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P48 Ebp1 acts as a downstream mediator of Trk signaling in neurons, contributing neuronal differentiation☆

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ABSTRACT

Two Ebp1 isoproteins, p48 and p42, regulate cell survival and differentiation distinctively. Here we show that p48 is the major isoform in hippocampal neurons and is localized throughout the entire neuron. Notably, reduction of p48 Ebp1 expression inhibited BDNF-mediated neurite outgrowth in hippocampal neurons. The p48 protein acts as a downstream effector of the Trk receptor, which mediates the functions of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in hippocampal cells. Trk receptor activation by both NGF and BDNF induced phosphorylation of Ebp1 at the S360 upon the activation of protein kinase Cδ (PKCδ) and triggered dissociation of p48 from retinoblastoma (Rb). Although both NGF and BDNF activate mitogen-activated protein kinase (MAPK; extracellular signal-related kinase (ERK)) as well as phosphatidylinositide 3-kinase (PI3K)/Akt, their activation is regulated in different time-frame upon growth factor specificity, especially, eliciting PKCδ mediated p48 S360 phosphorylation. Thus, p48 Ebp1 contributes to neuronal cell differentiation and growth factor specificity through the activation of PKCδ, acting as a crucial downstream effector of neurotrophin signaling.

1. Introduction

The ErbB3 binding protein Ebp1 is a member of the PA2G4 family of proliferation-regulated proteins (Radomski and Jost, 1995). Despite its initial isolation due to interaction with ErbB3 (Yoo et al., 2000), Ebp1 is ubiquitously expressed in all human tissues (Yoo et al., 2000), suggesting that Ebp1 may function as a general signaling molecule. It has two mRNA transcripts around 1.7 kb and 2.2 kb in size. The PA2G4 gene encodes for two alternatively spliced Ebp1 isoforms, p48 and p42, that correlate with two mRNA transcripts even though the gene possesses three in frame ATG codons (Liu et al., 2006). P48 initiates translation at the first ATG and appears at 48 kDa, which is 54 amino acids longer than p42 in the N-terminus, while p42 translation stems from third ATG because p42 mRNA skips an exon consisting of 29 nucleotides, which contain second ATG in p48 mRNA, and migrates at 42 kDa on SDS/PAGE (Liu et al., 2006). EGF strongly stimulates p42 to bind ErbB3. By contrast, p48 does not bind to ErbB3 regardless EGF treatment. Thus p48 and p42 possess distinctive cellular functions. In mammalian cells, p48 is the predominant form. The p42 isoform is barely detectable because it is polyubiquitinated and subsequently degraded in human cancers (Liu et al., 2009). In addition, the crystal structure of p42 suggests that this protein is unstable (Monie et al., 2007).

Recently, we showed that p48 protects DNA fragmentation in nerve growth factor (NGF)-treated PC12 cells. Survival was promoted by inhibiting apoptosis via the association between p48 and nuclear Akt, which is upregulated by protein kinase C (PKC) δ-mediated phosphorylation of the serine 360 (S360) aminoacyl residue (Ahn et al., 2006). However, the Ebp1 p42 isoform did not associate with Akt and did not prevent apoptosis. The longer p48 isoform, which is distributed in both the cytoplasm and the nucleus, enhanced cell growth, leading to attenuation of neurite outgrowth through downregulation of cyclin D1 expression after exposure to NGF in PC12 cells (Liu et al., 2006). In contrast, the shorter p42 isoform predominantly resides in the cytoplasm, barely suppressed apoptosis and stimulated PC12 cell differentiation. p42 binds to EbrB3 receptor in human breast cancer cell lines under serum starvation and dissociates from ErbB3 with heregulin treatment, leading to translocation to the nucleus where it binds to retinoblastoma (Rb) and effects on Rb transcriptional regulation (Yoo et al., 2000; Zhang et al., 2002), whereas p48 does not bind to ErbB3. Interestingly, p42 binds

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strongly to the ErbB3 receptor upon EGF stimulation and PKCδ-mediated p42 phosphorylation of a S360D phosphomimetic mutant tightly associated to the ErbB3 receptor (Liu et al., 2006). Very recently, it was shown that p48 constitutively associates with nucleophosmin (NPM)/B23 which is essential for cell proliferation and survival (Ahn et al., 2005), enhancing cell proliferation and suppressing apoptosis, whereas the p42 isoform interacts with B23 in response to EGF stimulation in mitotic arrested cells. An alignment of p42 expression with the cell cycle specific expression profile was identified (Okada et al., 2007), underscoring that the endogenous p42 protein level is much lower than that of p48.

The crystal structures of the two isoforms revealed that p42 lacks one and a half helices present at the amino-terminus of p48 (Monie et al., 2007), suggesting that the conformational changes of p48 due to the 54 N-terminal amino acids may be responsible for the different cellular functions of p48 and p42. Despite expanding interest in the possible differential roles of the two Ebp1 isoforms in cellular functions, little is known about their roles in neuronal cell signaling. In this study, we provide information on the functions of both Ebp1 proteins as general signaling molecules for neuron development. We demonstrate that p48 mRNA is predominantly expressed in most developing brain regions, even though both p48 and p42 mRNA are present, and p48 protein is preferentially expressed in cultured hippocampal neurons, while p42 is almost undetectable, correlating with the previous findings that the ErbB3 receptor was undetectable in hippocampal cells (Chen et al., 2005). Moreover, endogenous p48 resides throughout the neuron from the neural soma to neurites but not within glial cells, suggesting its potent role in the neuron. Reduction of Ebp1 by siRNA expression markedly attenuated the promoting effect of brain-derived neurotrophic factor (BDNF) on neurite extension in cultured hippocampal neurons. These findings reveal an essential role of Ebp1 in the downstream functions of neurotrophin signaling and provide a novel insight into the role of Ebp1 proteins in the nervous system.

2. Materials and methods

2.1. Materials

NGF, BDNF, K252a, p75NTR, LY294002, PD98059 and Rottlerin inhibitor were purchased from Calbiochem (Darmstadt, Germany). Anti-Akt, anti-phospho-Akt, anti-phospho-TrkA (Tyr490), anti-Rb, or anti-phospho-Rb (S780) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Ebp1, anti-TrkA, anti-TrkB and anti-p75 antibodies were obtained from Millipore (Billerica, MA, USA). Anti-β-actin, anti-tubulin, anti-Trk (Tyr680/Tyr681) and anti-GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-tubulin III antibody as neuronal marker was purchased from Covance Inc. (Princeton, NJ, USA). Anti-phospho-Ebp1 (Ahn et al., 2006) and anti-N-p48 (specific for p48 isoform) (Okada et al., 2007) antibodies were generated in our laboratory. Alexa Fluor 488-conjugated goat anti-mouse IgG or goat anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 5% horse serum and 100 units penicillin–streptomycin. Cells were cultured at 37 °C and 5% CO_2 in a humidified incubator. H19-7/IGF-IR rat hippocampal cell line was purchased from American Type Culture Collection (ATCC, catalogue number CRL-2526). The H19-7/IGF-IR cell line derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos was immortalized by retroviral transduction of temperature sensitive tsA58 SV40 large T antigen, and then were established by infecting with a retroviral vector expressing the human type I insulin-like growth factor receptor (IGF-IR). The cell was cultured at 34 °C with 5% CO_2 in complete DMEM (Life Technology, USA) supplemented with 10% FBS (Cellgro), 15 mM HEPES, 42.5 mM sodium bicarbonate (Sigma), 0.2 mg/ml G418 (Cellgro, Mediatech, Inc., Manassas, VA, USA) and 0.001 mg/ml puromycin (Sigma) (Kim and Kim, 2007). For primary neuronal cultures, the cells were prepared from 18-day-old rat embryos. Briefly, cerebrum, cerebellum, hippocampus, and brain stem were dissected in HBSS and mechanically triturated with micropipette and then plated onto poly-L-lysine-coated coverslips or culture dishes in neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA). The cells were further cultured at 37 °C for the times indicated in the figures.

2.3. Transfection and differentiation

H19-7/IGF-IR cells were transiently transfected with small interfering (si) RNA-Ebp1 or GFP-tagged p48- or p42-Ebp1 wild-type, a phosphorylation-abolished mutant S360A, and a phosphorylation mimetic mutant S360D using a microporator (Invitrogen). Twenty-four hours after transfection, the cells were replaced with N2 medium containing 10 ng/ml bFGF, and the temperature was adjusted to 39 °C in order to differentiate. In one study, we wished to determine if expression of p48- or p42-Ebp1 wild-type could rescue the phenotype obtained with siRNA-depletion of Ebp1. For these experiments, neurons were first transfected with si-Ebp1. Twenty hour after transfection with si-Ebp1, the cells were replaced with N2 medium containing 10 ng/ml bFGF and cultured for additional one day. Next day, the cells were transfected with either the wild-type GFP-p48- or -p42-Ebp1 and then replated on poly-L-lysine coated coverglasses, followed by differentiation.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Primary neurons were isolated from 18-day-old rat embryos and cultured in neurobasal medium supplemented with B27 for 3 days. Total RNA was extracted with QIAzol® reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The reverse transcription reaction was performed using a Superscript II™ kit (Invitrogen). Expressions of the two Ebp1 isoforms (Genbank accession number, NM_006191.2) were verified using GoTaq® Green Master mix (Promega, Madison, WI, USA). The RT-PCR primer sequences for Ebp1 were as follows: (forward) 5′-ACAGCCTGTGGCTGGGAAGGG-3′ (nucleotide 343 to 363), (reverse) 5′-CTTCAAAGGGGAGAAGTG-3′ (nucleotide 681–698) (the product size, 356 bp and 290 bp); for actin: (forward) 5′-CATGTTTGAGACCTTCAACACCCC-3′, (reverse) 5′-GCCATCTCCTGCTCGAAGTCGAG-3′.

2.5. Synthetic siRNA oligonucleotides

The siRNA for rat Ebp1 (Genbank accession number, NM_001004206.1) was obtained from Genolution Pharmaceuticals, Inc. (Republic of Korea). The siRNA sequences were as follows: duplex oligonucleotide #1, (sense) 5′-CGCAGCUCAGG-GAAAGUGAUU-3′, (anti-sense) 5′-UCACUUUCCCUGAGCUGCGUU-3′ (47–92).

2.6. Immunocytochemistry

The H19-7/IGF-IR or primary hippocampal neurons were fixed for 20 min at room temperature in 4% paraformaldehyde, permeabilized in 0.1% Triton-X 100 for 20 min and blocked in 2% BSA for 1 h at room temperature. Primary antibodies were diluted in blocking solution and incubated overnight with cells at 4 °C. After reaction with primary antibody, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG or goat anti-rabbit IgG in blocking solution. Nuclear staining was performed with DAPI (Roche, Mannheim, Germany). Images were acquired with an Axiophot II (Carl Zeiss, Oberkochen, Germany) microscope or an LSM510 confocal microscope (Carl Zeiss).

2.7. Immunoprecipitation and immunoblotting

The cells were rinsed in ice-cold 1× PBS, and then harvested with lysis buffer (50 mM Tris, pH 7.4; 40 mM NaCl; 1 mM EDTA; 0.5% Triton X-100; 1.5 mM Na_3VO_4; 50 mM NaF; 10 mM sodium pyrophosphate; 10 mM glycerophosphate; 1 mM PMSF; 10 mM protease inhibitor cocktail). For immunoprecipitation, 500 μg of cell lysates were incubated with appropriate antibodies and were captured by a protein A/G–agarose mixture (GenDEPOT, Barker, TX, USA). Bound proteins were resolved by SDS-PAGE and detected by the antibodies indicated in each figure. For immunoblotting, protein aliquots (30 μg) were analyzed via immunoblotting using a 1:1000 dilution of specific antibodies. Immunocomplexes were visualized using an enhanced chemiluminescence reagent (Santa Cruz, CA, USA) according to manufacturer's instructions.

2.8. Subcellular fractionation

The cytosolic and nuclear fractions of primary cultured hippocampal neuron were prepared from rat embryonic day 18. The method for fractionation was performed according to the previous protocol (Shapiro et al., 1988). Briefly, primary hippocampal neurons were cultured for 4 day-in vitro (DIV 4). The cells were washed once with ice-cold 1× PBS and once with 1 ml of lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 0.75 mM spermidine; 0.15 mM spermine; 0.5 mM PMSF; 10 μg/ml leupeptin), and then were centrifuged at 2500 rpm for 10 min at 4 °C. The cell pellet was lysed in 200 μl of lysis buffer and homogened with 26 GX 1/2 gauge syringe for several times, followed by mixing with 20 μl of sucrose restore buffer (1 volume of 10× sucrose restore buffer [50 mM HEPES, pH 7.9; 10 mM KCl; 0.2 mM EDTA; 0.75 mM spermidine; 0.15 mM spermine; 1 mM DTT; 0.5 mM PMSF; 10 μg/ml leupeptin] and 9 volume of 75%