HRP-conjugated secondary antibody (1:4000). Reactive bands were detected by using ECLplus per the manufacturer's instructions. Secondary antibodies for immunofluorescence were: Alexa488 goat anti-rabbit (1:1000) and Alexa568 rabbit anti-goat (1:1000).

RNAi experiments

Target and non-targeting control siRNA were designed by Ambion. Double-stranded rat Scribble siRNAs were as follows: si#1: 5′-CGAGATACCTGAGAGCAT-3′, si#2: 5′-GCATTGTAGGAGGTTCTG-3′ and si#3: 5′-TCAGTGACCTCACATAGCCGG-3′. Ahead of transfection PC12 cells were plated at a density of 5×10^4 cells on collagen coated coverslips and grown overnight. Cells were transfected with 2 μM of each siRNA (final concentration 10 nM) and grown in Opti-MEM without serum or antibiotics at 37 °C for further processing. For evaluation of knock-down efficiency, cells were treated with siRNA for 72 h and analyzed by Western blotting. For evaluation of ERK1/2 activation, NGF was applied to siRNA treated cells after 72 h for the indicated intervals and analyzed by Western blotting. For image analysis, cells were treated with siRNA for 48 h followed by combined treatment with siRNA and NGF for another 24 h.

Measurement of neurite density and length

PC12 cells treated with siRNA and NGF from three independent experiments were quantified. Neurite density and length was measured with Image J (NIH). The length of neurites was measured from the cell membrane at the starting point of the protrusion to the