R-Ras Controls Axon Specification Upstream of Glycogen Synthase Kinase-3β through Integrin-linked Kinase*

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The initial event in establishing a polarized neuron is the specification of a single axon. Spatially regulated glycogen synthase kinase-3β (GSK-3β) activity is critical for specifying axon-dendrite fate; however, the upstream signaling of GSK-3β in the determination of neuronal polarity still remains obscure. Here, we found that, in cultured hippocampal neurons, the small GTPase R-Ras selectively localized in a single neurite of stage 2 neurons and that its activity increased after plating and peaked between stages 2 and 3. Ectopic expression of R-Ras induced global inactivation of GSK-3β and formation of multiple axons, whereas knockdown of endogenous R-Ras by RNA interference blocked GSK-3β inactivation and axon formation. GSK-3β inactivation and axon formation by R-Ras required integrin-linked kinase (ILK), and subcellular localization of ILK was strictly regulated by R-Ras-mediated phosphatidylinositol 3-kinase activity. In addition, membrane targeting of ILK was sufficient to inactivate GSK-3β and to form multiple axons. Our study demonstrates a novel role of R-Ras and ILK upstream of GSK-3β in the regulation of neuronal polarity.

The establishment of the axon-dendrite polarity is important for unidirectional signal flow in the neuronal network and is the essential step in the differentiation of neurons. Typically, a neuron has a single long axon and multiple dendrites, and the initial event in establishing a polarized neuron is the specification of a single axon (1). A well established model system for studying neuronal polarity is the hippocampal pyramidal neurons (2, 3). Cultured hippocampal neurons first extend several undifferentiated neurites (at stages 1–2). At stages 2–3, one of the neurites begins to elongate rapidly to become the axon, whereas the remaining minor neurites develop into dendrites at stage 4 (4). One way for the symmetry breaking at stages 2–3 is the polarized distribution of the molecules affecting cytoskeleton dynamics between the axon and other neurites (5).

Glycogen synthase kinase-3β (GSK-3β)2 has been recently implicated in both the establishment and maintenance of neuronal polarity (6–9). GSK-3β is a multifunctional Ser/Thr kinase found in all eukaryotes, and in addition to the regulation of neuronal polarity, it also functions as a key regulator of a wide range of polarization processes such as polarization of migrating fibroblasts (10, 11). GSK-3β possesses a high basal kinase activity, and its signaling pathways act mainly by inhibition of its kinase activity by phosphorylation at Ser9. In neuronal cells, GSK-3β is locally phosphorylated in axons, and thus, the kinase activity of GSK-3β is higher in dendrites than in axons, which is critical for axon-dendrite polarity (8, 9). It has been shown that downstream targets of GSK-3β in axons are CRMP-2 (collapsin response mediator protein-2) and APC (adenomatous polyposis coli), both of which are microtubule-binding proteins that promote microtubule polymerization and stabilization (7, 9, 12). Phosphorylation of CRMP-2 and APC by GSK-3β suppresses their abilities to bind to microtubules. Thus, inactivation of GSK-3β leads to the promotion of microtubule polymerization and stabilization, thereby enhancing axon formation and elongation (13, 14). It is well known that GSK-3β is inactivated by a phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway in both neurons and non-neuronal cells (15), and local activation of PI3K is important for neuronal polarization (6, 8). However, upstream signaling that locally activates PI3K in the distal axon still remains obscure.

Here, we show that R-Ras is essential for axon specification and neuronal polarization in hippocampal neurons. R-Ras selectively localized in a single neurite of stage 2 neurons, and its activity increased after plating and peaked between stages 2 and 3. Spatial localization and activation of R-Ras during axon specification play important roles in the regulation of neuronal polarity in that overexpression of R-Ras led to multiple axon formation, whereas inactivation or knockdown of R-Ras by R-Ras-specific GTPase-activating protein (GAP) or RNA interference completely suppressed axon formation. GSK-3β inactivation and axon formation by R-Ras required integrin-linked kinase (ILK), and subcellular localization of ILK was strictly regulated by R-Ras-mediated PI3K activity. In addition, membrane targeting of ILK was sufficient to inactivate GSK-3β and to form multiple axons. We present here the evidence that R-Ras acts upstream of PI3K, ILK, and Akt to regulate GSK-3β activity in specifying axon-dendrite polarity.
R-Ras Controls Axon Specification

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Human R-Ras and R-Ras(Q87L), rat R-Ras, the glutathione S-transferase (GST)-fused Ras-binding domain of c-Raf-1 (RBD; amino acids 53–130), and the N-terminally hemagglutinin-tagged myristoylated form of R-Ras-GAP (amino acids 291–614) have been described previously (16). An effector mutant of human R-Ras, R-Ras(D64A), was generated by PCR-mediated mutagenesis. Rat ILK was obtained by reverse transcription-PCR from rat brain. The ILK pleckstrin homology (PH) domain (ILK-PH; amino acids 1–290) and kinase-dead ILK (ILK-KD; E359K) were generated by PCR-mediated mutagenesis. ILK was fused to Src myristoylated signal (MGSSSKS) to obtain the plasma membrane-targeting form. Each ILK construct with a Myc tag at the N terminus was subcloned into pcDNA3 (Invitrogen). The small interfering RNAs (siRNAs) for R-Ras were designed to target 19 nucleotides at nucleotides 359–377 (5’-gcaagtctctcactag-3’), nucleotides 426–444 (5’-caagcgacatgtagc-3’), and nucleotides 589–607 (5’-gacctcctctagccac-3’) of the rat rras transcript; the siRNAs for ILK were designed to target 19 nucleotides at nucleotides 660–678 (5’-gtgctgaagttgtagcag-3’), nucleotides 880–898 (5’-gtctgaatgttggtcag-3’), and nucleotides 1107–1125 (5’-cagctgctcagacagt-3’); and they were expressed using an siRNA expression vector (Ambion, Inc.) as described previously (16).

Materials—The pharmacological PI3K inhibitor LY 294002 and the GSK-3 inhibitor SB-216763 were purchased from Calbiochem and BIOMOL International, respectively, and they were dissolved in Me2SO. Laminin and poly-D-lysine were purchased from Sigma and used at 10 and 50 µg/ml, respectively. We used the following antibodies: a mouse monoclonal antibody against Myc (Upstate Biotechnology); mouse monoclonal antibodies against vesicular stomatitis virus, α-tubulin, and microtubule-associated protein (MAP)-2 (Sigma); a mouse monoclonal antibody against Tau-1 and rabbit polyclonal antibodies against GSK-3β and synapsin I (Chemicon); a rabbit monoclonal antibody against Akt phosphorylated at Ser 473 and GSK-3β phosphorylated at Ser 9 (Cell Signaling Technology); a rabbit polyclonal antibody against GSK-3β phosphorylated at Tyr216 (BD Transduction Laboratories); a rabbit polyclonal antibody against R-Ras (Santa Cruz Biotechnology, Inc.); a rabbit polyclonal antibody against ILK (Stressgen Bioreagents); a rabbit polyclonal antibody against GAP-43 (growth-associated protein of 43 kDa; Novus Biologicals); a rat monoclonal antibody against hemagglutinin (Roche Applied Science); and secondary antibodies conjugated to Alexa 594 (Molecular Probes) and to horseradish peroxidase (Dako).

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO2, at 37°C. Transient transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and the cultures were maintained in Dulbecco’s modified Eagle’s medium with 0.5% fetal bovine serum after transfection for 2.5 days. We added LY 294002 directly to the culture medium after transfection to a final concentration of 20 µM and changed it at every 12 h to reduce the basal levels of PI3K activity. Primary hippocampal neurons were isolated from day 17.5 rat embryos as described previously (16) and plated onto poly-D-lysine-coated coverslips (circular, 13 mm in diameter) or plastic dishes (60 mm in diameter) at a density of 3.5 × 104 cells/cm². To transfected neurons, the medium was changed to Opti-MEM I (Invitrogen) supplemented with 2% B-27 (Invitrogen), and the neurons were transfected using Lipofectamine 2000. LY 294002 (100 µM) was directly added to the neuronal culture medium after transfection, and SB-216763 (20 µM) was added 3 h after plating. Neurons were fixed with 4% paraformaldehyde and 15% sucrose in phosphate-buffered saline and processed for immunohistochemistry. The cotransfection efficiency of the each plasmid and green fluorescent protein (GFP) was >90% as revealed by immunostaining (data not shown).

Measurement of Neurite Length and Analysis of Neuronal Polarity—Neuronal morphology was analyzed from digital images acquired at magnifications of ×40 (neurons at 2.5 days in vitro (DIV)) or ×20 (neurons at 5 and 6 DIV) using a Leica DC350F digital camera system equipped with a Nikon Eclipse E800 microscope and Image-Pro Plus image analysis software (Media Cybernetics). The development of neuronal polarity was assessed by the combination of neurite length and Tau-1 staining. Neurites that were longer than two cells in diameter and that also showed Tau-1 immunoreactivity were counted as axons, and other neurites that were longer than one cell in diameter were counted as minor neurites. More than 20 cells were examined in each experiment, and the results are shown as the means ± S.E. of three independent experiments.

Separation of Membrane and Cytosolic Fractions—Transiently transfected COS-7 cells were suspended in homogenization buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, and 5% glycerol). The cell suspensions were then lysed by homogenization with a Potter-Elvehjem homogenizer, and the homogenates were centrifuged at 9300 × g for 5 min to remove the unbroken cells and nuclear fractions. The supernatants were further fractionated at 100,000 × g for 1 h. The particulate pellet was resuspended in the same volume as the cytosolic fraction, and equal volumes of each were analyzed by SDS-PAGE and immunoblotting.

Functional Characterization of Axons—Neurons were incubated for 3 min in Ringer’s solution (150 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 5 mM glucose, and 10 mM HEPES-NaOH (pH 7.5)) containing 45 mM KCl and 10 mM FM 4-64 dye (Molecular Probes) (8). The cells were then washed three times with Ringer’s solution, incubated at 37°C for 15 min, and fixed with 4% paraformaldehyde for 15 min at room temperature. FM 4-64 fluorescent images were collected using a Leica DC350F digital camera system equipped with a Nikon Eclipse E800 microscope.

Measurement of R-Ras Activity—Measurement of R-Ras activity in cells was performed as described previously (16). At the indicated times after plating, hippocampal neurons (1 × 10⁶ cells) were lysed directly on dishes with ice-cold cell lysis buffer (25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10% glycerol, 10 mM MgCl2,
1 mM EDTA, 25 mM NaF, 1 mM orthovanadate, 1 mM dithiothreitol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin) containing 75 mg of GST-RBD.

Nucleofection—We used an electroporation-based gene transfer method that shows low toxicity but high efficiency and that relies on the direct introduction of the DNA into the nucleus, “nucleofection” (17). Hippocampal neurons from day 17.5 rat embryos (1.5 × 10^6 cells) were suspended in 100 ml of Nucleofector solution (Amaxa Biosystems), mixed with total 3 mg of DNA (GFP/siRNA = 1:2), and nucleofected (program O-003, Nucleofector) prior to plating. At 2.5 DIV, cells were directly lysed on dishes with 1× Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting. Nucleofection efficiency was >75% as verified by counting GFP-positive cells (data not shown).

RESULTS

Spatial Accumulation and Temporal Activation of R-Ras during Axon Specification—To investigate a potential role of R-Ras in neuronal polarity, we used hippocampal neurons from day 17.5 rat embryos. To determine the distribution of R-Ras, hippocampal neurons seeded on poly-γ-lysine-coated coverslips were double-stained with dye-labeled phalloidin and anti-R-Ras antibody. The relative activity of R-Ras determined by the amount of R-Ras bound to GST-RBD normalized to the amount of R-Ras in cell lysates was analyzed by NIH Image software. Results are the means ± S.E. of triplicate experiments. Stimulation of axon formation by laminin. Hippocampal neurons grown for 18 h on laminin-coated coverslips were double-stained with dye-labeled phalloidin (upper panel) and Tau-1 (lower panel). Scale bar = 25 μm.

FIGURE 1. Activation and polarized distribution of R-Ras during specification of axon-dendrite polarity. A and B, distribution of R-Ras in stage 2 (18 h) and early stage 3 (36 h) neurons, respectively, grown on poly-γ-lysine-coated coverslips. A, hippocampal neurons were double-stained with anti-R-Ras antibody (upper panel) and a dye-labeled probe for F-actin (phalloidin) (lower panel). Arrows indicate the tip of the specific neurite that shows accumulated R-Ras. Scale bar = 25 μm. B, hippocampal neurons were double-labeled with anti-R-Ras antibody (upper panels) and the axonal marker Tau-1 or the dendritic marker MAP2 (lower panels). Arrows indicate the tip of the axon. Scale bar = 25 μm. C and D, activity of R-Ras in neurons at the indicated times after plating on dishes coated with poly-γ-lysine or laminin, respectively. GTP-bound R-Ras isolated with GST-RBD was detected with anti-R-Ras antibody. The relative activity of R-Ras determined by the amount of R-Ras bound to GST-RBD normalized to the amount of R-Ras in cell lysates was analyzed by NIH Image software. Results are the means ± S.E. of triplicate experiments. E, stimulation of axon formation by laminin. Hippocampal neurons grown for 18 h on laminin-coated coverslips were double-stained with dye-labeled phalloidin (upper panel) and Tau-1 (lower panel). Scale bar = 25 μm.
R-Ras Controls Axon Specification

A

GFP
GFP-R-RasWT
GFP-R-RasQL
GFP + Myr-R-RasGAP

Tau-1
Tau-1
Tau-1
Tau-1

No. axons
No. minor neurites
No. total neurites

GFP
R-RasWT
R-RasQL
R-RasGAP
GFP
R-RasWT
R-RasQL
R-RasGAP
GFP
R-RasWT
R-RasQL
R-RasGAP

B

GFP
GFP-R-RasWT

synapsin I
synapsin I

C

GFP
GFP-R-RasWT

GAP-43
GAP-43

D

GFP
GFP-R-RasWT

GFP
FM 4-64
GFP
FM 4-64

Neurite number

FM 4-64 uptake
No FM 4-64 uptake
R-Ras Controls Axon Specification

Overexpression of R-Ras Induces Multiple Axons—To examine whether ectopic expression of R-Ras affects neuronal polarity, hippocampal neurons were transfected with expression vectors for enhanced GFP (EGFP)-tagged wild-type (WT) or constitutively active (Q87L) R-Ras before polarization (12 h after plating) and then analyzed at 2.5 DIV (stage 3). Control hippocampal neurons (transfected with a vector for EGFP) formed normal axon-dendrite polarity, with most of them having a single axon and multiple dendrites at 2.5 DIV (0.97 ± 0.02 axons/cell and 3.8 ± 0.35 minor neurites/cell). On the other hand, expression of R-Ras-WT or R-Ras(Q87L) led to multiple axon formation (R-Ras-WT, 3.1 ± 0.20 axons/cell and 1.2 ± 0.18 minor neurites/cell; and R-Ras(Q87L), 3.5 ± 0.07 axons/cell and 0.93 ±

suggesting that spatial concentration of R-Ras precedes axon formation. In stage 3 polarized neurons, the axonal marker Tau-1 specifically recognized the axon; the dendritic marker MAP2 recognized all dendrites; and R-Ras was selectively restricted to a single Tau-1-positive neurite (arrows) and was absent from the other MAP2-positive neurites (Fig. 1B). We next measured the activity of endogenous R-Ras in cultured hippocampal neurons using a pull-down assay with the GST-RBD fusion protein, which selectively isolates active R-Ras (16, 18). Under our culture conditions, most hippocampal neurons were non-polarized at 18 h after plating (stage 2) and then became polarized by 36 h after plating (early stage 3) (Fig. 1, A and B). The activity of R-Ras increased after plating and peaked between stages 2 and 3 (Fig. 1C), when neuronal polarization occurs (19). In addition, when hippocampal neurons were seeded on the extracellular matrix ligand laminin, both R-Ras activity and neuronal polarization were stimulated, and neurons became polarized by 18 h after plating (early stage 3) (Fig. 1, D and E). These data suggest that spatial accumulation and temporal activation of R-Ras occur during axon specification.

R-Ras Controls Axon Specification

FIGURE 3. Essential role of R-Ras in axon specification. A, knockdown of endogenous R-Ras expression in hippocampal neurons. Cells were cotransfected with GFP and the R-Ras-specific siRNAs (siRNA-359, siRNA-426, or siRNA-589) at 0.5 DIV and stained with an antibody against R-Ras at 2.5 DIV (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows and arrowheads indicate transfected and untransfected cells, respectively. B, effects of R-Ras-specific siRNAs on exogenously expressed R-Ras. Lysates from 293T cells cotransfected with rat R-Ras and R-Ras-specific siRNAs were immunoblotted with an antibody against R-Ras or α-tubulin. C, effects of the R-Ras-specific siRNAs on neuronal polarity. Cells coexpressing GFP and the siRNA were immunostained with Tau-1 (lower panels), and neuronal polarity was analyzed. The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows indicate the transfected neurons, and arrowheads indicate axons of untransfected neurons. The numbers of axons, minor neurites, and total neurites are shown. Results are the means ± S.E. of triplicate experiments in which >20 cells were counted. Scale bar = 25 μm.

FIGURE 2. Overexpression of R-Ras induces multiple functional axons. A–C, effect of R-Ras expression on axon formation. A, neurons transfected with the indicated expression plasmids 0.5 DIV after plating were analyzed at 2.5 DIV by staining with Tau-1 (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows indicate the transfected neurons, and arrowheads indicate the untransfected neurons. The numbers of axons, minor neurites, and total neurites are shown. R-RasQL, R-Ras(Q87L). Scale bar = 25 μm. B and C, neurons transfected with GFP or GFP-R-Ras-WT at 0.5 DIV were stained at 2.5 DIV with the axonal markers synapsin I and GAP-43, respectively (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows indicate the transfected neurons, and arrowheads indicate the untransfected neurons. Scale bars = 30 μm. D, functional characterization of multiple axons induced by overexpressed R-Ras. Neurons transfected with GFP or GFP-R-Ras-WT at 0.5 DIV were loaded at 2.5 DIV with FM 4-64 and 45 mM K+ for 3 min. Boxed regions in the upper panels are shown at higher magnification in the lower panels. The numbers of FM 4-64 dye uptake-positive and -negative neurites/cell were counted. Results are the means ± S.E. of triplicate experiments in which at least 20 cells were counted. Scale bars = 50 μm.
0.066 minor neurites/cell) (Fig. 2A). In addition, the multiple axons induced by overexpressed R-Ras were also positive for other axonal markers, synapsin I and GAP-43 (Fig. 2, B and C) (8, 20, 21).

Although axons and minor neurites are usually distinguished by expression of molecular markers, we further examined whether the multiple axonal marker-positive neurites induced by overexpressed R-Ras are functional axons. A basic feature of functional axons is that they have active synaptic recycling, which can be monitored by visualizing FM 4-64 dye uptake (8). FM 4-64 was taken into the single neurite of the control GFP-transfected cells after stimulation by 45 mM K+/H11001, whereas little...
FM 4-64 was taken into the other neurites (1.02 ± 0.06 uptake-positive neurites/cell). On the other hand, multiple FM 4-64 uptake-positive neurites were observed in neurons ectopically expressing R-Ras (2.92 ± 0.044 uptake-positive neurites/cell) (Fig. 2D). These data suggest that overexpression of R-Ras induces multiple functional axons.

**R-Ras Is Essential for Axon Specification**—To investigate the involvement of endogenous R-Ras in the formation of normal axon-dendrite polarity, hippocampal neurons were transfected with R-Ras-specific siRNA expression vectors at 12 h after plating and then fixed and immunostained with the axonal marker Tau-1 at 2.5 DIV. An EGFP expression vector was cotransfected with R-Ras siRNA constructs to mark the transfected neurons. We prepared three siRNA constructs for R-Ras designed to target 19 nucleotides at nucleotides 359–377, 426–444, and 589–607 of the rat transcript (referred to as R-Ras siRNA-359, siRNA-426, and siRNA-589, respectively) and found that two of them (R-Ras siRNA-359 and siRNA-589) effectively reduced the amount of exogenously expressed rat R-Ras (Fig. 3B) or endogenous R-Ras protein in hippocampal neurons (Fig. 3A), whereas R-Ras siRNA-426 did not work. Neurons transfected with GFP alone or cotransfected with GFP and R-Ras siRNA-426 formed normal axon-dendrite polarity, with most of them having a single axon and multiple dendrites at 2.5 DIV (GFP, 0.97 ± 0.017 axons/cell and 3.8 ± 0.25 minor neurites/cell; and R-Ras siRNA-426, 1.0 ± 0.029 axons/cell and 4.3 ± 0.017 minor neurites/cell). By contrast, knockdown of R-Ras by R-Ras siRNA-359 or siRNA-589 led to a complete loss of polarity; the majority of the transfected neurons did not form any axons (R-Ras siRNA-359, 0.27 ± 0.017 axons/cell and 4.4 ± 0.060 minor neurites/cell; and R-Ras siRNA-589, 0.17 ± 0.033 axons/cell and 4.7 ± 0.093 minor neurites/cell) (Fig. 3C). We also confirmed these results by transfection of neurons with the GAP domain of R-RasGAP fused with the myristoylated (Myr) signal of Src (referred to as Myr-R-RasGAP), which exhibits a GAP-specific activity toward R-Ras and inactivates it (22).
R-Ras Controls Axon Specification

A. Control (DMSO) + SB216763 (20 μM)

B. GFP vs. GFP-R-RasWT

C. R-Ras siRNA-359, R-Ras siRNA-426, R-Ras siRNA-589

D. GFP vs. GFP-R-RasWT

E. R-Ras siRNA-359, R-Ras siRNA-426, R-Ras siRNA-589

F. Blot of p-Akt, Total Akt, p-GSK3β (Ser9), p-GSK3β (Tyr216), Total GSK3β, R-Ras, GFP, α-tubulin
Expression of Myr-R-RasGAP in stage 2 hippocampal neurons blocked axon formation (0.23 ± 0.033 axons/cell and 4.3 ± 0.16 minor neurites/cell) (Fig. 2A). These data indicate that R-Ras is required for axon formation and that its polarized localization and activation are essential for normal axon-dendrite polarity.

We also examined whether inhibition of axon formation by knockdown of R-Ras by R-Ras siRNA in primary rat hippocampal neurons could be rescued by overexpression of human R-Ras. Expression of GFP-tagged human R-Ras with R-Ras siRNA-359, which was effective for human R-Ras, was rescued by overexpression of GFP-tagged human R-Ras (R-Ras siRNA-359, 0.38 ± 0.033 axons/cell and 5.2 ± 0.044 minor neurites/cell; and R-Ras siRNA-589, 2.4 ± 0.10 axons/cell and 3.6 ± 0.033 minor neurites/cell) (Fig. 4, A and B). This result further supports the idea that R-Ras is essential for axon specification.

Role of R-Ras in Maintaining Neuronal Polarity—We further examined the maintenance role of R-Ras in axon-dendrite polarity after polarization. Hippocampal neurons were transfected after the establishment of axon-dendrite polarity (4 DIV) and then analyzed at 6 DIV. As shown in Fig. 5, control EGFP-expressing hippocampal neurons maintained normal axon-dendrite polarity, with most of them having a single long axon (1.0 ± 0.058 axons/cell and 6.3 ± 0.22 minor neurites/cell). On the other hand, overexpression of R-Ras WT or R-Ras (Q87L) induced surplus axons (R-Ras WT, 2.0 ± 0.058 axons/cell and 4.3 ± 0.14 minor neurites/cell; and R-Ras (Q87L), 2.1 ± 0.10 axons/cell and 4.6 ± 0.14 minor neurites/cell). By contrast, suppression of R-Ras activity by overexpression of Myr-R-RasGAP caused elimination of an axon (0.33 ± 0.044 axons/cell and 8.3 ± 0.44 minor neurites/cell). These results demonstrate that aberrant R-Ras activity can cancel the pre-existing axon-dendrite polarity, revealing that R-Ras is involved in the maintenance of axon-dendrite polarity after polarization.

R-Ras Functions Upstream of GSK-3β and Akt in the Formation of Neuronal Polarity—Spatially regulated GSK-3β activity in neurons is critical for specifying axon-dendrite fate (8, 9), and global inactivation of GSK-3β by treatment with the GSK-3β-specific inhibitor SB-216763 (20 μM) induced multiple axons (Fig. 6A) the same as R-Ras-induced ones. To examine whether R-Ras functions upstream of GSK-3β in the determination of axon-dendrite polarity, hippocampal neurons transfected with GFP-tagged R-Ras or R-Ras siRNAs were stained with an antibody against GSK-3β phosphorylated at Ser9 (p-GSK-3β). The majority of hippocampal neurons transfected with control EGFP had a single p-GSK-3β-positive neurite (1.13 ± 0.067 neurites/cell) at 2.5 DIV (stage 3). By contrast, overexpression of R-Ras induced multiple p-GSK-3β-positive neurites (3.17 ± 0.088 neurites/cell) (Fig. 6B). On the other hand, knockdown of R-Ras by R-Ras siRNA-359 or siRNA-589 led to loss of p-GSK-3β-positive neurites (R-Ras siRNA-359, 0.35 ± 0.050 neurites/cell; and R-Ras siRNA-589, 0.27 ± 0.017 neurites/cell), whereas most neurons transfected with R-Ras siRNA-426, which had no effect on R-Ras expression, formed a single p-GSK-3β-positive neurite (1.0 ± 0.0 neurites/cell) (Fig. 6C).

Akt, a kinase that functions downstream of PI3K, phosphorylates Ser473 of GSK-3β and is involved in many cellular events, including the axon-dendrite specification upstream of GSK-3β (8, 10). Akt is activated by phosphorylation at Thr308 and Ser473, and phosphorylated Akt is localized specifically in axons (6, 8). To examine whether R-Ras also mediates Akt phosphorylation in the determination of axon-dendrite polarity, hippocampal neurons transfected with GFP-tagged R-Ras or R-Ras siRNAs were stained with an antibody against Akt phosphorylated at Ser473 (p-Akt). Most hippocampal neurons transfected with control EGFP or R-Ras siRNA-426 had a single p-Akt-positive neurite (GFP, 0.83 ± 0.033 neurites/cell; and R-Ras siRNA-426, 0.83 ± 0.017 neurites/cell) at 2.5 DIV. However, p-Akt was detected in multiple neurites of neurons ectopically expressing R-Ras (3.02 ± 0.073 neurites/cell) (Fig. 6D). On the other hand, the number of neurons with a single p-Akt-positive neurite decreased when they were transfected with R-Ras siRNA-359 or siRNA-589 (R-Ras siRNA-359, 0.25 ± 0.050 neurites/cell; and R-Ras siRNA-589, 0.23 ± 0.017 neurites/cell) (Fig. 6E).

In addition, because knockdown of R-Ras by R-Ras siRNA inhibited axon formation (Fig. 3C), we also confirmed the requirement of endogenous R-Ras protein for phosphorylation of Akt and GSK-3β in hippocampal neurons by immunoblot analysis of whole cell lysates. Dissociated hippocampal neurons were transfected with R-Ras siRNAs prior to plating using nucleofection technology, and cells at 2.5 DIV were analyzed. Neurons nucleofected with the effective R-Ras siRNAs showed decreases in p-GSK-3β and p-Akt (Fig. 6F). This result confirms that the decrease in p-GSK-3β- or p-Akt-positive neurites by knockdown of R-Ras is not due to the lack of axon formation and that R-Ras is required for phosphorylation of Akt and GSK-3β in hippocampal neurons. GSK-3β activity is inhibited by direct phosphorylation at Ser9. On the other hand, GSK-3β also has a tyrosine phosphorylation site, and tyrosine phosphorylation of GSK-3β increases GSK-3β activity (23, 24). Neurons

**FIGURE 6. Regulation of localized phosphorylation of GSK-3β and Akt by R-Ras.** A, effect of GSK-3 inhibition on axon formation. Hippocampal neurons cultured in the presence of 20 μM GSK-3 inhibitor SB-216763 were analyzed by double staining with dye-labeled phalloidin (upper panels) and Tau-1 (lower panels) at 2.5 DIV. The numbers of axons, minor neurites, and total neurites are shown. Results are the means ± S.E. of triplicate experiments in which ≥20 cells were counted. Scale bar = 40 μM. DMSO, dimethyl sulfoxide. B and D, effect of ectopic expression of R-Ras on the distribution of p-GSK-3β and p-Akt, respectively. Hippocampal neurons transfected with GFP or R-Ras-WT at 0.5 DIV were analyzed at 2.5 DIV by staining with p-GSK-3β or p-Akt (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). C and E, effects of the R-Ras-specific siRNAs on the distribution of p-GSK-3β and p-Akt, respectively. Neurons coexpressing GFP and the R-Ras siRNAs were immunostained with p-GSK-3β (C) or p-Akt (E) (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows indicate the tips of R-Ras-positive axons. The numbers of the p-GSK-3β-positive (B and C) and p-Akt-positive (D and E) neurites/cell are shown. Results are the means ± S.E. of triplicate experiments in which ≥20 cells were counted. Scale bars = 25 μM. F, regulation of phosphorylation of Akt and GSK-3β by R-Ras in hippocampal neurons. Dissociated hippocampal neurons were transfected with GFP and R-Ras siRNAs prior to plating using nucleofection technology, and whole cell lysates at 2.5 DIV were analyzed by immunoblotting. The relative levels of p-Akt and p-GSK-3β were normalized to the amount of total Akt and GSK-3β in cell lysates. Results are the means ± S.E. of triplicate experiments.
**R-Ras Controls Axon Specification**

**A**

Time after plating (h) 0 3 12 36 72

| Blot: | p-Akt | Total Akt | p-GSK3β (Ser9) | Total GSK3β | ILK | R-Ras |
|-------|-------|-----------|----------------|--------------|-----|-------|
| Lysate |       |           |                |              |     |       |

![Graph showing relative p-Akt or p-GSK3β expression over time](image)

**B**

**Stage 3 (48 h)**

- ILK
- Tau-1
- Map2

**C**

- ILK siRNA-660
- ILK siRNA-880
- ILK siRNA-1107

**D**

- ILK siRNA

| Blot: | Myc-ILK | α-tubulin |
|-------|---------|-----------|

**E**

- ILK siRNA-660
- ILK siRNA-880
- ILK siRNA-1107

- Tau-1

**Graphs showing:**

- No. axons
- No. minor neurites
- No. total neurites

![Bar charts comparing GFP and ILK siRNA](image)
nucleofected with the effective R-Ras siRNAs showed a slight increase in phosphorylation of GSK-3β at Tyr216 (Fig. 6F).

Spatial Localization of ILK and Its Involvement in Axon Formation—We further studied downstream signaling of R-Ras leading to phosphorylation of Akt and GSK-3β and inducing subsequent axon specification. ILK has been shown to be an upstream kinase of Akt and GSK-3β (25). ILK is expressed in various brain regions, including hippocampal neurons (26).

Endogenous ILK protein in cultured hippocampal neurons was poor just after plating, and it sharply increased during axon formation. Moreover, in the same time course, phosphorylation of both Akt and GSK-3β was increased (Fig. 7A). ILK protein was spatially enriched in a single Tau-1-positive neurite in stage 3 neurons (Fig. 7B, arrows). Thus, we examined whether ILK is required for establishing normal axon-dendrite polarity.

Hippocampal neurons were cotransfected with ILK-specific siRNAs and an EGFP expression vector at 12 h after plating and then fixed and stained with the axonal marker Tau-1 at 2.5 DIV. Three siRNA constructs for ILK designed to target 19 nucleotides at nucleotides 660–678, 880–898, and 1107–1125 of the rat transcript (referred to as ILK siRNA-660, siRNA-880, and siRNA-1107, respectively) were prepared, and two of them (ILK siRNA-660 and siRNA-1107) effectively reduced the amount of exogenously expressed rat ILK (Fig. 7D) or endogenous ILK protein in hippocampal neurons (Fig. 7C), whereas ILK siRNA-880 did not work. Neurons transfected with GFP alone or cotransfected with GFP and ILK siRNA-880 formed normal axon-dendrite polarity at 2.5 DIV (GFP, 0.97 ± 0.017 axons/cell and 3.8 ± 0.025 minor neurites/cell; and ILK siRNA-880, 0.94 ± 0.01 axons/cell and 4.1 ± 0.049 minor neurites/cell). By contrast, knockdown of ILK by ILK siRNA-660 or siRNA-1107 led to a complete loss of neuronal polarity, and the majority of the transfected neurons did not form any axons (ILK siRNA-660, 0.39 ± 0.01 axons/cell and 4.0 ± 0.075 minor neurites/cell; and ILK siRNA-1107, 0.33 ± 0.037 axons/cell and 4.4 ± 0.11 minor neurites/cell) (Fig. 7E). These results suggest that ILK, an upstream kinase of Akt and GSK-3β, is essential for establishing neuronal polarity.

R-Ras Functions Upstream of PI3K and ILK in the Regulation of Neuronal Polarity—ILK contains the PH domain, and the kinase activity of ILK is stimulated by PI3K though the binding of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), the lipid product of PI3K, to the PH domain (25). We then examined whether PI3K and ILK function downstream of R-Ras in the regulation of neuronal polarity.

PI3K is the predominant effector of R-Ras (27, 28), and an effector loop mutant of R-Ras, R-Ras(D64A), fails to bind and activate PI3K (29). Western blot analysis of COS-7 cell lysates probed with phospho-specific antibodies for Akt and GSK-3β indicated that R-Ras(Q87L)-mediated phosphorylation of Akt and GSK-3β was abolished by the D64A mutation or the pharmacological PI3K inhibitor LY 294002 (8, A and B). We further observed that expression of R-Ras(D64A) failed to form multiple axons and that inhibition of PI3K activity by treatment with LY 294002 (100 μM) abolished the multiple axon formation by overexpression of R-Ras (R-Ras-WT, 3.1 ± 0.20 axons/cell and 1.2 ± 0.18 minor neurites/cell; and R-Ras(D64A), 0.18 ± 0.033 axons/cell and 4.9 ± 0.050 minor neurites/cell; LY 294002, 0.28 ± 0.017 axons/cell and 4.4 ± 0.016 minor neurites/cell) (Fig. 8C). These results indicate that PI3K is the downstream effector of R-Ras responsible for axon formation.

We next determined whether ILK functions downstream of R-Ras in axon formation. Two ILK mutant constructs (ILK-KD and ILK-PH) were generated (Fig. 8D). ILK-KD is a kinase-dead mutant that contains a mutation in its kinase domain, and ILK-PH lacks the C-terminal kinase domain; both function as dominant-negative mutants of ILK (25, 26). First, we investigated the effects of these ILK mutants on R-Ras-mediated phosphorylation of Akt and GSK-3β. Elevated phosphorylation of Akt and GSK-3β by R-Ras(Q87L) was not further enhanced by coexpression of ILK-WT with R-Ras(Q87L). However, coexpression of ILK-KD or ILK-PH completely blocked R-Ras(Q87L)-induced phosphorylation of Akt and GSK-3β (Fig. 8, E and F). Next, we transfected hippocampal neurons with ILK mutants and found that the formation of multiple axons induced by overexpression of R-Ras was suppressed by expression of ILK-KD or ILK-PH, whereas it was not affected by coexpression with ILK-WT (ILK-WT, 2.62 ± 0.017 axons/cell and 2.9 ± 0.29 minor neurites/cell; ILK-KD, 0.35 ± 0.029 axons/cell and 5.1 ± 0.018 minor neurites/cell; and ILK-PH, 0.35 ± 0.029 axons/cell and 4.4 ± 0.016 minor neurites/cell) (Fig. 8G). In addition, we examined the localization of endogenous ILK in cultured hippocampal neurons ectopically expressing R-Ras. As shown in Fig. 8H, in control GFP-transfected neurons, ILK accumulated in the single longest neurite (0.96 ± 0.20 ILK-positive neurites/cell). By contrast, expression of R-Ras-WT or R-Ras(Q87L), which induced multiple axon formation (Fig. 2A), caused localization of ILK to multiple long neurites (R-Ras-WT, 3.9 ± 0.41 ILK-positive neurites/cell; and R-Ras(Q87L), 5.6 ± 0.18 ILK-positive neurites/cell). Furthermore, LY 294002 treatment of R-Ras(Q87L)-expressing cells caused complete loss of ILK-positive neurites (0.22 ± 0.13 ILK-positive neurites/cell). These results indicate that ILK is
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**A**

| Treatment | + | DA | + |
|-----------|---|----|---|
| LY294002 (20μM) | - | + | + |

**B**

| Treatment | + | DA | + |
|-----------|---|----|---|
| LY294002 (20μM) | - | + | + |

**C**

- GFP-R-RasDA
- GFP-R-RasWT
- +LY294002

**D**

Integrin-Linked Kinase (ILK)

**E**

| HA-R-RasQL | + | + | + | + | + |
|------------|---|----|---|---|---|
| Myc-ILK    | - | WT | KD | PH |

**F**

| HA-R-RasQL | + | + | + | + | + |
|------------|---|----|---|---|---|
| Myc-ILK    | - | WT | KD | PH |

**G**

- GFP-R-RasWT +ILK-WT
- +ILK-KD
- +ILK-PH

**H**

- GFP
- GFP-R-RasWT
- GFP-R-RasQL
- +LY294002

- ILK
- +LY294002

**Legend**

- ANK = ankyrin repeats
- PH = pleckstrin homology domain
- E = kinase domain

| Blot: | p-Akt | Total Akt |
|-------|-------|-----------|
| HA    |        |           |
| Myc   |        |           |

| Blot: | p-GSK3β | Total GSK3β |
|-------|----------|--------------|
| HA    |          |              |
| Myc   |          |              |
R-Ras Controls Axon Specification

We further examined whether membrane translocation of ILK is associated with the activity of ILK to induce phosphorylation of Akt and GSK-3β. As shown in Fig. 9B, expression of ILK-WT did not induce phosphorylation of Akt and GSK-3β, whereas expression of Myr-ILK, the membrane-targeting form of ILK (Fig. 9A), by itself induced phosphorylation of Akt and GSK-3β (Fig. 9B). Furthermore, overexpression of ILK-WT in hippocampal neurons had little effect on the number of axons/cell ( GFP, 0.97 ± 0.017 axons/cell and 3.8 ± 0.025 minor neurites/cell; and ILK-WT, 1.2 ± 0.05 axons/cell and 3.1 ± 0.029 minor neurites/cell), whereas expression of Myr-ILK induced multiple axon formation (2.2 ± 0.06 axons/cell and 3.2 ± 0.015 minor neurites/cell) (Fig. 9C). These data suggest that ILK is activated by membrane localization through R-Ras-mediated PI3K activity to induce axon formation.

ILK Mediates Phosphorylation of Akt and GSK-3β in Hippocampal Neurons—Finally, we confirmed whether endogenous ILK protein mediates phosphorylation of Akt and GSK-3β in hippocampal neurons. As shown in Fig. 10 (A and B), the majority of hippocampal neurons transfected with ILK siRNA-880, which had no effect on ILK expression (Fig. 7, C and D), had a single p-Akt- and p-GSK-3β-positive neurite at 2.5 DIV (0.95 ± 0.055 p-Akt-positive neurites/cell and 1.0 ± 0.0 p-GSK-3β-positive neurite/cell). By contrast, knockdown of ILK by ILK siRNA-660 or siRNA-1107 led to loss of p-Akt- or p-GSK-3β-positive neurites (ILK siRNA-660, 0.28 ± 0.12 p-Akt-positive neurites/cell and 0.26 ± 0.082 p-GSK-3β-positive neurites/cell; and ILK siRNA-1107, 0.55 ± 0.082 p-Akt-positive neurites/cell and 0.48 ± 0.041 p-GSK-3β-positive neurites/cell). In addition, the requirement of endogenous ILK protein for phosphorylation of Akt and GSK-3β was also confirmed by immunoblotting of whole cell lysates of 2.5 DIV hippocampal neurons nucleofected with ILK

involved in Akt and GSK-3β phosphorylation and in axon formation downstream of R-Ras.

R-Ras Activates ILK by Membrane Translocation though PI3K Activity—We examined whether subcellular localization of ILK is regulated by R-Ras and R-Ras-mediated PI3K activity. We prepared crude membrane and cytosolic fractions from cellular homogenates of COS-7 cells expressing hemagglutinin-tagged R-Ras(Q87L) and Myc-tagged ILK and analyzed their distributions by immunoblotting. As shown in Fig. 9A, R-Ras(Q87L) was observed largely in the membrane fraction, whereas ILK presented mainly in the cytosolic fraction. ILK was translocated to the membrane fraction when it was coexpressed with R-Ras(Q87L), and membrane translocation of ILK by R-Ras(Q87L) was almost completely blocked by addition of LY 294002. These data indicate that R-Ras can regulate subcellular localization of ILK in a PI3K activity-dependent manner.
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A

B

C

FIGURE 10. ILK mediates phosphorylation of Akt and GSK-3β in hippocampal neurons. A and B, effects of the ILK-specific siRNAs on distribution of p-Akt and p-GSK-3β, respectively. Neurons coexpressing GFP and the R-Ras siRNAs were immunostained with p-Akt (A) or p-GSK-3β (B) (lower panels). The transfected neurons were shown by the fluorescence of GFP (upper panels). Arrows indicate the transfected neurons, and arrowheads indicate neurites of untransfected neurons. The numbers of the p-Akt-positive (A) or p-GSK-3β-positive (B) neurites/cell are shown. Results are the means ± S.E. of triplicate experiments.

C

siRNAs (Fig. 10C). Expression of a dominant-negative form of ILK in neuronal cells induces tyrosine phosphorylation and activation of GSK-3β (24), and neurons nucleofected with the effective ILK siRNAs showed a slight increase in phosphorylation of GSK-3β at Tyr216 (Fig. 10C). These results confirm that ILK mediates phosphorylation of Akt and GSK-3β in hippocampal neurons.

DISCUSSION

In summary, our results indicate that R-Ras is essential for axon specification upstream of GSK-3β in hippocampal neurons. R-Ras was selectively localized in a single neurite of stage 2 neurons, and its activity increased after plating and peaked between stages 2 and 3. Spatially regulated GSK-3β activity in neurons is critical for specifying axon-dendrite fate (8, 9). Overexpression of R-Ras led to global inactivation of GSK-3β and formation of multiple axons, whereas knockdown of endogenous R-Ras blocked GSK-3β inactivation and axon formation. We also found that GSK-3β global inactivation and multiple axon formation by overexpressed R-Ras required ILK activity and that R-Ras regulated subcellular localization of ILK in a PI3K activity-dependent manner. Membrane translocation of ILK was associated with the activity of ILK to induce phosphorylation of Akt and GSK-3β. We further demonstrated that endogenous ILK protein was required for inactivation of GSK-3β and formation of normal axon-dendrite polarity in hippocampal neurons.

PI3K is an effector of R-Ras, and activation of PI3K by R-Ras produces PI(3,4,5)P3 (27, 28). PI(3,4,5)P3 binds to the PH domain of ILK and stimulates its kinase activity (25). Activated ILK then activates Akt by phosphorylation of Akt at Thr308 and Ser473, and activated Akt phosphorylates GSK-3β at Ser9 and inactivates its kinase activity (10), resulting in dephosphorylation of CRMP-2 and APC and leading to microtubule polymerization and stabilization, thereby enhancing axon formation and elongation (7–9, 12–14). Inactivation of GSK-3β activity is also required for Par3 polarization, functionally establishing neuronal polarity (12). Thus, R-Ras functions as a key upstream regulator for neuronal polarity.

R-Ras is activated by stimulation of integrin with extracellular matrix ligands and is involved in the regulation of integrin-mediated neurite outgrowth (30, 31). In this study, we demonstrated that both endogenous R-Ras activity and axon formation were stimulated by laminin. In addition, we reported recently that R-Ras activated by the extracellular matrix ligands in turn enhances β1 integrin activity, implicating a positive feedback system during cell-substrate adhesion (32). Integrin stimulation enhances axonal growth and also regulates the development of polarity in cultured hippocampal neurons (33). Localized accumulation of PI3K and PI(3,4,5)P3 in response to extracellular matrix ligands is involved in neuronal polarization and axon formation (34). On the other hand, R-Ras activity is required for nerve growth factor-mediated neurite outgrowth in PC12 cells (26), and nerve growth factor also induces axonal growth and neuronal polarity by localized activation of PI3K at the growth cone (7). It has been recently reported that ectopic expression of a constitutively active form of H-Ras (H-Ras-V12) also induces forma-
tion of multiple axons in a PI3K activity-dependent manner (35). PI3K has emerged as the predominant effector for Ras family proteins, but R-Ras is a more potent activator of PI3K compared with H-Ras (27, 28). Therefore, we suppose that R-Ras might function as a primary regulator of axon specification via local activation of PI3K at the nascent axon downstream adhesion molecules or neurotrophins and mediate neuronal polarization.

Inhibition of GSK-3β activity by ILK has been suggested to be a major mechanism for neurite extension in cultured neurons (7, 26). Indeed, our results also show that endogenous ILK protein mediates phosphorylation of GSK-3β and that spatial regulation of subcellular localization of ILK by R-Ras-mediated PI3K activity is required for localized phosphorylation of GSK-3β and subsequent axon formation. However, it has been recently reported that depletion of ILK in the mouse forebrain using cre/lox technology has no effect on the proliferation of cortical cells or on the phosphorylation levels of Akt and GSK-3β and that ILK does not regulate cortical lamination via these kinases (36). We speculate that the discrepancy in the requirement of ILK for phosphorylation of Akt and GSK-3β may be due to cell-type specificity or that ILK may not be the unique upstream kinase of Akt and GSK-3β.

In conclusion, we have presented evidence that R-Ras plays a key role in axon specification and neuronal polarity regulation as the upstream regulator of GSK-3β. However, it will be important to determine more precisely how and when R-Ras is locally concentrated in one of the immature neurites during axon specification. Overwhelming levels of R-Ras in nascent axons may trigger a stabilized elongation of the neurite to become an axon. In our experiments, in addition to constitutively active R-Ras (R-Ras(Q87L))-transfected cells, R-Ras-WT-transfected cells also formed multiple axons. We speculate that there may be upstream molecules that govern more initial events of axon determination and induce selective R-Ras accumulation and activation and that, because of the limited amount of such molecules, overexpression of R-Ras may cause mislocalization of the protein, thus leading to formation of more than a single axon. In this regard, low levels of endogenous R-Ras in minor neurites seem to be important for suppression of unnecessary axonogenesis, which is critical for neuronal polarity. On the other hand, a positive feedback system exists during axon specification, and extracellular signals from adhesion can specify which neurite will become an axon (37). We suppose that once a small amount of overexpressed R-Ras-WT is activated, the activated R-Ras in turn induces activation of β1 integrins to induce further R-Ras activation, PI3K global activation, and resultant multiple axon formation.

When considering axon specification, emerging evidence indicates common molecules between polarity proteins and proteins implicated in neuronal growth (38). Indeed, in addition to axon specification, R-Ras and its downstream molecules PI3K, ILK, Akt, and GSK-3β are all reported to be involved in axonal growth (7, 26, 30). Further work will need to address the interplay between axon specification and axonal growth. In addition to neuronal polarity regulation, GSK-3β also functions as a key regulator of a wide range of polarization processes (10, 11). Because R-Ras functions in a variety of cells (39), we speculate that R-Ras may be involved in a wide range of polarization processes by regulating GSK-3β activity.

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