PBK/TOPK, a Proliferating Neural Progenitor-Specific Mitogen-Activated Protein Kinase Kinase

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We performed genomic subtraction coupled to microarray-based gene expression profiling and identified the PDZ (postsynaptic density-95/Discs large/zona occludens-1)-binding kinase/T-LAK (lymphokine-activated killer T cell) cell originating protein kinase (PBK/TOPK) as a gene highly enriched in neural stem cell cultures. Previous studies have identified PBK/TOPK as a mitogen-activated protein kinase (MAPK) kinase that phosphorylated P38 MAPK but with no known expression or function in the nervous system. First, using a novel, bioinformatics-based approach to assess cross-correlation in large microarray datasets, we generated the hypothesis of a cell-cycle-related role for PBK/TOPK in neural cells. We then demonstrated that both PBK/TOPK and P38 are activated in a cell-cycle-dependant manner in neuronal progenitor cells *in vitro*, and inhibition of this pathway disrupts progenitor proliferation and self-renewal, a core feature of progenitors. *In vivo*, PBK/TOPK is expressed in rapidly proliferating cells in the adult subependymal zone (SEZ) and early postnatal cerebellar external granular layer. Using an approach based on transgenically targeted ablation and lineage tracing in mice, we show that PBK/TOPK-positive cells in the SEZ are GFAP negative but arise from GFAP-positive neural stem cells during adult neurogenesis. Furthermore, ablation of the adult stem cell population leads to concomitant loss of PBK/TOPK-positive cells in the SEZ. Together, these studies demonstrate that PBK/TOPK is a marker for transiently amplifying neural progenitors in the SEZ. Additionally, they suggest that PBK/TOPK plays an important role in these progenitors, and further implicates the P38 MAPK pathway in general, as an important regulator of progenitor proliferation and self-renewal.

Key words: subventricular zone; microarray; neural stem cell; progenitor cell; P38 MAPK; external granular layer; rostral migratory stream

Introduction

Neural stem cells (NSCs) are an endogenous, self-renewing population of cells capable of generating all major cell types of the CNS (Lendahl et al., 1990; Reynolds and Weiss, 1992; Capela and Temple, 2002). NSCs exist throughout the germinal zones of the developing embryonic brain and persist into adulthood, providing for ongoing neurogenesis in select regions of the mammalian brain, offering hope for neural repair strategies (Palmer et al., 1999; Gage, 2000; Lie et al., 2004; Morshead and van der Kooy, 2004). Despite their rich potential for therapeutic applications, research on NSCs has been hampered by a lack of markers to identify NSCs prospectively and an incomplete understanding of

the pathways involved in the regulation of their proliferation and differentiation (Anderson, 2001; Lindvall et al., 2004).

To identify genes involved in NSC proliferation and differentiation, we performed extensive analysis of the gene expression in neural stem and progenitor cell cultures (Geschwind et al., 2001; Karsten et al., 2003). To provide additional functional annotation that would aid in identifying genes involved in NSC proliferation or self-renewal, we identified genes expressed in multiple CNS germinal zones and non-neural stem cell populations (Tersikh et al., 2001; Easterday et al., 2003). Postsynaptic density-95/Discs large/zona occludens-1 (PDZ)-binding kinase/T-LAK (lymphokine-activated killer T cell) cell-originating protein kinase (PBK/TOPK) was one transcript found to be consistently elevated in all progenitor cell populations examined consistent with a role in progenitor self-renewal.

PBK/TOPK was not previously known to be involved in any facet of CNS development, but work in non-neural cells suggested that it was expressed in a variety of specialized, proliferative cell types: PBK/TOPK expression was detected in male germ line progenitor cells, activated T-cells, and a variety of lymphomas and leukemias. However, it was absent in WiDr and HT-29 colon cancer cells, indicating that it was not ubiquitously expressed in cycling cells (Abe et al., 2000; Simons-Evelyn et al., 2001; Zhao et al., 2001). Previous work also suggested that PBK/

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TOPK was a member of the mitogen-activated protein kinase (MAPK) kinase (MAPKK) family (Abe et al., 2000; Gaudet et al., 2000; Matsumoto et al., 2004). Activated PBK/TOPK phosphorylated P38 MAPK but not JNK (c-Jun N-terminal protein kinase) MAPK or ERK (extracellular signal-regulated kinase) MAPK *in vitro*. Furthermore, activation of PBK/TOPK required phosphorylation by both the M-phase kinase complex cyclinB/cyclin-dependent kinase 1 (CDK1) and another unknown kinase, possibly RafC or RafA (Gaudet et al., 2000; Yuryev and Wennogle, 2003). These findings suggested that PBK/TOPK may play an important role in linking extracellular signals to an intracellular state, possibly allowing extracellular influence on the cell-cycle-related processes of proliferation or differentiation. Here, this was supported by an initial informatics-based annotation that suggested a role for PBK/TOPK in M-phase in neural cells. Therefore, we examined phosphorylation of PBK/TOPK and P38 MAPK and the impact of P38 inhibition in cycling neuronal progenitors *in vitro*, which provided evidence that this pathway is involved in progenitor proliferation and self-renewal. We then demonstrated PBK/TOPK expression in specific progenitor cells *in vivo*, showing by ablation and lineage mapping that PBK/TOPK is a novel marker for transiently amplifying progenitor cells likely to be involved in the regulation of their self-renewal.

Materials and Methods

In situ hybridization. *In situ* hybridization was performed as described previously (Geschwind et al., 2001). Probes from an antisense 384 bp fragment (GenBank accession number CA782113) and full-length PBK/TOPK had identical expression patterns. For all *in situ* hybridizations, sense RNA controls showed no labeling above background.

Analysis of microarray data. Data were downloaded from National Institute of Neurological Disorders and Stroke (NINDS)/National Institute of Mental Health (NIMH) database (<http://arrayconsortium.tgen.org>). This set included 85 gliomas from 79 patients hybridized onto Affymetrix (Santa Clara, CA) HG133A and B arrays (Freije et al., 2004). Arrays were normalized with dCHIP (www.dchip.org), and expression values were calculated (Li and Wong, 2001). We filtered for genes with a coefficient of variation >0.8 to identify genes that varied significantly across samples. After filtering, there were 2217 probe sets representing 1874 highly variable genes, including PBK/TOPK. Pearson's correlation was performed to identify genes with an expression that covaried with PBK/TOPK at a highly significant level across the samples. DAVID (database for annotation, visualization, and integrated discovery) was used to classify correlated and anticorrelated genes into level 5 biological processes gene ontologies (Dennis et al., 2003). Expression Analysis Systematic Explorer (EASE) was used to test for statistical overrepresentation of categories relative to a background of all 1874 analyzed genes (Hosack et al., 2003). Results are similar if less filtered or unfiltered gene sets are used or if either whole array or whole genome is used for background comparison in EASE analysis.

Phylogenetic analysis. Complete PBK/TOPK sequence for *Homo sapiens* and *Mus musculus* have been reported previously (Abe et al., 2000; Gaudet et al., 2000; Zhao et al., 2001). To identify key functional domains, we searched expressed sequence tag libraries with the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) and draft genome sequences with University of California, Santa Cruz Blat for PBK/TOPK sequence in all available vertebrate species. From these sequences, we were able to construct complete putative homologs for PBK/TOPK in *Rattus norvegicus*, *Xenopus laevis*, *Gallus gallus*, *Danio rerio*, *Oncorhynchus mykiss*, *Canis familiaris*, *Tetraodon nigroviridis*, and nearly complete sequences for *Pan troglodytes* and *Bos taurus* using the DNASTAR Seqman software. We aligned these sequences with clustalW, implemented on DNASTAR Megalign software.

Culture of cerebellar granule cell precursors. Cerebella were harvested from postnatal day 6 (P6) to P8 CD1 mouse pups and digested in Papain with DNase and dissociated in PBS BSA with fire-polished pipettes fol-

lowed by a cell strainer. Granule cell precursors were then separated on a 35/65% Percoll step gradient at 1500 g for 12 min as described previously (Wechsler-Reya and Scott, 1999). Cells were plated at 250 K cells/well onto poly-L-lysine-coated glass coverslips in 24-well plates in 330 μ l of Neurobasal media containing 2% B27 supplement, 1 mM sodium pyruvate, 2 mM glutamine, and 1% penicillin/streptomycin, supplemented with 2.5 μ g/ml mouse recombinant Sonic Hedge Hog (SHH) (461-SH-025; R & D Systems, Minneapolis, MN) as noted in our study.

Flow cytometry for cell cycle. Staining was as described previously (Krishan, 1975). Briefly, cells were lysed in a hypotonic buffer with Triton X-100, RNase, and propidium iodide. DNA content of >13,000 nuclei was measured using cellquest software and a Facs caliber cytometer from Becton Dickinson (Mountain View, CA), and data were analyzed with ModFit 3.1 with service pack 2 for Macintosh to determine percentages of cells in the various phases of the cell cycle. All CV and RCS values were below five.

Preparation and specificity of anti-PBK/TOPK antibodies. The phosphospecific (Thr9) and total PBK/TOPK antibodies were produced by immunizing New Zealand White rabbits with synthetic peptides. The following peptides, coupled to keyhole limpet hemocyanin, were used: Thr9(P) (NFKT*PSKLSEKC) and total PBK/TOPK (CTNEDPKDRP-SAAHIVE). Immunoglobulin was purified using protein A-Sepharose. To ensure phosphospecificity of the phospho-PBK/TOPK (Thr9) antibody, antibodies reactive with the nonphosphopeptide were removed by adsorption to a nonphosphopeptide affinity column. Antibodies that flowed through this column were passed over a column of immobilized phosphopeptide; after the column was washed, antibodies were eluted at low pH and dialyzed. For total PBK/TOPK, protein A-Sepharose purified antibodies reactive with the immunogenic peptide column were eluted and dialyzed. Analysis of the phosphospecificity of the resulting phospho-Thr9 antibody and phospho-independence of the resulting total PBK/TOPK antibody was performed by immunoblotting against whole-cell extracts from control and nocodazole-blocked ME-180 cells and recombinant activated glutathione S-transferase (GST)-PBK/TOPK or the protein dephosphorylated *in vitro* with λ phosphatase (catalog #P0753; New England Biolabs, Beverly, MA). The phospho-independence of the total PBK/TOPK antibody was further established by comparing whole-cell extracts from NIH-3T3 and PC12 cells that were treated with the Ser/Thr phosphatase inhibitor calyculin A (catalog #9902; Cell Signaling Technology, Beverly, MA) to extracts that were subjected to *in vitro* dephosphorylation with λ protein phosphatase.

Animals. Transgenic mice were created and treated as described previously (Bush et al., 1999; Imura et al., 2003; Garcia et al., 2004). All animal protocols were approved by the University of California at Los Angeles animal research committee.

Immunohistochemistry. P7, P12, P14, P21 CD1, and adult CD1, C57BL/6, and transgenic mice were perfused transcardially with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS, pH 7.4. Brains were removed, fixed in 4% paraformaldehyde overnight, sunk in 20% sucrose PBS, frozen in 4-methyl-butane, and stored at -80°C until use. Forty micrometer sections were cut on a cryostat and stored in PBS 0.1% azide at 4°C until use. Free-floating sections were incubated overnight in 24-well plates on a rotator at room temperature in the presence of 0.1% azide, 0.25% Triton X-100, and 5% normal goat serum in 500 μ l PBS and primary antibody at the following concentrations: anti-PBK/TOPK serum (1:500) (Gaudet et al., 2000), anti-PBK monoclonal (1:100; BD Transducin 612170), anti- β III tubulin (Tuj1) (1:1000; MMS-435P; Covance, Princeton, NJ), anti-Mash1 (1:20; 556604; BD Biosciences), anti-glutamate-aspartate transporter (GLAST; 1:5000; AB1782; Chemicon, Temecula, CA), anti-doublecortin (Dcx) (1:500; AB5910; Chemicon), anti-proliferating cell nuclear antigen (PCNA; 1:10000; M0879; Dako-Cytomation, Carpinteria, CA), anti-bromodeoxyuridine (BrdU; rabbit polyclonal; Upstate Biotechnology, Lake Placid, NY), anti-minichromosome maintenance-deficient 2 (MCM2; sc-9839; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NG2 (rabbit polyclonal; Chemicon). For BrdU and PCNA, antigens were retrieved by incubating sections for 1 h at 65°C in 50% formamide, $2\times$ SSX, and for 30 min in 2.0N HCl at 37°C . Secondary antibodies were diluted 1:1000 and included cyanine 2 (Cy2)-, Cy3-, and Cy5-conjugated antibodies (Jackson

ImmunoResearch, West Grove, PA) and Alexa 350-, 488-, 568-, and 594-conjugated antibodies (Invitrogen, San Diego, CA). For PBK/TOPK monoclonal antibody, Tyramide Signal Amplification (PerkinElmer Life Sciences, Emeryville, CA) was used occasionally to enhance signal. In all cases, no primary controls yielded no labeling, except in P7 animals, anti-mouse IgG Alexa 488 apparently labels some cells with a glial morphology. Where necessary, subtype-specific antibodies were used to avoid this confound.

Nuclei were counterstained with DAPI-containing mounting media (Vector Laboratories, Burlingame, CA) or with Topro-3-iodide (Invitrogen), a nuclear stain fluorescing in the far red range (650 nm), by exposing tissue sections for 5 min to a 20 μ M solution in PBS.

Immunocytochemistry. Coverslips were harvested and fixed in 4% paraformaldehyde, washed in PBS, and blocked for 30 min in 5% NGS 0.25% Triton X-100, and PBS. Cells were then exposed to primary overnight at room temperature at the following concentrations: anti-PBK/TOPK, 1:500 (serum) or 1:100 (monoclonal); neuronal-specific nuclear protein (NeuN), 1:250 (Chemicon MAB377); PCNA, 1:5000; doublecortin, 1:500; cyclinB, 1:500 (4125; Cell Signaling Technology); phospho-P38 monoclonal, 1:100 (9216; Cell Signaling Technology); phospho-P38 polyclonal, 1:500 (9211; Cell Signaling Technology). Secondaries and counterstaining were as above.

Microscopy. All fluorescent images were acquired on either a Leica (Heidelberg, Germany) TCS-SP MP confocal and multiphoton inverted microscope and a two-photon laser setup (Spectra-Physics, Fremont, CA) or Zeiss (Thornwood, NY) LSM 510 META confocal microscope using lasers and filters appropriate for the fluorophores, and pseudocolored images were overlaid with Zeiss software or Adobe Photoshop (Adobe Systems, Santa Clara, CA). Far-red wavelengths were most often pseudocolored blue. To confirm colocalization of antibodies, stacks of <1- μ m-thick high-power confocal images were acquired and examined both as three-dimensional reconstructions and as animations moving through the Z plane with Zeiss LSM Image Browser.

Cell counts. For immunocytochemistry, all cells in 10 random 40 $\times$ fields were counted. For the stem cell ablation experiments, we used a stereo investigator to count all PBK/TOPK cells in the subependymal zone (SEZ) ventral to the anterior horn at the level of the anterior commissure bilaterally in three replicate animals. *t* tests were performed in Microsoft Excel (Microsoft, Redmond, CA). Colocalization studies were performed as above in two to four animals. Percentages are given as mean $\pm$ SEM.

Results

PBK/TOPK transcript is expressed exclusively in neurogenic regions in embryonic and adult CNS

Previous array studies demonstrated that PBK/TOPK was expressed by a wide variety of stem and progenitor populations *in vitro*, consistent with a role in stem/progenitor cell proliferation (Geschwind et al., 2001; Terskikh et al., 2001; Easterday et al., 2003). Here, we verified continued expression of PBK/TOPK *in vivo*, in prenatal and postnatal regions of neurogenesis, including the forebrain germinal zone (Fig. 1A), developing hippocampus and dentate gyrus (Fig. 1B), and rostral migratory stream (RMS); (Fig. 1C,D). Especially striking was the expression in the external granular cell layer (EGL) region of the cerebellum in the P7 animal (Fig. 1C). At this age, this structure is populated strictly by granular cell precursors, suggesting that PBK/TOPK is expressed by these neuronal progenitors in addition to the subventricular zone (SVZ), RMS, and hippocampal progenitors.

PBK/TOPK is implicated in neural cell-cycle progression

Previous literature suggested a role for PBK/TOPK in the cell-cycle of non-neural cell types (Gaudet et al., 2000). We were curious whether this was the case in neural cells as well. To examine this hypothesis, we initially applied a bioinformatics-based functional annotation of PBK/TOPK in neural cells. This approach was based on the observation that genes that are involved

Figure 1. PBK/TOPK mRNA is specifically expressed in all germinal zones throughout neural development. **A–D**, Autoradiographic films of *in situ* hybridization with S^{35} -labeled PBK/TOPK antisense RNA in regions in which stem and progenitor cells are found. **A**, PBK/TOPK is expressed in the forebrain germinal zones at embryonic day 13 (E13) (sagittal, whole head), E17 (coronal, whole head), P1 (coronal), and P7 (coronal). **B**, **C**, PBK/TOPK is also expressed in the dentate gyrus (black arrow) and the posterior lateral ventricle (white arrow) at P7 (coronal) (**B**) and in the developing cerebellum (white arrow) and the RMS (black arrows) in sagittal sections at P7 (**C**). **D**, Expression continues in the subventricular zone and RMS of the sagittal adult brain (black arrow) but not the cerebellum (white arrow).

in the same biological process are often tightly coregulated (Eisen et al., 1998; Ren et al., 2000; Miki et al., 2001). If we identified genes falling into specific categories of biological processes that were highly coregulated with PBK/TOPK, this would generate testable hypotheses regarding PBK/TOPK function in neural tissue. In addition, this type of informatic screen should be of interest as a method to rapidly generate hypotheses regarding the function of unstudied or novel genes, as well as infer function for previously studied genes in new contexts.

To perform this analysis, we needed a large neural dataset ($n = 30$ or more) with variable PBK/TOPK expression, and thus we capitalized on array data from neural tumors available in the NINDS/NIMH microarray database. Although brain tumors are not normal progenitors, they are proliferative neural tissue, and data from several sources have demonstrated recently that some tumors, including gliomas, contain a multipotent tumor stem cell from which they derive (Ignatova et al., 2002; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004). We reasoned that using this rich neural tumor dataset to derive hypotheses about the function of a neural stem cell gene would be appropriate, provided that these hypotheses were then tested in the noncancerous, primary neural cells of interest. Furthermore, it was one of only a few datasets with enough distinct samples to permit such an analysis with sufficient precision. We therefore focused on a large-scale study of gliomas in this database (Freije et al., 2004). Although PBK/TOPK was detected in 79 of the 85 gliomas in this set, it showed a high degree of variability, ranging >50-fold, which made this data set amenable to assessing gene coregulation.

We created a gene coregulation matrix and observed that PBK/TOPK was strikingly correlated with a large number of genes involved in the cell-cycle machinery, including ki-67, aurora kinase B, and cyclin B1 (see Materials and Methods). To understand the level of significance of this finding in an unbiased manner, we performed statistical analysis of gene ontology categories using EASE (Hosack et al., 2003). This revealed that in the group of genes most correlated to PBK/TOPK, there is a highly significant overrepresentation of genes involved in the cell cycle (EASE statistic; $p < 10e-15$), especially M-phase genes (EASE statistic; $p < 10e-12$) (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). These data in neural tissues was parallel to data showing cell-cycle regulation of PBK/TOPK expression in synchronized HeLa cells (Whitfield et al., 2002; Matsumoto et al., 2004). This specific pattern of correlation

suggested that PBK/TOPK may play a fundamental role in the cell cycle in neural progenitor cells and tumors that derive from them, especially in the M phase of the cell cycle. In addition, an examination of phylogenetic conservation of the PBK/TOPK protein sequence across multiple vertebrate species demonstrated conservation of the cyclinB/CDK1 phosphorylation site and kinase domains, whereas the eponymous PDZ-binding motif is found only in primates (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material). Protein domains that are conserved across a variety of species may be important to the function of a protein, so we chose to focus our initial *in vitro* analysis on PBK/TOPK regulation in the cell cycle in neural cells and this site in particular (see below and Fig. 3).

PBK/TOPK is expressed by proliferating cerebellar granule cell precursors *in vitro*

For *in vitro* PBK/TOPK studies, we isolated primary cerebellar granule cell precursors (CGPs) from the EGL of the cerebellum, because PBK/TOPK was highly expressed in this region (Fig. 1C, white arrow), and these cells represent a relatively homogeneous neuronal progenitor pool that would simplify analysis. Previous work has shown that CGPs will proliferate in response to the mitogen SHH (Wechsler-Reya and Scott, 1999). We compared the expression of PCNA, PBK/TOPK protein, NeuN, and Dcx in CGPs cultured with or without mitogenic stimulus. PBK/TOPK expression overlapped highly with PCNA ($98.31 \pm 1.38\%$; $n = 138$), and expression of both PCNA and PBK/TOPK decreased dramatically in the absence of mitogen (Fig. 2A), in which case CGPs developed elaborate Dcx-positive process (data not shown). In contrast, relative expression of NeuN, a marker of more mature neurons, increased in the absence of SHH, and PBK/TOPK expression did not overlap with NeuN (Fig. 2B).

PBK/TOPK phosphorylation on cyclinB site is cell-cycle regulated

Having confirmed that PBK/TOPK was present in neuronal progenitor cells *in vitro*, we examined whether PBK/TOPK may be activated by phosphorylation in these cells. In non-neural cells, PBK/TOPK appears to require cyclinB/CDK1 phosphorylation for activation (Gaudet et al., 2000). Therefore, we produced an antibody against a phosphorylated form of the cyclinB/CDK1 target site (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material) as a method of gauging activation of PBK/TOPK. We initially tested this antibody with cell lines that express PBK/TOPK. This antibody recognized a PBK/TOPK-sized band, observed only in cells blocked in mitosis with nocodazole (Fig. 3A). This antibody also detected recombinant, activated GST-PBK/TOPK, and the signal decreased dramatically when recombinant GST-PBK/TOPK was phosphatase-treated (Fig. 3B), demonstrating specificity of the protein for the phosphorylated form of PBK/TOPK.

We then examined the phosphorylation of PBK/TOPK across the cell cycle. Flow cytometry revealed that phospho-PBK/TOPK-positive cells all had high DNA content, indicating that they had likely finished the S phase and were in either the G2 or M phase of the cell cycle (Fig. 3C). To determine whether the expression was in the G2 or M phase, we conducted immunocytochemistry on N2a neuroblastoma and P19 embryonic carcinoma cells. In both lines, phospho-PBK/TOPK was only detected in cells with condensed chromatin indicative of M-phase cells. Furthermore, it was clearly detected throughout all stages of mitosis, outlining condensed chromosomes, but its expression decreased abruptly and dramatically in telophase, and by late telophase,

Figure 2. PBK/TOPK is expressed in proliferating cerebellar granule cell precursors *in vitro*. **A**, Consistent with what is seen *in vivo* in the cerebellum, 72 h after dissection (72H), CGPs treated with mitogen SHH form proliferative clumps of PBK/TOPK (red) and PCNA (green) double-positive cells while the untreated cells stop proliferating and differentiate. Background signal is included in gray to show total cell population. **B**, Quantification of this effect reveals that CGPs in cultures treated with mitogen maintain expression of PBK/TOPK. Untreated cultures have proportionally more cells that express the neuronal maturation marker NeuN.

PBK/TOPK protein itself seemed to disappear (Fig. 3D). Finally, we confirmed this finding in primary cells (CGPs) (Fig. 3E). This suggests that PBK/TOPK is not only expressed in these cells but also phosphorylated and activated.

The PBK/TOPK target P38 MAPK is also phosphorylated in a cell-cycle-dependant manner in neural progenitor cells

Next, we wished to examine whether the PBK/TOPK target P38 MAPK is also phosphorylated in neural progenitors, because in non-neural cells, PBK/TOPK phosphorylates P38 MAPK, activating it (Abe et al., 2000). Because MAPK pathways are highly conserved, we examined the phosphorylation of P38 MAPK in relation to proliferation and the cell cycle in primary CGP cells. We found that differentiation induced by mitogen withdrawal significantly reduced the number of cells positive for phospho-P38 (paired *t* test; *p* < 0.01). Furthermore, strong phospho-P38 was only detected in cyclinB-positive CGPs (Fig. 4A). CyclinB is the G2/M phase-expressed cyclin that directs CDK1 to phosphorylate PBK/TOPK. Coexpression of cyclinB and phospho-P38 suggested that P38 is selectively activated in these cells in the G2/M phases of the cell cycle. Close examination of DNA in phospho-P38-positive cells revealed that they show the condensed chromatin characteristic of mitotic cells, suggesting activation in a cell-cycle-dependant manner. We performed double labeling with phospho-PBK/TOPK and phospho-P38 MAPK. Strikingly, all phospho-PBK/TOPK-positive cells were mitotic and phospho-P38 positive (Fig. 4B). Thus, the PBK/TOPK target P38 appears to be phosphorylated specifically during mitosis in proliferating CGPs, consistent with other reports of mitotically phosphorylated P38 in neuronal progenitors in other germinal zones (Campos et al., 2002). The complete overlap of P38 phosphorylation with PBK/TOPK phosphorylation in CGPs was also seen in N2a and P19 cell lines (data not shown).

P38 MAPK inhibition decreases proliferation of primary progenitors

The studies above suggested that the P38 MAPK pathway was being activated through PBK/TOPK in a cell-cycle-specific manner. We next wished to determine whether this activation had any relevance to the proliferation of primary progenitor cells. We found that treatment of cultured primary CGP with the specific

Figure 3. PBK/TOPK is phosphorylated exclusively during mitosis. **A**, An antibody raised against phosphorylated cyclinB/cdk1 site at threonine 9 (Thr 9) on PBK/TOPK only has signal in ME-180 cells treated with nocodazole, which blocks cells in mitosis (right blot). Probing with total PBK/TOPK antibody reveals equal amounts of PBK/TOPK protein in treated and untreated conditions (left blot). L, Molecular weight marker; +, treated; –, untreated. **B**, PBK/TOPK (left blot) and phospho-PBK/TOPK (right blot) antibodies recognize a recombinant activated GST-PBK/TOPK. Phospho-PBK/TOPK signal decreases with phosphatase treatment. L, Molecular weight marker; 1, ProQinase active GST-PBK (80 kDa); 2, GST-PBK after λ phosphatase treatment; 3, ME-180 (untreated) whole-cell lysate. **C**, Flow cytometric analysis of untreated Jurkat cells, using phospho-PBK/TOPK antibody labeled with FITC (y-axis) versus DNA content measured by propidium iodide (x-axis), which can be used to measure the position of a cell in the cell cycle. The boxed population indicates that phospho-PBK/TOPK-positive cells have 4N DNA content indicative of either G2 or M phase cells. **D**, A 100 $\times$ immunocytochemistry on N2A cells shows that phospho-PBK/TOPK is expressed specifically throughout the M phase, but expression of PBK/TOPK (green) and phospho-PBK/TOPK (red) decreases dramatically in late telophase. Notice that nonmitotic adjacent cells are phospho-PBK/TOPK negative. **E**, A 20 $\times$ immunocytochemistry, as with N2A in primary neuronal precursors (CGPs), reveals that phospho-PBK/TOPK (red) is detected only in cells undergoing mitosis as indicated by their condensed chromatin (arrowheads).

Figure 4. PBK/TOPK target P38 MAPK is also phosphorylated at mitosis in primary neuronal precursors and is required for normal proliferation. **A**, Strong P38 MAPK phosphorylation (red) occurs only in G2/M, cyclinB-positive primary CGPs (green), suggesting that P38 is activated primarily in the G2/M phase in these cells. **B**, Strong phospho-P38-positive (green) CGPs are always phospho-PBK/TOPK positive (red) and vice versa. Furthermore, they have the condensed chromatin of mitotic cells (arrowheads), suggesting that PBK/TOPK activates phospho-P38 at mitosis in these cells. **C**, The P38 MAPK-specific inhibitor SB203580 reduces a fraction of S-phase cells in proliferating CGP. Harvested CGPs were cultured with or without mitogen for 48 h, and 50 μ M SB203580, the specific P38 MAPK inhibitor, was added. After 24 h, cells were harvested and subjected to cell-cycle analysis via flow cytometric measurement of DNA content and immunocytochemistry. **D**, Cell counts of PBK/TOPK and NeuN-positive cells reveal that SB203580 reduces PBK/TOPK-positive proliferating progenitor cells and total number of cells, without significantly impacting nonproliferative, differentiated NeuN-positive cells. Error bars represent SD.

P38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) (Gallagher et al., 1997) resulted in a dramatic decrease in the fraction of cells in the S-phase (Fig. 4C). Twenty-four hours of P38 inhibition caused a reduction in the number of the PBK/TOPK-positive cells (the proliferating, PCNA-positive cells). However, in the absence of mitogen treatment on the primary progenitors, P38 inhibition had no impact on cell number (Fig. 4D) in NeuN-positive differentiating cells, indicating that P38 inhibition was not killing cells in a nonspecific manner but specifically impacting the proliferating cells. To extend these findings to other primary progenitor populations in addition to the CGPs, we also exposed attached neural progenitors cultured from E12 mouse telencephalic germinal zones to varying doses of SB203580 in the presence of the mitogen basic fibroblast growth factor (bFGF). These cells also express PBK/TOPK and phosphorylate P38 in a cell-cycle-dependent manner (supplemental Fig. 2A, available at www.jneurosci.org as supplemental material) (data not shown). We saw a dose-dependent decrease in cell number after exposure to drug (supplemental Fig. 2B, available at www.jneurosci.org as supplemental material) and a decrease in cycling cells as assessed

by flow cytometry. At long exposures or high doses, we also observed an accumulation of DNA-an euploid cells, suggesting that those cells that did survive were not completing cell division properly (supplemental Fig. 2C, available at www.jneurosci.org as supplemental material).

PBK/TOPK is not expressed by postmitotic neuroblasts or mature glia *in vivo*

These *in vitro* data suggested a role for PBK/TOPK in neural progenitor and immortalized cell line cell-cycle progression and division. To examine whether such a role was consistent with *in vivo* expression data, we performed a battery of immunohistochemical analyses with several markers of proliferation, differentiation, and progenitor states. Two different antibodies to PBK/TOPK in early postnatal and adult mice showed the same cytoplasmic and occasionally nuclear expression in the regions of ongoing neurogenesis: the subgranular layer of the dentate gyrus, the subependymal zone of the lateral ventricles, the rostral migratory stream in all ages, and the EGL of the early postnatal cerebellum, consistent with *in situ* hybridization results. Postnatal expression is summarized in Table 1. Note that, in our experiments, we did not

Table 1. Postnatal distribution of PBK/TOPK expression

	P7–P8	P12–P14	P21	Adult
Cerebellum	+	+		
External granule layer	+++	+		
White matter	++			
Cortex				
Subcortical white matter	+			
Hippocampus	++	+		
Subgranular layer	++	++	++	+
Lateral ventricles				
Subventricular zone	++	++	++	+
Anterior subventricular zone	+++	+++	++	++
Rostral migratory stream	+++	+++	++	++

observe PBK/TOPK expression in the adult corpus callosum, fornix, or other white matter regions where mature oligodendrocytes are predominantly found and thus concluded that mature oligodendrocytes do not generally express PBK/TOPK.

We examined the expression of PBK/TOPK in depth in two regions of postnatal neurogenesis, the EGL and the SEZ/RMS, to identify what cell type expresses PBK/TOPK *in vivo*. For the first 2 weeks after birth, cerebellar granule neurons continue to be born from the EGL. The EGL contains a mitotic layer with PCNA-positive proliferating progenitors (EGLa), a premigratory layer with postmitotic immature neurons (EGLb), and the radially oriented processes of Bergmann glia (Migheli et al., 1999). Double-label immunohistochemistry of PBK/TOPK and Tuj1 or Dcx (data not shown), both markers of immature neurons, reveal clear expression of PBK/TOPK in the PCNA-positive mitotic (Fig. 5B) but not the premigratory (Fig. 5D) zones of the EGL. Not surprisingly, there was no PBK/TOPK expression in the cerebellum in the P21 or adult animal, when neurogenesis in the cerebellum is complete (data not shown). In the EGL, scaffolding for cell division and migration are provided by GLAST-positive Bergmann glia (Furuta et al., 1997), which are PBK/TOPK negative (Fig. 5C). This pattern of expression is consistent with a role in proliferation of neuronal progenitors in the cerebellum.

In adult animals, PBK/TOPK is expressed sporadically within the subgranular layer of the dentate gyrus and strongly within the subependymal zone of the lateral ventricle. Especially striking is the postnatal PBK/TOPK expression in the neurogenic SEZ and the full extent of the rostral migratory stream from the anterior lateral ventricle to the beginning of the olfactory bulb (Fig. 6B). This expression is seen in all ages examined but decreases in intensity from P7 to adulthood. Most PBK/TOPK-positive cells were also PCNA positive when examined at high magnification. To further assess PBK/TOPK expression within migratory immature neuroblasts in the SEZ and RMS, we performed double labeling with Tuj1 and Dcx. Dcx expression had perfect overlap with strongly positive Tuj1 cells, so only Dcx is shown here. PBK/TOPK was strongly expressed in cells adjacent to, but not in, clusters of Dcx (Fig. 5E)-positive cells, consistent again with PBK/TOPK being expressed in proliferating neuronal progenitors but not in postmitotic immature neurons. There were a small fraction of cells that may have been positive for Dcx/Tuj1 and dimly positive PBK/TOPK, perhaps representing a transition state or the minority of Tuj1-positive cells that are still proliferative.

Recent evidence strongly suggests that the primary stem cell of the adult central nervous system is GFAP positive (Doetsch et al., 1999b; Imura et al., 2003; Garcia et al., 2004). We performed double-label immunohistochemistry for GFAP and PBK/TOPK to assess potential coexpression. We did not observe any double-

labeled cells in the adult subependymal zone (Fig. 5F), suggesting that if they did exist, they were rare.

PBK/TOPK expression overlaps with markers of proliferation and progenitor cells

To further examine the cellular context of PBK/TOPK expression, we performed double- and triple-label immunohistochemistry with neural progenitor cell markers and several markers of cell proliferation (Figs. 5B, 6B–E). After four injections of BrdU over 2 d, all PBK/TOPK-positive cells were BrdU positive (Fig. 6D). However, there were many BrdU-positive, but PBK/TOPK-negative cells. Most of these cells were Dcx positive. We surmised that the PBK/TOPK BrdU double-positive cells were currently proliferating population of cells, whereas the Dcx/BrdU-positive population represented primarily recently born neurons.

We tested this hypothesis in more detail, first, by colabeling with the PCNA antibody, which recognizes a subunit of the DNA-polymerase III complex involved in DNA synthesis during the S-Phase (Tsurimoto, 1999). Virtually all ($94.7 \pm 2.3\%$; $n = 387$) of the cells expressing PBK/TOPK in all regions, and at all ages, also expressed PCNA, suggesting that PBK/TOPK was expressed in cycling cells (Figs. 5B, 6B). However, not all of the PCNA-positive cells were PBK/TOPK positive, suggesting that the vast majority ($94.3 \pm 1.9\%$; $n = 392$), but not all, cycling cells in this region expressed PBK/TOPK (Fig. 6C). Next, we examined whether PBK/TOPK was expressed by rapidly cycling cells, or slowly cycling cells, by assessing PBK/TOPK expression several weeks after BrdU injections. This typical experimental design is based on the observation that slower cycling cells retain BrdU, whereas rapidly cycling cells dilute the BrdU beyond detection. The MCM2 protein, which is expressed in the G1 phase, was used to determine whether any of the slowly cycling cells were re-entering the cell cycle (Maslov et al., 2004). Four weeks after BrdU injection, we saw few BrdU-positive cells in the SEZ and did not see any BrdU/MCM2 double-positive cells, suggesting the BrdU-labeled cells were quiescent or postmitotic. There was no overlap between PBK/TOPK- and BrdU-positive cells, suggesting PBK/TOPK is not expressed in SEZ cells when they are not actively proliferative. There was, however, extensive overlap between MCM2 and PBK/TOPK, once again demonstrating that PBK/TOPK is expressed in actively cycling cells (Fig. 6E).

To examine PBK/TOPK expression in putative neuronal progenitors outside the EGL, we studied its colocalization with the proneural basic helix-loop-helix transcription factor Mash1. Mash1 has recently been shown to specify neuronal and oligodendritic fate in the postnatal brain *in vitro* and *in vivo* (Parras et al., 2004), and Mash1 knock-out mice have morphological defects in the olfactory bulb (Guillemot, 1995; Murray et al., 2003; Parras et al., 2004). This suggests that Mash1 may play a role in olfactory bulb neurogenesis, which occurs throughout a mammal's lifetime, because neurons born in the subependymal zone of the anterior lateral ventricle and RMS migrate to the olfactory bulb (Luskin, 1993). Consistent with this, we observed Mash1 expression for the extent of the SEZ and RMS in the adult animal, as was recently reported by Parras et al. (2004). Strikingly, at least 95% of Mash1-positive cells in adult SEZ/RMS ($96.3 \pm 1.0\%$; $n = 207$) were also clearly PBK/TOPK positive, suggesting that PBK/TOPK is expressed in a significant proportion of neuronal progenitors destined for the olfactory bulb (Fig. 6F). Mash1 and PBK/TOPK double-positive cells were seen in SEZ and RMS, but the PBK/TOPK-positive cells of the EGL of the cerebellum at P7 were clearly Mash1 negative (data not shown), demonstrating that not all neurogenic cells expressed Mash1, and not all PBK/

Figure 5. PBK/TOPK protein is not expressed in neurons or mature glia in the EGL or the SEZ and RMS. **A**, Schematics of tissue slices showing locations of **B–F** as red boxes. The RMS is in blue. **B**, PBK/TOPK (red) is expressed in the cytoplasm of cells in the PCNA-positive (green) mitotic layer of P8 EGL. Right, A $100\times$ magnification of the region similar to the box. Confocal reconstructions reveal that all PCNA-positive nuclei are surrounded by PBK/TOPK-positive cytoplasm. **C**, PBK/TOPK (red) is not expressed in GLAST (green)-positive Bergmann glia, the fibers of which provide scaffolding in P8 EGL. Right, $100\times$ magnification of region in box. **D**, PBK/TOPK (red) is not expressed in Tuj1 (green)-positive immature granule cell neurons in P12 EGL. Right, A $100\times$ magnification of the region similar to the box, with topiro-3-iodide (blue) added to label nuclei. **E**, PBK/TOPK (red) does not generally overlap with immature migrating neurons expressing Dcx (green) in a postnatal RMS. Right, A $63\times$ magnification of the box. **F**, PBK/TOPK (red) does not generally overlap with GFAP (green)-positive mature astrocytes in adult SEZ. Right, Magnification counterstained with topiro-3-iodide (blue). Scale bars, $20\ \mu\text{m}$. ML, Molecular layer; PL, Purkinje cell layer; IGL, internal granular layer.

Figure 6. PBK/TOPK is expressed exclusively in rapidly proliferating cells in the postnatal rodent brain. **A**, Schematics of tissue slices showing locations of **B–F** in red boxes. The RMS is in blue. **B**, Montage of 15 $10\times$ fields showing the front-third of a sagittally cut P8 brain. PBK/TOPK is expressed for the extent of the SEZ of the lateral ventricle and RMS around PCNA-positive nuclei. LV, Lateral ventricle; Olf, olfactory bulb. **C**, Most PBK/TOPK-positive cells have PCNA-positive nuclei: a $100\times$ section of adult SEZ showing PCNA single-labeled (blue arrow) and PCNA-PBK/TOPK double-labeled (yellow arrows) cells. **D**, In the adult RMS after four BrdU pulses in 2 d, all PBK/TOPK (red) cells have BrdU (green)-positive nuclei, showing recent birth or current proliferation (yellow arrows). Remaining BrdU-positive cells have Dcx (blue)-positive cytoplasm, a marker of immature neurons (blue arrows). Right, Magnification with Dcx included. **E**, Four weeks after a BrdU injection, there is still extensive overlap (blue arrows) between proliferation marker MCM2 (blue) and PBK/TOPK (red) but no overlap with MCM2 or PBK/TOPK and BrdU (green) in what are presumably slower cycling or quiescent cells. Right, Magnification of boxed region. **F**, There is extensive overlap between Mash1-positive (green) neuronal progenitor nuclei and PBK/TOPK (red) in adult RMS. Right, Magnification counterstained with topro-3-iodide: green arrow, MASH1 single-positive cell; red arrow, PBK/TOPK single-positive cell; yellow arrows, PBK/TOPK MASH1 double-positive cells. High Mag, High magnification.

TOPK cells were Mash1 positive. However, more than three-fourths of PBK/TOPK-positive cells were Mash1 positive in adult SEZ ($81.0 \pm 2.6\%$; $n = 243$), showing that PBK/TOPK is highly enriched in these progenitors.

We also compared PBK/TOPK expression with the expression of the epidermal growth factor (EGF) receptor (EGFR) and the NG2 chondroitin sulfate proteoglycan. EGF promotes the proliferation of neural progenitors *in vitro* and *in vivo*, and EGFR-positive cells are thought to be sphere-forming C cells (Doetsch et al., 2002). NG2 has traditionally been thought of as marker of oligodendrocyte or glial progenitors. Recently, a subset of NG2-positive cells in SEZ have also been identified as sphere forming, possibly C cells, but in contrast to EGFR, it is expressed in many regions beyond the neurogenic SEZ (Doetsch et al., 2002; Aguirre et al., 2004). Within the SEZ, $78.8\% (\pm 8.0\%)$; $n = 115$) of PBK/TOPK-positive cells were clearly EGFR positive, and $95.8\% (\pm 2.4\%)$; $n = 108$) of EGFR-positive cells were clearly PBK/TOPK positive (supplemental Fig. 3B, available at www.jneurosci.org as supplemental material), showing that the PBK/TOPK-positive population is highly overlapping with the EGFR expressing, sphere-forming cell population of the SEZ. Also, some PBK/TOPK-positive cells in the SEZ expressed NG2 (supplemental Fig. 3C, available at www.jneurosci.org as supplemental material). However, NG2 is also expressed broadly in the striatum and corpus callosum, two areas where virtually no PBK/TOPK signal is seen. In addition, NG2 labeling in the SEZ is generally dimmer than in the striatum. Overall, our results demonstrate that PBK/TOPK is only expressed in a subset of NG2-positive cells of SEZ, perhaps representing a proliferating subset of these progenitors, and is not generally expressed in NG2-positive progenitors outside the SEZ. In summary, PBK/TOPK-expressing cells comprise the cycling (MCM2+; short pulse BRDU+; PCNA+) neural progenitors (MASH1+, EGFR+, Tuj1-, Dcx-, GFAP-) of the SEZ *in vivo*.

PBK/TOPK-positive cells are mitotically active progenitors *in vivo*

The expression pattern *in vitro* as a mitotically active kinase, coupled with *in vivo* data, provided evidence that PBK/TOPK was expressed in several mitotically active progenitor cell populations in the CNS but not in quiescent, slower-cycling putative stem cells. To more conclusively establish PBK/TOPK expression in stem and progenitor cells *in vivo*, we examined PBK/TOPK in transgenic mice expressing the herpes simplex virus thymidine kinase gene (HSV-TK) under control of the GFAP promoter (Bush et al., 1999; Garcia et al., 2004). Because HSV-TK phosphorylates the drug ganciclovir and phosphorylated ganciclovir kills cells at mitosis, when these mice are given ganciclovir, all dividing GFAP-positive cells are killed. Previous work has shown that when these mice are treated with ganciclovir, there is a complete ablation of stem cell potential, as assessed by multipotent neurosphere forming ability *in vitro* (Imura et al., 2003; Morshead et al., 2003) and a complete ablation of neurogenesis *in vivo* (Garcia et al., 2004). Three transgenic mice were treated for 21 d with ganciclovir followed by four BrdU injections over 2 d and then processed for immunohistochemistry. This treatment resulted in a complete ablation of Dcx-positive cells in SEZ (data

Figure 7. PBK/TOPK cells are dramatically reduced when stem cells are ablated. **A**, Schematic of tissue slice showing the location of **B** in a red box. **B**, Representative images from subependymal zones from three replicate wild-type and transgenic animals that were treated with 21 d of ganciclovir to ablate neurogenesis and then given BrdU (green) show clear reduction of PBK/TOPK-positive cells (red). Quantification using StereoInvestigator reveals that this is a highly significant 70% decrease in PBK/TOPK-positive cells in transgenic animals.

not shown) and an $\sim 70\%$ reduction in PBK/TOPK-positive cells (independent samples *t* test; $p < 0.001$) in the SEZ, relative to nontransgenic controls. All remaining PBK/TOPK-positive cells were BrdU positive, demonstrating that they had been born after the discontinuation of ganciclovir treatment (Fig. 7), or were cycling cells not yet killed by the ganciclovir, which is lethal at mitosis. This finding provided direct *in vivo* evidence that PBK/TOPK-positive cells arise from proliferating GFAP-positive cells, consistent with PBK/TOPK being expressed exclusively by proliferating progenitor cells in the adult brain.

This finding raised several important questions, including an apparent paradox: an ablation of GFAP-positive cells leads to ablation of PBK/TOPK-positive cells, yet, at any given point in time, we were unable to identify any cells expressing both GFAP and PBK/TOPK. These data can be reconciled by considering a lineage relationship, such that TOPK/PBK-positive cells are generated from GFAP-positive cells but represent a different stage in progenitor lineage. Based on the previous data presented, this would be consistent with the PBK/TOPK-positive cells being expressed by a rapidly proliferating, GFAP-negative, population of cells called transient amplifying, or type C cells, that arise from more quiescent GFAP-positive cells, as suggested previously (Morshead et al., 1994, 1998, 2003; Doetsch et al., 1997, 1999a,b).

To test this hypothesis, we used a transgenic mouse that was generated by crossing a GFAP promoter-driven CRE with a floxed-stop-enhanced green fluorescent protein (eGFP) reporter (Garcia et al., 2004). In this mouse, all progeny of cells that have ever expressed GFAP become permanently eGFP positive. Virtually all ($94.7 \pm 2.6\%$; $n = 83$) of the PBK/TOPK-positive cells in the SEZ were eGFP positive, demonstrating conclusively that they arise from GFAP-positive cells (Fig. 8), even though they are themselves GFAP negative. These data support the general model, proposed by Doetsch et al. (1997), which contains a transient amplifying progenitor cell in adult SEZ neurogenesis.

Discussion

We previously initiated studies to discover and characterize genes that could provide functional insight into neuronal progenitor proliferation and their fundamental property of self-renewal by implementing a gene expression-profiling strategy that used representational difference analysis subtraction coupled to microarray screening (Geschwind et al., 2001). We further stratified that profile with extensive screening for overlapping expression in stem cell populations and germinal zones, identifying a few key genes with enriched expression in multiple stem cell populations,

Figure 8. PBK/TOPK cells are GFAP-negative progeny of GFAP-positive cells. **A**, Schematic of tissue slice showing the location of **B** in the red box. **B**, Subependymal zone from a transgenic in which all progeny of GFAP-positive cells express eGFP. Virtually all PBK/TOPK-positive cells (red) are GFAP negative (blue) but are progeny of GFAP-positive cells (green). Yellow arrows, Examples of PBK/TOPK and eGFP double-positive cells; green arrows, eGFP-positive cells; red arrows, an extremely rare PBK/TOPK single-positive cell.

suggesting a role in progenitor self-renewal (Easterday et al., 2003). Here, we have characterized extensively one of those candidates, PBK/TOPK, a MAPKK not previously known to be involved in CNS development. We show, using *in vitro* models, that PBK/TOPK is activated in a manner that suggests an important role in proliferation of progenitor populations. We also show, *in vivo*, that PBK/TOPK is expressed by a specific population of proliferating progenitors in the adult brain, the transient amplifying, or type C cell (Doetsch et al., 1997), as well as identifying other activated progenitor populations in postnatal cerebellar development. These data support a role for PBK/TOPK in neural progenitor proliferation and self-renewal and, in combination with our studies of other genes (Nakano et al., 2005), supports our general genomic screening approach to identify progenitor markers.

Previous literature in non-neural cell lines and our own bioinformatic exploration using microarray data from primary neural cells suggested that PBK/TOPK was a highly regulated protein, the function of which was most likely related to the M-phase in the cell cycle. Study of its sequence conservation across phylogeny from zebrafish to human supported this by identifying a highly conserved putative cyclinB phosphorylation site. Therefore, we explored this hypothesis *in vitro* by examining cell-cycle regulation of PBK/TOPK using a new phospho-specific antibody directed against the thr-9 site that comprised the target of cyclinB/CDK1. In both cell lines and primary neuronal progenitors from the cerebellum, we demonstrate that this site is specifically phosphorylated only in mitosis and that this phosphorylation rapidly disappears in late telophase. Additionally, P38 MAPK, the target of PBK/TOPK in non-neural tissue (Abe et al., 2000), is also phosphorylated at mitosis in neural cells; P38 phosphorylation is observed only in cyclinB-positive cells, with condensed chromatin, and its appearance is enhanced by mitogen. Finally, inhibition of P38 by the specific inhibitor SB203580 decreases the proliferation of these cells, providing preliminary evidence for a functional role of this pathway in progenitor self-renewal. It remains to be seen whether inhibition or ablation of PBK/TOPK will have the same effect or whether other MAPKKs may also activate P38 in this context. It also should be emphasized that not only are these data relevant for understanding PBK/TOPK function but they provide a confirmation of the bioinformatic approach that we used to generate functional inferences. This exploratory use of published microarray data sets is likely to be generally useful for developing new hypotheses about gene function that can then be tested at the bench, as we did here.

In vivo, the current studies also provide clear evidence that neuronal progenitors and possibly multipotent progenitors express PBK/TOPK while they are proliferating. First, PBK/TOPK is strongly expressed in the mitotic layer of the external granule layer in PCNA-positive, Dcx, Tuj1, and GLAST-negative cells. This structure only gives rise to cerebellar granule neurons; thus, PBK/TOPK must be expressed in the precursors of these neurons. This is supported by data showing that purified CGP *in vitro* expresses PBK/TOPK, which is mitogen dependent, parallel with the SSH requirement for CGP proliferation (Wechsler-Reya and Scott, 1999). Indeed, in culture, PBK/TOPK expression still overlaps strongly with PCNA, a marker of proliferation, and not with NeuN, a marker of maturing neurons. Furthermore, many proliferating cells in the SEZ and RMS, structures that give rise to neurons throughout the life of the animal (Luskin, 1993), are positive for both PBK/TOPK and the proneu-

ral Mash1 gene. Considered together, all of the evidence clearly demonstrates PBK/TOPK is expressed by multiple neuronal progenitor populations during development, and possibly multipotent progenitors as well (Parras et al., 2004).

Cytological investigations have identified three major morphologically defined populations of cells in the SEZ: ultrastructurally migrating neurons, GFAP-positive glial cells, and highly proliferative ultrastructurally immature cells (Doetsch et al., 1997; Doetsch et al., 2002). The current belief is that slow-cycling GFAP-positive cells give rise to rapidly cycling GFAP-negative progenitor cells, which then give rise to immature neurons that migrate to the olfactory bulb. This model has been supported by studies that demonstrate neurospheres must directly or indirectly arise from slow-cycling and GFAP-positive cells (Morshead et al., 1994, 1998; Doetsch et al., 1999b; Imura et al., 2003; Garcia et al., 2004). Using three different markers of proliferation, PCNA, MCM2, and short-pulse BrdU labeling, we show that PBK/TOPK cells are a highly proliferative population of cells. Using markers of migrating immature neurons, Tuj1 (Menezes et al., 1995) and Dcx, we have shown that PBK/TOPK-expressing cells are adjacent to but not overlapping with these immature neurons. Neither was there generally detectable overlap with quiescent GFAP-positive slow-cycling cells in adult animals.

These data lead to the following model for PBK/TOPK expression in the adult SEZ, illustrated in supplemental Figure 4 (available at www.jneurosci.org as supplemental material). A resting GFAP-positive cell in the SEZ enters the cell cycle and gives rise to a rapidly proliferating PBK/TOPK- and MASH1-positive and GFAP-negative progeny. These cells then give rise to PBK/TOPK-negative, Tuj1, Dcx-positive immature neuronal progeny that migrate to the olfactory bulb to become mature neurons. This model is strongly supported by the dramatic decrease in PBK/TOPK-positive cell number following ablation of GFAP-positive cycling cells and the lineage experiments that demonstrate that PBK/TOPK-positive cells must arise from GFAP-positive cells. These data demonstrate that the mitotic kinase PBK/TOPK is a marker of transiently amplifying, type C, progenitor cells in the SEZ and provide additional support for the models proposed previously (Doetsch et al., 1997, 2002).

It is also interesting to consider PBK/TOPK expression in progenitor cells *in vivo* compared with its detection in stem cell cultures *in vitro*. Our initial screening studies to identify PBK/TOPK were done with *in vitro* models of stem cells (Geschwind et al., 2001; Easterday et al., 2003). Yet, *in vivo*, PBK/TOPK appears to be expressed in the transiently amplifying progenitor cell but not in quiescent GFAP-positive stem cells. This observation may be explained by the differences between the behavior of neural stem cells *in vitro* versus *in vivo*. First, it is currently unclear whether the transiently amplifying cells can serve as a multipotent sphere forming cell *in vivo*, because there are studies both supporting (Doetsch et al., 2002) and questioning (Morshead et al., 1994) this role. Second, the GFAP-positive quiescent neural stem cell may express PBK/TOPK when activated, for example by bFGF or EGF in culture. Finally, it is unclear whether the transiently amplifying cell can revert into a quiescent GFAP-positive stem cell *in vivo* or *in vitro* and whether these are two distinct cell populations or simply the same population in two different states.

Outside of the SEZ, the other predominant region of expression of PBK/TOPK is the early postnatal cerebellum. The greatest expression is seen in the mitotic portion of the external granule cell layer (EGLa). Extensive studies have shown that during this period, this region produces granule cells exclusively (Altman, 1972; Lee et al., 2005). In addition to the expression of PBK/

TOPK in the EGL, there is moderate expression of PBK/TOPK in the cerebellar white matter (data not shown) in MASH1-positive cells. Recent studies have suggested that multipotent neural stem cells may be found in this region as late as the first postnatal week (Goldman et al., 1997; Milosevic and Goldman, 2002; Lee et al., 2005). Considered together with the results of our studies in the SEZ, this suggests that PBK/TOPK is expressed by proliferating, self-renewing stem and progenitor cells throughout the brain.

The identification of PBK/TOPK as significantly enriched in this critical mitotically active, self-renewing stem and progenitor population, its regulated phosphorylation by cyclinB/cdk1 during this process, and its relationship to P38 MAPK provides another tool with which to begin to understand molecular pathways of cell-cycle regulation and their coupling to cell fate decisions in the CNS (Anderson, 2001; Ohnuma and Harris, 2003). In this regard, the enrichment of PBK/TOPK, an M-phase, cell-cycle-related gene in a specific subpopulation of proliferating neuronal progenitor cells raises a number of interesting issues, including the issue of the role of M-phase regulation in progenitor cell self-renewal. Furthermore, how the process of cell division may provide critical windows in which daughter cell fate or the commitment to renew are integrated with environmental signals remains a fundamental question (Lu et al., 2000). Signaling proteins such as PBK/TOPK, which are relatively cell class enriched and preferentially active in M-phase, are poised to act as a coincidence detectors, providing a mechanism to combine extracellular signaling to intracellular state for cell fate decisions. Future studies specifically ablating PBK/TOPK expression will allow the examination of such a mechanism.

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