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BIG1, A BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN REGULATES NEURITE DEVELOPMENT VIA PI3K–AKT AND ERK SIGNALING PATHWAYS

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Abstract—The elongation of neuron is highly dependent on membrane trafficking. Brefeldin A (BFA)-inhibited guanine nucleotide-exchange protein 1 (BIG1) functions in the membrane trafficking between the Golgi apparatus and the plasma membrane. BFA, an uncompetitive inhibitor of BIG1 can inhibit neurite outgrowth and polarity development. In this study, we aimed to define the possible role of BIG1 in neurite development and to further investigate the potential mechanism. By immunostaining, we found that BIG1 was extensively colocalized with synaptophysin, a marker for synaptic vesicles in soma and partly in neurites. The amount of both protein and mRNA of BIG1 were up-regulated during rat brain development. BIG1 depletion significantly decreased the neurite length and inhibited the phosphorylation of phosphatidylinositide 3-kinase (PI3K) and protein kinase B (AKT). Inhibition of BIG1 guanine nucleotide-exchange factor (GEF) activity by BFA or overexpression of the dominant-negative BIG1 reduced PI3K and AKT phosphorylation, indicating regulatory effects of BIG1 on PI3K–AKT signaling pathway is dependent on its GEF activity. BIG1 siRNA or BFA treatment also significantly reduced extracellular signal-regulated kinase (ERK) phosphorylation. Overexpression of wild-type BIG1 significantly increased ERK phosphorylation, but the dominant-negative BIG1 had no effect on ERK phosphorylation, indicating the involvement of BIG1 in ERK signaling regulation may not be dependent on its GEF activity. Our result identified a novel function of BIG1 in neurite development. The newly recognized function integrates the function of BIG1 in membrane trafficking with the activation of PI3K–AKT and ERK signaling pathways which are critical in neurite development.

Key words: neurite, outgrowth, BIG1, PI3K, AKT, ERK.

INTRODUCTION

Neurons are highly polarized cells that require a number of mechanisms to initiate, maintain and regulate their biological functions. Proper growth, remodeling and maintenance of dendrites and axon are highly dependent on membrane trafficking. Disruption of membrane transport can block the formation and elongation of neurite (Sann et al., 2009). The secretory pathway is the major source of the plasma membrane. Lipids and proteins are synthesized in the endoplasmic reticulum (ER), then modified in the Golgi complex, segregated into vesicles and transported to the plasma membrane or enter the endosomal networks for further sorting (De Matteis and Luini, 2008). Post-Golgi secretory trafficking in neurons has been reported to be polarized toward longer dendrites and required for dendrites outgrowth (Horton et al., 2005). Disruption of vesicle trafficking from the Golgi complex to the plasma membrane by brefeldin A (BFA), an uncompetitive inhibitor of the guanine nucleotide-exchange factors (GEFs) activity of the high-molecular-weight ADP-ribosylation factor (Arf)-GEFs, interrupts neurite outgrowth and polarity development (Jareb and Banker, 1997).

The high-molecular-weight Arf-GEFs include GBF1, BFA-inhibited guanine nucleotide-exchange protein 1 (BIG1) and BIG2. Among them, BIG1 and BIG2 are mainly involved in the regulation of trafficking through the trans-Golgi network (TGN) and endosomes (Shen et al., 2006; Ishizaki et al., 2008), whereas GBF1 functions primarily in transport between the ER-Golgi intermediate and the cis-Golgi compartments (Zhao et al., 2006). An early study found that mutations in BIG2 disrupt human neural precursor proliferation and migration in the human cerebral cortex leading to a small brain and an abnormal arrest of cerebral cortical neurons in proliferative zones near the lateral ventricles

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Abbreviations: AKT, protein kinase B; Arf, ADP-ribosylation factor; BFA, brefeldin A; BIG1, BFA-inhibited guanine nucleotide-exchange protein 1; DIV, days *in vitro*; ECL, Dulbecco's modification of Eagle's medium; ER, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; GEF, guanine nucleotide-exchange factor; GTP, guanosine triphosphate; LDH, lactate dehydrogenase; MAP2, microtubule-associated protein 2; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; PI3K, phosphatidylinositide 3-kinase; RIPA, radio immunoprecipitation assay; RT, room temperature; RT-qPCR, reverse-transcribed polymerase chain reaction; SD, Sprague–Dawley; TGN, trans-Golgi network.

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(Sheen et al., 2004). BIG2 null mice developed periventricular heterotopia with impaired neural migration and increased phosphorylation of filamin A (Zhang et al., 2012). GSK-3β signaling was recently implicated in suppression of inhibitory synaptic transmission resulting from dopamine-induced inhibition of BIG2-dependent delivery of GABA receptors to their synaptic lici (Li et al., 2012). BIG1 and BIG2 are 74% identity in amino acid sequence and 90% identity in Sec7 domain that is responsible for Arf activation. Both of them are involved in the membrane trafficking between the Golgi apparatus and the plasma membrane, and are sensitive to BFA, which inhibited neurite outgrowth and polarity development (Jareb and Banker, 1997; Ishizaki et al., 2008). Therefore, we question whether BIG1 is also involved in neurite development.

We report here studies of BIG1 effects on neurite outgrowth, and potentially related regulatory signaling pathways showing that the content of both BIG1 mRNA and protein increased during embryonic brain development. Selective depletion of BIG1 in primary cultured cortical neurons using siRNA impaired neurite outgrowth. As phosphatidylinositide 3-kinase (PI3K)–protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) signaling pathways are important for neurite outgrowth (Brunet et al., 2001; Samuels et al., 2008), we further explored whether the two signaling pathways were involved in the neurite outgrowth mediated by BIG1. Our results identified a novel function of BIG1 in regulating neurite outgrowth through PI3K–AKT and ERK signaling pathways.

EXPERIMENTAL PROCEDURES

Animals, antibodies, plasmids, and reagents

Specific pathogen-free (SPF) Sprague–Dawley (SD) rats were provided by the Laboratory Animal Center, Sun Yat-Sen University. SH-SY5Y cells were obtained from ATCC. Affinity Purified Rabbit anti-BIG1/Arf-GEF1 antibody was purchased from BETHYL Laboratories, USA. Rabbit polyclonal antibodies against phospho-AKT (Ser473), AKT, PI3K, phospho-PI3K, phospho-ERK1/2, and ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse Monoclonal anti-α-Tubulin antibody was from Sigma Aldrich (St. Louis, MO, USA), and Mouse monoclonal anti-β-Actin antibody from Abcam (Cambridge, MA, USA). Mouse monoclonal anti-GAPDH antibody, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) and lactate dehydrogenase (LDH) release assay Kit were purchased from Beyotime (Jiangsu, China). Polyclonal rabbit anti-MAP2 antibody and monoclonal anti-Synaptophysin antibody were from Meck Millipore (Billerica, MA, USA). Alexa Fluor 488, 594-conjugated secondary antibodies were from Molecular Probes invitrogen, (Eugene, OR USA); Enhanced chemiluminescence (ECL) kit was from GE Healthcare (South Burlington, VT, USA); EDTA-free protease inhibitor was from Roche. EGFP-BIG1-WT and HA-BIG1-E793K plasmids were kind gifts from Dr. Martha Vaughan in National Heart, Lung, and Blood Institute.

Extraction and Western blotting of embryonic brain proteins

Cortices of E11, E15, E18 embryos and 1-day-old SD rats were excised and homogenated in ice-cold radio immunoprecipitation assay (RIPA) buffer (Beyotime, China) supplemented with EDTA-free protease inhibitor cocktail. Samples of lysate proteins (20 μg) were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Little Chalfont, UK). After blocking with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h at room temperature (RT), the membranes were incubated with appropriately diluted primary antibodies at 4 °C overnight, and then probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were detected by ECL and visualized with the Las4000 (GE Healthcare). The intensity of the blots was quantified by densitometry using MULTI GAUGE software according to manufacturer's instruction as described (Li et al., 2011).

Primary cortical neuron culture, siRNA transfection and BFA treatment

Cortical neurons were dissociated from embryos of E18 SD rats. Briefly, cortices were aseptically dissected and incubated with 2 mg/ml papain (Sigma) for 15 min at 37 °C. Dissociated neurons were plated at a density of 2.0×10^6 cells/ml onto poly-L-lysine-treated coverslips or cell culture plates. Cultures were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin for 8 h at 37 °C. Subsequently, the medium was removed and neurobasal medium containing 1% B27 supplement (Invitrogen, Carlsbad, CA, USA) was added. Experiments were carried out on neurons after 7–10 days *in vitro* (DIV).

Three siRNA targeting BIG1, designated D09 (AAUACUUGCUAUCUGUUUCCAGUCC), F04 (UUGAG AGUGCAACACAAAGAUACUG), and F08 (UAAAGUG CAUGAUUGCAAGUUGUUG), were synthesized by Invitrogen (Carlsbad, CA, USA). Neurons (6–7 DIV) were transfected with D09, F04, F08, or control scrambled siRNA (RIBOBIO, Guangzhou, China) using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction before fixation for immunofluorescence microscopy or homogenization for Western blot analysis.

For MTT and LDH release assay, primary cultured cortical neuron cells (DIV 6) were transfected with or without siRNA (mock), or with control scrambled siRNA (NC), or BIG1 siRNA F04, respectively. The cell viability was examined with MTT assay, and the conditional medium was harvested for LDH assay according to the manufacturer's instruction.

To assay the effect of BFA on PI3K–AKT and ERK signaling pathway, cortical neurons (7 DIV) were treated with 0.5 μg/ml BFA for 0.5, 1, 3, 6 h, respectively. Dimethyl sulfoxide (DMSO) was used as a menstruum control. Then, total proteins were extracted by Western blot analysis.