The Cyclin-dependent Kinase Inhibitors p57 and p27 Regulate Neuronal Migration in the Developing Mouse Neocortex*

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Neuronal precursors remain in the proliferative zone of the developing mammalian neocortex until after they have undergone neuronal differentiation and cell cycle arrest. The newborn neurons then migrate away from the proliferative zone and enter the cortical plate. The molecules that coordinate migration with neuronal differentiation have been unclear. We have proposed in this study that the cdk inhibitors p57 and p27 play a role in this coordination. We have found that p57 and p27 mRNA increase upon neuronal differentiation of neocortical neuroepithelial cells. Knockdown of p57 by RNA interference resulted in a significant delay in the migration of neurons that entered the cortical plate but did not affect neuronal differentiation. Knockdown of p27 also inhibits neuronal migration in the intermediate zone as well as in the cortical plate, as reported by others. We have also found that knockdown of p27 increases p57 mRNA levels. These results suggest that both p57 and p27 play essential roles in neuronal migration and may, in concert, coordinate the timing of neuronal differentiation, migration, and possibly cell cycle arrest in neocortical development.

In the developing mammalian cerebral cortex, cell proliferation, differentiation, and migration are highly coordinated and temporally linked. Neocortical neurons are generated in the proliferative pseudostratified ventricular zone, where neural precursor cells undergo massive expansion before they differentiate into cortical neurons and exit the cell cycle. Newborn neurons leave the ventricular zone and move toward the cortical pial surface in a highly ordered process of neuronal migration (1, 2). The precise temporal coupling between neuronal differentiation, cell cycle arrest, and migration is essential for determining the number and location of each type of neurons generated through orchestrated waves of cortical neurogenesis and thus for the formation of the organized layers of the neocortex. Although the proneural basic helix-loop-helix protein Neurogenin2 has been reported to be involved in cell migration (3, 4), the mechanism that coordinates neuronal differentiation and cell cycle arrest with migration remains largely unclear.

One candidate that may couple neuronal differentiation, cell cycle arrest, and cell migration is p27, a member of the cyclin-dependent kinase (cdk)3-interacting protein/kinase inhibitor protein (Cip/Kip) family of the cdk inhibitors. Nuclear p27 negatively regulates G1 cell cycle progression by sequestering and inactivating cyclin E or A-cdk2 complexes. p27 protein levels are increased upon induction of neuronal differentiation, and this is implicated in the cell cycle arrest of neural precursor cells during cortical development (5–7). However, emerging data also suggest a role for cytoplasmic p27 in the regulation of cell migration, independent of cyclin-cdk inhibition. For example, mouse embryonic fibroblasts deficient in p27 exhibited impaired cell motility, whereas expression of p27 has been shown to promote migration of HepG2 hepatocellular carcinoma, embryonic fibroblasts, endothelial cells, and vascular smooth muscle cells (8). The C-terminal domain of p27 is capable of rescuing the migration defect of p27-deficient fibroblasts and thus appears to be responsible for the regulation of cell motility (9). Very recently, it has been shown that knockdown of p27 by RNA interference or p27 gene deletion results in a delay in neuronal migration in the developing neocortex (10, 11). We also found that acute knockdown of p27 by RNA interference inhibits neuronal migration, although deletion of p27 had less of an inhibitory effect (see “Results and Discussion”). This observation prompted us to examine the possibility that some related molecule might compensate for the function of p27 to regulate neuronal migration within the developing neocortex when the p27 gene was deleted.

The member of the cdk inhibitor family closest to p27 is p57 (12, 13). Indeed, p27 and p57 function redundantly to control cell cycle arrest and the differentiation of lens fiber cells and placental trophoblasts, as revealed by the phenotypes of single and double mutants (14). p57 is expressed in the neocortex during development, but its functions in the developing neo-
cortex are largely unknown (15–17). In addition to a well-conserved cdk inhibitor domain in the N-terminal half, p27 and p57 share a QT domain with a low similarity in the C-terminal half, which is important for recognition by SKP1-CUL1-F-box protein complex (SCF)Kip2-dependent ubiquitylation and for degradation of each protein (18, 19). However, it has been unknown whether p57 is involved in cell migration. In this study, we found that the level of p57 transcript was elevated when p27 was knocked down in neuroepithelial cells. We also observed an increase in p57 and p27 mRNA after induction of neuronal differentiation. Moreover, we found that knockdown of p57 resulted in impaired neuronal migration in the cortical plate of developing neocortex, although it did not affect neuronal differentiation. These results suggest that p57 is involved in neocortical neuronal migration and possibly compensates in part for the functions of p27 in this process.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—To construct short hairpin RNA (shRNA)-expressing vectors, oligonucleotides targeting the coding sequence and their complementary sequences were inserted into pSIREN vector (BD Biosciences). All contain a nine-base hairpin loop sequence (5′-TTCAGAGA-3′). The targeting sequences used in this study are as follows: p27 shRNA (5′-GTGGAGAATTTCCATTTCCAG-3′), p57 shRNA number 1 (5′-GCA- GGACGAGAATCAGAAG-3′), p57 shRNA number 2 (5′-GAGA- AACTGCGAGAGGAGAAC-3′), control shRNA (5′-GCACTGG- CGGGATATGGAA-3′) (Fig. 1), control shRNA (5′-GTGC- GTTGCTAGTACCAC-3′) (Fig. 2), and control shRNA (5′- TGGCAACGATCTTTCGCCG-3′) (Figs. 3 and 4 and supplemental Figs. S3 and S5). We used control shRNA plasmids that were originally designed to target the p57 or p57 coding sequence but did not reduce p27 or p57 expression (Fig. 1 and 3, respectively). We used shRNA that targets the luciferase gene product as a control shRNA plasmid (Fig. 2). Under the conditions used in this study, no detectable effect on neuronal migration was observed by the expression of these control shRNAs (supplemental Fig. S6). We also verified that neither of the control shRNAs used in this study affected the expression of p27 or p57 (data not shown).

**Retrovirus Production**—Recombinant retroviruses were obtained by transfection of pSIREN vectors into the packaging cell line PLAT-E. Retrovirus particles were collected by centrifugation of culture supernatants of the transfected cells at 6,000 × g for 24 h. Retrovirus particles were collected by centrifugation of culture supernatants of the transfected cells at 6,000 × g for 24 h.

**RESULTS AND DISCUSSION**

Two recent studies have reported that knockdown of p27 in the developing mouse neocortex by the introduction of shRNA targeting p27 causes delayed neuronal migration (10, 11). We independently obtained similar results by the use of a different shRNA against p27, as shown in Fig. 1. p27 shRNA (but not control shRNA) effectively reduced the amount of endogenous p27 protein when introduced into NIH3T3 cells (Fig. 1A). When a plasmid expressing p27 shRNA, together with that of EGFP, was introduced into neuroepithelial cells in the developing neocortex of ICR mice at E14 by in utero electroporation, the immunoreactivity of p27 was reduced only in the GFP-positive cells at E18 (Fig. 1B). Remarkably, in this experiment, expression of p27 shRNA, together with EGFP, caused a significant delay in the radial migration of neocortical cells at E18 compared with the expression of control shRNA with EGFP or EGFP alone (Fig. 1, C and D). Cells expressing p27 shRNA accumulated in the intermediate zone and the lower cortical plate.
suggesting that cell migration from the intermediate zone to and through the cortical plate was impaired. A delay in neuronal migration was also observed when p27 shRNA was introduced into the neocortical neuroepithelium of mice of a C57/BL6J background at E15, and the cellular position was analyzed at E19 (supplemental Fig. S1).

We also examined the role of p27 in neocortical neuronal migration by analyzing mice in which the p27 gene had been deleted. Neuronal migration was investigated by a pulse administration of BrdUrd at E15 and subsequent detection of BrdUrd-positive cells in the brain at E19. To our surprise, neuronal migration was only slightly affected in homozygous (p27−/−) C57/BL6J mice compared with their wild-type littermates (Fig. 1E). Although the number of BrdUrd-positive cells in the upper cortical plate decreased slightly in p27−/− mice (Fig. 1F), this might be attributable to the enlargement of the brain and increase in the migration distance between the ventricular zone and cortical plate in these mice, as previously reported (5–7). Deletion of the p27 gene had little effect on the percentage of cells localized at the intermediate zone (Fig. 1F). We also observed only subtle defects in neuronal migration in p27−/− deficient mice when BrdUrd was administered at E14 and detected at E17 or E18 (supplemental Fig. S2; data not shown). Thus, long term loss of the p27 gene might induce some compensatory mechanism that substitutes for its function in neuronal migration.

We therefore examined whether reduction of p27 expression affects the levels of p27-related molecules in neocortical cultures. The Cip/Kip family of Cdk inhibitors consists of three members, p21, p27, and p57. We focused on p57, because p27 and p57 are structurally related and genetically interact in the developing retina (14). When endogenous p27 was knocked down by RNA interference in neuroepithelial culture prepared from E11 mouse neocortex, the level of p57 mRNA was significantly increased, whereas that of p21 mRNA was unaffected (Fig. 2A). This suggested that p57 could play a compensatory role in p27−/− mice in differentiating neurons. Supporting a possible role for p57 in neurons (together with p27), we observed that the amounts of p57 and p27 mRNA (but not those of p21

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FIGURE 2. Expression patterns of p27 and p57 during neocortical development. A, primary neuroepithelial cells were infected with retrovirus expressing GFP alone or GFP with control or p27 shRNA in the presence of FGF2 and EGF for 24 h. Three days after withdrawal of FGF2 and EGF, cells were harvested and subjected to RT-PCR analysis for the indicated mRNAs. Error bar, S.E. B, primary neuroepithelial cells prepared from E11 mouse neocortex were plated onto a poly-L-lysine-coated dish and cultured with (open bar) or without (closed bar) FGF2 and EGF. One or two days later, cells were harvested and subjected to RT-PCR analysis for the indicated mRNAs. Error bars, S.E. C and D, immunohistochemistry of p57 at E15 (C) or p27 at E14 (D) mouse neocortex. Scale bars, 100 μm (C and D).

mRNA were increased upon neuronal differentiation induced by removal of growth factors (fibroblast growth factor (FGF) 2 and epidermal growth factor (EGF)) in these neuroepithelial cultures (Fig. 2B). In the developing neocortex, p57 protein was mainly localized in the cortical plate and to a lesser extent in the intermediate zone (Fig. 2C), as described by a previous report (11). On the other hand, p27 staining was found to be intense in the intermediate zone and cortical plate and faint in the subventricular and ventricular zones, as previously reported (Fig. 2D) (11). These results were consistent with the possibility that p57 may be up-regulated when p27 is absent or when neurons exit the cell cycle, and p57 may thus partially substitute for deletion of the p27 gene.

Because p57 is expressed in newly differentiated neurons, we next investigated whether p57 plays a role in neuronal migration. We designed two different shRNAs targeting p57 (p57 shRNA numbers 1 and 2), which effectively, although not completely, reduced the amounts of endogenous p57 protein in mouse embryonic fibroblasts (Fig. 3A) and endogenous p57 mRNA in primary neuroepithelial cultures (Fig. 3B). This partial reduction of p57 might be in part because of the inefficient viral infection under the conditions used in this study (60–70% efficiency) (Fig. 3, A and B). When p57 shRNA numbers 1 and 2 (but not control shRNA) was introduced into E14 mouse cortex by in utero electroporation, we observed impaired radial neuronal migration when the neuronal localization was analyzed at E18 (Fig. 3, D and E). In brains expressing control shRNA together with EGFP or EGFP alone, most GFP-positive cells migrated to reach the superficial layer. On the other hand, many cells expressing p57 shRNA number 1 or 2 still had not reached the brain surface (Fig. 3, D and E). We confirmed by immunohistochemistry that the immunoreactivity of p57 was indeed reduced in the migrating neurons harboring p57 shRNA number 2 and GFP but not GFP alone (Fig. 3C). In contrast to the effects of p27 knockdown (see the effects of p27 shRNA in Fig. 1, C and D), most of the cells expressing p57 shRNA number 1 or 2 migrated beyond the intermediate zone but accumulated in the middle and lower cortical plate (Fig. 3, D and E).

We next asked what might have caused impaired neuronal migration within the cortical plate in the absence of p57. Neuronal migration can be affected by radial scaffolds (radial fibers of neural precursor cells) that support neuronal migration in a non-cell autonomous manner. We performed immunostaining of radial fibers with an antibody against Nestin, a marker of neural precursor cells. We found no distinguishable differences in the radial fibers of neuroepithelial cells expressing p57 shRNA number 2 versus those of normal neuroepithelial cells (supplemental Fig. S3). In addition, because migration defects could be observed even when p57 was knocked down in only a small number of neuroepithelial cells in the neocortex (data not shown), it is likely that cell-autonomous mechanisms are crucial for the effects of p57 on neuronal migration. It has been established that cytoskeletal organization plays a central role in regulating neuronal morphology and migration in a cell-autonomous manner. We, however, observed no gross morphological changes of neurons expressing p57 shRNA number 2 (supplemental Fig. S4A). Moreover, expression of p57 shRNA number 2 had little effect on F-actin organization, as detected by staining with Alexa 594-labeled phalloidin (supplemental Fig. S4B). Localization of microtubule-associated protein 2, which identifies microtubule networks, was also unaffected by the reduction of p57 protein by shRNA number 2 (supplementary Fig. S4C). We then examined the possibility that p57 affects neuronal migration through its potential to regulate neuronal differentiation. To test this,
we electroporated a plasmid expressing p57 shRNA into the neuroepithelial cells of mouse neocortex at E14 and examined the neuronal differentiation of the plasmid-expressing (GFP-positive) cells at E16. Neuronal differentiation was examined by immunohistochemistry using the pan-neuronal markers βIII-tubulin (detected by an antibody called TuJ1) and HuC/D, which are expressed both in immature and mature neurons. βIII-tubulin is localized mainly within the microtubule network of neurons, whereas HuC/D is localized predominantly to the cell soma (23). Knockdown of p57, however, did not affect neuronal differentiation (Fig. 4 and supplemental Fig. S5). These results do not necessarily mean that p57 is dispensable for neuronal differentiation or establishment of cell morphology, given that the knockdown of p57 was not complete, but suggest that the migration defects observed by p57 knockdown were not a secondary effect caused by gross changes in either of these processes. These results together suggest that p57 directly controls neuronal migration in the developing neocortex.

In this study, we found that p57 regulates radial migration of newly differentiated neurons within the neocortex. Knockdown of p57 caused delayed migration within the cortical plate. This delay appeared to be independent of radial fiber formation or the timing of neuronal differentiation, because knockdown of p57 had little effect on either of these events. It is thus unclear how p57 regulates neuronal migration.

p57 has been shown to sequester LIM-kinase into the nucleus and inactivate it, resulting in the activation of actin-depolymerizing factor cofilin, which is usually phosphorylated and inactivated by LIM-kinase in the cytoplasm (24). However, we could not detect gross changes in F-actin organization caused by p57 knockdown (supplemental Fig. S4B), although this does not exclude the possibility that actin organization is affected locally, such as in the leading process of migrating neurons. Further study is necessary to elucidate the function of p57 and its downstream effectors in neuronal migration.

In addition to p57, we have also revealed that p27 regulates neuronal migration by introducing p27 shRNA into developing mouse neocortex, in agreement with two recent reports (10, 11). p27 has been shown to affect actin organization by inhibiting the interaction between RhoA and its guanine-nucleotide exchange factors via its C-terminal domain (9). Because p57 and p27 share a common QT domain in their C-terminal domains, it would be interesting to examine whether the QT domain contributes to the regulation of actin organization and

FIGURE 3. p57 plays an important role for neuronal migration in the developing mouse neocortex. A and B, reduction of p57 by RNA interference. Mouse embryonic fibroblasts (A) or primary neuroepithelial cells prepared from E11 mouse neocortex (B) were left uninfected (mock) or infected with retrovirus expressing either GFP alone or GFP with control or p57-targeting shRNA (number 1 or 2). In A, 4 days after infection, cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. In B, 24 h after infection in the presence of FGF2 and EGF, cells were further cultured in the absence of these growth factors for 3 days and then harvested. The cell lysates were subjected to RT-PCR analysis for the indicated mRNAs. Error bars, S.E. C–E, plasmids expressing GFP alone or GFP with control or p57 shRNA (number 1 or 2) were injected into the lateral ventricle of E14 ICR mouse and electroporated into the dorsolateral region of the neocortex. Brains were excised 4 days later and examined. C, brain sections were stained with anti-p57 antibody. Arrows indicate both GFP (green) - and p57 (red)-positive cells, whereas arrowheads indicate GFP-positive but p57-negative cells. D shows typical results, and E shows distribution of GFP-positive cells. Data are the mean ± S.E. of values from five samples, and similar results were obtained in three independent experiments. Scale bars, 50 μm (C), 200 μm (D).
neuronal migration in future studies. p57 and p27 may have overlapping functions in the regulation of neuronal migration, but they may also have distinct functions, because the phenotypes resulting from their respective knockdowns are somewhat different (Figs. 1C and 3D); knockdown of either p57 or p27 resulted in a delayed neuronal migration within the cortical plate, whereas knockdown of p27 (but not of p57) resulted in accumulation of migrating neurons in the intermediate zone. It is possible that the differential expression patterns of these proteins account for this difference, because p27 (but not p57) is expressed in the newly differentiating neurons localized in the intermediate zone, whereas both proteins are expressed in the neurons in the cortical plate (Fig. 2, C and D).

In the developing neocortex, neuronal fate commitment is linked to cell cycle exit and radial migration. We found that p57, in addition to p27, is induced upon neuronal differentiation and that p57 plays an essential role in neuronal migration. We therefore propose that the cell cycle inhibitor p57 may act in concert with p27 in coordinating the timing of three events that are key to neocortical development, neuronal differentiation, cell cycle exit, and neuronal migration, and thereby contribute to the establishment of the finely organized neocortical structure.

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FIGURE 4. Effects of p57 knockdown on neuronal differentiation. A–C, expression plasmids of GFP alone or GFP with control or p57 shRNA (number 1 or 2) were injected into a lateral ventricle of E14 ICR mouse and electroporated into the dorsolateral region of the neocortex. Brains were excised 36 h (A) and examined. The neocortical coronal sections were subjected to immunohistochemistry with antibodies to the pan-neuronal marker HuC/D (A) or TuJ1 (B and C). The percentage of TuJ1- or HuC/D-positive cells among GFP-positive cells was determined. Data are the mean ± S.E. of values from five samples.
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Regulation of Neuronal Migration by cdk Inhibitors