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MAGI-1 acts as a scaffolding molecule for NGF receptor-mediated signaling pathway

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ABSTRACT

We have recently found that the membrane-associated guanylate kinase with inverted organization-1 (MAGI-1) was enriched in rat nervous tissues such as the glomeruli in olfactory bulb of adult rats and dorsal root entry zone in spinal cord of embryonic rats. In addition, we revealed the localization of MAGI-1 in the growth cone of the primary cultured rat dorsal root ganglion cells. These results point out the possibility that MAGI-1 is involved in the regulation of neurite extension or guidance. In this study, we attempted to reveal the physiological role(s) of MAGI-1 in neurite extension. We found that RNA interference (RNAi)-mediated knockdown of MAGI-1 caused inhibition of nerve growth factor (NGF)-induced neurite outgrowth in PC12 rat pheochromocytoma cells. To clarify the involvement of MAGI-1 in NGF-mediated signal pathway, we tried to identify binding partners for MAGI-1 and identified p75 neurotrophin receptor (p75NTR), a low affinity NGF receptor, and Shc, a phosphotyrosine-binding adaptor. These three proteins formed an immunocomplex in PC12 cells. Knockdown as well as overexpression of MAGI-1 caused suppression of NGF-stimulated activation of the Shc-ERK pathway, which is supposed to play important roles in neurite outgrowth of PC12 cells. These results indicate that MAGI-1 may act as a scaffolding molecule for NGF receptor-mediated signaling pathway.

1. Introduction

The membrane-associated guanylate kinase with inverted organization (MAGI) proteins belong to a subfamily of membrane-associated guanylate kinase (MAGUK) proteins, and consist of three members, MAGI-1, MAGI-2/synaptic scaffolding molecule (S-SCAM) and MAGI-3. They usually harbor six PSD-95/Discs Large/Zona Occludens (PDZ) domains, a GUK domain and two WW motifs [1–3]. MAGI-2/S-SCAM is the neural isoform of MAGI protein and interacts with various synaptic molecules such as NMDA receptors, neuroligins and AMPA receptors [2,4,5]. Physiological functions of MAGI-1 have been investigated mainly in non-neuronal tissues; it is localized at tight junction of epithelial cells [6] and cell–cell contacts of vascular endothelial cells [7]. Although MAGI-1 is thought to act as a scaffold for various proteins such as β-catenin, RapGEP and JAM4 at non-neuronal cell junctions [8–10], the physiological significance of MAGI-1 in neuronal tissues is yet obscure. We have recently developed the specific antibody for MAGI-1 and found that MAGI-1 was enriched in rat neural tissues such as the glomeruli in olfactory bulb of adult rats and dorsal root entry zone in spinal cord of embryonic rats. In addition, we found the localization of MAGI-1 in the growth cone of primary cultured rat dorsal root ganglion neurons [11]. These results appeared to indicate the possible participation of MAGI-1 in neurite extension or guidance.

Neurotrophins are a family of proteins that influence various activities of neuronal cells such as proliferation, growth and differentiation [12,13]. They interact with two distinct classes of receptors, tropomyosin-related kinase (Trk) family consisting of TrkA, TrkB and TrkC, and p75 neurotrophin receptor (p75NTR). Four neurotrophins, NGF, BDNF, NT-3 and NT-4, exhibit specificity for Trk receptors, and NGF interacts only with TrkA [14]. The cytoplasmic domain of Trk can interact with various molecules involved in signaling cascade such as Ras-extracellular signal-regulated kinase (ERK), phosphatidylinositol-3 (PI3) kinase and phopholipase C (PLC)-γ1 signaling. In PC12 rat pheochromocytoma cells, NGF binds to TrkA with high affinity and induces Ras-mediated activation of ERK that leads to subsequent proliferation and differentiation [12]. On the other hand, p75NTR does not possess a cytoplasmic tyrosine kinase domain and interacts with Trk receptors. As a Trk co-receptors p75NTR binds each of neurotrophins with relatively low affinity [15], and has been shown to enhance Trk receptor activity mediated by neurotrophins [16,17]. While p75NTR does not have intrinsic catalytic activity, this protein interacts with diverse signaling molecules such as tumor necrosis factor receptor-associated factor-6 (TRAF6), RhoA

Abbreviations: NGF, nerve growth factor; MAGI-1, membrane-associated guanylate kinase with inverted organization-1; MAGUK, membrane-associated guanylate kinase; PDZ, PSD-95/Discs Large/Zona Occludens; Trk, tropomyosin-related kinase; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MBP, maltose binding protein; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; RNAi, RNA interference; PTB, phosphotyrosine binding; JNK, c-Jun N-terminal kinase

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and Nogo receptors and exerts diverse signaling pathways leading to apoptosis, cell survival and neurite outgrowth [18–20].

Here, we demonstrate that MAGI-1 regulates NGF-stimulated neurite extension of PC12 cells. Moreover we found that MAGI-1 interacts with p75NTR and Shc, and acts as a scaffolding molecule for NGF signaling pathway.

2. Materials and methods

2.1. Plasmid construction

pRK5-myc-mouse MAGI-1 was obtained from Dr. K. Patrie (University of Michigan). Mouse MAGI-2 and MAGI-3 were from Drs. Y. Hata (Tokyo Medical and Dental University, Japan) and E. Peles (The Weizmann Institute of Science, Israel), respectively. Full length and various fragments of MAGI proteins, p75NTR and Shc were produced by PCR and subcloned into pCAG-GFP-MCS2, pCAG-myc-MCS2, pCAG-FLAG-MCS2 [21], EGFP-C1(Clontech Laboratories, Inc., CA, USA), pMal (New England Biolab. Inc., MA) and pGEX-4T3 (GE Healthcare Bio-Sciences, Uppsala, Sweden) vectors. All constructs were verified with DNA sequencing. GFP-ERK was obtained from Dr. N. Kioka (Kyoto University, Japan).

2.2. Antibodies and reagents

Using glutathione S-transferase (GST)-fused p75NTR fragment (p75-intracellular domain, aa 274–472) expressed in *Escherichia coli* as an antigen, a rabbit polyclonal antibody for p75NTR, anti-p75, was generated and affinity-purified on a column to which the antigen had been conjugated. A MAGI-1-specific antibody, anti-MAGI-1, and pan-MAGI antibody, anti-MAGI1/2/3, were prepared as described previously [11]. Polyclonal rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr/204), anti-Shc, anti-phospho Shc (Tyr239/240) (Cell Signaling Technology Inc., Danvers, MA, USA), and anti-GFP (Medical & Biological Laboratories Co., Nagoya, Japan) were purchased. Rat monoclonal anti-GFP was obtained from Nacalai Tesque (Kyoto, Japan). Monoclonal mouse anti-p75NTR (clone 192-IgG, Millipore), anti-GFP (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) and anti-myc 9E10 [22] were also used.

2.3. Cell culture and transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. PC12 cells were cultured in DMEM supplemented with 10% horse serum, 5% FBS and antibiotics. Transient transfection was carried out with the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.4. Immunofluorescent analysis

Immunofluorescent analysis was done as described [23]. Alexa Fluor® 488 and 568 (Invitrogen) were used as secondary antibodies. Fluorescent images were obtained using FV-1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

2.5. RNA interference

The following target sequences were inserted to pSUPER-puro or pSUPER-GFP vector (OligoEngine, Seattle, WA, USA), rat MAGI-1; 5′-GTCGGCACCTATGAAGGAA-3′ (534–552, MAGI#1), 5′-AGTGATCACGAC GGATGCC-3′ (599–617, MAGI#2). Numbers indicate positions from transcription start sites. We usually used pSUPER-puro vector for biochemical analysis and pSUPER-GFP vector for cell biological analysis. To generate RNAi-resistant mutants of MAGI-1, MAGI-1#1mt and MAGI-1#2mt, we introduced three silent mutations, as underlined, in the target sequences of pSUPER-MAGI-1#1 (5′-GTCGGGACCTACGAG GGAA-3′) and pSUPER-MAGI-1#2 (5′-AGTCATCACCACCGATGCC-3′).

2.6. Quantification of neurite-bearing cells

PC12 cells were transfected with various vectors and cultured for 48 h. Then, neurite extension was induced by DMEM with 1% horse serum, 0.5% FBS and 100 ng/ml NGF for 48 to 72 h. Cells were fixed and stained as described. Images were obtained using a confocal microscope. Morphometric measurements were performed using ImagePro PLUS software (Media Cybernetics, Silver Spring, MD, USA). For quantification of neurite bearing cells, 100–150 cells were randomly chosen and length of processes was measured. Cells with a process longer than one cell diameter were defined as neurite-bearing cells. Statistical analyses were performed by the Student's *t*-test.

2.7. Immunoprecipitation

Immunoprecipitation was done as previously described [24]. Briefly, cells expressing tagged proteins were harvested with immunoprecipitation (IP) buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4 and protease inhibitor cocktail (Nacalai Tesque). Insoluble material was removed by centrifugation at 4 °C for 10 min at 10,000 ×*g* and the resultant supernatants were subjected to immunoprecipitation using indicated antibody and proteinA-sepharose beads. After washing the beads with IP buffer, the precipitates were subjected to SDS-PAGE followed by western blotting. Immunoreactive bands were visualized as described [25]. In the experiment of the binding of MAGI-1 and Shc, we used IP buffer without NaCl. To determine the complex formation in PC12 cells, cells were lysed in a buffer consisting of Tris–buffered saline (pH 8.0) with 0.1% Triton X-100, 60 mM octylglucoside, 1 mM Na_3VO_4 and protease inhibitor cocktail.

2.8. In vitro binding assay

Full length MAGI-1 was constructed into pMal vector. The fusion protein was then purified by one-step affinity purification specific for maltose-binding protein (MBP) according to the manufacturer's instruction. GST-fused proteins were immobilized on to glutathione–sepharose 4B (GE Healthcare Bioscience) and incubated with MBP or MBP-fused MAGI-1 in IP buffer for 1 h at 4 °C. The beads were then washed with IP buffer and bound proteins were analyzed by western blotting.

2.9. Analyses of ERK and Shc activation

PC12 cells were transfected with GFP-ERK or GFP-Shc in the presence or absence of a MAGI-1 knockdown vector and cultured for 48 h. Cells were then serum-starved for 24 h and stimulated by 100 ng/ml NGF for the indicated time periods. Cells were fixed with 10% trichloroacetic acid, washed with PBS, suspended in lysis buffer (50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaF, 5 mM EDTA, 2% SDS and protease inhibitor) and centrifuged at 10,000 ×*g* for 10 min. Cleared supernatants were subjected to SDS-PAGE followed by western blot analyses using anti-phospho p44/p42 MAP kinase, anti-phospho Shc or anti-GFP. Relative intensities of bands were quantified using ImagePro PLUS software. Statistical analyses were performed by the Student's *t*-test.

3. Results

3.1. Knockdown of MAGI-1 caused the suppression of NGF-induced neurite outgrowth in PC12 cells

Based on our pervious study [11], we explored possible role of MAGI-1 in neurite extension. Effect of MAGI-1 knockdown on the NGF-induced neurite extension was examined in PC12 rat pheochromocytoma cells since this cell line is widely used to study the mechanism of neurite extension [26]. We constructed two RNAi vectors,