Cdc42-mTOR Signaling Pathway Controls Hes5 and Pax6 Expression in Retinoic Acid-dependent Neural Differentiation

Received for publication, October 7, 2008, and in revised form, November 26, 2008 Published, JBC Papers in Press, December 19, 2008, DOI 10.1074/jbc.M807745200

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The conditional knockout of the small GTPase Cdc42 from neuroepithelial (NE) and radial glial (RG) cells in the mouse telencephalon has been shown to have a significant impact on brain development by causing these neural progenitor cells to detach from the apical/ventricular surface and to lose their cell identity. This has been attributed to the requirement for Cdc42 in establishing proper apical/basal cell polarity and cell-cell adhesions. In the present study, we provide new insights into the role played by Cdc42 in the maintenance of neural progenitor cells, using the mouse embryonal carcinoma P19 cell line as a model system. We show that the ability of P19 cells to undergo the transition from an Oct3/4-positive, undifferentiated status to microtubule-associated protein 2-positive neurons and glial fibrillary acidic protein-positive astrocytes, upon treatment with retinoic acid (RA), requires RA-induced activation of Cdc42 during the neural cell lineage specification phase. Experiments using chemical inhibitors and RNA interference suggest that the actions of Cdc42 are mediated through signaling pathways that start with fibroblast growth factors and Delta/Notch proteins and lead to Cdc42-dependent mTOR activation, culminating in the up-regulation of Hes5 and Pax6, two transcription factors that are essential for the maintenance of NE and RG cells. The constitutively active Cdc42(F28L) mutant was sufficient to up-regulate Hes5 and Pax6 in P19 cells, even in the absence of RA treatment, ultimately promoting their transition to neural progenitor cells. The ectopic Cdc42 expression also significantly augmented the RA-dependent up-regulation of these transcription factors, resulting in P19 cells maintaining their neural progenitor status but being unable to undergo terminal differentiation. These findings shed new light on how Cdc42 influences neural progenitor cell fate by regulating gene expression.

In vertebrates, central nervous system development starts with the formation of the neural tube from the embryonic ectoderm (1, 2). At its earliest stage, the neural tube consists of single-layered neuroepithelial (NE)2 cells. As embryogenesis proceeds, these single-layered structures undergo expansion into multilayered structures, mediated through the asymmetric division of NE cells in the ventricular zone and the directional cell migration of their daughter cells. In the later stages of development of the mouse telencephalon, two groups of cells, radial glial (RG) cells and basal progenitor cells, reside in different layers. RG cells, like NE cells, remain in the ventricular zone near the apical/inner surface, throughout embryogenesis. In contrast, basal progenitor cells reside in the subventricular zone, close to the basal layer of NE cells, and transiently amplify during embryogenesis and gradually disappear (1, 2). The establishment and maintenance of neural progenitor cell populations are essential for proper central nervous system development, and knockout and mutant mice of several genes show defects in this process. For example, Hes family and Pax6 transcription factors are specifically expressed in the ventricular zone-residing apical progenitor cells, including NE and RG cells, but not in basal progenitor cells of the mouse forebrain (3–7). Mice lacking these genes as a result of knockout or mutation show defects in the maintenance of apical progenitor cells (3–6).

The small GTPase Cdc42 has also been reported to participate in the proper maintenance of apical progenitor cells. Once Cdc42 is depleted from these cells in the mouse telencephalon, they detach from the apical/ventricular surface (8, 9), lose their cellular identity, and finally change into randomly distributed basal progenitor cells (9). Previous reports suggested that these phenotypes were due to the loss of epithelial structures at the apical/ventricular surface, as a result of the absence of Cdc42 and its ability to control apical/basal polarity and cell-cell adhesions in apical progenitor cells (8, 9). Indeed, the roles played by Cdc42 in the establishment of cell polarity and cell-cell adhesions are central to many aspects of organogenesis (10–12). However, Cdc42 is also involved in a wide range of intracellular activities by regulating intracellular trafficking, cell cycle progression, and gene expression (13–16). Thus, it was of interest to see whether Cdc42 plays other roles in neural differentiation, especially when cells commit to neural cell lineages from their pluripotent undifferentiated status.

One downstream signaling target of Cdc42 that is of particular interest is the mammalian target of rapamycin (mTOR), a serine/threonine kinase (17, 18). mTOR is a key regulator of cell

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S9, Table S1, and references.

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2 The abbreviations used are: NE, neuroepithelial; RG, radial glial; mTOR, mammalian target of rapamycin; RA, retinoic acid; FGFR, fibroblast growth factor receptor; DAPT, N-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine t-butyler ester; siRNA, short interfering RNA; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TuJ1, βIII-tubulin; MAP2, microtubule-associated protein-2; GFAP, glial fibrillary acidic protein; APP, amyloid precursor protein; EGFR, epidermal growth factor receptor; RAGE, receptor for advanced glycation-end products; α-MEM, α-minimal essential medium; PBD, Cdc42/Rac-binding domain from PAK; GST, glutathione S-transferase.
growth and proliferation and has also been implicated in the survival of neural stem/progenitor cells as mediated by Notch/Delta proteins through their regulation of the expression of Hes family transcription factors (19). Although both Cdc42 and mTOR are essential for the maintenance of the neural stem/progenitor cell population in developing or adult brains, thus far a direct functional connection has not been established between Cdc42 and mTOR in central nervous system development. In this study, we have set out to determine whether such a link exists, and if so, how it might impact neural differentiation, using P19 cells as a model system.

The embryonal carcinoma P19 cell line was established from a teratocarcinoma caused by the transplantation of a mouse embryo (20, 21). P19 cells express the pluripotent marker Oct3/4, similar to embryonic stem cells. However, in response to specific extracellular stimuli and differentiation conditions, they lose Oct3/4 expression and differentiate into ectodermal (neurons and glia cells) (20–24) or mesodermal cell lineages (skeletal muscle cells and cardiomyocytes) (20, 21, 25, 26), as well as into single-layered epithelia (24). By taking advantage of P19 cells that show a retinoic acid (RA)-dependent transition from an Oct3/4-positive, undifferentiated status to neural progenies, we demonstrate that Cdc42, in response to RA-induced extrinsic factors (i.e., fibroblast growth factors (FGFs) and Delta ligands), activates mTOR to up-regulate the expression of the Hes5 and Pax6 transcription factors. Cdc42 depletion inhibits mTOR activation and the resultant up-regulation of Hes5 and Pax6, thereby disrupting RA-induced neural differentiation. The ectopic expression of the constitutively active mutant Cdc42(F28L) is sufficient for mTOR activation and up-regulation of Hes5 and Pax6, stimulating P19 cells to differentiate into Oct3/4−/Nestin+ neural progenitor cells in the absence of RA stimulation. We also show that the ectopic expression of Cdc42 significantly augments the RA-dependent up-regulation of these transcription factors and, consequently, maintains cells at the neural progenitor stage and blocks terminal differentiation. These findings highlight a potentially important role for Cdc42, by working together with mTOR, to influence apical progenitor cell fate by regulating the expression of key transcription factors.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal anti-mouse Nestin repeat (Nestin18) antibody was from Covance. Monoclonal anti-Cdc42 antibody was from Upstate. The neuron-glial cell marker sampler kit containing antibodies for MAP2, βIII tubulin (TuJ1), and GFAP was from Chemicon. Monoclonal anti-Oct3/4 (C10), GAP43 (B5), and polyclonal anti-Cdc42 (P1) antibodies were from Santa Cruz Biotechnology. Monoclonal RC2 and anti-myosin heavy chain antibodies (MF20) were obtained from Developmental Studies Hybridoma Bank (The University of Iowa). Monoclonal anti-vinculin antibody was from Sigma.

**Cell Culture and Differentiation**—P19 cells (ATCC) were cultured at 37 °C in 5% CO2, with α-MEM supplemented with 7.5% CS and 2.5% fetal bovine serum (Invitrogen) (10% serum growth medium). Cells were sub-cultured by trypsinization and used within 10 passages after thawing from liquid nitrogen stocks.

The procedures used for RA-induced neural differentiation are depicted in Fig. 1A. Briefly, 1 × 106 cells were cultured on a 100-mm bacterial grade dish in α-MEM supplemented with 3.5% calf serum, 1.5% fetal bovine serum, and 1 μM RA (neural-induction medium) (day 0). On day 2, cells were collected by centrifugation and placed into a bacterial grade dish with fresh neural induction medium. On day 4, the cells were again collected by centrifugation and trypsinized to disrupt cell aggregation, and then seeded (250 cells/mm2) onto poly-l-lysine-(Sigma) coated 100-mm tissue culture plates in 10% serum growth medium without RA. On day 6, the medium was changed to neurobasal medium containing B27 supplement (Invitrogen) and 2 mM glutamine (terminal differentiation medium). Although the B27 supplement included vitamin A, we confirmed that the terminal differentiation medium alone was not sufficient to induce neural differentiation in the absence of additional RA.

The FGF receptor inhibitor PD173074 (final concentration, 20 nM), the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Calbiochem, final concentration, 200 nM), and the mTOR inhibitor rapamycin (Calbiochem, final concentration, 20 nM), were added on days 0 and 2 during the RA-dependent neural differentiation protocol. For the −RA culture conditions, the cells were cultured on bacterial grade dishes in 5% serum-containing α-MEM and then trypsinized on day 4.

For DMSO-induced myogenic differentiation, cells (1 × 106) were cultured on a bacterial grade dish in 5% serum-containing α-MEM with 1% DMSO. On day 2, the DMSO-containing medium was replenished. On day 4, cells were collected, trypsinized, and then seeded onto non-coated tissue culture plates in 10% serum growth medium without DMSO. The medium was exchanged daily, and cells were cultured until the indicated day.

In some cases, we were interested in examining whether ectopically expressed Cdc42 was capable of inducing P19 cells to undergo RA-independent terminal differentiation into neural cells. For these experiments, the cells were cultured in bacterial grade dishes with 5% serum-containing α-MEM without RA, and then trypsinized on day 4 and grown on poly-l-lysine-coated tissue culture plates with 10% medium, before switching on day 6 to neurobasal medium.

**Transfection and RNA Interference**—Constructs expressing Myc-tagged wild-type Cdc42 and Cdc42(F28L) were generated as previously described (27). Transfections were carried out using Lipofectamine as suggested by the manufacturer’s instructions (Invitrogen). To establish stable cell lines, cells were selected with 500 μg/ml G418 (Research Products International) and then maintained in growth medium supplemented with 250 μg/ml G418, with the drug being removed prior to experiments.

P19 cells (that were 50–70% confluent) were transfected with Cdc42-targeting siRNAs (Invitrogen) or control siRNA using Lipofectamine 2000. The following day, the cells were transferred to bacterial grade dishes to induce differentiation. The transfection efficiency (~90%) was measured using Block-iT Fluorescent Oligo (Invitrogen).
PBD Pulldown Assays—Cdc42-activation assays were performed in pulldown experiments using glutathione S-transferase (GST) fused to the limit Cdc42/Rac-binding domain from PAK (called the PBD) as described previously (27). Briefly, whole cell lysates were collected with lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 20 mM NaF, 20 mM β-glycerol phosphate, 10 μg/ml leupeptin and aprotinin) containing 2 mM MgCl₂. Lysate proteins (300 μg) were incubated with glutathione-agarose beads bound to 50 μg of recombinant GST or GST-PBD at 4 °C for 90 min. The beads were washed three times with lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting.

Immunofluorescence Microscopy—Undifferentiated cells were seeded on 2-well LabTech chambers (Nalge Nunc International). Cells subjected to the differentiation protocols were transferred on day 4 from bacterial grade dishes to poly-L-lysine-coated chamber slides or to 60-mm tissue culture plates. After culturing for the indicated times, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, blocked with phosphate-buffered saline containing 2% bovine serum albumin, and incubated with primary antibodies. The cells were then washed and stained with Texas Red, Oregon Green 488, or Alexa Fluor 633-conjugated anti-mouse or anti-rabbit IgG antibodies (Molecular Probes). Chromatin was stained with 4,6-diamidino-2-phenylindole (Sigma). Epifluorescence images were captured using an Axioskop inverted microscope (Carl Zeiss) equipped with an AxioCam or Sensicam qe charge-coupled device camera system (The COOKE Corp.).

RT-PCR—Total RNA was extracted from cells at the indicated time points using the RNeasy kit (Qiagen). Reverse transcriptase (RT) reactions were performed with SuperScript III enzyme (Invitrogen), using oligo(dT) nucleotides as a primer. Following the RT reactions, the DNA products were subjected to the PCR using primer sets to amplify specific genes. To exclude the possibility that we are amplifying contaminating genomic DNA, RT reactions lacking the SuperScript III enzyme were performed on the RNA samples. The negative control samples were subjected to PCR using the primer set for Gapdh (glyceraldehyde-3-phosphate dehydrogenase) and are indicated at the bottom of each figure as −RT GAPDH. The DNA primers, annealing temperature, and number of PCR cycles used to amplify each gene, are listed in supplemental Table S1. For semi-quantitative RT-PCR, the DNA products were subjected to real-time PCR using Power SYBR Green PCR Master Mix and the 7500 Fast Real-Time PCR system (Applied Biosystems). Each reaction was performed in triplicate, and the amount of target RNA was normalized relative to the amount of Gapdh mRNA.

RESULTS

Characteristics of the Neural Differentiation of Mouse Embryonic P19 Cells—Fig. 1A depicts the protocol used for the RA-dependent neural differentiation of P19 cells. Prior to treatment with RA, P19 cells strongly express the pluripotent transcription factor Oct3/4 but show little or no expression of neural-specific differentiation markers like GAP43, βIII-tubulin (TuJ1), and microtubule-associated protein-2 (MAP2) (Fig. 1B). To induce neural differentiation, the cells were cultured in bacterial grade dishes to undergo embryoid body-like aggregation. Upon stimulation with RA, the expression of Oct3/4 was dramatically reduced (Fig. 1B). We also monitored the mRNA levels of several transcription factors necessary for neural differentiation, using RT-PCR. The Hes family of transcription factors and Pax6 are highly expressed in NE and RG cells and play a role in establishing and maintaining the neural stem/progenitor cell population (3–7). Fig. 1C shows that the mRNA levels of each of these transcription factors were quite low in the undifferentiated P19 cells, but were highly up-regulated in response to RA during the neural cell lineage specification phase (day 4). At the terminal differentiation phase, i.e. day 11 in the RA-induced neural differentiation protocol, the expression levels of these transcription factors were slightly down-regulated (Fig. 1C).

By day 7 of the RA-induced neural differentiation protocol, the expression of the neuronal markers GAP43 and TuJ1 was detected (Fig. 1B), and the cells began to extend neurites (see the TuJ1 staining in supplemental Fig. S1, second column of panels from the left). To promote terminal differentiation, the medium was changed to serum-free neurobasal medium with B27 supplement at day 6 (Fig. 1A). This resulted in the expression of the mature neuron-specific gene product, MAP2 (Fig. 1B), and culminated in the formation of axons (stained by GAP43; see supplemental Fig. S1, third column of panels from the left) and dendrites (stained by MAP2; Fig. 1D, top set of panels; and supplemental Fig. S1, fourth column of panels from the left) after 7–9 days, and 9–11 days, respectively.

GFAP-positive cells appeared during days 9–11 of the RA-induced differentiation protocol (Fig. 1D, top set of panels; and supplemental Fig. S1, fifth column of panels from the left). GFAP is used as a glial marker protein and is also expressed in primate RG cells (28). However, when using the RA-dependent protocol, we did not detect RC2-positive staining (see supplemental Fig. S2), which is a characteristic of both primate and rodent RG cells in vivo (28). Because these RC2−/GFAP+ cells often showed astrocyte-specific star-like cell morphology (especially obvious in supplemental Fig. S1, bottom-right panel), it seems likely that most of the GFAP-positive cells in this culture condition were mature astrocytes.

Upon 11 days of RA treatment, ~34% (±3%) of the cells had differentiated into MAP2-positive neurons, whereas ~15% (±7.5%) of the cells became GFAP-positive astrocytes (Fig. 1E). This period represents the terminal phase of neural differentiation (Fig. 1A). When cells were continuously exposed to 10% serum after stimulation by RA (Fig. 1A, indicated by +RA/+serum), instead of being cultured in serum-free neural medium, they did not undergo terminal differentiation but maintained the neural progenitor status that showed a high expression level of the neural progenitor marker Nestin (Fig. 1D, middle set of panels). In the absence of RA treatment (Fig. 1A, indicated by −RA), the cells did not lose their ability to express Oct3/4 and remained in an undifferentiated state in 10% serum-containing medium (Fig. 1, B and D, bottom set of panels).

Cdc42 Is Necessary for the RA-dependent Up-regulation of Hes5 and Pax6 in P19 Cells and Neural Cell Differentiation—In P19 cells, the expression level of Cdc42 was dependent on the
FIGURE 1. RA-induced neural differentiation of P19 cells. A, schematic describing the protocols used for RA-induced neural differentiation and control experiments. The details for each of the protocols are described under “Experimental Procedures.” B, Western blots showing the expression patterns for pluripotent and neural differentiation markers, upon RA-induced neural differentiation. Vinculin served as a protein-loading control. C, DNA electrophoretogram showing the expression patterns of the indicated transcription factors. Total RNA was isolated from non-stimulated or RA-stimulated P19 cells at the indicated day. RT-PCR was performed on the RNA samples using specific sets of primers to amplify the indicated transcripts. D, epifluorescence images showing the differentiation status of P19 cells for the different culture conditions. Cells were fixed at day 11 for each culture condition outlined in A, and then stained with anti-Oct3/4, Nestin, MAP2, and GFAP antibodies. The RA-dependent differentiation condition is shown in the top six panels. For the +RA/+serum condition (middle six panels), the culture conditions were the same as those for normal RA-induced differentiation (+RA) until day 6, at which point the cells were cultured in 10% serum-containing MEM medium until day 11. In the absence of RA treatment (bottom six panels), P19 cells exhibited an Oct3/4-positive, undifferentiated phenotype. E, histograms showing the percentage of MAP2-positive neurons or GFAP-positive astrocytes at day 11 of the RA-induced differentiation protocol. Error bars indicate ±S.D. (n = 3).
FIGURE 2. RA-dependent activation of Cdc42 is necessary for neural differentiation in P19 cells. A, Western blots showing the results of the GST-PBD-binding assays to detect the amount of activated GTP-bound Cdc42 in P19 cells. Cells were cultured using the RA- or DMSO-dependent differentiation protocol, and the lysates were collected at day 4 and subjected to PBD assays. The top panel shows the expression of Cdc42 in cell lysates as detected by an anti-Cdc42 antibody. The middle panel shows the relative amounts of GTP-bound Cdc42 as precipitated by GST-PBD, and the lower panel shows the relative amounts of GST-PBD used in each assay. The last lane in the figure represents a control in which lysates from RA-treated cells were incubated with GST instead of GST-PBD. B, epifluorescence image shows the MF20 (myosin heavy chain antibody)-staining of the DMSO-treated cells. C, Cdc42-targeting siRNAs suppressed Cdc42 expression (bottom panel) but did not affect Oct3/4 down-regulation (middle panel) in RA-treated P19 cells. Vinculin served as a protein-loading control (top panel). D, cells transfected with control and Cdc42-siRNAs were stimulated with RA, and the relative mRNA levels of the indicated transcription factors were determined by RT-PCR. The mRNA levels are represented relative to that of each transcription factor in control siRNA-treated cells at day 4. Error bars indicate ±S.D. (n = 3). Significant differences are indicated by * (p < 0.1) and ** (p < 0.05) using the t test. E, Western blots showing the expression pattern of neural differentiation markers at day 11. Vinculin was used as a protein-loading control. F, histograms showing the percentage of MAP2-positive neurons or GFAP-positive astrocytes at day 11 of P19 cells transfected with control or Cdc42-targeting siRNAs. Error bars indicate ±S.D. (n = 3).
extent of cell-cell contact and was influenced by the state of confluency of the cells (supplemental Fig. S3). The formation of embryoid body-like aggregates caused a progressive increase in Cdc42 expression that started on days 2–4, regardless of the absence or presence of differentiation-inducing agents like RA and DMSO (Fig. 2A, top panel). We checked the Cdc42 activation status for each culture condition, as a read-out using GST-PBD pulldown assays. Cdc42 was effectively activated during RA-induced neural cell lineage specification (Fig. 2A, middle panel), compared with the weak activation observed with DMSO treatment, which induces myogenic cell lineages (see Fig. 2B), or the lack of detectable activation in undifferentiated cells (designated as “non-stimulated” in Fig. 2A, middle panel). These findings suggest that Cdc42 has specific functions in RA-dependent neural cell lineage specification.

To determine if Cdc42 is in fact necessary for RA-dependent neural differentiation, we knocked down its expression using siRNA. Cells in which Cdc42 was knocked down still showed RA-dependent down-regulation of Oct3/4 expression at day 4 (Fig. 2C), indicating that Cdc42 was not necessary for this outcome. However, in Cdc42-siRNA-treated cells, the mRNA levels for Hes5 and Pax6 were significantly lower at day 4 (Fig. 2D). The effects of knocking down Cdc42 were sustained throughout the time period required for terminal differentiation, as indicated by the markedly reduced ability of RA to call-up the neuronal-specific markers, GAP43 and MAP2 (Fig. 2E). A significantly smaller percentage of Cdc42-siRNA-treated cells exhibited MAP2- or GFAP-staining as detected by fluorescence microscopy, compared with control-siRNA-treated cells (Fig. 2F and supplemental Fig. S4A).

To determine whether knocking down Cdc42 simply delayed neural differentiation or blocked the ability of P19 cells to differentiate into neural cells, we extended the time period for differentiation from 11 to 15 days. This allowed the recovery of Cdc42 expression in the Cdc42-siRNA-treated cells (supplemental Fig. S4B). Nonetheless, significant differences in neural cell morphology and differentiation efficiencies, as a read-out by MAP2- and GFAP-staining, were still evident between control- and Cdc42-siRNA-treated cells (supplemental Figs. S4C and S4D). These results suggest that Cdc42 depletion does not simply delay RA-dependent neural differentiation but completely impairs it.

Inhibitors of FGFR Kinase Activity and γ-Secretase Suppressed RA-induced Cdc42 Activation and Up-regulation of Hes5 and Pax6—The results described above suggest that Cdc42 depletion disrupts RA-dependent neural differentiation and that the impairment is already manifested during the time frame for the RA-dependent up-regulation of transcription factors, when cells commit to neural cell lineages. We next examined which signaling pathways activate Cdc42 in an RA-dependent manner in P19 cells. Because RA serves as a ligand for nuclear receptors that promote gene transcription, we suspected that Cdc42 is activated by extrinsic factors whose expression was up-regulated by RA. A number of extrinsic factors, through their ability to initiate cellular signaling events, have been implicated in neural differentiation. Two such signaling systems in particular are the FGF- and Delta/Notch-coupled pathways. FGF-dependent signaling activities play a critical role in central nervous system development (29) and can be inhibited by FGF receptor (FGFR) kinase inhibitors. Delta/Notch-dependent signaling pathways are essential for cell proliferation and survival in adult stem cells (30). They control the expression of the Hes family of transcription factors and can be suppressed by the γ-secretase inhibitor, DAPT, because the Notch proteins are activated as a result of their being cleaved by γ-secretases (30). Indeed, we found that the mRNA expression levels of several FGF and Delta genes were up-regulated in an RA-dependent manner in P19 cells (supplemental Fig. S5). After 4 days of RA stimulation, cells were treated with either the FGFR inhibitor PD173074 or DAPT for 3 h, and then Cdc42 activation was examined using PBD pulldown assays. Treatment with either PD173074 or DAPT reduced the amount of GTP-bound Cdc42 in cells by ~50% (Fig. 3A, middle panel), suggesting that FGF/FGFR and Delta/Notch proteins are up-stream regulators of Cdc42 during the neural cell lineage specification phase.

We also examined the effects of these chemical inhibitors on the RA-induced up-regulation of Hes5 and Pax6 transcription factors. Cells treated with the inhibitors showed the normal RA-mediated reduction of Oct3/4 expression (Fig. 3B). However, both inhibitors showed a significant reduction in the mRNA levels for Hes5 and a moderate but consistent reduction in Pax6 mRNA levels (Fig. 3C).

mTOR Is a Downstream Signaling Target of Cdc42 and Is Involved in the RA-induced Up-regulation of Hes5 and Pax6—The results described above suggest that FGF/FGFR- and Delta/Notch-coupled signals activate Cdc42 to up-regulate Hes5 and Pax6 expression in RA-stimulated P19 cells. We also found that Cdc42-depletion reduced the phosphorylation levels of mTOR in RA-stimulated P19 cells (Fig. 4A, bottom panel), suggesting that mTOR is a downstream signaling target of Cdc42 in these cells. This finding was especially interesting because mTOR functions as a signal transducer in a Delta/Notch-coupled signaling pathway that up-regulates Hes family transcription factors in adult neural stem cells (19). In RA-stimulated P19 cells, the phosphorylation level of mTOR was also suppressed by treatment with PD173074 as well as DAPT (Fig. 4B, bottom panel). These results suggest that mTOR is activated in FGF/FGFR- and Delta/Notch-coupled signaling pathways during the RA-dependent neural differentiation of P19 cells. To examine if mTOR is involved in the RA-dependent up-regulation of Hes5 and Pax6, we checked the mRNA expression levels of these transcription factors in the presence of rapamycin, a chemical inhibitor of mTOR. Cells treated with rapamycin inhibited the RA-dependent activation of mTOR but showed the normal RA-mediated reduction of Oct3/4 expression (Fig. 4C). However, rapamycin treatment caused a significant reduction in the Hes5 and Pax6 mRNA levels (Fig. 4D), suggesting that mTOR is involved in the RA-induced up-regulation of these transcription factors in P19 cells.

Ectopically Expressed Cdc42 Up-regulates Hes5 and Pax6 in P19 Cells in an mTOR-dependent Manner and Promotes Their Transition to Neural Progenitor Cells—We next examined the effects of the ectopic expression of wild-type and constitutively active Cdc42 on the neural differentiation of P19 cells. We established P19 cell lines that stably expressed Myc-tagged
FIGURE 3. Effects of FGFR and γ-secretase inhibitors on RA-dependent Cdc42 activation and up-regulation of transcription factors. A, Western blots showing the results of GST-PBD assays conducted on cells cultured using the RA-dependent differentiation protocol for 4 days and then treated with inhibitors of the FGFR kinase activity (PD173074, 20 nm) and/or γ-secretase (DAPT, 200 nm) for 3 h prior to being lysed. Top panels show the relative expression of Cdc42 in whole cell lysates using an anti-Cdc42 antibody. The middle panels show the relative amounts of GTP-bound Cdc42 as precipitated by GST-PBD and the bottom panels show the GST-PBD inputs. The first lane in the figure represents a control in which lysates from untreated cells were incubated with GST instead of GST-PBD. B, Western blots showing the expression levels of Oct3/4 from cells cultured using the RA-dependent differentiation protocol for 4 days, with or without PD173074 and DAPT. The inhibitors were added to the differentiation medium at day 0 and day 2, and cell lysates were collected after 4 days. Vinculin served as a protein-loading control. C, histograms showing the relative mRNA levels of Hes5 and Pax6 at day 4, in response to RA treatment. The mRNA levels of each transcription factor are plotted relative to the expression levels of each transcription factor in the RA-stimulated cells without chemical inhibitors. Error bars indicate ± S.D. (n = 3). Significant differences are indicated by * (p < 0.1) and ** (p < 0.05) using the t test.

We also checked the differentiation status of the cell lines stably expressing Cdc42. After culturing the cells in bacterial grade dishes in 5% serum-containing medium, for 4 days in the absence of RA, they were then grown in tissue culture plates with 10% serum (i.e. the −RA protocol depicted in Fig. 1A). Under these conditions, nearly all of the vector control cells still showed Oct3/4 expression, whereas some of the Myc-Cdc42- and Myc-Cdc42(F28L)-expressing cells did not show Oct3/4 expression and instead exhibited Nestin-positive staining, indicative of their having reached neural progenitor status (supplemental Fig. S6A).

We went on to examine whether these Oct3/4⁻/Nestin⁺ neural progenitor cells could terminally differentiate into neurons or glial cells. P19 cells stably expressing Myc-Cdc42 or Myc-Cdc42(F28L) were cultured in bacterial grade dishes in the absence of RA for 4 days and then were plated onto poly-L-lysine-coated tissue culture plates containing 10% serum. On day 6, the medium was changed to neurobasal medium with B27 supplement (i.e. the condition used for terminal neural differentiation; designated as “−RA/terminal” in supplemental Figs. S6B–E).

Cdc42-expressing cells that were cultured under these conditions exhibited an epithelial-like morphology; this was particularly evident for cells expressing the Cdc42(F28L) mutant, which showed a significant up-regulation of the neural progenitor protein Nestin (supplemental Figs. S6B and S6C). Supplemental Fig. S6D quantifies the reduction in Oct3/4 expression that occurred in the Cdc42-expressing cell lines for these conditions (i.e. in the absence of RA treatment). Neither the expression of wild-type Cdc42 nor Cdc42(F28L) induced the up-regulation of neuronal marker proteins (i.e. GAP43, βIII-tubulin (TuJ1), or MAP2), unlike the case when vector control cells were stimulated by RA to undergo terminal neural differentiation (supplemental Fig. S6E). In contrast, vector control cells remained undifferentiated and showed Oct3/4 expression under these culture conditions (supplemental Fig. S6D), but ultimately underwent apoptosis in the serum-starved neurobasal medium. Thus, the ectopic expression of Cdc42 in P19 cells, and in particular the constitutively active Cdc42(F28L) mutant, triggers the initial phases of neural differentiation as indicated by the reduced expression of Oct3/4, and the up-

forms of wild-type Cdc42 and the constitutively active mutant Cdc42(F28L) (Fig. 5A, top panel). Myc-Cdc42(F28L)-expressing P19 cells when cultured in low serum exhibited higher levels of mTOR activation, compared with vector control or wild-type Cdc42-expressing P19 cells (Fig. 5A, bottom panel). We then examined whether the ectopic expression of Cdc42 was sufficient to alter the levels of transcription factors of interest in an RA-independent manner. The cells were grown on bacterial grade dishes with 5% serum for 4 days in the absence of RA. This resulted in an up-regulation of Hes5 mRNA levels in Myc-Cdc42- and, especially in Myc-Cdc42(F28L)-expressing cells (Fig. 5B, left panel). Pax6 was also highly up-regulated in Myc-Cdc42(F28L)-expressing cells, but not in Myc-Cdc42-expressing cells (Fig. 5B, right panel). Rapamycin treatment then effectively reduced the RA-independent up-regulation of Hes5 and Pax6 in Myc-Cdc42(F28L)-expressing cells. Taken together, these results demonstrate that the expression of the constitutively active Cdc42 is sufficient to up-regulate Hes5 and Pax6 in an mTOR-dependent manner.

We then examined whether the ectopic expression of Cdc42 was sufficient to alter the levels of transcription factors of interest in an RA-independent manner. The cells were grown on bacterial grade dishes with 5% serum for 4 days in the absence of RA. This resulted in the expression of the constitutively active Cdc42 is sufficient to up-regulate Hes5 and Pax6 in an mTOR-dependent manner.

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Cdc42-mTOR Up-regulates Hes5 and Pax6

FIGURE 4. Cdc42-dependent mTOR activation and effects of rapamycin on RA-dependent up-regulation of transcription factors. A, Western blots showing the expression and activation levels of mTOR in cells treated with Cdc42-targeting siRNAs. Cells transfected with control and Cdc42-siRNAs were stimulated with RA for 4 days, and the lysates were collected. Cdc42 was knocked down in Cdc42-targeting siRNA-treated cells but not in cells treated with control siRNA. Vinculin served as a protein-loading control. The relative values for the phospho-mTOR signals, as measured by densitometry using ImageJ (NIH) (n = 3), are shown below the panels. Signal intensities were indicated relative to that of control siRNA-treated cells. B, Western blots showing the expression and activation levels of mTOR from cells cultured using the RA-dependent differentiation protocol for 4 days, with or without PD173074 (20 nM) or DAPT (200 nM). The inhibitors were added to the differentiation medium at day 0 and day 2, and cell lysates were collected after 4 days. The relative values for the phospho-mTOR signals are shown below the panels. Signal intensities were indicated relative to that of cells without chemical inhibitors. C, Western blots showing the expression and/or activation levels of Oct3/4 and mTOR from cells cultured using the RA-dependent differentiation protocol for 4 days, with or without rapamycin (20 nM). Rapamycin was added to the differentiation medium at days 0, 2, and 3, and cell lysates were collected after 4 days. D, histograms showing the relative mRNA levels of Hes5 and Pax6 at day 4, in response to RA treatment. The mRNA levels of each transcription factor are plotted relative to the expression levels of each transcription factor in the RA-stimulated cells without rapamycin. Error bars indicate ± S.D. (n = 3). Significant differences are indicated by ∗(p < 0.05) using the t-test.

regulation of Nestin, Hes5, and Pax6, but cannot drive terminal neural differentiation.

The Ectopic Expression of Cdc42 Augments the RA-dependent Up-regulation of Hes5 and Pax6 and Prevents Cells from Terminal Differentiation—The studies described in the preceding section raised the question as to whether induction by RA might enhance the effects of Cdc42 and enable cells ectopically expressing this protein to terminally differentiate into mature neurons or glial cells. Thus, we examined the effects of ectopically expressed Cdc42 on RA-dependent neural differentiation. Vector control cells showed the expected up-regulation of Hes5 and Pax6 on RA-dependent manner (Fig. 6A). However, upon exposure to RA, both Myc-Cdc42-expressing cell lines showed a further enhancement in the degree of up-regulation of both transcription factors, compared with vector control cells. These stimulatory effects were suppressed when the kinase activity of mTOR was inhibited by rapamycin (Fig. 6B). Vector control cells as well as the Myc-tagged Cdc42-expressing cells showed RA-induced Oct3/4 down-regulation (Fig. 6C, see days 9 and 15). The vector control cells also exhibited normal RA-induced neural differentiation into Nestin-positive neural progenitor cells, TuJ1- or MAP2-positive neurons, and GFAP-positive astrocytes (Fig. 6, D and E). In contrast, Myc-Cdc42-expressing cells ultimately exhibited an epithelial cell morphology (Fig. 6D) and strong Nestin-staining (Fig. 6E) but failed to show signs of terminal differentiation into either mature neurons or glial cells (Fig. 6, C and E). Overall, these results suggest that the ectopic expression of Cdc42 significantly augmented the RA-dependent up-regulation of Hes5 and Pax6, thus maintaining the neural progenitor status of P19 cells, while inhibiting their terminal differentiation.

DISCUSSION

It has been well established that RA stimulates the mouse embryonal carcinoma P19 cell line to commit to becoming neural cell lineages (20–24). However, it remains to be elucidated how RA-mediated transcriptional activation leads to this transition. Here, we show that Cdc42 plays an essential role in RA-induced neural differentiation, at the stage where P19 cells commit to differentiate into neural cell lineages. This occurs as an outcome of Cdc42 regulating the activation of mTOR and the resultant up-regulation of transcription factors that are necessary for the generation of apical progenitor cells in vivo. Fig. 7 summaries how RA might work through Cdc42 to mediate some of the events necessary for neural differentiation.

RA induces the up-regulation of several forms of FGF together with members of the Delta family of proteins (supplemental Fig. S5), which can promote the activation of Cdc42, and various reports have suggested that both FGFRs and Delta ligands contribute to RA-induced neural differentiation in P19 cells (31–33). Thus far, we have not been able to identify a specific FGF molecule or Delta protein that is sufficient for triggering either full Cdc42 activation or the up-regulation of Hes5 and Pax6. Therefore, it seems likely that the activation of endogenous Cdc42 is regulated in an RA-dependent manner through the cooperative effects of multiple members of the FGF and Delta families of proteins. Although the γ-secretase inhibitor DAPT is often used to inhibit Delta/Notch-coupled signaling pathways (30), the γ-secretase proteolytic complex is also important for the processing of other proteins, including amyloid precursor protein (APP). Recent studies have shown that...
APP is cleaved in a γ-secretase-dependent manner in the NE/RG cell-enriched lateral ventricles of mouse brains, modulating neurogenesis (34). Furthermore, RAGE (receptor for advanced glycation-end products), which functions as a receptor for β-amyloid (a proteolytic product of APP), is important for the RA-induced up-regulation of Pax6 as well as for Cdc42 activation in P19 cells (35). Although as yet there is no direct evidence to show that the RAGE-dependent activation of Cdc42 leads to the up-regulation of Pax6 expression, we cannot rule out the possibility that an APP-dependent signaling pathway is involved in the RA-dependent Cdc42 activation and resultant up-regulation of transcription factors. It is also possible that Cdc42 is activated by distinct extracellular factors whose expression levels are controlled by RA (Fig. 7, indicated by X), given that inhibiting FGFR kinase activity, as well as γ-secretase activity, only partially mimicked the effects of Cdc42-depletion in P19 cells. Delineating exactly how Cdc42 is activated downstream of RA will be the subject of future studies.

Our work shows that Cdc42 activates mTOR during the RA-dependent neural differentiation of P19 cells. In several cell lines, Cdc42-dependent activation of mTOR leads to S6 kinase activation to promote ribosomal translation (17, 18). However, the phosphorylation status of S6 kinase did not change in response to RA stimulation, nor did we see any significant changes in the activation status of S6 kinase due to Cdc42 activation in P19 cells (supplemental Figs. S7A and S7B). We have also examined a number of conventional Cdc42-signaling partners that are involved in gene expression, including Erk, JNK (c-Jun N-terminal kinase), p38, glycogen synthase kinase 3-β, and β-catenin; however, thus far we have not detected significant differences in the expression or activities of these proteins, due to Cdc42 activation in P19 cells. We also checked EGF-coupled signaling pathways for several reasons. EGF promotes cell proliferation and survival in adult neural stem cells (36) and activates Cdc42 in several cell lines (27). Furthermore, constitutively active Cdc42(F28L) protects EGFRs from ubiquitin-dependent protein degradation in fibroblasts (37). In P19 cells, EGFR expression levels were quite low and undetectable by Western blot analysis during RA-dependent neural differentiation. The mRNA levels of EGF also showed only modest reductions upon stimulation with RA (supplemental Fig. S7C). Furthermore, we found that the EGFR kinase inhibitor AG1478 did not inhibit the RA-dependent neural differentiation of vector control cells, nor did it affect the terminal differentiation of Myc-Cdc42-expressing cells (supplemental Fig. S7D). These results suggest that EGF-dependent signaling pathways are not essential for RA-dependent neural differentiation.
Cdc42-mTOR Up-regulates Hes5 and Pax6

**A**

![Graph showing relative mRNA expression levels for Hes5 and Pax6 with RA treatment](image)

**B**

![Graph showing relative mRNA expression levels for Hes5 and Pax6 with Rapamycin treatment](image)

**C**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative mRNA levels</th>
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<tbody>
<tr>
<td>WT</td>
<td></td>
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<tr>
<td>Rapamycin</td>
<td></td>
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<tr>
<td>+Rapamycin</td>
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**D**

![Images of cell culture with RA treatment](image)

**E**

![Images of immunostaining with RA treatment](image)
Cdc42-mTOR Up-regulates Hes5 and Pax6

**FIGURE 7. Model for the Cdc42-dependent regulation of neural differentiation in P19 cells.** RA induces the expression of members of the FGF and Delta families of ligands, as well as possibly additional proteins (X), resulting in the activation of Cdc42. This gives rise to the activation of mTOR and the up-regulation of the expression levels of Hes5 and Pax6 (see details in text).

The ectopic expression of Cdc42 promoted the RA-independent differentiation of P19 cells into Oct3/4+/Nestin+ neural progenitor cells, even in the absence of RA treatment. Therefore, Cdc42 is involved in the down-regulation of Oct3/4 during RA-induced neural differentiation? This appears to be unlikely, given that the down-regulation of Oct3/4 accompanying the ectopic expression of Cdc42 is much slower than the down-regulation stimulated by RA (see Fig. 1B, and Figs. S6A, S6C, and S6D). For example, Oct3/4 expression was undetectable on day 2 of the RA-induced neural differentiation protocol, whereas half of the Myc-Cdc42(F28L)-expressing cells still showed Oct3/4 staining on day 7 in the absence of RA treatment. Because the knock down of Cdc42 by RNA interference did not affect the RA-dependent down-regulation of Oct3/4 (Fig. 2C), the RA-independent reduction in Oct3/4 expression by Cdc42 might be a side-effect caused by the up-regulation of Hes5 and Pax6, because the overexpression of tissue-specific transcription factors has been reported to induce differentiation (38–42).

Cdc42-dependent activation of mTOR mediates signaling to the nucleus, up-regulating the expression of the transcription factors Hes5 and Pax6. The ectopic expression of Cdc42 in P19 cells, and in particular, the constitutively active Cdc42(F28L) mutant, can bypass the requirement for RA, FGFs, and Delta proteins and increase the expression levels of Hes5 and Pax6. We checked the mRNA levels of several transcription factors, aside from Hes5 and Pax6, that are essential for the generation of NE and RG cells in central nervous system development (e.g. Sox1–3, Hes1, and Hes3). However, the expression levels of these transcription factors were either not up-regulated by RA stimulation of P19 cells or responded to RA but showed little or no dependence on Cdc42 (supplemental Figs. S8A and S8B). We also examined the involvement of Cdc42 in the RA-induced up-regulation of the neuronal precursor cell-specific transcription factor Mash1 (38). Mash1 was up-regulated by RA in an FGF-, Delta/Notch-, Cdc42-, and mTOR-independent fashion in P19 cells (Figs. S8C–E). Apparently, RA activates several distinct signaling pathways to promote the expression of specific sets of transcription factors that contribute to neural cell lineage specification in P19 cells.

The results obtained when ectopically expressing Cdc42 in P19 cells suggest that the tight regulation of Cdc42 expression and activation is essential for prompting neural progenitor cells to undergo terminal differentiation. We examined whether the inability of Myc-Cdc42-expressing cells to undergo terminal differentiation was the outcome of the overactivation of mTOR. However, treating cells with rapamycin during the terminal differentiation phase (days 6–9) did not answer this question. Such treatment inhibited the neural differentiation of vector control cells and failed to restore Myc-Cdc42-expressing cells with the capability to undergo neural differentiation (supplemental Figs. S9A and S9B). This suggests that mTOR activity is essential for terminal differentiation as well as for neural cell lineage specification.

RA-dependent neural differentiation mainly occurs in the caudal-ventral areas of the spinal cord (43), suggesting that the signaling pathway that we delineated in this study (RA–FGF/FGFR and Delta/Notch–Cdc42–mTOR–(Hes5 and Pax6)) might be especially applicable to this specific region of the developing nervous system. However, considering that FGF/FGFR and Delta/Notch are highly expressed and indispensable in broad regions of central nervous system development (29, 30), Cdc42 might also play important roles in activating mTOR in the FGF/FGFR- and/or Delta/Notch-coupled signaling pathways that are independent of RA. Overall, our findings, when taken together with the results of previous studies (8, 9), suggest that Cdc42 serves two important functions in the maintenance of the apical progenitor cell population in mouse central nervous system development. One involves the proper localization of apical progenitor cells, whereas the other is the regulation of the expression of specific transcription factors.

Total Cdc42 knockout mice died at very early stages of embryogenesis at embryonic stage 5.5 (E5.5)–E7.5, i.e. before the onset of neurogenesis (44). One possible reason for this outcome is that Cdc42 is essential for cell-cell adhesion and directional cell movement during this stage of embryogenesis, as shown in Cdc42-null embryonic stem cells (11, 44). However, another explanation, which is not mutually exclusive with the former possibility, is that Cdc42 mediates intracellular signals at the early stages of cellular differentiation, during cell lineage specification, as shown by our finding that...
Cdc42 is necessary for the RA-dependent neural cell lineage specification of Oct3/4-positive P19 cells. It will be interesting to see whether Cdc42 is essential for the establishment of the apical progenitor cell population at the early stage of neural tube formation as well as for the maintenance of apical progenitor cells during later stages of mouse forebrain development.

Acknowledgments—We acknowledge Cindy Westmiller for expert secretarial assistance. We also thank Dr. Kristin F. Wilson-Cerione and Dr. Xu Peng for reading the manuscript and for helpful discussions.

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