The Scaffold Protein JIP3 Functions as a Downstream Effector of the Small GTPase ARF6 to Regulate Neurite Morphogenesis of Cortical Neurons

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Abstract

The small GTPase ARF6 plays crucial roles in a wide variety of cell functions. To better understand the molecular mechanisms of the ARF6-mediated signaling and cellular functions, we sought new ARF6-binding proteins in the mouse brain. Here, we identified the signaling scaffold protein JIP3, which is exclusively expressed in neurons, as a downstream effector of ARF6. Overexpression of a unique dominant negative mutant of ARF6, which was unable to interact with JIP3, and knockdown of JIP3 in mouse cortical neurons stimulated elongation and branching of neurites. These results provide evidence that ARF6/JIP3 signaling regulates neurite morphogenesis.

Key words: ARF6; JIP3; JLP; neurite morphogenesis

Abbreviations: ARF, ADP-ribosylation factor; GFP, green fluorescent protein; GTPγS, guanosine 5'-*O*-(thio)triphosphate; JIP, JNK-interacting protein; JLP, JNK-interacting leucine zipper protein; JNK, c-Jun N-terminal kinase; LZ, leucine zipper; PIP5K, phosphatidylinositol 4-phosphate 5-kinase

1. Introduction

The mammalian small GTPase ADP-ribosylation factor (ARF) family consists of six related gene products, ARF1-6, which are subdivided into three classes based on their sequence homology. Classes I and II of ARFs, ARF1-3 and ARF4-5, respectively, localize primarily to perinuclear organelles and are implicated in intracellular vesicle trafficking. The class III of ARF6 localizes mainly at the plasma membrane and plays roles in a wide variety of cellular events, such as endocytosis, exocytosis, membrane ruffle formation, cell migration, and cytokinesis [1, 2]. However, the molecular mechanisms through which ARF6 regulates these cell events remain largely unknown.

One of possible approaches to better understand the molecular mechanisms of the ARF6-mediated signaling is to identify downstream effectors of ARF6. By this approach, we have successfully demonstrated that the lipid kinase phosphatidylinositol 4-phosphate 5-kinase (PIP5K) functions as a downstream effector of ARF6 to couple the signal of epidermal growth factor to the membrane ruffle formation [3]. Although several target molecules of ARF6 had been identified [1, 2], it is still required to identify new downstream effectors of ARF6 for clear understanding of multi-functions of ARF6.

In this study, we identified JIP3, a signaling scaffold protein exclusively expressed in neurons, as a novel partner protein of ARF6. Finally, we demonstrated that interaction of ARF6 with JIP3 regulates neurite morphogenesis of mouse cortical neurons.

2. Material and Methods

2.1. Antibodies, plasmids, recombinant proteins, and analysis of protein interaction in the cell

Antibodies, plasmids, recombinant proteins, and analysis of interaction in the cell were described in “Supplementary Materials and Methods”.

2.2. Detection of binding proteins of ARF6 in mouse brain cytosol

The active (ARF6Q67L) and inactive (ARF6T27N) mutants of ARF6 tagged with glutathione *S*-transferase and FLAG at N- and C-terminus, respectively, were expressed in *E.coli*. After lysis of *E. coli*, these ARF6 mutants were trapped on the Glutathione-Sepharose resin (GE Healthcare) and incubated with PreScission protease (GE Healthcare) at 4°C for 12 h to release ARF6-FLAG mutants from the resins. ARF6-FLAG mutants released were immobilized on the anti-FLAG M2 resin (Sigma). ARF6Q67L- and ARF6T27N-resins thus prepared were incubated with guanosine 5’-*O*-(thio)triphosphate (GTPγS) and GDP, respectively, at 30°C for 20 min and then with mouse brain cytosol (7.6 mg of protein) at 4°C for 12 h in the buffer consisting of 20 mM Tris-HCl, pH7.5, 1 mM EGTA, 2 mM $MgCl_2$, 1% Triton X-100, and 1 mM PMSF. After the resins were thoroughly washed, proteins bound to these ARF6 mutant-resins were separated on 12% gels by SDS-PAGE, and visualized by SYPRO Ruby staining (Bio-Rad) or immunoblotting.

2.3. Identification of ARF6Q67L-binding proteins by MALDI-QIT/TOF MS

Proteins bound to the ARF6Q67L-resin, which were visualized on gels by SYPRO Ruby staining, were subjected to in-gel tryptic digestion, followed by MS and MS/MS analyses with an AXIMA-QIT/TOF (Shimadzu Biotech, Kyoto, Japan) as described previously [4]. To identify proteins, MS and MS/MS spectra were searched against NCBInr databases using the MASCOT search engine (peptide mass fingerprint and MS/MS ion search, Matrix science Ltd.).

2.4. Primary culture of mouse cortical neurons, transfection, and confocal immunofluorescent microscopy

Primary culture of cortical neurons and transfection were described in “Supplementary Materials and Methods”. The immunofluorescent analyses were performed by the method described previously with minor modifications [5]. The detail was described in “Supplementary Materials and Methods”.

3. Results

3.1. Identification of JIP3 and JLP as novel target proteins of ARF6

We sought ARF6-interacting proteins in mouse brain cytosol using affinity resins immobilized with ARF6Q67L and ARF6T27N (Fig. 1A). A protein with an apparent molecular weight of 170 kDa (p170), which bound to ARF6Q67L, but not to ARF6T27N, was identified as the c-Jun N-terminal kinase (JNK)-interacting leucine zipper protein (JLP), a member of the JNK-interacting protein (JIP) family [6], by MALDI-QIT/TOF mass spectrometry, indicating that JLP is a novel target protein of the active form of ARF6.

JIP family is composed of JIP1, JIP2, JIP3, and JLP. JIP3 and JLP are highly homologous in their sequences and domain structures (Fig. 1B), indicating that ARF6Q67L also binds JIP3 as well as JLP. As expected, the p170 proteins bound to the ARF6Q67L-resin were recognized by both anti-JIP3 and -JLP antibodies (Fig. 1C). Specific interaction of the active form of ARF6 with JLP and JIP3 was also observed in the cell. Immunoprecipitation of ARF6Q67L-FLAG coexpressed with GFP-tagged JIP family members in HEK293T cells coprecipitated JLP and JIP3, of four JIP family members (Fig. 1D). We also found that the LZ region in JIP3 and LZII region in JLP are the binding sites for ARF6 (for details, see Supplementary Results).

In order to examine the specificity of ARF isoforms in the interaction with JIP3 and JLP, the active mutants of ARF1, ARF5, and ARF6, which are the representatives of class I, II and III of ARFs, respectively, were coexpressed with JIP3 or JLP in HEK293T cells, and interaction was examined. As shown in Fig. 1E, the active mutant of ARF6 specifically interacted with JIP3 and JLP.

3.2. Generation of a unique ARF6 mutant lacking the ability to interact with JIP3 and JLP

As an ARF6 mutant that can interferes with the interaction specifically with JIP3 and JLP would be a very useful tool to investigate the ARF6/JIP3- and ARF6/JLP-mediated cellular functions, we determined amino acid residues in ARF6 critical for the binding to JIP3 and JLP.

Since ARF5 failed to interact with JIP3/JLP, we constructed cDNAs for an active mutant of ARF6/5 chimera ARF(N6-C5)Q67L, which is composed of 1-76 amino acid residues of ARF6 and 81-180 amino acid residues of ARF5, and for the deletion mutant ARF6(Δ1-11)Q67L (Fig. 2A), and the interaction was examined by immunoprecipitation from HEK293T cells. Both the ARF(N6-C5)Q67L and ARF6(Δ1-11)Q67L interacted with JIP3 and JLP (Fig. 2B), indicating that the region corresponding to 12-76 amino acid residues of ARF6 is important for the interaction. Since eight amino acid residues in this region of ARF6 are divergent from those of ARF5, those in ARF6Q67L were point-mutated, and the interaction was examined (Fig. 2C). Point mutations of L19V, T53E, and K58C weakened the interaction with JIP3 and JLP. In addition, the point mutation of N60T of ARF6Q67L reduced the interaction with JLP. These results suggest that these four amino acid residues in ARF6 are substantially critical for the interaction with JIP3/JLP. It has been reported that three amino acid residues of T53, K58, and N60 in ARF6 locate close to the interswitch effector domain surface, and an amino acid residue of L19 is buried inside the ARF6 molecule [7]. This report led us to examine the interaction of the T53E/K58C/N60T triple mutant of ARF6Q67L (ARF6Q67LTriM). ARF6Q67LTriM completely lost its ability to interact with JIP3/JLP (Fig. 2D). Importantly, interaction of ARF6Q67LTriM with other ARF6 effectors, PIP5K (Fig. 2E) and GGA3 (data not shown), was not impaired. These results conclude that three amino acid residues, T53, K58, and N60, in ARF6 are crucial especially for the interaction with JIP3 and JLP, consistent with the report for the crystal structure of the ARF6/JLP complex [8]. Thus, ARF6TriM could be a useful dominant negative mutant to investigate physiological significance of ARF6/JIP3 and ARF6/JLP signaling.

3.3. ARF6/JIP3 signaling regulates neurite morphogenesis of cortical neurons

As JIP3 is specifically expressed in neurons [9], we speculated that the ARF6/JIP3 signaling couples to neurite morphogenesis. To address this issue, the effects of ARF6 mutants on morphology of mouse cortical neurons were examined. When ARF6T27N and ARF6TriM

were overexpressed in neurons, elongation and branching of neurites were stimulated, while they were inhibited by overexpression of wild type ARF6 (Fig. 3A-C), consistent with the reports with hippocampal neurons [10, 11]. The number of neurites per neuron was slightly increased, if any, by overexpression of ARF6T27N, but not affected by ARF6TriM (Fig. 3D). Similarly, knockdown of JIP3 enhanced both neurite elongation and branching, although the number of neurites was not affected (Fig. 3E-H). These effects by the knockdown of JIP3 were blocked by the cotransfection of the siRNA-resistant plasmid for JIP3 (Fig. 3E-H). Knockdown of overexpressed and endogenous JIP3 by siRNA were confirmed with HEK293T cells and cultured neurons, respectively (Supplementary Fig. 2 and 3), and the recovery of the JIP3 expression by the siRNA-resistant plasmid for JIP3 was confirmed with HEK293T cells (Supplementary Fig. 2). These results provide evidence that the ARF6/JIP3 signaling negatively regulates elongation and branching of neurites.

3.4. ARF6/JIP3 signaling regulates elongation of axons and dendrites and branching of axons.

Previous report has suggested that ARF6 regulates axonal and dendritic formation through distinct downstream cascades [11]. Therefore, we examined which of axonal or dendritic morphogenesis is regulated by ARF6/JIP3 signaling. Axons and dendrites were distinguished by their morphological characteristics (Fig. 4A). Quantification of their morphologies (Fig. 4B and C) was conducted with cortical neurons immunostained with anti-Tau1 and MAP2 antibodies for axons and dendrites, respectively (data not shown). When ARF6T27N and ARF6TriM were overexpressed in neurons, elongation of axons and dendrites and branching of axons were stimulated (Fig. 4A-D). ARF6T27N also stimulated dendrite branching, but ARF6TriM failed to do so (Fig. 4E). These results indicate that ARF6 negatively regulates not only elongation of axons and dendrites but also axon branching through the interaction with JIP3: it regulates dendrite branching through a distinct downstream signaling pathway(s).

Discussion

In the present study, we identified JIP3 as a novel partner protein of ARF6, and demonstrated that ARF6/JIP3 signaling negatively regulates elongation of axons and dendrites. JIP3 functions as a scaffold protein for JNK cascade molecules to efficiently activate JNKs [9, 12]. The activation of JNK has been implicated in the elongation and branching of neurites by phosphorylating various cytoskeleton-associated proteins [13-15]. These observations raised a possibility that ARF6 controls neurite morphology by regulating JNK activity through the interaction with JIP3. However, this point remains to be clarified.

Recently, Montagnac et al. independently identified JIP3 and JLP as partner proteins of ARF6, and demonstrated that JLP functions as an adaptor between the anterograde motor protein Kinesin-1 and the endosomal cargo [16]. They also showed that the active form of ARF6 competes with Kinesin-1 for binding to JLP (and JIP3), thereby moving away the JLP-associated cargos from Kinesin-1 complex to the dynein-dynactin retrograde motor complex, which in turn transfer the cargos back to the cell body. Hernández-Deviez et al. have reported that the inactive form of ARF6 promotes redistribution of endosomal proteins to the cell surface in rat hippocampal neurons, which results in enhancement of neurite growth and branching [17]. These reports, together with our results, indicate that ARF6 moves away the JIP3-associated endosomal cargos, which contain membrane-constituting materials essential for neurite elongation, from tips of neurites to the cell body, thereby negatively regulating not only elongation of axons and dendrites but also axon branching.

Although we demonstrated that JIP3 is crucial for the regulation of neurite morphogenesis as a downstream effector of ARF6, it is also plausible that another downstream molecule(s) of ARF6 is involved in the neurite morphogenesis. A candidate for such a molecule is PIP5Kα, since ARF6 has been reported to inhibit the neurite morphogenesis by interacting with PIP5Kα [11]. It would be of interest to know how these different partner proteins cooperate to control neuronal morphology at the downstream of ARF6.

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Figure legends

Fig. 1. Identification of JIP3 and JLP as novel target molecules of ARF6. (A) Detection of ARF6-binding proteins in mouse brain cytosol. Proteins bound to ARF6Q67L- and ARF6T27N-resins were detected by SDS-PAGE and following SYPRO Ruby staining. (B) Schematic representation of domain structures of JIP family members. JB, JNK-binding domain; SH3, Src-homology domain 3; PTB, phosphotyrosine-binding domain; CC, coiled-coil domain; LZ, leucine zipper domain. (C) Detection of JIP3 and JLP in p170 proteins bound to the ARF6Q67L. Protein samples obtained in A were immunoblotted with anti-JIP3 and -JLP antibodies. (D) Specific interaction of JIP3 and JLP with the active form of ARF6. JIP family members and ARF6Q67L or ARF6T27N were coexpressed in HEK293T cells, and their interaction was detected by immunoprecipitation. (E) Specificity of ARF isoforms for the interaction with JIP3 and JLP. Binding of JIP3 and JLP to the active mutants of ARF1, ARF5 and ARF6 were detected as described in D.

Fig. 2. Identification of critical amino acid residues in ARF6 for the interaction with JIP3/JLP. (A) Schematic representation of ARF6Q67L mutants employed in this study. Asterisk denotes the position of Q67L mutation on ARF6 or Q71L mutation on ARF5. (B) Interaction of ARF6 mutants with JIP3 and JLP, which were analyzed as described in Fig. 1D. (C) Determination of critical amino acid residues in ARF6 for the interaction with JIP3/JLP. Interaction of point-mutated ARF6Q67Ls with JIP3/JLP was analyzed as described in Fig. 1D. (D) Complete loss of interaction for ARF6Q67L with JIP3/JLP by triple mutations in ARF6Q67L. Interaction of ARF6Q67LTriM and ARF6Q67L with JIP3/JLP was analyzed as described in Fig. 1D. (E) Ability of ARF6Q67LTriM to interact with PIP5Kγ661. Interaction of ARF6Q67LTriM and ARF6Q67L with PIP5Kγ661 was determined as described in Fig. 1D.

Fig. 3. ARF6/JIP3 regulates neurite morphogenesis of cortical neurons. Mouse cortical neurons

were cotransfected with GFP and HA-tagged wild type or mutants of ARF6 (A), or JIP3 siRNA with or without siRNA-resistant JIP3 (E), and stained with the anti-GFP antibody to observe the morphology under the confocal fluorescent microscope (A and E). Knockdown of endogenous JIP3 and the recovery of the JIP3 expression by the JIP3 siRNA-resistant plasmid were confirmed with cultured cortical neurons and HEK293T cells (Supplementary Fig. 2 and 3). Neurite branch points per neurite (B and F), total neurite length (C and G), and number of neurites bared from a cell body (D and H) of transfected neurons were analyzed. Scale bars, 20 μm. Data are the means ± S.E. of at least three independent experiments. **P* < 0.05; ***P* < 0.01

Fig. 4. Regulation of axonal and dendritic morphology by ARF6/JIP3. Mouse cortical neurons were cotransfected with GFP and HA-tagged wild type or mutants of ARF6, and immunostained with the anti-GFP antibody. Morphology of the neurons was then observed under fluorescent microscope (A). Total axonal (B) and dendritic length (C), number of axonal tips (D), and dendritic tips per dendrite (E) of the transfected neurons were analyzed. Axons and dendrites were identified by their morphological characteristics (A). For quantifications of the data (B-E), neurons were immunostained with anti-Tau1 and -MAP2 antibodies. Scale bars, 80 μm. Data are the means ± S.E. of at least three independent experiments. **P* < 0.05

Supplementary Materials and Methods

Antibodies. Primary antibodies used were M2 (Sigma) for FLAG, 16B12 (Covance) for hemagglutinin (HA), 26H1 (Cell Signaling Technology) for glutathione *S*-transferase (GST), 598 (MBL) and AB16901 (MILLIPORE) for green fluorescent protein (GFP), F-6 and H-140 (Santa Cruz Biotechnology) for JIP3, ab12331 (Abcam) for JLP, MAB3420 (Chemicon International) for Tau1, and HM-2 (Abcam) for MAP2.

Plasmids. pEGFP-C2, pcDNA3, pGEX-6P-2 and pFLAG-MAC were obtained from Clontech, Invitrogen, GE Healthcare and Sigma, respectively. The pCAGGS vector was kindly provided by Dr. J. Miyazaki, Osaka University [1]. Mouse JIP1 cDNA was generous gift of Dr. R. Kobayashi (Kagawa University Faculty of Medicine, Japan). cDNAs encoding JIP2, JIP3, and JLP were amplified by RT-PCR from mouse brain total RNA, and inserted into the mammalian expression vector pEGFP-C2. DNA fragments encoding deletion mutants of JIP3 and JLP were constructed by PCR-based method using full length of JIP3 and JLP cDNAs as templates, and inserted into either pEGFP-C2 or the bacterial expression vector pGEX-6P-2. cDNAs encoding mouse ARFs and their constitutively active and inactive mutants were generous gifts of Dr. K. Nakayama (Kyoto University, Japan), and were used to construct pcDNA3-ARF-HA or pcDNA3-ARF-FLAG and pGEX-6P-2-ARF6-FLAG. cDNAs encoding ARF6(Δ1-11)Q67L and ARF(N6-C5)Q67L were constructed by PCR-based method. Expression plasmids for ARF6Q67L were prepared by using the Site-directed Quick Change Mutagenesis Kit (Stratagene). pcDNA3-FLAG-PIP5Kγ661 was previously described [2]. The sequences of all constructs were confirmed.

Preparation of recombinant proteins. GST-ARF6Q67L-FLAG and GST-ARF6T27N-FLAG proteins were expressed in *E. coli* BL21 and purified by glutathione-sepharose resin.

Analysis of interaction in the cell. HEK293T cells were cotransfected with expression vectors by using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were lysed in the lysis buffer consisting of 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, 1% TritonX-100, and protease inhibitor cocktail (Roche). FLAG-tagged proteins were immunoprecipitated with the anti-FLAG M2 resin, and the precipitated proteins were analyzed by immunoblotting.

In vitro binding experiments. Bacterially expressed FLAG-ARF6 was immobilized on the anti-FLAG M2 resin. The resin was incubated with either of the purified GST-LZ or GST-LZII peptide in the buffer consisting of 20 mM Tris-HCl, pH7.5, 1 mM EGTA, 2 mM $MgCl_2$, 1% Triton X-100, and 1 mM PMSF. After the resin was washed, bound GST-fusion peptides were analyzed by immunoblotting using the anti-GST antibody.

Primary culture of mouse cortical neurons, transfection, and confocal immunofluorescence microscopy. Cerebral cortices dissected from E17 ICR mice were treated with 10 U/ml papain and 100 U/ml DNase in Dulbecco's modified Eagle's medium at 37°C for 20 min. The dissociated cortical neurons were cultured on poly-L-lysine-coated coverslips at 1.3-2.6 x 10^4 cells/cm^2 in Neurobasal media (GIBCO) supplemented with B-27 (GIBCO), 0.5 mM L-glutamine, and penicillin/streptomycin. For the experiment to examine neurite morphology, neurons cultured for 4 h were cotransfected with pCAGGS-EGFP and pcDNA3-ARF6-HA using the calcium phosphate method and further cultured for 48 h. For the experiment to distinguish the axon and dendrite, neurons cultured for 5 d were cotransfected with pCAGGS-EGFP and pcDNA3-ARF6-HA with Lipofectamine 2000 and further cultured for 48 h. For the knockdown experiment, neurons cultured for 4 h were cotransfected with pCAGGS-EGFP and either of 2 different JIP3 siRNAs with or without the plasmid encoding siRNA-resistant JIP3 with Lipofectamine 2000 and cultured for another 48 h. Neurons were immunostained, and confocal

immunofluorescent images were acquired as previously described [3]. For statistical analysis, more than 20 transfected neurons were analyzed in each experiment, and statistical significance was evaluated using double-tailed Student's *t* test.

Supplementary Results

The leucine zipper region of JIP3 and JLP is the binding site for ARF6

To identify the binding region of JIP3 and JLP for ARF6, a series of deletion mutants of JLP were prepared (Supplementary Fig. 1A), and their interaction with ARF6 was investigated by coimmunoprecipitation of these mutants from HEK293T cells with ARF6Q67L or ARF6T27L. The N-terminal, but not C-terminal, fragment of JLP interacted with ARF6Q67L (Supplementary Fig. 1B). Furthermore, the N3 fragment, which corresponds to the leucine zipper (LZ) II region, bound to ARF6Q67L (Supplementary Fig. 1C). Since JIP3 has only one LZ region with high homology (75%) to the LZII region of JLP (Supplementary Fig 1D), it is plausible that JIP3 binds to the active form of ARF6 via the LZ region. As expected, ARF6Q67L, but not ARF6T27N, interacted with the LZ region of JIP3, as well as the LZII of JLP (Supplementary Fig. 1E). Finally, we confirmed the direct interaction of JIP3 and JLP with ARF6 in an *in vitro* reconstitution system using the purified recombinant proteins (Supplementary Fig. 1F).

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Supplementary Fig. 1. The leucine zipper region of JIP3 and JLP is the binding site for ARF6. (A) Schematic representation of various deletion mutants of JLP employed in this study. The black boxes represent leucine zippers, LZI and LZII. (B and C) Binding of various deletion mutants of JLP to the active form of ARF6. Deletion mutants of JLP and ARF6Q67L or ARF6T27L were coexpressed in HEK293T cells, and their interaction was examined by the immunoprecipitation, followed by immunoblotting. (D) Comparison of amino acid sequences of the LZ region of JIP3 and LZII region of JLP. Underlined amino acid residues indicate the leucine zipper motifs. (E) Interaction of the LZ region of JIP3 and the LZII region of JLP with the active form of ARF6. Interaction was detected as described in B. (F) Direct interaction of the active form of ARF6 with the LZ region of JIP3 and the LZII region of JLP. Recombinant peptides expressed in and purified from *E. coli* were incubated with the ARF6-resins, and interaction was assessed by immunoblotting.

Supplementary Fig. 2. Knockdown of overexpressed JIP3 by siRNA. Plasmid for HA-tagged JIP3 or siRNA-resistant JIP3 was cotransfected with the control siRNA or either of 2 different siRNAs for JIP3 into HEK293T cells. The cells were harvested 24 h after transfection, and subjected to immunoblotting probed with antibody against HA.

Supplementary Fig. 3. Knockdown of endogenous JIP3 in mouse cortical neurons by siRNA. (A) Mouse cortical neurons were cotransfected with pCAGGS-EGFP and either of 2 different siRNAs for JIP3, immunostained with the anti-GFP and -JIP3 antibodies, and observed under the confocal fluorescent microscope. (B) The ratio of the number of JIP3-positive neurons to that of GFP-positive neurons was calculated from the data of A. Arrows indicate cell soma. Scale bars, 20 μm. Data shown in B are the means ± S.E. of at least three independent experiments.