Apical Accumulation of Rho in the Neural Plate Is Important for Neural Plate Cell Shape Change and Neural Tube Formation

Nagatoki Kinoshita,* Noriaki Sasai,[†] Kazuyo Misaki,* and Shigenobu Yonemura*

*Electron Microscope Laboratory and [†]Organogenesis and Neurogenesis Group, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan

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Although Rho-GTPases are well-known regulators of cytoskeletal reorganization, their *in vivo* distribution and physiological functions have remained elusive. In this study, we found marked apical accumulation of Rho in developing chick embryos undergoing folding of the neural plate during neural tube formation, with similar accumulation of activated myosin II. The timing of accumulation and biochemical activation of both Rho and myosin II was coincident with the dynamics of neural tube formation. Inhibition of Rho disrupted its apical accumulation and led to defects in neural tube formation, with abnormal morphology of the neural plate. Continuous activation of Rho also altered neural tube formation. These results indicate that correct spatiotemporal regulation of Rho is essential for neural tube morphogenesis. Furthermore, we found that a key morphogenetic signaling pathway, the Wnt/PCP pathway, was implicated in the apical accumulation of Rho and regulation of cell shape in the neural plate, suggesting that this signal may be the spatiotemporal regulator of Rho in neural tube formation.

INTRODUCTION

Rho-GTPases, primarily made up of Rho, Rac, and Cdc42, are well known as regulators of cytoskeletal reorganization and other cellular events (Van Aelst and D'Souza-Schorey, 1997; Jaffe and Hall, 2005). The GTP-bound active form of Rho-GTPases can interact with their effector proteins and thus control various aspects of cell behavior. To date, numerous studies have examined the multiple signal transduction pathways of Rho-GTPases in extensive detail at the biochemical and cellular levels, but little is known yet about how these proteins work *in vivo* in vertebrate morphogenesis. Several lines of Rho-GTPase knockout mice showed early embryonic lethality (Sugihara *et al.*, 1998; Chen *et al.*, 2000), indicating an essential role for such regulators, although the results also suggested that a genetic approach is not ideal for analysis of the functions of these proteins throughout development. In addition, the precise endogenous distribution of Rho-GTPases has not been investigated because of the lack of reliable antibodies for staining. We have been successful recently in developing specific antibodies and suitable staining conditions for visualizing the distribution of Rho (Yonemura *et al.*, 2004). Here we report that Rho accumulates in the apical region of neural plate cells during neural tube formation.

The neural plate—the thickened ectoderm—bends and fuses to form the neural tube through a series of precisely orchestrated morphogenetic movements (see Figure 1A; Smith and Schoenwolf, 1997). Rearrangement of neural plate

cells drives mediolateral narrowing and rostrocaudal elongation of the neural plate. Further mediolateral narrowing and bending (or rolling) of the neural plate result from changes in the shape of the neural plate cells, especially by apical constriction. Because the driving forces are generated by the coordinated remodeling of cell shape and adhesion, cytoskeletal reorganization and regulation are essential to neurulation. Several lines of knockout mice lacking cytoskeleton-associated proteins, such as p190 RhoGAP, shroom, Mena, MARCKS, and vinculin, have shown defects in neurulation (Wu *et al.*, 1996; Xu *et al.*, 1998; Hildebrand and Soriano, 1999; Lanier *et al.*, 1999; Brouns *et al.*, 2000). Exactly how these proteins spatiotemporally regulate the cytoskeleton and how such activities are regulated during neurulation remain elusive.

Rho is the leading candidate as the regulator of this cytoskeletal reorganization, but whether Rho activation during neurulation is required has not yet been determined. When p190 RhoGAP, a negative regulator of Rho, was lost, knockout mice showed defects in neural tube formation despite apparently high Rho activity (Brouns *et al.*, 2000). One of the members of the shroom family, shroom3, is associated with both the actomyosin cytoskeleton and apical constriction of neural plate cells (Hildebrand and Soriano, 1999; Haigo *et al.*, 2003; Hildebrand, 2005), but is regulated by Rap1, not Rho (Haigo *et al.*, 2003). Seen in this light, therefore, the molecular mechanisms of cytoskeletal reorganization during neurulation and the involvement of Rho remain issues for further exploration.

Rho-GTPases are regulated by several morphogenetic signals, such as Wnt, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF; Habas *et al.*, 2003; Harada *et al.*, 2005; Theriault *et al.*, 2007; Zhang *et al.*, 2007), among which the Wnt/planar cell polarity (PCP) signaling pathway is the most studied (Habas *et al.*, 2003; Veeman *et al.*, 2003). Wnt signals include the canonical signaling pathway that

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Address correspondence to: Shigenobu Yonemura (yonemura@cdb.riken.jp).

leads to β -catenin-mediated transcriptional regulation and several noncanonical signaling pathways (Kikuchi *et al.*, 2006). One noncanonical pathway, the Wnt/PCP pathway, is an important element of cell rearrangement within single-cell layers. In *Xenopus* gastrulation, it regulates convergent extension movement (Tada *et al.*, 2002), and activation of the Rho-GTPases downstream of the Wnt/PCP signal is required for this morphogenesis (Habas *et al.*, 2001, 2003). Recent studies indicate that the Wnt/PCP signal during neurulation is an important element of neural plate cell rearrangement (Wallingford and Harland, 2002; Ciruna *et al.*, 2006; Wang *et al.*, 2006; Ybot-Gonzalez *et al.*, 2007), although further investigation is required to explore the involvement of Rho-GTPases downstream of the Wnt/PCP signal in this process.

In this study we report on the coordination between Rho activity, myosin activity, and neural plate dynamics and the need for Rho and Rho-kinase activity for correct neurulation. In addition, activation of Rho is required for its own apical accumulation in neural plate cells. Inhibition of the Wnt/PCP pathway causes disruption of the apical accumulation of Rho in neural plate cells, abnormal morphology of neural plate cells, and the failure of neural tube development. Our data, based on endogenous protein distribution, delineate the physiological function and importance of Rho in the morphogenetic movement of the neural plate and further our understanding of the molecular mechanisms of cytoskeletal reorganization in neural plate cells during neurulation.

MATERIALS AND METHODS

Plasmid Constructs

A bacterial expression vector for production of a probe protein for pulldown assays, pGEX-3X-Rhotekin-RBD (Rho-binding domain; Reid *et al.*, 1996), was kindly donated by Dr. S. Narumiya (Kyoto University, Japan). pGEX-4T-1-N-Tat-C3 was constructed as follows. Tat-tag is derived from human immunodeficiency virus and is a cell-penetrating peptide tag (Suzuki *et al.*, 2002). A DNA fragment containing the region coding Tat-tag (oligonucleotides 5'-GATCAGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCTCAAGGATCCCCGG-3' and 5'-AATTCGGGGATCCTTGAGGAGGCTTCGCTCGTGTCTCCGCTTCTCTGCCT-3' were annealed) was inserted into the BamHI-EcoRI site of pGEX-4T-1 (GE Healthcare, Little Chalfont, United Kingdom) to construct pGEX-4T-1-N-Tat. This has the same multicloning site as pGEX-4T-1, and Tat-tag is located just after the thrombin digestion site and immediately before the multicloning site. The sequence encoding botulinum toxin C3 (Kumagai *et al.*, 1993) from the vector pEGFP-C3 (donated by Dr. S. Narumiya) was subcloned into the BamHI site of pGEX-4T-1-N-Tat. The chick expression vector pCA MCS (-) (Niwa *et al.*, 1991) was kindly donated by Dr. H. Niwa (RIKEN CDB, Japan) and was amino-terminally tagged with enhanced green fluorescent protein (EGFP) to generate pCA-EGFP-C1 by inserting the NheI-BamHI-digested EGFP-containing fragment from pEGFP-C1 (Clontech, Mountain View, CA) into the NheI-BglII site of pCA MCS (-). pEF-BOS-Myc-RhoA, carrying a constitutively active Rho mutant (Rho-CA, V14G), was kindly donated by Dr. K. Kaibuchi (Nagoya University, Japan). Rho-CA and C3 were subcloned into the BglII site of pCA-EGFP-C1. For *Xenopus* microinjection experiments, we used the mRNA production vectors pCS2-Venus (a GFP mutant kindly donated by Dr. A. Miyawaki, RIKEN BSI, Japan; Nagai *et al.*, 2002), pCS2-Xdsh (*Xenopus* dishevelled wild type), pCS2-Xdd1 (*Xenopus* dishevelled dominant negative form; Sokol, 1996), pCS2-XWnt11-dC (*Xenopus* carboxy-terminal region lacking Wnt11 dominant negative form; Tada and Smith, 2000), and pCS2-XFrz7-dC (*Xenopus* carboxy-terminal region lacking Frizzled7 dominant negative form; Djiane *et al.*, 2000).

Antibodies

Anti-Rho rat mAb was raised and characterized as described previously (Yonemura *et al.*, 2004). Anti-chick β -catenin rabbit serum was kindly donated by Dr. M. Takeichi (RIKEN CDB, Japan). Anti-myosin light chain 2 (MLC2) and anti-phospho-MLC2 (Thr18, Ser19) rabbit polyclonal antibodies were purchased from Cell Signaling (Danvers, MA). Anti-GFP rabbit serum and Alexa-Fluor 488-, 647-phalloidin were purchased from Invitrogen (Tokyo, Japan).

Immunocytochemistry

Chick and *Xenopus* embryos cultured by conventional methods were fixed with 4% paraformaldehyde in 0.1 M HEPES buffer (pH 7.5) or 10% trichloro-

roacetic acid (TCA) for 2 h at 4°C. For cryoprotection, specimens were placed into 0.1 M HEPES buffer (pH 7.5) containing 30% sucrose for 12 h at 4°C. The specimens were frozen in OCT compound (Sakura, Tokyo, Japan) and cut into sections (16 μ m thickness) by cryostat. The sections were permeabilized with phosphate-buffered saline (PBS; pH 7.2) containing 30 mM glycine (G-PBS) and 0.2% Triton X-100 for 5 min and blocked with G-PBS containing 4% normal donkey serum for 30 min. The sections were incubated with the primary antibody for 1 h (anti-Rho, 1:200; anti- β -catenin 1:200, anti-phospho-MLC2 1:100, and anti-GFP 1:500), washed, and incubated with secondary antibodies for 30 min. All antibodies were diluted with G-PBS containing 4% normal donkey serum. Specimens were observed through a confocal microscope (LSM 510, Carl Zeiss, Tokyo, Japan).

Pulldown Assay of GTP-bound Rho-GTPases

Neural plate regions were cut from chick embryos with fine tungsten needles and immediately frozen in liquid nitrogen. In the case of Hamburger and Hamilton stage 8–9 (HH 8–9) embryos, only neural plate regions (uncloned neuroepithelial regions) were isolated, whereas in the case of HH 12–13 embryos, only neural tube regions (closed neuroepithelial regions) were isolated. For each assay, 40–50 HH 5–6 embryos, ~25 HH 8–9 embryos, and ~20 HH 12–13 embryos were collected. The G-LISA RhoA Activation Assay Biochemistry Kit (Cytoskeleton, Denver, CO) was used for the detection of Rho activity. For immunoblotting data, however, we performed the assay with glutathione S-transferase (GST)-RBD protein purified from *Escherichia coli* (80 μ g of GST-RBD protein and 20 μ l [bed volume] of glutathione Sepharose 4B [GE Healthcare] per experimental point). All procedures and buffers were the same as those for the G-LISA Kit, except that bound proteins were eluted with 100 μ l of Laemmli buffer and were detected by immunoblotting.

Detection of Phosphorylation of MLC

Chick embryos were fixed in 10% TCA for 1 h at 4°C. This treatment allowed specific neuroepithelial cells to be isolated easily with forceps. In the case of HH 8–9 embryos, only neural plate regions were isolated, whereas for HH 12–13 embryos, only neural tube regions were isolated, as above. For each assay, ~25 HH 5–6 embryos, 15 HH 8–9 embryos, and 12 HH 12–13 embryos were collected. The samples subsequently underwent immunoblot analysis with anti-MLC and anti-phospho-MLC.

Inhibitor Experiments with Chick Embryos

Chick embryos were cultured on albumen-agar plates as described previously (Sundin and Eichele, 1992), but with slight modifications. Inhibitors (cytochalasin D [CD], Y27632, and (-) blebbistatin; Merck Calbiochem, Tokyo, Japan) were diluted with Hanks' balanced salt solution (Invitrogen) containing 0.01% saponin instead of with Yolk-Tyrode medium. Inhibitor solutions were added individually to albumen-agar plates (0.5 ml per 6-cm dish) 30 min before use. A filter paper circle carrying an HH 5–6 embryo dorsal side up was placed on the agar plate and overlaid with 20 μ l of inhibitor solution. The vitelline membrane was then broken with forceps to expose the embryo to the inhibitor solution. The embryo was preincubated at room temperature for 2 h to enable the inhibitor to penetrate while development was suspended. Excess inhibitor solution was then removed, 20 μ l of fresh inhibitor solution was added carefully, and the plate was incubated at 38.5°C (time 0) to resume embryonic development. Every 3 h, the inhibitor solution was replaced. After 8–9 h of incubation, when the development of control embryos had reached about HH 9, the samples were fixed. Purified GST-Tat and GST-Tat-C3 were obtained from a bacterial protein expression system, GST Gene Fusion System, according to the manufacturer's instructions (GE Healthcare), and Tat-C3 was obtained by using thrombin to remove GST.

Electroporation of Chick Embryos

HH 5–6 embryos were electroporated as described previously (Kobayashi *et al.*, 2002), but at 5 V, on a model CUY21 EDIT electroporator (NEPA GENE, Ichikawa, Japan).

Microinjection of *Xenopus* Embryos

Embryos were microinjected with mRNA as described previously (Mizuseki *et al.*, 1998). Embryos were fixed in 10% TCA (4°C, 2 h) at neurula stage.

Scanning Electron Microscopy

Neurula *Xenopus* embryos were fixed in 1% glutaraldehyde in PBS (4°C, 8 h). They were postfixed in 0.5% osmium oxide for 90 min at 4°C, dehydrated through a graded ethanol series, and transferred into isopentyl acetate. The specimens were then critical-point dried, platinum coated, and analyzed under scanning electron microscope (JSM-5610LV, JEOL, Tokyo, Japan).

RESULTS

Rho Accumulates in the Apical Region of Neural Plate Cells

Because cell migration and morphogenesis occur far more dynamically in embryos than in adult tissues, the dynamics of Rho in tissue morphogenesis can be observed easily during development. We therefore searched for regions in the chick embryo where Rho accumulates and found that it accumulated predominantly in the apical regions of the neural plate (Figure 1Ba). Considering that apical constriction and changes in the morphology of neural plate cells are essential for correct neurulation (Figure 1A) and that Rho regulates actomyosin constriction and cytoskeletal reorganization, this apical accumulation of Rho likely coincides with apical constriction of neural plate cells concomitant with cytoskeletal reorganization. We also found outstanding accumulation of Rho in the apical regions of lens, nasal, and otic placodes (Figure 1C). Placodes are the primordium of every sensory organ and are thickened regions of the epidermal ectoderm, which curl and invaginate in a similar

manner to the neural plate. Taking this evidence together, it seems that Rho generally accumulates in the apical regions of ectodermal cells where a sheet of columnar epithelial cells is either bending or invaginating.

Interestingly, we also found that after neural tube closure, the apical accumulation was reduced (Figure 1Bb). These results suggest that the Rho accumulation in the apical region of the neural plate coincides with morphogenetic activity.

Rho Localizes at Active Actomyosin-enriched Sites

The apical constriction of neural plate cells is thought to be based on an actomyosin network associated with junctional complexes. Rho has been implicated in the formation of adherens junctions and in actomyosin contraction through phosphorylation of MLC (Jaffe and Hall, 2005). Accumulated Rho likely reorganizes or maintains adherens junctions and their associated actomyosin bundles in neural plate cells that exert apical constriction force. The distribution of Rho partly overlapped with actin filaments in the apical region of neural plate cells (Figure 2A), and Rho further accumulated to spot-like sites largely merged with β -catenin, a known constituent of adherens junctions (Figure 2B). The colocalization of Rho and phosphorylated MLC (Figure 2C) indicates that Rho accumulated in sites where myosin II was activated.

Dynamics of Rho Distribution in the Neural Plate Coordinates with Morphogenetic Movement of the Neural Plate

To confirm the coordination between accumulation of Rho and constriction activity in the neural plate, we investigated the dynamics of Rho distribution during neurulation. Although apical accumulation of Rho, phosphorylated MLC, and actin filaments were all present even in flat neural plates before the onset of bending (i.e., HH 5), their immunoreactivities were relatively weak (Figure 3A). As the neural plate began to bend, we noted their slightly increased accumulation in the apical region of the median hinge point (Figure 3A). At later stages, in the anterior part of the chick neural plate, Rho and phosphorylated MLC accumulated predominantly in the dorsolateral region, including the second bending region where dynamic morphogenesis was occurring, but decreased in the first bent point (Figure 3B). After neural tube closure, the apical accumulation of Rho decreased significantly and that of phosphorylated MLC dropped slightly, but actin filaments remained at high concentration (Figure 3B).

In the posterior neural groove also (Figure 3C), Rho and phosphorylated MLC accumulated in the dorsolateral apical region. As seen during anterior neurulation, this posterior accumulation increased as compaction and bending progressed and decreased after neural tube closure.

Activities of Rho and Myosin II in the Neural Plate Are Related to the Dynamics of Neurulation

Activated Rho-GTPases localize at plasma membranes (Takai *et al.*, 1995; Van Aelst and D'Souza-Schorey, 1997), and the Rho that accumulated apically certainly seemed to be active. To confirm this speculation, we collected neural plates dissected from embryos and biochemically measured the activity of Rho in the neural plate. The activity of Rho was highest at HH 8–9 (Figure 4A), in agreement with the histochemical data of Rho accumulation. We next examined myosin II activity in the neural plate by measuring the amount of phosphorylated MLC. Like Rho, the activity of myosin II in the neural plates was highest at HH 8–9 (Figure 4B). Because the morphogenetic movement of primary neu-

Figure 1. Apical accumulation of Rho in neural plate cells of chick embryos. (A) Diagrammatic transverse sections illustrating morphogenesis of chick neural tube from flat neural plate. (a) First, the ectoderm thickens into the neural plate. (b) The neural plate bends into a "V" shape. (c and d) The anterior region of the neural plate, which later differentiates into the brain, bends again to form a diamond-like shape with a larger lumen than in the posterior region. (e) The posterior region of the neural plate, which later differentiates into the spinal cord, lacks an obvious second bending point and forms a slit-like smaller lumen. Arrowheads indicate the bending points. (B and C) Chick embryos were transversely sectioned and immunostained for Rho. (B) The posterior part of the neural plate during (HH 8–9, a) and after (HH 13, b) neural tube closure. (C) Rho also accumulates in the apical region of the lens (a), nasal (b), and otic (c) placodes of chick embryos. Dotted lines represent basal boundaries of placodes. Scale bars, 100 μ m.

Figure 2. Rho colocalizes with actomyosin bundles associated with cell-cell adhesion. The anterior parts of chick neural plates were transversely sectioned and stained for Rho, actin filament (F-actin), β -catenin, and phosphorylated MLC (MLC-P). (A) F-actin (a and a') partly overlaps with Rho (b and b') in the apical region of the neural plate. (B) Both β -catenin (a and a') and Rho (b and b') are enriched in cell-cell adhesion sites of the neural plate. (C) Both MLC-P (a and a') and Rho (b and b') accumulate in the apical region (mainly at cell-cell adhesion sites) of the neural plate. (A–C) (c) Merged image of a and b. (a'–c') Magnified images of the square regions in a–c. (c') Merged image of (a') and (b'). Scale bars, (c) 100 μ m, (c') 20 μ m.

neurulation is most dynamic at HH 8–9, the peak activities of Rho and myosin II are coincident with the morphogenetic activity of neurulation.

Figure 3. Dynamics of localizations of Rho, activated myosin, and F-actin during neurulation. (A) Localization of Rho, phosphorylated MLC (MLC-P), and F-actin during the early stages of chick neurulation. Arrows indicate the points of onset of the first bending. (B) Localization of Rho, MLC-P, and F-actin at later stages of chick neurulation. Arrowheads indicate second bending points. (C) Localization of Rho, MLC-P, and F-actin in the neural plate of posterior spinal cord region at HH 9. (a) Dorsal view of a HH 9 chick embryo. Numbers and lines indicate levels of transverse sections as shown in b. (b) Localizations of Rho, MLC-P, and F-actin in each transverse section. Scale bars, 100 μ m.

Figure 4. Rho and myosin activity during neurulation are highest at HH 8–9, coinciding with movement in neural plate activity. (A) Rho activity in neural plates manually isolated at each step of neurulation was detected by using pull-down assays for GTP-bound Rho. Data are shown as mean $\pm$ SEM ($n = 11$, seven independent experiments); $**p < 0.01$. (B) Levels of myosin activation (level of MLC phosphorylation) in the neural plate manually isolated at each step. Data are shown as mean $\pm$ SEM ($n = 6$, six independent experiments); $**p < 0.01$.

Inhibition of Actomyosin and Rho Causes Failure of Neural Tube Formation

We next assayed the functional significance of Rho and the Rho-associated cytoskeleton in neurulation by using specific inhibitors. The effects of several inhibitors of cytoskeleton-associated proteins on neurulation have already been reported (Schoenwolf *et al.*, 1988; Park *et al.*, 2003); however, in many cases, the concentration of inhibitor was extremely high compared with concentrations often used in cell biology, perhaps because inhibitors cannot penetrate into embryos as easily as cultured cells. Because we cannot exclude the possibility that high concentrations of inhibitors might lead to undesirable side effects, we developed a more effective drug delivery protocol for use in chick embryos. Some specific aspects of this improved system include direct exposure of the dorsal surface of embryos to inhibitor solutions by cutting the vitelline membrane, addition of a mild detergent in inhibitor solutions to increase penetration, and preincubation of embryos in inhibitor solutions at room temperature before resumption of development by shifting the incubation temperature to 38.5°C. In the vast majority of

Table 1. Effects of various inhibitors on chick neurulation

	Neural tube		
	Unclosed (%)	Closed, but abnormally (%)	Total defective (%)
Control ($n = 34$)	6	6	12
Cytochalasin D (1 μ M; $n = 20$)	100	—	100
Cytochalasin D (0.2 μ M; $n = 11$)	82	0	82
Blebbistatin (100 μ M; $n = 32$)	47	44	91
Y27632 (50 μ M; $n = 22$)	55	23	77
GST-Tat (0.1 mg/ml; $n = 32$)	13	0	13
Tat-C3 (0.1 mg/ml; $n = 33$)	67	18	85

HH 5–6 chick embryos were put on albumen–agar plates, and inhibitors were added to the embryos, which were preincubated and then cultured until control embryos grew to $\sim$ HH 9. The embryos were fixed and the phenotypes were analyzed. Unclosed, at least the anterior forebrain region was open even if the midbrain region was closed; Closed, but abnormally, the anterior neural tube was closed, but its shape was abnormal or it had ectopic structures, as seen in Figure 5C.

cases (88%), control embryos treated according to this protocol but in the absence of inhibitors developed normally (Table 1).

As previously reported (Schoenwolf *et al.*, 1988), neurulation of the chick embryo is affected by CD, which inhibits actin polymerization (Figure 5B and Table 1). In most cases, exposure to 1 μ M CD caused severe defects, including nearly halting neurulation and thinning and flattening of the neural plate (Figure 5B, c and d). These results indicate that the actin cytoskeleton is necessary to maintain the columnar shape of neural plate cells. Several embryos showed mild defects (mainly in experiments using 0.2 μ M CD), successfully completing neural tube closure at the midbrain level but failing to complete closure at the forebrain and spinal cord levels (Figure 5Ba). These mild phenotypes are the same as those described in the previous report (Schoenwolf *et al.*, 1988), even though the concentration of CD used in the previous study was much higher (1.6 μ g/ml $\approx$ 3.2 μ M).

Blebbistatin inhibits myosin II motor activity. Exposure to 100 μ M blebbistatin also caused a failure of neural tube closure (Figure 5C and Table 1). Even in cases in which closure appeared to be successful, we often noted several abnormal structures in the neural tube, which can be described as ectopic protrusions of the neural tube into the lumen (Figure 5Cc). In addition, the morphology of the neural plate was abnormal, with a variety of wavy or disordered shapes (Figure 5Cd). These extra or loose structures may result from elongation of the apical surface due to inhibition of apical constriction or mediolateral elongation of the neural plate caused by abnormal rearrangement of neural plate cells.

C3 is a component of a botulinum toxin and an inhibitor of Rho. Tat-tag is a penetrating peptide tag; Tat-C3 has proved to be an effective tool for inhibiting Rho (Park *et al.*, 2003). Exposure to Tat-C3 caused a severe phenotype (Figure 5D and Table 1), and the apical accumulation of Rho was

Figure 5. Acto-myosin, Rho, and Rho-kinase activation are necessary for correct neural tube formation. (A) Control. (a) Dorsal view of a chick HH 5–6 embryo at the beginning of inhibitor experiments. (b) Dorsal view of a normal control embryo (HH 9) after incubation with DMSO-containing medium only. (c) A transverse section of anterior neural tube at line 1 in panel b and stained for F-actin. (d) A transverse section of posterior neural plate cut at line 2 in panel b and stained for F-actin. (B) Effects of CD. Dorsal views of severe (a) and mild (b) phenotypes. Transverse sections of severe specimens cut at lines 1 (c, anterior region) and 2 (d, posterior region) and stained for F-actin. (C) Effects of blebbistatin. Dorsal views of severe (a) and mild (b) phenotypes. Transverse sections of severe specimens cut at lines 1 (c, anterior region) and 2 (d, posterior region) and stained for F-actin. (D) Effects of Tat-C3. (a) An embryo (HH 5–6) at the beginning of this experiment. (b–d) Control embryos (HH 9) incubated with GST-Tat. A transverse section cut at a line (b, posterior region) was stained for F-actin (c) and Rho (d). Arrowheads indicate apical accumulation of Rho. (e) Dorsal view of an embryo incubated with Tat-C3 for 8 h. A transverse section cut at a line (e, posterior region) was stained for F-actin (f) and Rho (g). (h) Immunoblot of whole embryo lysates shows the reduction of myosin II activity due to Tat-C3. (E) Effects of Y27632. (a) Dorsal view of a Y27632-exposed chick embryo with the severe phenotype. Transverse sections of severe specimens cut at lines 1 (b, anterior region) and 2 (c, posterior region) and stained for F-actin. (d) Exposure to Y27632 decreased myosin II activity. Immunoblot of whole embryo lysates of control and Y27632-exposed chick embryos indicates levels of MLC phosphorylation. Green dotted lines represent outlines of the neural plate or neural tube. Scale bars, 100 μ m.

decreased dramatically (Figure 5Dg), indicating that activation of Rho is necessary for its localization in the apical region. Exposure to C3 also inhibited MLC phosphorylation (Figure 5Dh). These results suggest that active Rho localizes in the apical region of the neural plate and generates myosin-mediated apical constriction, which is necessary for correct neurulation.

Y27632 inhibits Rho-kinase, a key effector of Rho and regulator of MLC phosphorylation (Zhao and Manser, 2005). Although a previous study cursorily assessed the effect of 200 μ M Y27632 on neurulation (Wei *et al.*, 2001), the high concentration used might have inhibited several other kinases in addition to Rho-kinase. In the current study, we succeeded in reducing the concentration to levels similar to those used in cell biology (50 μ M). Exposure to Y27632 caused failure of neural tube closure or resulted in an abnormal neural plate, and we saw wavy neural plates (Figure 5E and Table 1). These phenotypes were all very similar to those produced by treatment with blebbistatin. Subsequent immunoblots showed a Y27632-induced reduction in MLC

phosphorylation (Figure 5Ed). These data suggest that the Rho–Rho-kinase pathway plays an important role in correct neurulation via regulation of myosin II activity.

Correct Regulation of Rho Activity Is Important for Neurulation

Although it is reasonable to assume that actomyosin constriction is essential for correct neurulation, the importance of the activation of Rho has remained unclear because the p190 RhoGAP knockout mouse showed neurulation defects (Brouns *et al.*, 2000). We observed the effects of overstimulation of Rho on neurulation (Figure 6). Overstimulation of Rho by expression of its constitutively active mutant (Rho-CA) caused rounding of cells, some of which were extruded from the neural tube into the apical lumen (Figure 6, C and D), whereas neural plate cells expressing only GFP after electroporation were columnar in shape, just as were normal neural plate cells (Figure 6, A and B). Inhibition of Rho due to expression of C3 also caused rounding of cells and extrusion from the neural tube to the apical lumen (Figure 6, E

Figure 6. Both activation and inactivation of Rho are important for correct neural tube formation. (A and B) Only GFP-electroporated neural plate cells showed columnar cell shape as seen in normal nonelectroporated neural plates. (C and D) Expression of the GFP-tagged Rho constitutively active mutant (GFP-Rho-CA) caused rounding of cells, some of which were extruded from the neural tube into the apical lumen. (E and F) Inhibition of Rho by expression of GFP-tagged C3 (GFP-C3) also caused rounding of cells and extrusion from the neural tube into the apical lumen. (A, C, and E) Dorsal views of electroporated chick neural tubes (HH 10, spinal cord region). (B, D, and F) Transverse sections of electroporated chick neural tubes were immunostained with anti-GFP antibody (HH 10, spinal cord region). Dotted lines show outline of neural tube. Scale bar, 50 μ m.

and F). At sites in the neural tube that contained many Rho-CA- or C3-expressing cells, the neural tube was thin or disordered (Figure 6, D and F). These results suggest that not only activation of Rho, but also its inactivation, is important for correct neural tube cell shape and neural tube formation.

The Wnt/PCP Pathway Is Involved in the Apical Accumulation of Rho in the Neural Plate of Embryos

We next turned to the relationship between morphogenetic signals and the distribution of Rho in the neural plate. The Wnt/PCP pathway leads to the activation of Rho-GTPases via its primary signal transducer, dishevelled (Habas *et al.*, 2003). The Wnt/PCP signal is also important for proper neurulation throughout vertebrates (Wallingford and Harland, 2002; Ciruna *et al.*, 2006; Wang *et al.*, 2006; Ybot-Gonzalez *et al.*, 2007). Therefore, it is reasonable to think that activation and accumulation of Rho in the neural plate are regulated by the Wnt/PCP pathway. Mutant molecules that affect the Wnt signal transduction pathway have been established in the *Xenopus* system, and we detected apical accumulation of Rho in the *Xenopus* neural plate (Figure 7C, a–c). We therefore decided to use *Xenopus* embryos in an attempt to explore the relationship between the Wnt/PCP signal and the accumulation of Rho.

Xdd1 is a dominant-negative mutant of *Xenopus* dishevelled (Xdsh) and acts as an inhibitor of the Wnt/PCP path-

way (Sokol, 1996). The expression of Xdd1 caused neurulation defects with broad neural plates, as previously reported (Figure 7B; Wallingford and Harland, 2002). These broad neural plates had a reduced apical accumulation of Rho (Figure 7C, d–f), and these Xdd1 phenotypes were partially rescued by coexpression of wild-type Xdsh (Figure 7C, g–j). These results implicate Xdsh activity in the apical accumulation of Rho in the *Xenopus* neural plate, probably through Rho activation.

To further confirm the relationship between the Wnt/PCP pathway and apical accumulation of Rho, we inhibited the Wnt/PCP pathway by using other specific molecules. A Wnt receptor, *Xenopus* frizzled7 (XFrz7), and one of its ligands, *Xenopus* Wnt11 (XWnt11), both specifically stimulate the Wnt/PCP pathway (Tada *et al.*, 2002), and the C-terminal deletion mutants of these proteins (XFrz7-dC and XWnt11-dC) are their dominant negative forms (Djiane *et al.*, 2000; Tada and Smith, 2000). Like Xdd1, expression of XFrz7-dC and XWnt11-dC affected neurulation and decreased the apical accumulation of Rho (Figure 7D).

The Wnt/PCP pathway regulates cell rearrangement in the neural plate in the same way as it regulates convergent extension in gastrulation, because inhibition of the Wnt/PCP pathway causes the neural plate to have a smaller length-to-width ratio than that of wild-type embryos, with no effect on tissue differentiation (Wallingford and Harland, 2002; Wang *et al.*, 2006; Ybot-Gonzalez *et al.*, 2007). However, little is known about cell shape in the neural plate in Wnt/PCP-inhibited embryos. We used scanning electron microscopy to assess the cell surface of the neural plate. Normal neural plate cells were arranged tightly and densely in the neural groove, and the apical cell surface was long and narrow along the anterior–posterior axis. In contrast, Xdd1-injected neural plate cells were arranged irregularly (Figure 7E), they were wider than normal, and their area was increased. This observation suggests that the smaller length-to-width ratio of the neural plate in Wnt/PCP-inhibited embryos results not only from inhibition of cell rearrangement, but also from the smaller length-to-width ratio and the increased surface area of each neural plate cell. These changes in cell shape are likely caused by inhibition of actomyosin apical constriction downstream of the Wnt/PCP-Rho pathway.

DISCUSSION

Rho-GTPases constitute a group of evolutionarily conserved proteins from yeast to mammals that regulate a variety of cellular processes associated with dynamic cytoskeletal reorganization. Rho-GTPases have also been implicated in the morphogenesis of tissues in developing multicellular organisms. In *Drosophila*, the Rho–Rho-kinase pathway is involved in a number of morphogenetic-related PCP events, such as cellular rearrangement at germ band elongation and establishment of orientation of ommatidia and hairs (Winter *et al.*, 2001; Bertet *et al.*, 2004); and Rho, Rac, and Cdc42 regulate cytoskeletal reorganization of dorsal closure (Harden *et al.*, 1999). In mice, the Rho–Rho-kinase pathway is involved in eyelid formation (Shimizu *et al.*, 2005). Many studies of the function of Rho-GTPases in vertebrate morphogenesis focus on *Xenopus* gastrulation, in which the Wnt/PCP pathway leads to cell rearrangement through Rho activation (Tada *et al.*, 2002; Veeman *et al.*, 2003).

Genetic approaches like those just described are not always sufficient for analysis of the spatiotemporal-specific function of Rho-GTPases, because the phenotypes are the sum of all effects during development. In addition, the fun-

Figure 7. Inhibition of Wnt/PCP pathway causes neurulation defects and disrupts the apical accumulation of Rho in neural plate cells in *Xenopus* embryos. (A) mRNA injection experiment in embryos. mRNA was injected into two right-side blastomeres of the animal hemisphere at the eight-cell stage. Venus mRNA (50 pg) was coinjected as a marker. At the neurula stage, injected mRNA was distributed mainly to the right side of the ectoderm. (B) Phenotypes of embryos at the neurula stage producing mutant molecules that inhibit the Wnt/PCP pathway. Top row contains bright microscopy images; middle row shows images of Venus distribution; bottom row contains sketches depicting the morphology as transverse sections. Normal, a Venus-expressing control neurula embryo. Mild and Severe, dominant-negative dishevelled (Xdd1)-expressing embryos with mild and severe phenotypes, respectively. In mild cases, the medial line is almost straight and the neural

damental nature of Rho-GTPases in so many cellular processes means that they are likely to be implicated throughout morphogenesis, and the morphogenetic event that most depends on Rho-GTPase remains undetermined. In the current study, we show the physiological importance of Rho in neurulation, based on our observations of when, where, and to what extent Rho accumulates.

We found that Rho accumulates predominantly near the apical region of bending neural plates. Its overlapping localization with actin filaments, β -catenin, and phosphorylated MLC supports the idea that its accumulation causes apical constriction and concomitant cytoskeletal reorganization in the neural plate. In the anterior dorsolateral region, the accumulation of Rho was maintained during second bending, but decreased after neural tube closure. This spatiotemporal-specific accumulation suggests the functional significance of Rho in the morphogenesis of neurulation. Further, the apical accumulation of Rho at the first bending point was less than and in a narrower region than that seen at the second bending. This pattern suggests that the lower constriction force produced by a smaller number of cells is sufficient for the first bending, probably because forces from outside the neural plate (extrinsic forces) are dominant for this bending, indicating that the first bending of the neural plate may be a rather passive movement. Extrinsic forces are caused by the proliferation and expansion of epidermal ectodermal cells adjacent to the neural plate. In the posterior part of the bending neural plate, Rho accumulated throughout the apical region of dorsolateral cells, which have no obvious hinge point. The posterior spinal cord neural tube has a much smaller diameter than has the anterior brain neural tube, whereas the thicknesses of both the anterior and posterior neural plates are similar. Curving of a layer of the same thickness with a smaller radius of curvature requires a greater apical constriction force. Even though the dorsolateral regions of the posterior neural plate lack an obvious

hinge point, apical constriction at levels similar to those during the second bending of the anterior neural plate may be required on account of the decreased radius posteriorly.

In this study we demonstrate that the high levels of Rho activity as measured by biochemical assays are related to dynamic movement during neurulation. We have also been able to use specific inhibitors of actin, myosin II motor, Rho, and Rho-kinase during neurulation to show that each caused various defects in neural tube closure. In addition, we showed that the activation of Rho is necessary for its own apical localization in neural plate cells and confirmed that inhibition of Rho or Rho-kinase leads to a biochemical reduction in myosin II activity. Together our findings indicate that the active form of Rho that accumulates near the apical region regulates the cytoskeletal reorganization required for correct neurulation. Inhibition of Rho kinase or myosin II caused wavy neural plates; this wavy phenotype is indicative of a loose neural plate, which is probably due to failure of apical surface constriction and cellular rearrangement. Because the second bending does not occur in the posterior part of the neural plate (Figure 1A), the ω -like structures seen in Figure 5, Cc and Ec, did not result from normal bending, but suggest that both the right and left side of the loose neural plate were pushed upward by extrinsic forces. Our observations also suggest that the myosin II and Rho kinase activity is important in generating intrinsic forces in the neural plate, but is less so for extrinsic forces.

In addition to the transient accumulation and activation of Rho at the neural plate cells during neural tube formation and the requirement of Rho activity for neural tube formation, we showed that continuous activation or inhibition of Rho in neural plate cells by using RhoA-CA or C3, respectively, results in defects in neurulation (Figure 6). These results indicate that not only Rho activity, but also its correct spatiotemporal regulation, is required for neural tube formation.

shroom3 is one of the most important actin-associated proteins involved in the apical constriction of neural plate cells (Hildebrand and Soriano, 1999; Haigo *et al.*, 2003; Hildebrand, 2005), and one study has reported that shroom3 is regulated by Rap1 but not by Rho (Haigo *et al.*, 2003). However, because both Rho and shroom3 coordinately contribute to apical constriction of epithelial cells (Hildebrand, 2005), they might also work together in different pathways in neurulation. Because the functional link between shroom3 and Rap1 was examined only in blastula epithelial cells in the cited study, the possibility remains that shroom3 is regulated by Rho in neuroepithelial cells at the neurula stage. Further study is needed to explore the regulatory pathway of shroom3-mediated apical constriction of neural plate cells.

The most-studied morphogenetic signaling pathway underlying Rho activation is the Wnt/PCP pathway, which also is involved in neurulation. However, the link between Rho activation and neurulation has remained obscure. Although some reports suggest the involvement of Rho-kinase in neural tube formation (Wei *et al.*, 2001; Ybot-Gonzalez *et al.*, 2007), the changes in Rho activity and its downstream target activity in neural plate regions had not been measured directly. In the current study, we used biochemical means to quantify the temporal activation of Rho and Rho-kinase in neural plate regions and found that Rho activity is essential for its apical accumulation. Considering that inhibition of the Wnt/PCP pathway disrupted the apical accumulation of Rho and apical constriction, this pathway likely regulates Rho activity in the neural plate during neurulation.

Figure 7 (cont). plate of the mRNA-injected side is broader than that of uninjected control side. In severe cases, medial lines are curved toward the injected side, and the neural plates of the injected side are much broader than those of the control side. Black lines indicate sides of neural folds. Dotted lines represent medial line of embryos. (C) Transverse sections of control (a–c), Xdd1 mRNA (200 pg)-injected (d–f), and Xdd1 plus wild-type disheveled (Xdsh, 400 pg) mRNA-injected (g–i) embryos. Venus (a, d, and g), Rho (b, e, and h), and merged (c, f, and i) images. (j) Statistics of the phenotypes induced by Xdd1 injection and coinjection with Xdsh. Venus: n = 138; Venus + Xdd1: n = 130; Venus + Xdd1 + Xdsh: n = 102. (D) Transverse sections of XFrz7-dC mRNA (400 pg)-injected (a–c) and XWnt11-dC mRNA (400 pg)-injected (d–f) embryos. Venus (a and d), Rho (b and e), and merged (c and f) images. (g) Statistics of the phenotypes induced by XFrz7-dC- or Xwnt11-dC-injection. Venus: n = 138; Venus + XFrz7-dC: n = 93; Venus + XWnt11-dC: n = 90. Broken lines in C and D represent outlines of neural plates. Dotted lines represent medial lines of embryos. (C and D) Arrowheads, the region of apical accumulation of Rho; open arrowheads, disruption of apical accumulation of Rho. Scale bars, 50 μ m. (E) Dorsal views of neural plate cells in control and Xdd1 mRNA-injected embryos. (a and b) Arrangements of neural plate cells of (a) Venus- and (b) Venus + Xdd1 mRNA-injected *Xenopus* embryos. (c and d) Dorsal scanning electron microscopic views of the neural grooves of Venus- (c) and Venus + Xdd1 (d) mRNA-injected *Xenopus* embryos. Scale bar, 10 μ m. (e) Statistics of apical surface area of neural plate cell. In the Xdd1-injected side, the apical surface area of neural plate cells is much larger than in the uninjected control side, whereas in Venus-injected embryos, neural plate cells on both sides have almost the same apical surface area. Venus: n = 20, 4 specimens; Xdd1: n = 20, 3 specimens. Bars, mean $\pm$ SEM.

PCP causes orientated cell migration and changes in cell shape within cell layers and often directs the morphogenetic movement of epithelium-like cell layers. The noncanonical Wnt signaling pathway regulates this morphogenetic movement through several developmental events (Veeman *et al.*, 2003). During neurulation, PCP leads to rearrangement of neural plate cells, thus causing mediolateral narrowing and rostrocaudal lengthening of the neural plate (Wallingford and Harland, 2002). This cell rearrangement in the neural plate is similar to the convergent extension during gastrulation, which is regulated by Rho-GTPases under the control of the Wnt/PCP signal (Habas *et al.*, 2001, 2003; Tada *et al.*, 2002). Taking these findings together with our data, we think it likely that the cell rearrangement during neurulation also is regulated by the Wnt/PCP signal via Rho activation. Neural plate cells in Wnt/PCP-inhibited embryos had increased surface area (Figure 7E), perhaps due to inhibition of Rho-mediated actomyosin apical constriction. Because the Wnt/PCP signal induces both cell rearrangement and changes in the shape of individual cells in the neural plate, the two events may be linked. In fact, the orientated constriction of actomyosin that is regulated by Rho-kinase drives cell rearrangement in *Drosophila* germ band elongation (Bertet *et al.*, 2004). Our data suggest that the actomyosin constriction generated by the Rho-Rho-kinase pathway also involves rearrangement of neural plate cells during neurulation.

The experimental approaches and findings we have presented will help explain the diverse physiological functions of Rho and their comprehensive features and hierarchy. In this study, we found a similar apical accumulation of Rho in multiple placodes. Because placodes, like the neural plate, are areas of thickened ectoderm that are curving and invaginating, our study is a useful starting point for further exploration of more general morphogenetic mechanisms, despite the numerous specific differences between individual cases.

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