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Pituitary Adenylate Cyclase Activating Polypeptide Anti-Mitogenic Signaling in Cerebral Cortical Progenitors Is Regulated by $p57^{Kip2}$-Dependent CDK2 Activity

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Generation of distinct cell types and numbers in developing cerebral cortex is subject to regulation by extracellular factors that positively or negatively control precursor proliferation. Although signals stimulating proliferation are well described, factors halting cell cycle progression are less well defined. At the molecular level, production and association of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) regulate cycle progression. We now report that the endogenous peptide, pituitary adenylate cyclase activating polypeptide (PACAP), negatively regulates the cell cycle by inhibiting $p57^{Kip2}$-dependent CDK2 activity in embryonic cortex. Protein levels of CDK2 and members of the CIP/KIP family of CKIs ($p27^{Kip1}$, $p57^{Kip2}$) were detected in developing rat cortex from embryonic day 13.5 through postnatal day 2. With advancing development, CDK2 protein levels decreased, whereas CKI expression increased, suggesting that stimulatory and inhibitory cycle proteins control cell cycle exit. Using a well defined, nonsynchronized, 8 hr precursor culture, PACAP decreased the fraction of cells crossing the G_1/S boundary, inhibiting DNA synthesis by 35%. CDK2 kinase activity was inhibited 75% by PACAP, whereas kinase protein and its regulatory cyclin E subunit were unaffected. Moreover, decreased kinase activity was accompanied by a twofold increase in levels of $p57^{Kip2}$ protein, but not $p21^{Cip1}$ or $p27^{Kip1}$, suggesting that $p57^{Kip2}$ mediates PACAP anti-mitogenic effects. Indeed, immunoprecipitation of CDK2 complex revealed increased $p57^{Kip2}$ association with the kinase and concomitant reduction in free inhibitor after PACAP exposure, suggesting that $p57^{Kip2}$ interactions directly regulate CDK2 activity. These observations establish a mechanism whereby anti-mitogenic signals actively induce cell cycle withdrawal in developing cortex.

Key words: PACAP; neurogenesis; proliferation; stem cells; neuronal precursors; cyclin-dependent kinase inhibitors (CKIs)

The mammalian cerebral cortex comprises six distinct layers of neurons generated from the underlying proliferative ventricular zone (VZ). Although precursors sequentially exit the cell cycle to produce layer-specific neurons, mechanisms remain undefined (Angevine and Sidman, 1961; McConnell, 1988; Rakic, 1988; Caviness et al., 1995). Current models suggest that proliferation is controlled by interacting pro-mitogenic and anti-mitogenic signals (Temple and Qian, 1995; Cameron et al., 1998; Suh et al., 2001). Factors stimulating proliferation in culture and *in vivo* are well documented, including bFGF, EGF, and IGF-I (Ghosh and Greenberg, 1995; Kilpatrick and Bartlett, 1995; Vaccarino et al., 1995, 1999; Burrows et al., 1997; Cavanagh et al., 1997; Qian et al., 1997; Lillien and Raphael, 2000), whereas inhibitory signals, GABA and glutamate, have been defined only *in vitro*, acting after embryonic day (E) 15 (LoTurco et al., 1995; Antonopoulos et al., 1997).

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Recently, anti-mitogenic signaling in developing cortex has been defined. Pituitary adenylate cyclase activating polypeptide (PACAP) is widely expressed during neurogenesis, exhibiting population-specific inhibition (Lu and DiCicco-Bloom, 1997; DiCicco-Bloom et al., 1998, 2000; Waschek et al., 1998; Nicot and DiCicco-Bloom, 2001). In cortex, PACAP ligand and receptor are expressed (Tatsuno et al., 1994; Lu et al., 1998; Suh et al., 2001) as neurons are first born on E13.5 (Hicks and D'Amato, 1968; Konig et al., 1977). In cultures and embryos, PACAP activates its G-protein-coupled receptor, eliciting cAMP in minutes and inhibition in 4–8 hr. Furthermore, endogenous PACAP provides ongoing anti-mitogenic signaling as precursors progressively exit the cycle, functioning in an autocrine manner (Lu and DiCicco-Bloom, 1997; Suh et al., 2001). To investigate mechanisms, we defined PACAP effects on cycle machinery.

Antagonistic extracellular signals are mirrored by positive and negative intracellular regulators, coordinating cycle stages G_1, DNA synthetic S-phase, G_2, and mitosis. Extracellular signals act during G_1 to determine commitment to DNA synthesis and mitosis. Cycle progression is controlled by pro-mitogenic cyclin-dependent kinases (CDKs) complexed with regulatory cyclins, and anti-mitogenic CDK inhibitors (CKIs). CDK proteins remain constant throughout the cycle, whereas their activity is modulated by cyclin and CKI association. Although early G_1 depends on cyclin D-dependent kinases, the late G_1/S transition critically depends on cyclin E/CDK2 complexes, which phosphorylate ret-

inoblastoma protein, allowing S-phase gene activation (Sherr and Roberts, 1999).

Although several components underlie mitotic withdrawal, CKIs are major targets of anti-mitogenic growth factors and cAMP (Koff et al., 1993; Kato et al., 1994; Polyak et al., 1994). Of two CKI families, INK4 and CIP/KIP, the latter, including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, bind and inhibit all G_1 CDKs, especially cyclin E/CDK2, preventing G_1/S transition (Sherr and Roberts, 1999). Although CIP/KIP proteins are expressed in dynamic and restricted ontogenetic patterns, only p57 is required for development (Lee et al., 1995; Matsuoka et al., 1995; Parker et al., 1995; Fero et al., 1996; Kiyokawa et al., 1996; Yan et al., 1997; Zhang et al., 1997). Furthermore, $p57^{Kip2}$ expression colocalizes with PACAP systems in E15.5 cortex, suggesting possible interactions (van Lookeren Campagne and Gill, 1998). We now report that PACAP inhibits precursor mitosis and CDK2 activity by selectively increasing $p57^{Kip2}$ protein and kinase association. These observations establish one mechanism whereby endogenous anti-mitogenic signals actively induce cell cycle withdrawal in cortical precursors and suggest cell lineage-specific proliferation control.

MATERIALS AND METHODS

Antibodies. Antibodies against CDK2 (SC-163), cyclin E (SC-481), $p27^{Kip1}$ (SC-528), actin (SC-1615), $p57^{Kip2}$ (SC8298; SC1039), and $p21^{Cip1}$ (SC-397) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against $p21^{Cip1}$ (65951A) was purchased from PharMingen (San Diego, CA). Polyclonal antibody against p57 was a gift from Dr. Pumin Zhang (Baylor College of Medicine, Houston, TX). Monoclonal antibody KP10 against $p57^{Kip2}$ was a gift from Dr. Ed Harlow (Harvard Medical School, Boston, MA). Biotin-conjugated secondary antibodies were purchased from Boehringer Mannheim (Indianapolis, IN), Santa Cruz Biotechnology, and Promega (Madison WI).

Cortical cell culture and characterization. Time-mated pregnant Sprague Dawley rats were obtained from Hilltop Labs (Philadelphia, PA). At E14.5, embryonic skull and meninges were removed and dorsolateral cerebral cortex was dissected, mechanically dissociated, and plated, at $3–4 \times 10^6$ cells, 2×10^6 cells, or 10^5 cells, on poly-D-lysine (1 μg/ml)-coated 60 mm dishes, 35 mm dishes, or 24-multiwell plates, respectively, in defined medium (insulin omitted), as described previously (Lu and DiCicco-Bloom, 1997). Culture medium was composed of a 50:50 (v/v) mixture of DMEM and F12 (Invitrogen, Grand Island, NY) containing penicillin (50 U/ml) and streptomycin (50 μg/ml) and supplemented with transferrin (100 μg/ml) (Calbiochem, La Jolla, CA), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), and bovine serum albumin (10 mg/ml). Unless stated otherwise, components were obtained from Sigma (St. Louis, MO). Cultures were maintained in a CO_2 incubator. PACAP (American Peptide, Sunnyvale, CA) or vehicle (1 mg BSA/ml PBS) was added at plating.

To characterize the phenotype of precursors that were mitotic during the 8 hr incubation period, two distinct strategies were used. The very low poly-D-lysine (1 μg/ml) concentration used to enhance proliferation had the disadvantage of reducing adhesion to such an extent that precursors were lost during the multiple washes required for immunocytochemistry. Furthermore, high cell density precluded analysis of cells in clumps, especially after several days in culture, which promoted differentiation. To address these issues, cells were incubated at usual high density, 2×10^6 cells/35 mm dish, and low adhesion conditions, in the presence of bromodeoxyuridine (BrdU; 10 μM) for the entire 8 hr incubation, labeling all cells in S-phase potentially responsive to PACAP. To remove BrdU after the 8 hr, cells were lifted by gentle pipetting, pelleted at 2500 rpm for 10 min, resuspended twice in PBS, and replated in two conditions. To promote adhesion and single cell analysis, cells were plated at low density, $1–2 \times 10^5$/35 mm dish, on standard poly-D-lysine (100 μg/ml), and incubated for 2 and 3 d before fixation. However, because these conditions may possibly lead to different outcomes than the maintenance of cell–cell contact, another group was replated under the usual high density and low adhesion conditions. Then at 2 and 3 d, cells were lifted and dissociated using a trypsin (0.05%)/EDTA (0.5 mM)/saline solution, exposed to trypsin inhibitor (2 mg/ml), replated at low density with standard poly-D-lysine (100 μg/ml), and fixed 3 hr later for characterization by double immunocytochemistry. Cells were processed for markers of precursors, nestin (1:1000; developed by S. Hockfield, provided by Developmental Studies Hybridoma Bank, University of Iowa), neurons, βIII tubulin (1:1000, TuJ1, clone TU-20; Biogenesis, Poole, UK), and MAP-2 (polyclonal, 1:1000; I. Fischer, Medical College of Philadelphia), astrocytes, GFAP (polyclonal, 1:1000, Chemicon AB5040; Chemicon, Temecula, CA), and oligodendrocytes, MBP (monoclonal, 1:50, MCA184S; Serotec, Kidlington, Oxford, UK) and NG2 (polyclonal, 1:750, Chemicon AB5320) as described previously (Nicot and DiCicco-Bloom, 2001). Staining was visualized using a Vectastain ABC Kit and DAB reaction (Vector, Burlingame, CA). To visualize BrdU labeling, cells were fixed again, exposed to 2N HCl (30 min), rinsed twice in PBS, incubated with monoclonal anti-BrdU (1:100; Becton-Dickinson, San Jose, CA), followed by FITC-conjugated secondary antibody, as described previously (Lu and DiCicco-Bloom, 1997; Nicot and DiCicco-Bloom, 2001). Approximately 100 BrdU (+) cells in two to three dishes per antigen and condition were analyzed under epifluorescent microscopy and scored for expression of neural markers under bright field at both 2 and 3 d of incubation. Negative controls consisted of no primary antibody or glial and neuronal cultures for the neuronal and glial antigens, respectively. Positive controls for glia (kindly provided by C. Dreyfus, Robert Wood Johnson Medical School) were virtually pure P1 astrocytes or oligodendrocytes. Because the data at 2 and 3 d were similar, the latter data alone are reported. In addition, the percentages of cellular expression were no different under the two culture paradigms, so the data were summed and are presented in Table 1.

DNA synthesis. Incorporation of [^{3}H]thymidine ([^{3}H]dT) into polymerized DNA was used as a marker of cells in the mitotic cycle (Lu and DiCicco-Bloom, 1997). For time course experiments, cells were incubated with [^{3}H]dT (1 μCi/ml; Amersham, Arlington Heights, IL) for 1 hr for the 1 hr point, and the terminal 2 hr for other time points. For standard 8 hr experiments, cells were incubated with [^{3}H]dT for the terminal 3 hr. Subsequently, cells were collected with a semiautomatic harvester (Skatron, Sterling, VA), and incorporation was assessed by scintillation spectroscopy, as described previously.

Cell cycle analysis. Cells were detached from plates using trypsin (0.05%)/EDTA (0.5 mM)/saline and transferred to a polypropylene tube. A single cell suspension was made by repeated pipetting through a 1000 μl pipette tip. Cells were pelleted and resuspended in 0.1 M PBS. One hundred microliters of RNase (1 mg/ml) were added to the tube and incubated for 5 min at 37°C. Next, 5 μl of propidium iodide was added and incubated for an additional 5 min. at 37°C. Cells were then analyzed by a flow cytometer.

Protein extract preparation. Cells were detached from 60 mm culture plates using a rubber policeman in PBS, pH 7.4. Cells were pelleted and resuspended in buffer (20 mM HEPES-KOH, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$), 0.5 mM dithiothretiol (DTT), 2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 3.5 μg/ml aprotinin (Tikoo et al., 1997). Cells were lysed by sonication and centrifuged to separate the pellet. Protein extracts were adjusted to 0.1 M NaCl and then stored at −80°C.

Western blotting. Protein extracts (30–100 μg per lane) were analyzed by 12% SDS-PAGE. Protein was transferred from gel to polyvinylidene difluoride (PVDF) membrane by transfer apparatus at 30 V for 4 hr. The membrane was blocked with 5% BSA or 5% milk and incubated with primary antibody against cyclin, CDK, or inhibitor. After incubation with anti-rabbit, anti-goat, or anti-mouse horseradish peroxidase-conjugated secondary antibody, protein was visualized using enhanced chemiluminescence system (Pierce, Rockford, IL).

Histone H1 kinase assay. Protein extracts (20–40 μg per sample) were incubated for 30 min at 37°C in the presence of assembly buffer containing 20 mM HEPES-KOH, pH 7.5, and 7 mM $MgCl_2$, and 40 mM phosphocreatine, 0.16 mg/ml creatine phosphokinase (1:1, glycerol/H_2O), 12 mM ATP, 2 mM DTT (Sigma), and exogenous recombinant cyclin E (gift of Moses Chao, Skirball Institute, New York University) (Tikoo et al., 1997). Exogenous cyclin E allowed better detection of kinase reaction without changing the intrinsic activity of protein extracts (Koff et al., 1993). The assembled extracts were subjected to immunoprecipitation in 0.5% Nonidet P-40-radioimmune precipitation (NP40-RIPA) buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA) for 1 hr at 4°C in the presence of antibody against CDK2 followed by a 1 hr incubation with immobilized protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ). The beads were washed twice with NP40-RIPA buffer and then four times with kinase assay buffer (20 mM Tris,

pH 7.4, 7.5 mM $MgCl_2$, 1 mM DTT). Phosphorylation of histone H1 was performed by incubating beads in a 50 μl reaction mixture containing 10 μCi of [γ-^{32}P] ATP (DuPont NEN, Boston, MA), 30 μM disodium ATP (Sigma), and 1 μg of histone H1 (Boehringer Mannheim) at 37°C for 30 min. After incubation, the supernatants were resolved by 12% SDS-PAGE. The gel was dried and subjected to autoradiography.

Immunoprecipitation. Cortical cell lysates (200 μg) were incubated on ice for 30 min with several dilutions of CDK2 primary antibody. Fifty microliters of protein A-Sepharose (Pharmacia Biotech) were added for an additional 1 hr at 4°C. Precipitates were washed five times with 900 μl of 0.5% NP40-RIPA buffer before electrophoresis and Western transfer.

Densitometric analysis and image production. Autoradiographic films were analyzed for intensity using the Bio-Rad Gel Doc 2000 quantifying signal with Quantity One version 4.2.1 software. Images were entered into a Macintosh Power Mac G4 computer using the UMAX PowerLook 1100 scanner, using magic scan 4.4 program, and converted to Adobe Photoshop files using version 5.0 for composition and printing.

RESULTS

Expression of cell cycle proteins during cerebral cortical development

Previous studies have examined mRNA expression patterns of CIP/KIP family members during rodent embryogenesis (van Lookeren Campagne and Gill, 1998; Delalle et al., 1999). p57^{Kip2} mRNA was detected in the VZ, the subventricular zone (SVZ), and the cortical plate (CP) of the neocortex at E15.5 and E17.5. Similarly, p27^{Kip1} mRNA expression was intense in the VZ at E13.5 and in VZ and SVZ from E15.5 through E20. In contrast, p21^{Cip1} mRNA was absent at E15.5 and E17.5 with exception of the ependymal layer and choroid plexus. To further characterize expression of CIP/KIP family members, we examined protein levels in fresh brain tissue extracts throughout the period of cortical neurogenesis by immunoblot. p57^{Kip2} protein was expressed in rat cortex at E14.5, the earliest age examined. Protein levels increased from E14.5 to E15.5, remained constant through E18.5, and increased again by postnatal day (P) 2 (Fig. 1). p27^{Kip1} protein levels were also detected at E14.5 and exhibited a sustained increase thereafter (Fig. 1). In contrast, we were only able to detect low levels of p21^{Cip1} at E14.5, using multiple antibodies, consistent with other expression studies (Parker et al., 1995; van Lookeren Campagne and Gill, 1998). The progressive increase in CIP/KIP family protein expression raises the possibility of regulatory functions during neurogenesis, as cells progressively exit the cycle.

Progress through early G_1 phase depends on CDK4/6 activity, whereas activation of CDK2 is essential to pass the G_1/S boundary and initiate DNA synthesis. We therefore examined CDK2 protein levels and kinase activity throughout development, as an index of proliferative cells. CDK2 kinase activity was intense at E14.5 and decreased to barely detectable levels by P2 (Fig. 2), paralleling the decrease in precursor proliferation in developing cortex (Caviness et al., 1995; Takahashi et al., 1999). Interestingly, CDK2 protein levels decreased in parallel with kinase activity; however, by P2, CDK2 protein was present without detectable activity (Fig. 2). The absence of CDK2 activity may be expected as a result of the presence of both p57^{Kip2} and p27^{Kip1} inhibitors, because association with the CDK2 complex (see below) is known to inhibit kinase activity (Sherr and Roberts, 1999). Alternative although less likely reasons for different levels of protein and kinase activity may include differential assay sensitivities or different levels of CDK2 phosphorylation, which defines catalytically active and inactive forms (Koff et al., 1993).

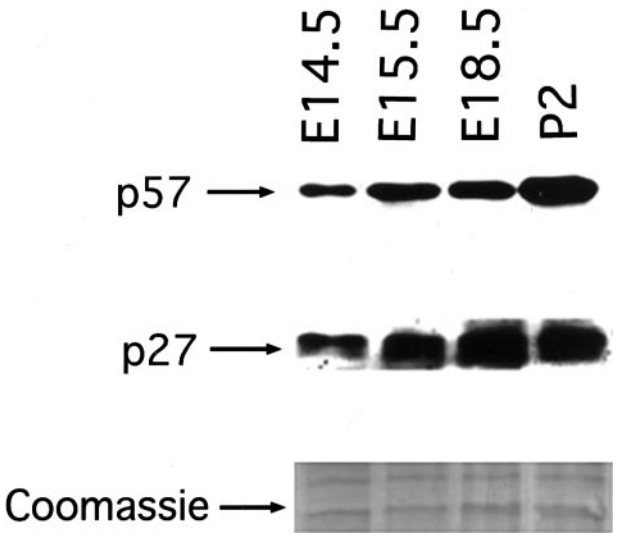

Figure 1. Developmental time course of p57^{Kip2} and p27^{Kip1} protein expression. Cell extracts were prepared from rat embryo cortices at E14.5, E15.5, E18.5, and P2 and were separated (50 μg per sample) by 12% SDS-PAGE, transferred to PVDF membrane, and then incubated with antibody to p57^{Kip2} (KP10 mouse monoclonal) or p27^{Kip1}. Visualization was by the enhanced chemiluminescence technique. The experiment was repeated three times with identical results. p57^{Kip2} and p27^{Kip1} protein levels increased during development. Coomassie blue staining is shown at the *bottom* to demonstrate equal protein loading, because reprobing of immunoblots with actin antibody revealed developmental regulation of the cytoskeletal protein.

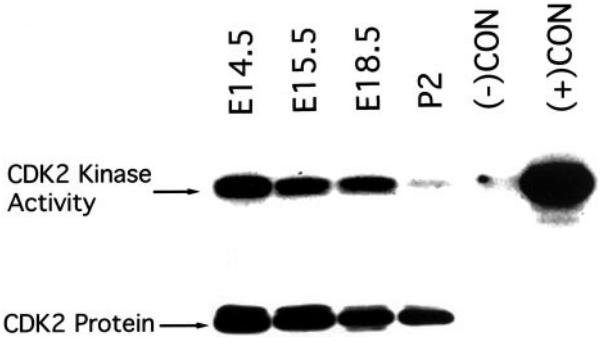

Figure 2. Developmental time course of CDK2 kinase activity and protein levels. Cell extracts were prepared from rat cortices at various ages and subjected to histone H1 kinase assay as detailed in Materials and Methods. Hela cells were used as positive control [*(+)CON*], and no extract was used as negative control [*(−)CON*]. CDK2 activity decreased during development. Extracts were subjected in parallel to Western blot analysis. CDK2 protein levels decreased during development. Data represent one of three experiments that yielded similar results.

PACAP decreases S-phase entry and DNA synthesis in cortical progenitor cells

In previous studies of cortical precursors in low-density culture (10^5 cells per dish), >90% of the cells expressed MAP2 and NSE by 24 hr, whereas all cells exhibited these markers by 2 d. PACAP inhibited precursor DNA synthesis without inducing cell death, acting via the cAMP pathway (Lu and DiCicco-Bloom, 1997). Although mitotic inhibition reached a peak of 43% at 24 hr, effects were initiated rapidly, with 25% inhibition by 6 hr. Furthermore, in addition to effects of exogenous PACAP, the endogenous peptide apparently plays a neurogenetic role, because blockade of PACAP receptor activity elicited mitotic stimulation,

A

Group	G_0/G_1 (%)	S-Phase (%)	G_2/M (%)
Control	60.0	13.9	26.2
8-Br-cAMP	71.7	11.6	16.7
PACAP	71.0	11.1	17.9

B

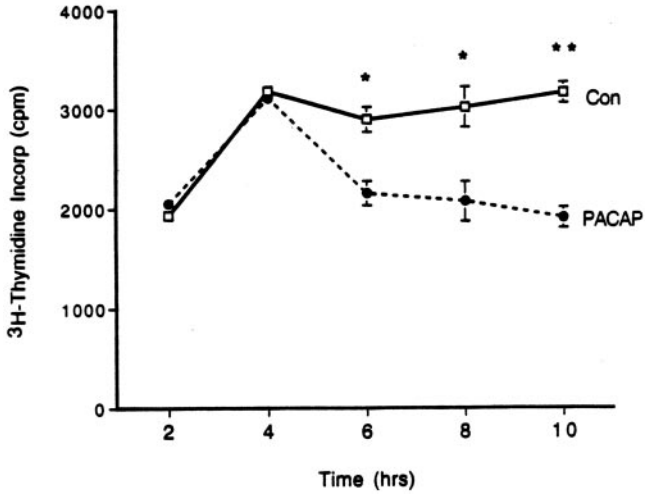

Figure 3. Effect of PACAP on cortical precursor S-phase entry and DNA synthesis. *A*, In low-density cultures, cells were incubated in control medium or medium containing PACAP (10^{-8} M) or 8-Br-cAMP (1 mM) for 24 hr, and DNA content was analyzed by flow cytometry. Although cells in control conditions progressively exited the cycle, PACAP and 8-Br-cAMP elicited enhanced G_1/S blockade, increasing G_1 cells by 20%. *B*, In conditions of increased cell density and reduced substrate adhesiveness, PACAP elicited a time-dependent decrease in DNA synthesis, consistent with diminished progress through the cell cycle. Data are expressed as mean cpm ± SEM. $^*p < 0.05$, $^{**}p < 0.001$; by ANOVA and Scheffe-f test.

both in culture and *in vivo*, consistent with an autocrine regulatory function (Lu and DiCicco-Bloom, 1997; Suh et al., 2001). The rapid anti-mitogenic effects of the peptide raised the possibility that G_1 cell cycle molecules were targets of PACAP receptor activation.

In the current study, we found that PACAP and its downstream signal, cAMP, increased the proportion of cells in G_o/G_1 from 60 to 72%, a 20% enhancement, suggesting that peptide signaling may inhibit DNA synthesis by blocking the G_1/S transition (Fig. 3*A*). To begin examining possible peptide effects on CDK activity *in vitro*, we optimized previous culture conditions to promote precursor proliferation, by increasing cell density to 2–3 × 10^5 cells/cm^2 and decreasing substrate adhesiveness. Using the new model, we performed analysis to define a time course of the effect of PACAP. Although PACAP elicited no inhibition during the first 4 hr, there was a 26% reduction in DNA synthesis by 6 hr, and further inhibition thereafter (Fig. 3*B*). This rapid inhibition of DNA synthesis is comparable with previous 6 hr culture studies and parallels the rapid anti-mitogenic action of PACAP observed

Table 1. Colocalization of BrdU with neural markers at 3 d in culture

Neural antigen	Colocalization on day 3 (%)
MAP2	84.0 ± 2.0
TuJ1	67.5 ± 1.8
Nestin	9.7 ± 1.4
GFAP	0
MBP	0
NG2	0

Cortical precursors were incubated at high density (2–3 × 10^5 cells/cm^2) in the presence of BrdU (10 μM) for 8 h. After rinsing twice in defined medium, cells were replated and further incubated in the absence of BrdU until day 3, at which time cultures were fixed and analyzed by double immunocytochemistry.

recently in living embryos (Lu and DiCicco-Bloom, 1997; Suh et al., 2001). Consequently, we chose an 8 hr culture paradigm to define the role of late G_1 pro-mitogenic and anti-mitogenic machinery in PACAP neurogenetic activity. However, because the changes in culture conditions potentially alter the composition of the cultures compared with previous characterization, we analyzed the neural fate of mitotic cells in this modified system. Specifically, we labeled cells that entered S-phase using BrdU for the entire 8 hr incubation, because this cohort of precursors is the target of PACAP anti-mitogenic action. Subsequently we analyzed their fate by performing double immunocytochemistry to colocalize BrdU nuclear labeling with cytoplasmic markers for neurons and glia (Table 1). At 2 and 3 d, the overwhelming majority of cells entering S-phase during the 8 hr incubation exhibited neuronal markers: 81 and 84%, respectively, exhibited MAP2, whereas 68% expressed TuJ1. Significantly, an additional 10% expressed the precursor marker nestin. In contrast, although we detected only rare GFAP-positive cells in our cultures, 0.01% at 3 d, and no cells expressing oligodendrocyte markers, NG2 and MBP, there was no colabeling with BrdU, indicating that the culture model was composed primarily of neuronal precursors.

PACAP decreases cyclin E/CDK2 complex activity but not protein levels in cortical progenitor cells

As the transition from G_1 into S-phase depends on cyclin E/CDK2 activity, we defined PACAP effects on these pro-mitogenic regulators. Furthermore, this kinase complex is a useful index for inhibitory action of CIP/KIP family members. Cortical cells were incubated in control or PACAP-containing medium for 8 hr, and cultures were assessed for DNA synthesis and changes in CDK2 activity. PACAP exposure inhibited DNA synthesis by 35% (Fig. 4, *top*). In parallel culture extracts, we found that PACAP treatment decreased CDK2 kinase activity by ~75% compared with control (Fig. 4, *middle*). This was a change in enzyme-specific activity, because PACAP treatment did not alter CDK2 protein levels during the 8 hr incubation. These observations suggest that the reduction in cortical precursor mitosis induced by PACAP depends in part on inhibition of this critical regulator of S-phase entry.

The stimulatory activity of CDK2 kinase depends on the its G_1 phase-specific regulatory subunit, cyclin E. Potentially, PACAP may induce rapid loss of cyclin E, the levels of which are subject to ubiquitin-dependent degradation (Singer et al., 1999; Nakayama et al., 2001). However, examination of cultures after 8 hr exposure to PACAP demonstrated no change in cyclin E protein levels by Western analysis (Fig. 4, *bottom*), indicating that decreased levels of pro-mitogenic regulators, cyclin E and CDK2

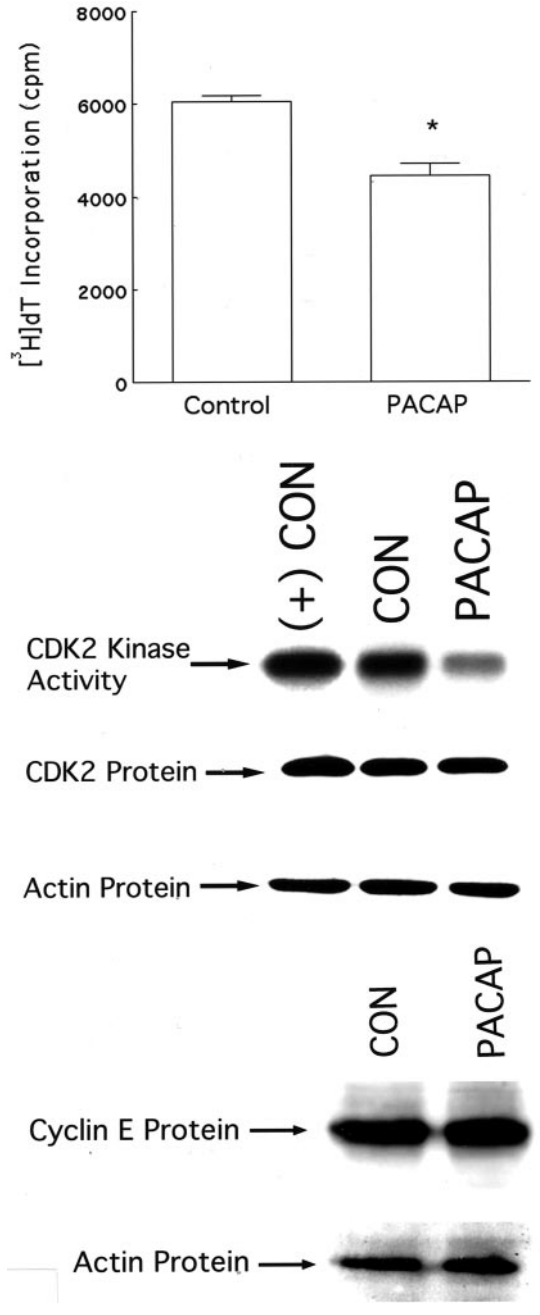

Figure 4. Effect of PACAP on mitosis and cyclin E/CDK2 kinase activity and protein levels in cortical precursor culture. *Top*, PACAP inhibited DNA synthesis by 35% in 8 hr cortical cultures. [^{3}H]thymidine was added at 5 hr, and incorporation was assayed at 8 hr. Data are expressed as mean cpm ± SEM. *$p < 0.05$. *Middle*, Using parallel cultures, extracts (30 μg per sample) of control and PACAP (10^{-8} M)-treated cells were analyzed for CDK2 kinase activity, using methods as in Figure 2. PACAP decreased CDK2 activity by ~75%. Hela cells were used as positive control [*(+)CON*]. Extracts were also subjected to Western blot analysis to determine whether PACAP regulated CDK2 protein levels. No change was observed in total CDK2 protein levels after PACAP treatment. Immunoblots were reprobed with actin antibody to verify equal protein loading. Experiments were performed three times yielding similar results. *Bottom*, Parallel extracts were analyzed for cyclin E protein levels by immunoblotting, indicating no change, a result obtained in four separate experiments.

protein, did not underlie PACAP anti-mitogenic activity. In turn, we evaluated potential roles of the CKIs.

PACAP selectively stimulates p57^{Kip2} levels and association with CDK2

The pro-mitogenic functions of CDKs are balanced by anti-mitogenic regulators, including CIP/KIP inhibitors p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}. Thus, in parallel experiments, we examined the effects of PACAP on CKIs. Although PACAP treatment decreased CDK2 kinase activity, the peptide increased levels of p57^{Kip2} protein by more than twofold (Fig. 5). In contrast, PACAP had no effect on p27^{Kip1} protein levels, and p21^{Cip1} protein was not detected, as observed in fresh tissue extracts. The selective increase in p57^{Kip2} protein elicited by PACAP raises the possibility that it may be a specific intracellular mediator of PACAP effects. Because CKI–CDK association is a prerequisite for the regulation of kinase activity, we next examined the effect of PACAP on p57^{Kip2} association with the CDK2 kinase complex. After incubating precursors in control or PACAP-containing medium, cell extracts were subjected to kinase immunoprecipitation using CDK2 antibody, and complexes were separated and examined for CDK2-associated proteins. We found increased levels of p57^{Kip2} protein associated with CDK2 after PACAP treatment compared with control (Fig. 6*A*), suggesting that p57^{Kip2} interacts directly with CDK2 to inhibit cell cycle progression. Moreover, after PACAP treatment, p57^{Kip2} protein unassociated with the CDK2 complex was no longer detectable in the supernatant (Fig. 6*B*). In marked contrast, the inhibitor remained unassociated with the kinase in control cells, indicating that PACAP regulated p57^{Kip2} protein association in addition to total protein levels.

DISCUSSION

Our results define a mechanism whereby an endogenous anti-mitogenic signal regulates intrinsic cell cycle machinery in neuronal precursors to elicit cell cycle exit. PACAP induced a rapid increase in p57^{Kip2} levels while decreasing CDK2 kinase activity, S-phase entry, and DNA synthesis. Furthermore, PACAP enhanced p57^{Kip2} association with the kinase complex, consistent with its anti-mitogenic activity. In contrast, there were no changes in levels of CDK2, cyclin E, or CKIs p21^{Cip1} and p27^{Kip1}, suggesting that p57^{Kip2} plays a specific role in cortical neurogenesis. Finally, a selective function of p57^{Kip2} in neuronal progenitor cell cycle exit contrasts markedly with that of p27^{Kip1} in glial precursors, raising the possibility that CIP/KIP members play lineage-specific roles in neural cell cycle regulation.

Mitogenic regulation in developing cerebral cortex

Although extracellular factors play major roles in proliferation control, little is known about underlying cell cycle mechanisms. Recent evidence suggests that the precise temporal schedule of proliferation and cell cycle exit reflects interaction among positive and negative extracellular signals. bFGF, EGF, and IGF-I stimulate mitogenesis in culture and *in vivo*, increasing neuron numbers and brain growth (Gensburger et al., 1987; Drago et al., 1991; Ghosh and Greenberg, 1995; Kilpatrick and Bartlett, 1995; Vaccarino et al., 1995, 1999; Burrows et al., 1997; Cavanagh et al., 1997; Qian et al., 1997; Lillien and Raphael, 2000). The persistent expression of cortical mitogens during neurogenesis, however, raises questions regarding mechanisms by which precursors cease proliferating. Culture studies indicate that developing cortex contains anti-mitogenic signals: GABA and glutamate potentially act as paracrine signals after E15, released from cortical plate neu-

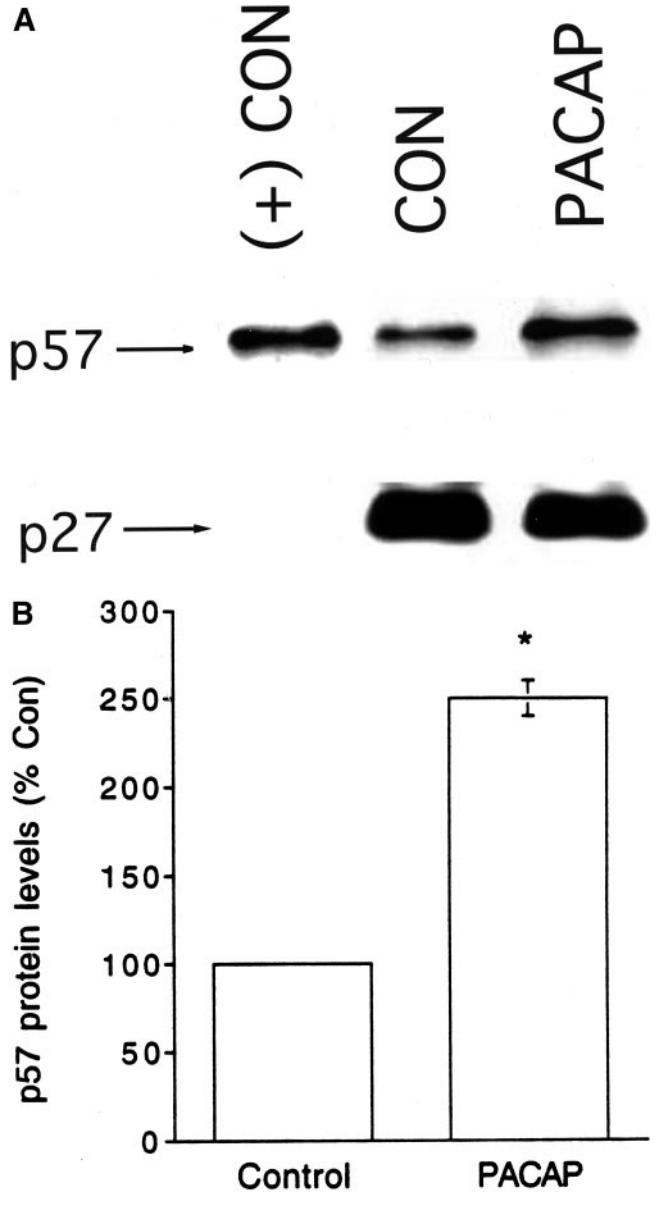

Figure 5. Effect of PACAP on CKI protein levels. *Top*, Cortical precursors were incubated in control medium or medium containing PACAP (10^{-8} M) for 8 hr. Cell extracts (50 μg per sample) were prepared by harvesting and sonication and analyzed by Western blot. Hela cell extracts were used as positive control [*(+)CON*]. Membranes were cut at ~42 kDa, and each half was incubated with $p57^{Kip2}$ or $p27^{Kip1}$ and $p21^{Cip1}$ antibody as appropriate. PACAP increased $p57^{Kip2}$ protein levels more than twofold. In contrast, PACAP did not alter levels of $p27^{Kip1}$ protein. $p21^{Cip1}$ protein was barely detectable and exhibited no change with PACAP exposure (data not shown). Filters were reprobed with actin antibody or stained with Coomassie blue to verify equal protein loading (data not shown). *Bottom*, Histogram of relative $p57^{Kip2}$ protein levels obtained in three separate experiments, expressed as percentage of control densitometric signal. $p < 0.001$.

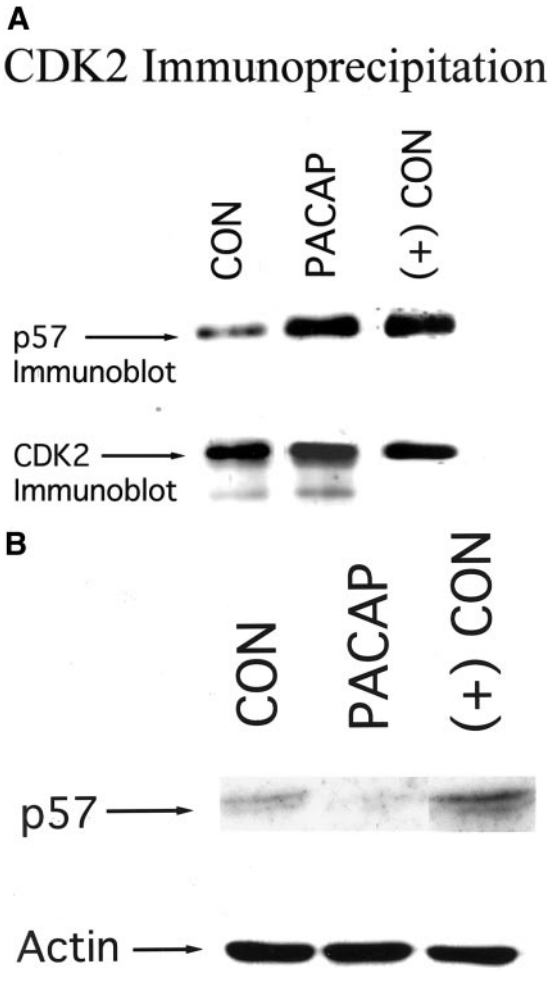

Figure 6. Effect of PACAP on CDK2 associated proteins. Cell extracts (200 μg per sample) from control and PACAP-treated cortical cultures were incubated on ice for 30 min with CDK2 antibody, control antibody, or no antibody and then immunoprecipitated at 4°C for 1 hr. Precipitates and supernatants were separated on 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were cut at ~42 kDa, and each half was incubated with $p57^{Kip2}$ or CDK2 antibody as appropriate. *A*, PACAP increased the amount of $p57^{Kip2}$ protein associated with CDK2 (*p57 Immunoblot*). Probing of immunoprecipitates with CDK2 antibody indicated that similar levels of the complex from control and PACAP-treated groups were obtained and effectively separated and transferred to filters for Western analysis. *B*, $p57^{Kip2}$ protein unassociated with the CDK2 complex was no longer detected in supernatant from PACAP-treated cultures, whereas residual inhibitor remained in control cell extracts. Equal loading is demonstrated by actin probing of the immunoblot.

rons to inhibit VZ precursors (LoTurco et al., 1995; Antonopoulos et al., 1997). However, at E13.5, when preplate neurons are first generated (Hicks and D'Amato, 1968; Konig et al., 1977; Bayer and Altman, 1991), endogenous PACAP inhibits proliferation *in vitro* (Lu and DiCicco-Bloom, 1997) and, more importantly, provides ongoing anti-mitogenic signaling in the developing embryo cortex (Suh et al., 2001). Because PACAP counters endogenous mitogen stimulation, the evidence supports a model of interacting antagonistic extracellular signals regulating neurogenesis.

Although stimulatory and inhibitory proliferative factors are known, negative regulation of neuronal cell cycle machinery by extracellular signals has not been described previously. The mammalian cell cycle is a complex, coordinated array of steps. Under pro-mitogenic signaling, cyclin D-dependent kinases initiate retinoblastoma protein (Rb) phosphorylation in mid-G_1, after which cyclin E/CDK2 becomes active, phosphorylating Rb at additional sites (for review, see Sherr and Roberts, 1999). Rb hyperphosphorylation in late G_1 disrupts its association with E2F transcription factors, promoting activation of S-phase genes. Regulation at multiple steps may induce cell cycle exit. First, with-

drawal of pro-mitogenic signals halts cyclin D synthesis, leading to inactivation of cyclin D-dependent kinases and release of bound CKIs, which in turn inhibit cyclin E/CDK2 complexes and induce arrest (Reynisdottir et al., 1995; Nagahara et al., 1999). Second, negative signals may stimulate CKI levels, which inhibit cyclin D-dependent kinases and cyclin E/CDK2 complexes, actively inducing cell cycle exit. Because cortical mitogens are expressed throughout neurogenesis (Drago et al., 1991; Powell et al., 1991; Vaccarino et al., 1999), presumably maintaining cyclin D levels, it is likely that additional anti-mitogenic pathways are required for cell cycle exit.

CKIs act as primary effectors of signaling pathways controlling cell cycle exit, especially CIP/KIP members, which inhibit all kinases involved with G_1/S transition (Koff et al., 1993; Kato et al., 1994; Polyak et al., 1994a,b; Lee et al., 1995; Matsuoka et al., 1995). For example, the anti-proliferative signals, serum withdrawal, cAMP, prostaglandins, and rapamycin, all increased synthesis and levels of $p27^{Kip1}$ by two- to fourfold in 3–15 hr, demonstrating rapid changes in inhibitor levels and interaction with CDK complexes, whereas antisense blockade of inhibitors restored cycle progression (Kato et al., 1994; Coats et al., 1996). In the current studies, we characterized cycle components critically regulating the late G_1/S transition. CDK2 kinase was most highly expressed and active at E14.5–15.5, when VZ cells divide symmetrically to produce additional precursors, whereas levels diminished progressively as cells exited the cycle to produce neurons (Caviness et al., 1995; Takahashi et al., 1995, 1999). Conversely, CIP/KIP proteins expressed in developing brain, $p27^{Kip1}$ and $p57^{Kip2}$, increased progressively with ontogeny, consistent with mRNA expression (van Lookeren Campagne and Gill, 1998; Delalle et al., 1999), suggesting roles in mitotic withdrawal.

We found that PACAP increased $p57^{Kip2}$ expression and association with the cyclin E/CDK2 complex, reducing kinase activity. Consequently, fewer cells entered S-phase, accumulating in G_1, which in turn resulted in decreased DNA synthesis. In contrast, PACAP did not alter levels of cyclin E, $p21^{Cip1}$, or $p27^{Kip1}$, suggesting that $p57^{Kip2}$ plays a selective role in withdrawal of neuronal precursors. Furthermore, these rapid changes in cycle machinery are entirely consistent with the timing of peptide effects observed previously in cultures and living embryos (Suh et al., 2001). These studies describe the first evidence of a G_1 kinase in mammalian neuronal precursors being negatively regulated by a physiological agent.

Anti-mitogenic function for p57 has been defined previously in culture and *in vivo*. Unlike other CKIs, including $p21^{Cip1}$, $p27^{Kip1}$, p15, p18, and p16, $p57^{Kip2}$ is essential for development (Yan et al., 1997; Zhang et al., 1997). In its absence, animals die, exhibiting proliferative disorders in multiple tissues, including lens, adrenal gland, bone, and placenta. Mitotic labeling is increased more than twofold in chondrocytes, whereas ectopic mitoses occur in lens fibers, suggesting aberrant cycling during differentiation. In the nervous system, twice as many retinal precursors undergo mitosis at E14.5 in deletion mutants, whereas $p57^{Kip2}$ overexpression elicits premature cell cycle exit (Dyer and Cepko, 2000). Finally, $p57^{Kip2}$ likely plays additional roles in postmitotic survival and/or differentiation. For example, major $p57^{Kip2}$ expression in retina occurs from E14.5 to E20.5 as precursors exit the cycle, whereas subsequent postnatal expression suggests differentiation functions in an amacrine subpopulation (Dyer and Cepko, 2000). Similarly, $p57^{Kip2}$ is expressed during prenatal and early postnatal cortical neurogenesis, decreasing markedly thereafter (van Lookeren Campagne and Gill, 1998; Cunningham and Roussel, 2001; this study). Localization of $p57^{Kip2}$ to VZ and CP suggests multiple roles in the transition from proliferation to differentiation, although functions await definition. Our current observations identify one upstream signal regulating $p57^{Kip2}$ anti-mitogenic activity during brain development.

Although our studies support an anti-mitogenic role for $p57^{Kip2}$, understanding precise inhibitor function in ventricular zone precursors awaits cellular analysis. For example, at E14.5, progressively more neurons are born as a consequence of asymmetric precursor division, yielding one postmitotic neuron and one proliferative stem cell (Caviness et al., 1995; Takahashi et al., 1995, 1999). Potentially, $p57^{Kip2}$ is expressed in the mitotic precursor and subsequently distributed asymmetrically to one daughter cell to elicit withdrawal, a pattern recently described for TIS21/PC3, a protein apparently determining cell cycle exit (Iacopetti et al., 1999; Malatesta et al., 2000). Alternatively, $p57^{Kip2}$ is downstream of TIS21 or similar regulatory signals, blocking G_1 progression and maintaining and promoting differentiation by preventing cycle reentry (Cunningham and Roussel, 2001). In current experiments, PACAP–$p57^{Kip2}$ signaling elicited rapid inhibition of G_1 progress and DNA synthesis, consistent with the latter alternative. On the basis of intriguing overexpression studies, a similar role may be considered for $p27^{Kip1}$, which rapidly reduced mitotic labeling 25–32% by apparently increasing G_1 length, whereas effects on neurogenesis await definition (Mitsuhashi et al., 2001). Interestingly, manipulations of diverse extracellular factors and intrinsic cycle proteins yield similar ~25% changes in precursor mitosis, implicating a complex and resilient regulatory network (LoTurco et al., 1995; Antonopoulos et al., 1997; Lu and DiCicco-Bloom, 1997; Mitsuhashi et al., 2001; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001).

Although PACAP modulates $p57^{Kip2}$ function to inhibit CDK2 activity and G_1/S transition, other cycle machinery may be involved. PACAP may act on early G_1, cyclin D-dependent kinases, CDK4 and CDK6, by altering INK4 proteins or cyclins. Recent work indicates critical roles for early G_1 kinases in cortical precursors, in contradistinction to tumor lines (Ferguson et al., 2000). However, because CIP/KIP proteins bind and inhibit all G1 kinases, cyclin D-dependent kinases are likely targets of elevated $p57^{Kip2}$ (Lee et al., 1995; Matsuoka et al., 1995). Furthermore, compound deletion mutants suggest that cooperative interactions among pro-mitogenic and anti-mitogenic machinery contribute to tissue specific regulation of cell cycle withdrawal (Zindy et al., 1997, 1999; Zhang et al., 1998, 1999; Caspary et al., 1999; Cheng et al., 1999; Gomez Lahoz et al., 1999).

Population selectivity

Does selective $p57^{Kip2}$ regulation provide insight into cortical cellular diversity? In rat, neuronal populations are generated between E13 and E20, whereas gliogenesis proceeds postnatally. Potentially, progenitors are multipotent, the changing environment determining cell fate, including use of specific CKIs. Alternatively, cells may be intrinsically diverse as neurogenesis commences, with specific lineage dictating both CKI use and final cell fate. We found that both $p27^{Kip1}$ and $p57^{Kip2}$ were expressed at E14.5, and levels increased with development. However, the levels and CDK2 association of $p57^{Kip2}$ alone were regulated by environmental signal PACAP. Because the precursors differentiate to express MAP2, NSE, tau, and class III β-tubulin (Table 1) (Lu and DiCicco-Bloom, 1997; Suh et al., 2001), $p57^{Kip2}$ may elicit

cell cycle exit in the neuronal lineage. In marked contrast, $p21^{Cip1}$ and $p27^{Kip1}$ play important roles in gliogenesis. $p27^{Kip1}$ knock-out mice exhibited increased mitotic precursors of glia in embryonic spinal cord and oligodendrocytes in neonatal forebrain (Casaccia-Bonnefil et al., 1997, 1999). Moreover, in oligodendrocyte cultures, cell cycle arrest caused by mitogen withdrawal or transmitter receptor activation increased $p21^{Cip1}$ and $p27^{Kip1}$ levels and CDK2 association (Tikoo et al., 1997; Ghiani et al., 1999), whereas $p27^{Kip1}$ deletion enhanced proliferation, suggesting that different neural lineages use distinct CKIs. However, roles for $p27^{Kip1}$ in control of neuronogenesis have been suggested (Zindy et al., 1997, 1999; Delalle et al., 1999; Dyer and Cepko, 2001). Thus, as an alternative, PACAP and cAMP may induce withdrawal of a precursor subpopulation (Suh et al., 2001) through $p57^{Kip2}$. In contrast, factors stimulating different intracellular pathways in alternative neuronal or glial lineages may act via $p27^{Kip1}$, an issue to address by comparing PACAP with other inhibitory signals.

In summary, the endogenous anti-mitogenic signal, PACAP, elicited rapid cell cycle exit by acting on G_1 phase progression. PACAP increased levels of $p57^{Kip2}$ and promoted its association with the CDK2 complex, reducing kinase activity. The selective response of $p57^{Kip2}$ to PACAP in neuronal progenitors suggests that lineage-specific expression and function of CKIs is involved in generating cellular diversity.

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