p57Kip2 regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina

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Accepted 2 June; published on WWW 20 July 2000

SUMMARY

A precise balance between proliferation and differentiation must be maintained during retinal development to obtain the correct proportion of each of the seven cell types found in the adult tissue. Cyclin kinase inhibitors can regulate cell cycle exit coincident with induction of differentiation programs during development. We have found that the p57Kip2 cyclin kinase inhibitor is upregulated in G1/G0 in a subset of retinal progenitor cells exiting the cell cycle between embryonic day 14.5 and 16.5 of mouse development. Retroviral mediated overexpression of p57Kip2 in embryonic retinal progenitor cells led to premature cell cycle exit. Retinae from mice lacking p57Kip2 exhibited inappropriate S-phase entry and apoptotic nuclei were found in the region where p57Kip2 is normally expressed. Apoptosis precisely compensated for the inappropriate proliferation in the p57Kip2-deficient retinae to preserve the correct proportion of the major retinal cell types. Postnatally, p57Kip2 was found to be expressed in a novel subpopulation of amacrine interneurons. At this stage, p57Kip2 did not regulate proliferation. However, perhaps reflecting its role during this late stage of development, animals lacking p57Kip2 showed an alteration in amacrine subpopulations. p57Kip2 is the first gene to be implicated as a regulator of amacrine subtype/subpopulation development. Consequently, we propose that p57Kip2 has two roles during retinal development, acting first as a cyclin kinase inhibitor in mitotic progenitor cells, and then playing a distinct role in neuronal differentiation.

Key words: p57Kip2, Cyclin kinase inhibitor, Mouse, Retina, Proliferation, Amacrine cell, Apoptosis

INTRODUCTION

The retina is a thin sheet of neural tissue lining the back of the eye. It is made up of seven major cell types organized into three cellular layers (reviewed in Dowling, 1987). These major retinal cell types can be further classified into distinct subtypes, which serve unique roles in the intricate circuitry of the retina. Light passing through the cornea is focused by the lens on the outer segments of the rod and cone photoreceptors whose cell bodies constitute the outer nuclear layer (ONL). Photoreceptors transmit their signals through the bipolar interneurons found in the inner nuclear layer (INL) to the ganglion cells whose cell bodies make up the ganglion cell layer (GCL). The signals from the ganglion cells are sent to the brain through the optic nerve. These direct signaling circuits can be modulated by two types of cells that make lateral connections in the INL – the horizontal cells and the amacrine cells (reviewed in Masland, 1986). At least 22 morphologically distinct amacrine cell subtypes have been described in the vertebrate retina and each subtype is believed to play a unique role in visual signal processing (Kolb, 1997; MacNeil and Masland, 1998). Broader amacrine cell subpopulations made up of two or more subtypes have also been described based on immunoreactivity for a variety of antigens (discussed in Kolb, 1997). The one non-neuronal cell type found in the retina, Müller glial cells, spans all three layers of the retina and is believed to play a supportive role for photoreceptors and perhaps other retinal cells as well (Newman and Reichenbach, 1996; Willbold and Layer, 1998).

Birthdating studies (reviewed in Altshuler, 1991) have shown that each of the seven retinal cell types is generated at characteristic times during development from multipotent progenitor cells (Fekete et al., 1994; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). Considerable experimental evidence suggests that, at any given stage of retinal development, progenitor cells are only competent to generate a subset of the differentiated cell types (reviewed in Cepko et al., 1996). Consequently, it was proposed that the birth order of retinal cell types results from unidirectional changes in progenitor cell competence and environmental cues during development (discussed in Cepko, 1999). Beyond these changes that affect cell fate decisions, the decision to exit the cell cycle must also be precisely regulated during retinal development in order to obtain the correct proportion of each of the retinal cell types. If too many cells were to exit the cell cycle during the early stages of development there might be an increase in the proportion of early born cell types at the expense of later born cell types. Despite its fundamental importance, little is known about how retinal progenitor cell proliferation is regulated during development.
Studies conducted in vitro have demonstrated that cyclin kinase inhibitors (CKIs) of the Cip/Kip family regulate cell cycle progression by blocking phosphorylation of the retinoblastoma protein (reviewed in Sherr and Roberts, 1995). Results from \textit{D. melanogaster} and \textit{C. elegans} have extended these observations by showing that CKIs play a critical role regulating proliferation during development (de Nooij et al., 1996; Hong et al., 1998; Lane et al., 1996). Three Cip/Kip family members have been identified in mammals – p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2} (reviewed in Sherr and Roberts, 1995). Each is expressed in a dynamic and restricted pattern during development and in postmitotic cells (Lee et al., 1995; Matsuoka et al., 1995; Nakayama et al., 1996; Parker et al., 1995; Zhang et al., 1998, 1999). Mice deficient for one or more of the Cip/Kip family members exhibit tissue-specific defects that are believed to result from increased proliferation during development, increased cell death, ectopic cell division in mature tissues or some combination of the three (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et al., 1997; Zhang et al., 1997, 1998, 1999). However, it is also possible that these promiscuous kinase inhibitors (discussed in Chellappan, 1996) more directly influence cell fate specification and/or differentiation long after terminal mitosis. Such a dual role would be consistent with the structural diversity of the Cip/Kip family, the developmental expression patterns and the phenotypes observed in knockout mice.

Recent work in \textit{Xenopus} has provided the first evidence that cyclin kinase inhibitors can directly influence cell fate decisions during development (Kroll and Amaya, 1996; Ohnuma et al., 1999).

We have found that p57\textsuperscript{Kip2} is expressed at two distinct times during retinal development and suggest that it plays a different role during each phase of its expression. It is expressed in a subset of mitotic progenitor cells during embryonic stages and we show that it is required for proper exit from the cell cycle at this time. In the absence of p57\textsuperscript{Kip2} protein expression, embryonic cells that would normally exit the cell cycle inappropriately re-enter S-phase and subsequently undergo apoptosis. Later in development, when mitosis has ceased, p57\textsuperscript{Kip2} is expressed in a restricted subpopulation of amacrine interneurons. Mice lacking p57\textsuperscript{Kip2} show a specific defect in the number of cells in at least one amacrine subpopulation suggesting that p57\textsuperscript{Kip2} is required for the proper allocation of amacrine cells to different subpopulations. This is the first mutation reported to affect the distribution of amacrine cells into their subpopulations and subtypes.

MATERIALS AND METHODS

Animals

C57BL/6 and ICR mice were purchased from Tacdon Farms, Inc. p57\textsuperscript{Kip2} knockout mice (Zhang et al., 1997) were crossed to ICR or C57BL/6 mice with equivalent results. Genotypes were determined by performing PCR amplification of the wild-type and mutant alleles from tail DNA (Zhang et al., 1998).

In situ hybridization

Sense or antisense digoxigenin (Boehringer Mannheim)-substituted riboprobes were incubated at 50 ng/ml with 20 μm cryosections of staged retinas (E12.5, E14.5, E16.5, E18.5, P0, P3, P6, P9, P12, Adult) from C57BL/6 and ICR mice as described previously (Riddle et al., 1993). Probes correspond to the full-length 1.4 kb cDNA of mouse p57\textsuperscript{Kip2} (Matsuoka et al., 1995).

Immunohistochemistry, microscopy and imaging

Retinal cryosections or dissociated cells (see below) were fixed in parafomaldehyde (4% in PBS), washed and treated with hydrogen peroxide (1% in PBS) prior to incubation in blocking solution (PBS containing 0.1% Triton X-100 and 2% normal serum; Vector Laboratories). Normal donkey serum was used for the following antibodies: anti-rhodopsin, Rho4D2 (mouse monoclonal, 1:250; Molday and MacKenzie, 1983), anti-parvalbumin, PA-235 (mouse monoclonal, 1:5000, Sigma), anti-choline acetyltransferase, 1E6 (mouse monoclonal, 1:3000, Chemicon), anti-calretinin, (mouse monoclonal, 1:5000, Chemicon), anti-glial fibrillary acidic protein, GA5 (mouse monoclonal, 1:400, Sigma), anti-HNK-1, VC1.1 (mouse monoclonal, 1:5000, Sigma), anti-syntactin, HPC-1 (mouse monoclonal, 1:5000, Sigma), anti-calbindin-D28K, CL-300 (mouse monoclonal, 1:5000, Sigma), anti-bipolar antigen, 115A10 (mouse monoclonal, undiluted; Onoda and Fujita, 1987), and anti-cone opsins (mouse monoclonal, 1:10,000; Chiu et al., 1994; Wang et al., 1992). Normal rabbit serum was used for the anti-p57\textsuperscript{Kip2}, E-17 (goat polyclonal, 1:50, Santa Cruz Biotech) antibody. Biotin-conjugated secondary antibodies (donkey anti-mouse IgG, rabbit anti-goat IgG, goat anti-rabbit IgG; Vector Laboratories) were used at a dilution of 1:500 in blocking solution. Following secondary antibody binding, an avidin-biotin-peroxidase complex (Vectastain ABC, Vector Laboratories) was incubated with the sections or dissociated cells followed by dianaminobenzidine detection (Vector Laboratories) or Cy-3 tyramide detection (NEN) according to the manufacturer's instructions. For nuclear staining, DAPI (Sigma) was added to the final wash solution at 0.0005%. Labeled cells were visualized using a Zeiss Axiosplan-2 microscope using 10×, 20× and 40× Plan NEOFLUAR objectives or a 100× Plan APOCHROMAT objective with adjustable iris. Images were captured with a SPOT digital camera (Diagnostic Instruments Inc.).

RNA isolation and RT-PCR assay

Three independent retinas were removed from staged embryonic (E14.5, E16.5, E18.5), postnatal (P0, P3, P6, P9, P12) and adult (6 weeks) ICR mice and immediately dissolved in 500 μl lysis solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol). RNA was prepared as described (Chomczynski and Sacchi, 1987). Expression of p57\textsuperscript{Kip2} and β-actin was examined in each sample by performing semiquantitative multiplex RT-PCR as described previously (Farrington et al., 1997). Sequence for the β-actin primers can be found in Farrington et al. (1997). Oligonucleotide primers for mouse p57\textsuperscript{Kip2} were: (5′): 5′-TTCAGATCTGAAGCTTGATT-3′ (Tm=57°C) (3′): 5′-TGCTTTGATCTCGTCC-3′ (Tm=57°C).

Retinal explant culture and dissociation

Retinas were dissected away from the surrounding tissue in prewarmed (37°C) explant culture medium (45% Dulbecco’s Modified Eagle’s Medium (Gibco) 45% F12 Nutrient Mix (Gibco), 10% Fetal Calf Serum (HyClone), 2 mM L-glutamine (Gibco), Penicillin/ Streptomycin (Gibco) and 5 μg/ml insulin (Sigma)). Immediately following dissection, retinas were placed on polycarbonate filters (13 mm diameter, 1.0 μm pore size; Corning) in explant culture medium at 37°C and 5% CO2. 20 μl of conditioned retinal supernatant was added to the explants every 24 hours (protocol available at http://hkimin3.med.harvard.edu/~cepko/). For overexpression studies using replication incompetent retroviral vectors (Fig. 3), E14.5 mouse retinas were cultured for 10 days following infection at the time of dissection. Retinas from newborn mouse pups were cultured 10 days for the analysis of cell type distribution in p57\textsuperscript{Kip2}-deficient animals (Figs 5, 6). Tissue dissociation was carried out as described previously (Morrow et al., 1998).
Several observations have demonstrated that retinal proliferation and differentiation are normal using this explant culture system. (1) The total number of cells and the percentage of mitotic cells closely mimics data obtained in vivo for the first 5 days in culture (M. A. D. and C. L. C., unpublished data). (2) Clone size distribution data presented here (Fig. 2E) are similar to data obtained previously from in vivo infection of embryonic retinae by performing exo-utero surgery (Turner et al., 1990). (3) The distribution and proportion of all the retinal cell types examined in this work (rods, cones, amacrine, horizontals, glia, bipolars) are indistinguishable from in vivo data. Notably, even the distribution and proportion of highly restricted amacrine subpopulations remained unchanged (Figs 5, 6).

[3H] thymidine and BrdU labeling

To label retinal progenitor cells in S-phase, retinae were incubated in 1 ml explant culture medium containing [3H] thymidine (NEN, 5 μCi/ml, (89 Ci/mmol)) or 10 μM bromodeoxyuridine (BrdU) (Boehringer Mannheim) for 1 hour at 37°C. For continuous labeling experiments (Table 2), explant culture medium was removed and fresh medium containing [3H]thymidine was added every 24 hours. Autoradiography and BrdU detection were carried out as described previously (Morrow et al., 1998).

Replication incompetent retroviral vector constructs and viral stocks

Oligonucleotides encoding the FLAG-His cassette were synthesized, annealed and cloned into the pNIN replication incompetent retroviral vector (C. L. C., unpublished) to make pNIN-E. Oligonucleotide sequences were as follows:

Flag.His.top 5'-CTAGAACCATGGACTACAAGGACGACGATG-ACAGCGCCCGCCGACATCATCATCATCATCATATT-3'
Flag.His.bottom 5'-CTAGATTAATGTGAAGATGTAGATGATGTCGGCCTTTGCTGTGTAGTCCCATGGT-3'

Mouse p57Kip2 was PCR amplified, sequenced and cloned into pNIN-E to generate pNIN-Ep57. Oligonucleotide primers for p57Kip2 PCR amplification were as follows:
p57-amino, 5'-TAGAGCCGCGCAGAAGCTTGGCCCTCCAGC-3'
p57-carboxy, 5'-TAGAGCCGCGCCTCTCACAGGTTGGCCCGG-3'

To prepare high titer retroviral stocks, the plasmid constructs were transiently transfected into a 293T cotropic producer cell line (Phoenix-E) by calcium phosphate co-precipitation as described (Cepko et al., 1998). Supernatant containing the viral particles was harvested at 48 hours post-transfection and viral titer was determined on NIH-3T3 cells as described (Cepko et al., 1998).

Recombinant p57Kip2 purification and immunoblotting

E14.5 ICR retinae (approx. 30 each) were cultured as explants and infected with 20 μl of a high titer stock (>105/ml) of pNIN-E or pNIN-Ep57 containing polybrene (6 μg/ml, Sigma). Approximately 8 hours later, an additional 20 μl of the same stock was added to achieve optimal infection of embryonic retinal progenitor cells. After 48 hours in culture, retinae were transferred to 1.0 ml of sonication buffer (50 mM sodium phosphate (pH 7.8), 300 mM NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF (Sigma), protease inhibitor cocktail (Sigma), 5% glycerol) and a cleared lysate was prepared by sonication using a Branson Sonifier 250 with microtip attachment. Purification of histidine-tagged p57Kip2 on Ni2+-NTA agarose resin (Qiagen) was carried out according to the manufacturer’s instructions for non-denaturing conditions. Proteins bound to the recombinant histidine-tagged p57Kip2 were eluted in 50 μl of wash buffer (50 mM sodium phosphate pH 6.0, 300 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol) containing 6 M guanidine hydrochloride. Eluates were dialyzed and concentrated as described previously (Dyer et al., 1998). Recombinant cyclin D1 was prepared using a baculovirus expression vector system according to the manufacturer’s instructions (Pharmingen). For analysis of amacrine cell markers, cultured retinae were boiled for 5 minutes in 100 μl SDS-PAGE loading dye (NEB).

Immunoblotting was performed according to the antibody manufacturers recommendations. Antibodies used for these experiments include: anti-FLAG (M2 mouse monoclonal, 1:2000, Sigma), anti-cyclin D1 (rabbit polyclonal, 1:100, Santa Cruz Biotecnology), anti-α-tubulin (B-5-1-2 mouse monoclonal, 1:2000, Sigma), anti-calbindin-D28K (CL-300 mouse monoclonal, 1:2000, Sigma), and anti-calretinin (mouse monoclonal, 1:2000, Chemicon). Secondary biotinylated antibodies (donkey anti-mouse IgG or goat anti-rabbit IgG, Vector Laboratories) were used at a dilution of 1:1000. Amplification was achieved by incubating the blot with an avidin-biotin-peroxidase complex (Vectastain ABC, Vector Laboratories) followed by diaminobenzidine detection (Vector Laboratories).

TUNEL assay

The colorimetric and fluorescent apoptosis detection systems (Promega) were used on 10 μm cryosections according to the manufacturer’s instructions.

Dissociated cell scoring and statistical methods

Criteria for dissociated cell scoring and examples are posted on the Cepko laboratory web site (http://hhmiw3.med.harvard.edu/~cepko/). To evaluate the significance of differences in the proportion of cell types between wild-type and p57Kip2-deficient retinae, the average and standard deviation were calculated for counts of retinae from each genotype and a t-test was performed. All P values are one-sided unless otherwise indicated. Formulas and more detailed explanations can be found at http://www.statistics.com/.

RESULTS

p57Kip2 is expressed at two distinct stages of retinal development

The expression pattern of p57Kip2 was examined by performing in situ hybridization, immunohistochemistry and RT-PCR on mouse retinae from 10 different stages of development. In situ hybridization studies using an antisense probe corresponding to the p57Kip2 coding sequence revealed that p57Kip2 mRNA was first detected around embryonic day 14.5 (E14.5) in a subset of retinal progenitor cells (Fig. 1A,B). At this stage, postmitotic cells are found in the inner neuroblastic layer near the vitreal surface while mitotic progenitor cells make up the broad outer neuroblastic layer. Cells migrate within the outer neuroblastic layer according to cell cycle phase: mitosis occurs near the pigmented epithelium (PE) and S-phase occurs near the boundary between the outer and inner neuroblastic layers (Sauer, 1937). While p57Kip2 mRNA was detected in cells within the outer neuroblastic layer, p57Kip2 immunoreactivity was primarily restricted to the region where cells are in the process of exiting the cell cycle, or have already left the cell cycle and are differentiating (Fig. 1C). This pattern is consistent with p57Kip2 mRNA expression during G2 or G1 of the terminal cell cycle followed by p57Kip2 protein accumulation, which is coincident with cell cycle exit. Over the next 2 days, p57Kip2 expression moved from the central to peripheral retina (Fig. 1D), mimicking the temporal progression of retinal development. Expression dropped off at approximately E17.5 and no mRNA was detected at E19.5 or P0 (Fig. 1E), even using a sensitive RT-PCR assay (Fig. 1K).

Beginning around postnatal day 2 (P2), p57Kip2 mRNA and protein were readily detected in an evenly distributed population of cells in the lower half of the inner nuclear layer.
Fig. 1. p57Kip2 is expressed at two distinct stages of retinal development. The distribution and temporal expression of p57Kip2 mRNA and protein was examined by non-radioactive in situ hybridization (A,B,D-I), semiquantitative multiplex RT-PCR (K) and immunohistochemistry (C,J). (A) Expression of p57Kip2 mRNA was first detected in retinal progenitor cells at E14.5. (B) A magnified region from A. (C) Nuclear localized p57Kip2 protein was present in cells between the outer neuroblastic layer and the inner neuroblastic layer at E14.5. (D) Expression of p57Kip2 mRNA progressed to the peripheral retina by E16.5 and tapered off completely by P0 (E). Several days later, p57Kip2 mRNA expression was upregulated in a restricted subpopulation of inner nuclear layer cells at P2 (F), P6 (G) and P12 (H). (I) A magnified region from H. Nuclear localized protein expression was detected at all postnatal stages where mRNA was expressed; a representative example (P12) is shown in J. (K) Semiquantitative multiplex RT-PCR was performed to demonstrate that p57Kip2 expression was transiently silenced between the embryonic and postnatal stages (P0). β-actin served as an internal control for the efficiency of RNA isolation and cDNA synthesis. Arrows indicate cells expressing p57Kip2 mRNA in the cytoplasm (A,B,D,F-I) or p57Kip2 protein in the nucleus (C,J). Abbreviations are as follows: inbl, inner neuroblastic layer; onbl, outer neuroblastic layer; inl, inner nuclear layer; onl, outer nuclear layer; gcl, ganglion cell layer; ipl, inner plexiform layer; opl, outer plexiform layer; pe, pigmented epithelium. –RT, reverse transcriptase. Scale bars, 50 μm.

(INL), where amacrine cells were differentiating (Fig. 1F,G). This highly restricted expression persisted throughout postnatal stages (Fig. 1H-J), peaking around P10-P12, at the time when other amacrine interneuron subpopulation markers also show peak expression (Oguni et al., 1998). Extensive analysis has revealed that p57Kip2 protein is expressed in a subpopulation of amacrine cells that have not been previously described (M. A. D. and C. L. C., unpublished data). This expression pattern is remarkable for a component of the cell cycle control machinery because there is little mitosis at this stage of development (Alexiades and Cepko, 1996). In summary, embryonic expression of p57Kip2 was consistent with a role in regulating progenitor cell cycle exit, while postnatal expression raised the possibility that p57Kip2 plays an additional role in the specification and/or differentiation of a new subpopulation of amacrine interneurons.

A subset of retinal progenitor cells upregulate p57Kip2 as they exit the cell cycle during embryonic development

To determine if the timing of expression of p57Kip2 within a cell cycle might suggest that it regulates embryonic progenitor cell proliferation, the expression of p57Kip2 was examined during different phases of the cell cycle. S-phase cells were pulse-labeled by incubating E14.5 retinae with [3H]thymidine for 1 hour (see Materials and Methods). After labeling, retinae were cultured as explants for different lengths of time, dissociated and reacted with antisera specific for p57Kip2. Immediately after labeling (T=0), the vast majority of cells expressing p57Kip2 (>99%) were not labeled with [3H]thymidine and were therefore not in S-phase (Fig. 2A-C; Table 1). Only 1 out of 1,543 (0.06%) [3H]thymidine-labeled cells was found to express p57Kip2 (Table 1). Progenitor cells in S-phase at the time of labeling should begin to enter G1 by 8 hours (T=8) after labeling (Alexiades and Cepko, 1996). While a slight increase in the proportion of [3H]thymidine-labeled cells expressing p57Kip2 (0.7%) was observed by this time,
A subset of retinal progenitor cells upregulate p57Kip2 during G1/G0. Immunofluorescent and autoradiographic analysis was performed on dissociated cells from embryonic retinae incubated with [3H]thymidine. Arrows indicate cells expressing p57Kip2. Immediately after [3H]thymidine labeling (T=0), the majority of p57Kip2 expressing cells (A) do not contain silver grains as visualized by bright-field microscopy (B). (C) DAPI staining highlights the nuclei of cells in A,B. 1 day after [3H]thymidine labeling (T=24), p57Kip2 immunoreactive cells (D) containing silver grains (E) were readily detected. (F) DAPI staining of D,E. Scale bar, 10 µm. For quantitation, see Table 1.

Table 1. p57Kip2 is upregulated during late G1 in a subset of embryonic retinal progenitor cells

<table>
<thead>
<tr>
<th>Stage</th>
<th>Culture time* (hours)</th>
<th>[3H]thymidine/total‡</th>
<th>p57Kip2/total§</th>
<th>p57Kip2+ [3H]thymidine+</th>
<th>(counts; mean %±s.d.)</th>
<th>(counts; mean %±s.d.)</th>
<th>(counts, %)</th>
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<tbody>
<tr>
<td>E14.5</td>
<td>0</td>
<td>822/2500, 721/2500; 30.8%±2.8%</td>
<td>43/2500, 55/2500; 1.9%±0.34%</td>
<td>1/1543, 0.06%</td>
<td></td>
<td></td>
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<tr>
<td>E14.5</td>
<td>4</td>
<td>870/2500, 788/2500; 33.1%±2.3%</td>
<td>58/2500, 53/2500; 2.2%±0.14%</td>
<td>4/1658, 0.2%</td>
<td></td>
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<tr>
<td>E14.5</td>
<td>8</td>
<td>970/2500, 905/2500; 37.5%±1.8%</td>
<td>63/2500, 44/2500; 2.1%±0.52%</td>
<td>13/1875, 0.7%</td>
<td></td>
<td></td>
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<tr>
<td>E14.5</td>
<td>16</td>
<td>833/2500, 1071/2500; 38.0%±6.7%</td>
<td>117/2500, 90/2500; 4.1%±0.76%</td>
<td>58/1904, 3.0%</td>
<td></td>
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</tr>
<tr>
<td>E14.5</td>
<td>24</td>
<td>935/2500, 1130/2500; 41.3%±5.5%</td>
<td>124/2500, 101/2500; 4.4%±0.63%</td>
<td>69/2065, 3.3%</td>
<td></td>
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<tr>
<td>P2</td>
<td>0</td>
<td>457/2500, 406/2500; 17.2%±1.4%</td>
<td>20/2500, 15/2500; 0.7%±0.1%</td>
<td>0/863, 0%</td>
<td></td>
<td></td>
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<tr>
<td>P2</td>
<td>16</td>
<td>481/2500, 558/2500; 20.7%±2.2%</td>
<td>32/2500, 24/2500; 1.1%±0.24%</td>
<td>2/1039, 0.2%</td>
<td></td>
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<tr>
<td>P2</td>
<td>24</td>
<td>485/2500, 500/2500; 19.7%±0.4%</td>
<td>18/2500, 27/2500; 0.9%±0.26%</td>
<td>0/985, 0%</td>
<td></td>
<td></td>
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<tr>
<td>P2</td>
<td>36</td>
<td>531/2500, 483/2500; 20.2%±1.3%</td>
<td>22/2500, 28/2500; 1.0%±0.17%</td>
<td>0/1014, 0%</td>
<td></td>
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*Freshly dissected retinae (6-8) were incubated with [3H]thymidine in culture medium for 1 hour, washed and cultured as explants for the amount of time indicated.
‡The number of grains for 14 randomly selected [3H]thymidine-labeled cells varied from 8 to 65 grains per cell (mean=29.5±16.1). The number of grains for 14 randomly selected unlabeled cells varied from 0-3 grains per cell (mean=0.5±0.9).
§These cells were borderline between labeled and unlabeled cells. One cell contained 13 grains, and the other contained 14 grains.
¶Data for the 36 hour and subsequent (data not shown) time points were taken from a separate experiment in which the number of grains found in 14 randomly selected [3H]thymidine-labeled cells varied from 9-61 grains per cell (mean=27±17). The number of grains for 14 randomly selected unlabeled cells varied from 0-5 grains per cell (mean=0.8±1.4).

s.d.=standard deviation.
when p57Kip2 is not normally expressed (see Fig. 1). Immediately after labeling, at T=0, only 1 cell out of 5,000 was found to express p57Kip2 and this cell did not accumulate [3H]thymidine (Table 3). After 12 hours in culture, a few more p57Kip2 immunoreactive cells (3/5000) were detected and the proportion increased steadily during the first 48 hours when the levels reached that seen in the adult retina (Table 3). At all timepoints examined, [3H]thymidine-labeled p57Kip2 immunoreactive cells were rare or not detected at all (Table 3). Taken together, the pulse labeling and the continuous labeling experiments indicate that p57Kip2 is not used in the postnatal retina to mediate cell cycle exit and that the majority of p57Kip2 immunoreactive amacrine cell subpopulation completed their final mitosis during the embryonic stages of development.

### Forced expression of p57Kip2 leads to premature cell cycle exit

The results described above show that p57Kip2 is upregulated in mitotic progenitor cells during G1/G0, consistent with a role in cell cycle exit. This effect is likely to be mediated through binding of p57Kip2 to the major D-type cyclin in the retina (cyclin D1, Fantl et al., 1995; Sicinski et al., 1995) and inactivation of its associated kinase. To test if excess p57Kip2 can force premature cell cycle exit, embryonic retinae (E14.5) were infected with a replication incompetent retrovirus (pNIN-Exp57) carrying the p57Kip2 cDNA and a nuclear lacZ reporter gene (Fig. 3A). The p57Kip2 protein expressed from this retrovirus contains an epitope tag (FLAG) on the amino terminus and a purification tag (6×His) on the carboxy terminus (Fig. 3A). Cultured fibroblasts (NIH-3T3) infected with pNIN-Exp57 express high levels of p57Kip2, which can be readily purified on Ni2+-NTA agarose resin and detected using a monoclonal antibody specific for the FLAG epitope (Fig. 3B). In addition, infected cells exit the cell cycle but do not undergo apoptosis (data not shown). Retinae (n=21) infected with pNIN-Exp57 or the control virus (pNIN-E) were cultured for 10 days (see Materials and Methods). Following this culture period, retinae were stained for nuclear β-galactosidase expression, and the size of each clone derived from individual infected progenitor cells was scored. The number of cells per clone, even in very large clones, can be reliably scored using this virus due to the intense nuclear localized staining (Fig. 3C,D). A distribution in clone size ranging from 1 to 30 cells was observed in retinae infected with each virus (Fig. 3E). However, the proportion of single cell clones in retinae infected with pNIN-Exp57 (73/208, 35%±2.8%) was significantly increased compared to those infected with the control virus (21/190, 11%±2.5%; P=0.0054; Fig. 3E). No apoptotic nuclei

### Table 3. p57Kip2 immunoreactive cells increase in culture but do not accumulate [3H]thymidine

<table>
<thead>
<tr>
<th>Culture time* (hours)</th>
<th>p57Kip2 +/total§ (counts; mean %±s.d.)</th>
<th>p57Kip2 +/d [3H]thymidine +/Antigen§ (counts; mean %±s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12/2500, 0/2500; 0.02%±0.03%</td>
<td>0/1</td>
</tr>
<tr>
<td>12</td>
<td>0/2500, 2/2500; 0.06%±0.03%</td>
<td>0/3</td>
</tr>
<tr>
<td>24</td>
<td>4/2500, 2/2500; 0.12%±0.05%</td>
<td>1/68</td>
</tr>
<tr>
<td>48</td>
<td>12/2500, 15/2500; 0.54%±0.08%</td>
<td>2/27§</td>
</tr>
<tr>
<td>96</td>
<td>11/2500, 14/2500; 0.50%±0.08%</td>
<td>2/25</td>
</tr>
</tbody>
</table>

*Freshly dissected retinae (2-3) from postnatal day 0 mouse pups were incubated continuously with [3H]thymidine in culture medium as explants for the amount of time indicated. Culture medium with fresh [3H] thymidine was changed every 24 hours of culture.

†The number of grains for 15 randomly selected [3H] thymidine labeled cells varied from 18 to >80 grains per cell (mean=47±30). The number of grains for 14 randomly selected unlabeled cells varied from 0-6 grains per cell (mean=1.5±1.2).

‡Due to the heavy labeling of cells incubated in the presence of [3H]thymidine for 6 days, these slides were exposed for approximately half the time of the slides from samples cultured for 2 and 4 days.

§The labeled, p57Kip2 -immunopositive cell in this sample contained 15 grains which was borderline between labeled and unlabeled cells.

||The [3H]thymidine-labeled cells detected at 96 hours contained 12 and 20 silver grains.

s.d.=standard deviation.
Fig. 3. p57kip2 drives mitotic retinal progenitor cells out of the cell cycle and interacts with cyclin D1 in the retina. Mouse p57kip2 was overexpressed in retinal progenitor cells using a replication incompetent retrovirus. (A) Retroviral construct showing the p57kip2 cDNA flanked by epitope (FLAG) and purification (6xHis) tags. The bicistronic mRNA produced from this virus encodes p57kip2 and nuclear localized β-galactosidase. (B) An anti-FLAG immunoblot of crude lysate (lane 1) and Ni2+-NTA agarose-purified eluate (lane 2) from NIH-3T3 cells infected with pNIN-Ep57 showed high levels of expression of full-length p57kip2 protein. No protein was detected when 3T3 cells were infected with the control virus lacking the p57kip2 cDNA (pNIN-E) (B, lanes 3, 4). The number of cells in small (C) and large (D) retinal clones infected with pNIN-E were readily scored on sections stained for β-galactosidase expression. Scale bar, 10 μm. Approximately 200 clones for each virus were scored from 2-3 independent experiments to obtain clone size distribution data (E). p57kip2 protein was purified from retinae infected with pNIN-Ep57 on Ni2+ -NTA agarose resin using non-denaturing conditions. An anti-cyclin D1 immunoblot was performed on eluates from the Ni2+ resin containing proteins bound to p57kip2 (F). Lane 1, recombinant cyclin D1; lane 2, guanidine-HCl eluate from pNIN-Ep57 infected explants; lane 3, guanidine-HCl eluate from pNIN-E infected explants. The arrow indicates cyclin D1. Mr, protein markers, relative molecular mass from bottom: 26, 36, 42, 55, 66, 97, 116, 158 kDa; LTR, long terminal repeat; IRES, internal ribosome entry site.

were observed in retinal clones (n>50) expressing p57kip2, as assessed using the TUNEL assay (data not shown).

To determine if the reduction in clone size that resulted from p57kip2 overexpression might be mediated through interactions with cyclin D1, we took advantage of the 6xHis purification tag on the carboxy terminus of p57kip2 expressed from pNIN-Ep57 (Fig. 3A). Embryonic day 14.5 retinae (62 retinae) were infected with high titer stocks (>10^7/ml) of pNIN-Ep57 or pNIN-E and cultured for 2 days to facilitate maximum expression of p57kip2 from the viral LTR (Fig. 3A). Retinal lysates were prepared and the histidine-tagged p57kip2 protein was bound to Ni2+ agarose resin. Proteins associated with p57kip2 were eluted (see Materials and Methods) and immunoblotted with an anti-cyclin D1 antibody (Fig. 3F). Eluates from pNIN-Ep57-infected explants contained cyclin D1 (Fig. 3F), suggesting that p57kip2 can bind cyclin D1 in mitotic retinal progenitor cells. These data do not rule out the possibility that p57kip2 interacts with other components of the cell cycle machinery in vivo.

Retinae from mice deficient for p57kip2 showed increased mitosis during embryonic development

A targeted disruption of the mouse p57kip2 gene has been described previously (Yan et al., 1997; Zhang et al., 1997). This gene is imprinted such that the paternal allele is inactivated and half the progeny from a p57kip+/− female will be null for p57kip2 expression (Zhang et al., 1997). Offspring that inherit the disrupted allele of p57kip2 from such a cross are designated p57kip+/-m. To determine if retinal progenitor cells undergo additional rounds of cell division in the absence of p57kip2, the proportion of mitotic cells was calculated during embryonic development by performing bromodeoxyuridine (BrdU) pulse-labeling (Fig. 4A,B). At least 500 cells were scored from 6-8 retinae at each stage of development. After scoring, the genotypes were determined and the data from the wild-type or p57kip2-deficient retinae were averaged (Fig. 4C). The proportion of mitotic cells observed at E13.5, prior to the peak in p57kip2 expression, was not significantly different between the wild-type and knockout retinae (22.2%±0.9% and 21.3%±1.2%; Fig. 4C). Several days later (P0), when p57kip2 is no longer expressed, the proportion of mitotic cells was indistinguishable between the wild-type and knockout retinae (6.0%±1.4% and 6.2%±0.45%; Fig. 4C).
While postnatal progenitor cells do not express p57Kip2 and thus do not use it to mediate cell cycle exit (Table 1), it is possible that p57Kip2 expression is required in a subset of developing amacrine cells to prevent them from re-entering the cell cycle. To test this possibility, BrdU labeling was performed on wild-type and p57Kip2-deficient retinae from later stages of development. Loss of the maternally inherited allele of p57Kip2 results in neonatal lethality (Yan et al., 1997; Zhang et al., 1997). Therefore, to facilitate complete retinal differentiation (see Materials and Methods), retinae from newborn pups were cultured as explants. After 2, 4 or 6 days in vitro (DIV), no difference in the proportion of mitotic cells was observed between explant retinae from wild-type and p57Kip2-deficient neonates (Fig. 4C).

Apoptosis compensates for extra rounds of cell division observed in p57Kip2-deficient retinae

Previous reports have demonstrated that lens epithelial cells and lens fiber cells express p57Kip2 (Zhang et al., 1997). As in the retina, additional rounds of mitosis were observed during the peak period of p57Kip2 expression in the lens of p57Kip2 knockout mice (Zhang et al., 1997). Inappropriate S-phase entry led to apoptosis in the lens epithelial and fiber cells (Zhang et al., 1997). To test if inappropriate S-phase entry leads to apoptosis in the retina, a TUNEL assay was performed on retinal sections from wild-type and p57Kip2-deficient mice at E13.5 and E14.5. Apoptotic nuclei were observed in lens fiber cells and anterior lens epithelium of p57Kip2-deficient embryos at E13.5 as described previously (Fig. 4D,E), but no apoptotic nuclei were found in the retina at this stage (Fig. 4D). By E14.5, as p57Kip2 expression is upregulated in the retina, apoptotic nuclei were observed in the inner neuroblastic layer of retinae from knockout mice where p57Kip2 protein is normally expressed (Fig. 4F,G).

The BrdU labeling experiments described above demonstrate that embryonic retinal progenitor cells continue to divide in mice lacking p57Kip2. In the absence of any compensatory process, such continued proliferation should result in perturbations in the proportion of retinal cell types. However, the increase in apoptosis seen in the p57Kip2 retinae may provide compensation by selectively eliminating these extra cells. To test this possibility directly, the proportion and distribution of several retinal cell types were examined in retinal explants (see Materials and Methods) from p57Kip2-deficient and wild-type littermates using cell-type-specific antibodies. Mice were genotyped after cell counting and data from wild-type or p57Kip2-deficient neonates were pooled to obtain the mean and standard deviation for each pair of samples (Fig. 5I). Neither the distribution nor the proportion of the major retinal cell types (rod and cone photoreceptors, bipolar cells, amacrine cells, horizontal cells, Müller glia) was affected in the mutant animals (representative sections are shown in Fig. 5A-H and cell counts are summarized in Fig. 5I).

Loss of p57Kip2 results in an increase in the calbindin population of amacrine interneurons

Several of the developmental defects described in the initial characterization of the p57Kip2 knockout mouse could not be
explained as the result of deregulated proliferation (Zhang et al., 1997). This led to the proposal that p57 Kip2 might serve a non-CKI role in cell fate specification and/or differentiation (Zhang et al., 1997). In the postnatal retina, p57Kip2 is expressed in a novel subpopulation of amacrine cells (M. A. D. and C. L. C., unpublished data), and because it is not involved in regulating cell cycle exit or maintenance of cells in a postmitotic state (see Table 1; Fig. 4), we set out to test if p57Kip2 is required for amacrine cell development. The proportion and distribution of several amacrine cell populations in retinae from wild-type and p57 Kip2-deficient mice were compared.

As shown in Fig. 5, the proportion (8.1%±0.3%; Fig. 5I) and distribution (Fig. 5E-H) of amacrine cells in retinal explants were very similar to those seen in vivo (7.6% (Jeon et al., 1998)). These data were obtained using two antibodies against pan-amacrine epitopes, syntaxin-1 (Barnstable et al., 1985) and VC1.1 (Naegele and Barnstable, 1991). We next asked whether the distribution of the p57Kip2 subpopulation of amacrine cells was similar in explants relative to in vivo. This highly restricted subpopulation was distributed across the lower portion of the INL in retinal explants and showed occasional displacement to the GCL (Fig. 6A), as was seen in vivo between postnatal day 10-12 (Fig. 1J). In addition, retinae from p57+/m mice lacked p57 Kip2 immunoreactivity (Fig. 6B) verifying that, as expected from studies on other tissues (Zhang et al., 1997), the paternal allele is silenced in retinal cells.

A systematic survey of amacrine subpopulations in wild-type and p57Kip2-deficient retinae revealed a significant increase (1.77%±0.3%, versus 0.74%±0.16, P<0.0001) of calbindin amacrine cells in the p57Kip2-deficient retinae (Fig. 6C,D). Three other well-characterized amacrine subpopulation markers (calretinin, parvalbumin and choline acetyltransferase) were examined and showed no difference in p57Kip2-deficient explants (Fig. 6E-H,I). As an independent measure of the observed increase in calbindin amacrine cells, an immunoblot was performed on crude retinal lysates prepared from wild-type and p57Kip2-deficient retinal explants cultured for 10 days (Fig. 6J). Consistent with the data from cell counts, an increase in calbindin protein was observed in samples prepared from knockout retinae whereas the subpopulation marker calretinin was not increased (Fig. 6J).

Calbindin is expressed in horizontal cells as well as amacrine cells (Ogini et al., 1998; Uesugi et al., 1992). The additional calbindin immunoreactive cells in the p57Kip2-deficient retinae resembled amacrine interneurons morphologically (disassociated cell scoring, see Materials and Methods) and were found in the lower half of the INL where amacrine cells normally reside. Furthermore, other horizontal cell markers such as parvalbumin (Ogini et al., 1998) were not increased in the p57Kip2-deficient retinae (Fig. 6I). Taken together these observations suggest that the calbindin subpopulation of amacrine interneurons are specifically increased in the retinae from p57Kip2-deficient mice.

**DISCUSSION**

We have presented evidence that the p57Kip2 cyclin kinase inhibitor plays distinct roles at different stages of retinal development. In embryonic retina, p57Kip2 regulates the cell cycle exit of a subset of progenitor cells and is sufficient to drive retinal progenitor cells out of the cell cycle, perhaps by interacting with cyclin D1. In the absence of p57Kip2, programmed cell death compensates for the extra cell divisions seen in retinal cells of the inner neuroblastic layer. In contrast...
to this role in the embryonic retina, p57Kip2 does not appear to regulate proliferation in the postnatal retina. Instead, the expression pattern of p57Kip2 is consistent with a role in amacrine subpopulation development. In the p57Kip2-deficient retinae, there is an alteration in at least one subpopulation of amacrine cells. A mutation that results in such an alteration has not been previously reported.

**Retinal progenitor cells use distinct mechanisms to exit the cell cycle**

We found that p57Kip2 is upregulated during the G1/G0 phase of the cell cycle in embryonic retinal progenitor cells. Of particular interest was the observation that only a subset (approx. 16.5%) of retinal cells that become postmitotic during this time do so through upregulation of p57Kip2. Another Cip/Kip family member, p27Kip1, is broadly expressed in the retina at this stage of development and is likely to work in other retinal progenitor cells to regulate their proliferation (Levine et al., 2000; Zhang et al., 1998) (M. A. D. and C. L. C., unpublished). This suggests that progenitor cells differ in the mechanisms they use to withdraw from the cell cycle and it is possible that the way in which progenitor cells exit the cell cycle (p57Kip2 versus p27Kip1) may affect the fates of their progeny. Alternatively, progenitor cells may exhibit bias in their production of different retinal cell types before they exit the cell cycle and this is reflected in their distinct use of CKIs. Indeed, progenitor cell heterogeneity has been demonstrated at this stage of development by virtue of the expression of two markers for amacrine and horizontal cells (Alexiades and Cepko, 1997). It is possible that these distinct progenitor cell types use distinct mechanisms to control cell cycle exit.

**Apoptosis compensates for deregulated proliferation in the absence of p57Kip2**

Perturbation of progenitor cell proliferation should affect the proportion of the various retinal cell types because of their birth order during development (Altshuler, 1991). For example, if exit from the cell cycle early in development was blocked, then the neurons normally produced at this time may be lost or reduced in the mature retina. We found that embryonic progenitor cells that would normally exit the cell cycle by upregulating p57Kip2 instead continue to divide in retinae from

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**Fig. 6.** Calbindin amacrine cells were specifically increased in retinae from mice lacking p57Kip2. Dissociated retinae and retinal sections were stained with amacrine subpopulation-specific antibodies. (A,B) p57Kip2 immunoreactive cells (arrows) were distributed normally in retinal explants from wild-type animals (A) but were absent from p57Kip2, deficient explants (B). Scale bar, 10 μm. (C,D) Calbindin immunoreactive amacrine cells (arrows) are increased in the inner nuclear layer of retinal explants from p57Kip2-deficient retinae. (E-H) Calretinin-immunoreactive cells were distributed normally in the p57Kip2 retinae (H) as compared to the wild-type explants (F). (E,G) DAPI staining of the sections shown in F,H. (I) The proportion of calbindin immunoreactive cells were increased in the p57Kip2 knockout retinae as measured by dissociated cell scoring. Each bar represents the average of 2,500 cells scored from 3 (calretinin, parvalbumin, choline acetyltransferase (ChAT)) or 18 (calbindin) independent retinae. (J) Immunoblots of crude lysates prepared from wild-type (lanes 1,2,3,7,8) and p57Kip2-deficient (lanes 4,5,6,9) retinal explants. Tubulin was used as an internal control for protein loading. Three of the neonates were phenotypically p57Kip2-deficient as indicated by lens vacuolization (arrows) and one knockout animal (lane 4) exhibited normal lens morphology. Variable penetrance has been reported previously (Zhang et al., 1997). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; h, horizontal cells.
p57Kip2-deficient mice. However, contrary to the above prediction, the proportion of both early born and late born retinal cell types was unaffected in these animals. Although a small difference in proportion of cells that populate the majority of the retina (such as rods, 79%; Jeon et al., 1998) might be missed in our analysis, small differences in the other cell types (such as cone photoreceptors, 2.2%) would have been detected. Therefore, a compensatory mechanism must be operating in the p57Kip2-deficient retinae. Data presented here strongly suggest that this compensation is achieved through immediate selective apoptosis of the extra cells generated during the peak period of p57Kip2 expression. There may be a regulatory mechanism that monitors the production of postmitotic daughter cells throughout development or, alternatively, these cells may undergo immediate apoptosis because of an autonomous mechanism that is triggered when they attempt to re-enter the cell cycle while initiating a program of differentiation. Such a mechanism has been proposed to account for the widespread apoptosis in the central nervous system of the retinoblastoma (Rb)-deficient mice (Jacks et al., 1992; Lee et al., 1992).

**p57Kip2 is required for development of amacrine cell subpopulations**

Several lines of evidence suggest that Cip/Kip family members in general, and p57Kip2 in particular, may be involved in developmental processes beyond their prescribed roles as cyclin kinase inhibitors. The Cip/Kip family members are promiscuous kinase inhibitors and may regulate kinases required for cell fate specification and/or differentiation during development (Chellappan, 1996). In addition to their shared cyclin kinase binding domains, the Cip/Kip proteins have distinct, structurally complex domains which may prove to contain novel biochemical properties (Matsuoka et al., 1995). This is especially true for p57Kip2, which is the most structurally complex family member (Matsuoka et al., 1995).

Several of the abnormalities observed in the p57Kip2 knockout mice (including defects in muscle, kidney, palate and chondrocyte development) appear to not be linked to proliferation defects. It was therefore proposed that this protein directly influences cell fate specification and/or differentiation in these tissues (Zhang et al., 1997). Recently, the *Xenopus* cyclin kinase inhibitor p27Xic1, which is a member of the Cip/Kip family and is believed to be related to the mammalian protein p27Kip1, was shown to induce the Müller glial cell fate during retinal development (Ohnuma et al., 1999). Consistent with the idea that cyclin kinase inhibitors may be bifunctional molecules, the Müller-inducing portion of the p27Xic1 was found to be separable from the portion of protein that induces cell cycle exit (Ohnuma et al., 1999). Cyclin kinase inhibitors are not the only molecules that may regulate both cell cycle progression and cell fate specification. A novel protein cloned from *Xenopus* called geminin was found to contain a neutralizing domain that was separable from a domain that has been shown to be involved in the regulation of DNA replication (Bastians et al., 1999; Kroll et al., 1998; McGarry and Kirschner, 1998). Taken together, these reports indicate that cell cycle exit and cell fate specification are not only coordinated temporally during development but that individual molecules can play roles in both processes through distinct protein domains.

We have presented data that support the idea that p57Kip2 plays a role in the development of amacrine interneurons. First, p57Kip2 is expressed during the period of amacrine cell differentiation (P2-P10; (Fletcher and Kalloniatis, 1997; McArdle et al., 1977; Oguni et al., 1998)) in a restricted subpopulation of amacrine cells (M. A. D. and C. L. C., unpublished data). Second, at this stage, p57Kip2 does not regulate progenitor cell proliferation, nor is it required to prevent amacrine interneurons from re-entering the cell cycle. Third, and most importantly, a specific increase in the calbindin-expressing amacrine cell subpopulation was identified in p57Kip2-deficient retinae. The increase in calbindin cells is relatively small (approx. 2.5 fold) but it is precisely what would be expected for an amacrine subpopulation defect based on the restricted expression of p57Kip2 and calbindin. We showed that the defect in calbindin subpopulation genesis does not reflect a generalized defect in amacrine cell development because the proportion of amacrine cells labeled by two different antibodies against pan-amacrine markers and three different antibodies against subpopulation markers showed no differences in the knockout mice. p57Kip2 is not expressed in the same cells as calbindin (M. A. D. and C. L. C., unpublished data) so we propose that there is either a switch in amacrine subpopulation fate or a defect in communication between subpopulations during development.

**p57Kip2 may act embryonically or postnatally to regulate amacrine subpopulation genesis**

Some amacrine cell subtypes may be specified at or around the time that they exit the cell cycle (E13-P2), while others may be specified later as they further differentiate in the postnatal period (P2-P12). This idea is suggested by the distinct kinetics of different amacrine subpopulations markers. Some markers of subpopulation differentiation appear shortly after cells exit the cell cycle (ChA T (Galli-Resta et al., 1997); calbindin (Uesugi et al., 1992)) while others appear somewhat later (parvalbumin (Uesugi et al., 1992)). p57Kip2 is expressed during both of these periods of development and therefore, p57Kip2 may influence amacrine cell genesis embryonically or postnatally. Based on the work presented here and previous studies on amacrine cell development, we propose two models to account for the specific increase in calbindin amacrine cells in the p57+/− mice.

In the first model, the decision to differentiate into a specific amacrine cell subtype is made around the time that these cells commit to the amacrine cell fate during the embryonic period of retinal development. If the cells that become postmitotic by upregulating p57Kip2 normally go on to become calbindin amacrine cells, then the extra rounds of cell division observed in the knockout retinae would account for the increase in the number of these cells. In favor of this idea is the observation that calbindin is normally expressed in amacrine cells in the embryonic period suggesting that specification of this subpopulation occurs early (Uesugi et al., 1992). However, against this notion is the observation that too many cells express p57Kip2 during the embryonic period to be only one subpopulation of amacrine cells as each is only about 5% of the total amacrine population and approximately 20% of all cells express p57Kip2 during cell cycle exit. In addition, apoptosis appeared to compensate for the extra cell divisions in the embryonic period. Nonetheless, it is still possible that a
small fraction of cells escape apoptosis and then these cells go on to calbindin amacrine cells.

In the second model, at least some amacrine cells may not commit to specific subpopulations until the postnatal stages when neurotransmitters are produced (Fletcher and Kalloniatis, 1997) and synaptic connections are formed (McArdle et al., 1977). In this scenario, p57Kip2 serves as a determinant for a unique amacrine subpopulation. If specification of the p57Kip2 subpopulation is cell autonomous, then, in the absence of this protein, these cells switch fates and become calbindin amacrine cells. Alternately, the role of p57Kip2 in amacrine subtype specification may not be cell autonomous. In this case, absence of p57Kip2 results in perturbation of cell signaling and thus a secondary increase in calbindin amacrine cells.

These models provide a valuable foundation for understanding how the amacrine interneuron network is organized during development. We have demonstrated for the first time a connection between the specification and/or differentiation of two amacrine cell subpopulations during development. Our results, combined with previous studies, suggest that the orderly expression of amacrine subpopulation markers reflects the orderly commitment and differentiation of individual amacrine subtypes. Such a mechanism would ensure the appropriate formation of the complex amacrine cell subtype mosaics that are required for visual processing in the vertebrate retina.

We wish to thank Dr. M. H. Baron for many helpful discussions and support throughout this project, Drs. S. Elledge, W. Harper and P. Zhang for cDNAs and knockout mice, J. Zitz for technical support, M. Peters and Drs M.H. Baron and V. Twombly for critical reading of the manuscript. M. A. Dyer was supported by NRSA fellowship no. EY06803-02. This work was supported by National Eye Institute Grant no. EY0-8064.

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