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A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord

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Abstract

Control over cell cycle exit is fundamental to the normal generation of the wide array of distinct cell types that comprise the mature vertebrate CNS. Here, we demonstrate a critical role for Cip/Kip class cyclin-kinase inhibitory (CKI) proteins in regulating this process during neurogenesis in the embryonic spinal cord. Using immunohistochemistry, we show that all three identified Cip/Kip CKI proteins are expressed in both distinct and overlapping populations of nascent and post-mitotic neurons during early neurogenesis, with $p27^{Kip1}$ having the broadest expression, and both $p57^{Kip2}$ and $p21^{Cip1}$ showing transient expression in restricted populations. Loss- and gain-of-function approaches were used to establish the unique and redundant functions of these proteins in spinal cord neurogenesis. Using genetic lineage tracing, we provide evidence that, in the absence of p57, nascent neurons re-enter the cell cycle inappropriately but later exit to begin differentiation. Analysis of $p57^{Kip2};p27^{Kip1}$ double mutants, where p21 expression is confined to only a small population of interneurons, demonstrates that Cip/Kip CKI-independent factors initiate progenitor cell cycle exit for the majority of interneurons generated in the developing spinal cord. Our studies indicate that p57 plays a critical cell-autonomous role in timing cell cycle exit at G1/S by opposing the activity of Cyclin D1, which promotes cell cycle progression. These studies support a multi-step model for neuronal progenitor cell cycle withdrawal that involves $p57^{Kip2}$ in a central role opposing latent Cyclin D1 and other residual cell cycle promoting activities in progenitors targeted for differentiation.

Keywords: Neurogenesis; Stem cells; Proliferation; Neural tube; Differentiation; Progenitor; Cyclin; $p57^{Kip2}$; $p27^{Kip1}$; $p21^{Cip1}$

Introduction

The vertebrate central nervous system (CNS) contains thousands of functionally distinct neuronal cell types that are produced within a brief period during embryogenesis. Normal CNS function depends critically on the generation of both the correct types and numbers of neurons in their proper positions and at the proper time. Post-mitotic neurons arise from multipotent progenitors that can give rise to multiple distinct progeny types, making it critical to balance proliferation with cell cycle withdrawal so that the appropriate number of cells are generated at any given interval while also leaving sufficient numbers of progenitors available to generate subsequent neuronal, and glial, types. Although the core cellular mechanisms regulating progenitor cell cycle exit and differentiation in the CNS are similar to other developing organ systems, it is unclear whether similar factors play equivalent roles in all tissues or whether context-dependent differences exist in their function.

The current study addresses these issues using the vertebrate spinal cord as a model system. In this structure, distinct classes of neurons are generated during neurogenesis in discrete domains along the dorsoventral (DV) axis, and derive from similarly organized progenitor domains (Jessell, 2000). In the spinal cord, the production of post-mitotic neurons from neuronal progenitors (neurogenesis) begins shortly after the formation of the neural tube, and is largely complete by mid-gestation. As in most

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other regions of the developing CNS, proliferating cells in the spinal cord reside in the ventricular zone (VZ) and undergo interkinetic nuclear migration during progression through the cell cycle. During this process, cell nuclei translocate between the lumenal and pial sides of the VZ, with the position of the nucleus correlating with phases of the cell cycle: nuclei close to the lumen medially are in M-phase, those located laterally towards the pial surface are in S-phase, and those that are in transit are in G1 or G2.

Studies in cell culture have shown that the decision to continue or withdraw at each cycle is controlled by mitogen availability and is mediated by D-type cyclins at a point late in G1 phase, termed R (for restriction point; Sherr and Roberts, 1999). Many signals are thought to exert their control over the cell cycle at R. For example, various mitogens and integrin-mediated ECM signals stimulate cell cycle progression, while factors such as TGF-β can induce cell cycle withdrawal (Assoian and Schwartz, 2001). Completion of a cell cycle (passage through M-phase) after crossing R is independent of further inputs (Sherr and Roberts, 1999). In the spinal cord, nascent neurons that withdraw from the cell cycle enter a G0 state and migrate out of the VZ into the mantle zone (MZ), an area of accumulating neurons that becomes the adult gray matter. This transition takes place when their nuclei are positioned laterally in the VZ during late G1 phase.

A number of factors have been identified whose expression is either initiated in or confined to cells near the lateral margins of the VZ during neurogenesis, making them good candidates for regulating the transition of progenitor cells into neurons. Among these are $p27^{Kip1}$ and 57^{Kip2}, two of the three members of the vertebrate Cip/Kip family of cyclin-dependent-kinase inhibitor (CKI) proteins (Sherr and Roberts, 1999; Zhang, 1999). The expression and function of the third member, $p21^{Cip1}$, has not been characterized extensively in the developing CNS.

Several lines of evidence indicate that Cip/Kip factors play an important role in many tissues to prevent cell cycle progression at the G1/S restriction point (R) via control of Rb phosphorylation, a factor that is a critical regulator of the E2F factors that control the transcription of S-phase DNA synthesis genes (Macleod, 1999; Hamel et al., 1992). First, the expression of these factors is largely confined to post-mitotic cells (Nagahama et al., 2001; Westbury et al., 2001). Second, loss-of-function studies in mice have demonstrated that $p27^{Kip1}$ and $p57^{Kip2}$ mutants exhibit various proliferative defects consistent with their requirement in arresting mitosis in various organs of the body (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et al., 1997; Zhang et al., 1998). Third, gain-of-function experiments have shown that these proteins are capable of driving proliferating cells out of the cell cycle (Sherr and Roberts, 1999; Dyer and Cepko, 2000, 2001).

In the mammalian CNS, the role of p27 and p57 has been studied in retinal histogenesis. In the absence of each of these CKIs, additional rounds of cell division are seen, along with changes in the proportions of retinal sub-types depending on which Cip/Kip CKI is lost (Dyer and Cepko, 2000, 2001). These studies are consistent with a model where individual Cip/Kip CKIs are required for cell cycle exit in unique sub-populations of cells, and suggest that different populations employ distinct mechanisms to control cell cycle exit. However, a number of questions remain unanswered. First, it is unclear whether differences in the requirement for these factors represent functional differences between the proteins, or are related to specific patterns of expression in different tissues. Although previous studies have shown that there is some functional redundancy among and between the two major families of CKI proteins (Cip/Kip and INK4), it has not yet been determined whether all CKI activity is required for cell cycle exit. Second, prior studies have not established whether the requirement for Cip/Kip CKIs is cell-autonomous. Because loss of cell cycle control in vivo often leads to apoptosis and non-cell-autonomous tissue defects, this question bears not only on understanding Cip/Kip CKI function but also on the broader mechanisms that balance neuronal proliferation with differentiation. Finally, although transfection studies in cell culture have demonstrated the ability of Cip/Kip CKI factors to block cell cycle progression via inhibition of G1 cyclin (cyclin D) activities, it is unclear whether similar mechanisms are responsible for this effect in vivo (Dyer and Cepko, 2000).

In this study, we set out to address these issues using a combination of in vivo approaches in the embryonic mouse and chick spinal cord. We find that all three identified Cip/Kip proteins are expressed in subsets of neurons in the spinal cord during neurogenesis, with both p21 and p57 being expressed transiently in the nuclei of distinct sub-classes of nascent interneurons exiting the ventricular zone, while p27 expression is initiated and maintained in the nuclei of post-mitotic neurons in the mantle zone. Thus, most or all post-mitotic neurons within the mouse spinal cord express at least one, and sometimes two, Cip/Kip CKI proteins at early neurogenic stages. In contrast, some INK4-family CKIs are only expressed in neuronal progenitor cells at these stages and therefore are not likely to function in controlling cell cycle exit or differentiation decisions (Zindy et al., 1997a,b). To determine the requirement for Cip/Kip CKI proteins in spinal cord neurogenesis, we examined the development of single and compound mutant mice. We find that loss of *p57* results in the excess production of most, but not all, classes of early-generated interneurons. In contrast, loss of *p27* has no consequences on early neurogenesis. Utilizing a method to genetically mark cells that express p57, we find that in the absence of this factor, many interneurons re-enter the cell cycle for at least one additional round of cell division before ultimately differentiating. We also demonstrate the requirement of the N-terminal CKI domain of p57 in functioning downstream of Cyclin D1 to arrest proliferation in progenitors. Finally, our data shows that neuronal differentiation can proceed in the spinal cord, albeit abnormally, in the absence of all Cip/Kip CKI proteins. Our studies support a model whereby cell cycle exit involves multiple steps, with exit being triggered upstream of Cip/Kip CKIs, which function primarily to control the specific timing and number of spinal interneurons by opposing latent Cyclin D1 and other residual cell cycle promoting activities in progenitors targeted for differentiation.