Development/Plasticity/Repair

Critical Role of Integrin-Linked Kinase in Granule Cell Precursor Proliferation and Cerebellar Development

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Integrin-linked kinase (ILK) is a serine/threonine protein kinase that plays an important role in integrin signaling and cell proliferation. We used Cre recombinase (Cre)-loxP technology to study CNS restricted knock-out of the ilk gene by either Nestin-driven or gfap-driven Cre-mediated recombination. Developmental changes in ilk-excised brain regions are similar to those observed in mice lacking the integrin β1 subunit in the CNS, including defective laminin deposition, abnormal glial morphology, and alterations in granule cell migration. Decreases in 6-bromodeoxyuridine (BrdU) pulse labeling and proliferating cell nuclear antigen expression in the external granule cell layer of the cerebellum demonstrated that proliferation is disrupted in granule cells lacking ILK. Previous studies have shown that laminin-sonic hedgehog (Shh)-induced granule cell precursor (GCP) proliferation is dependent on β1 integrins, several of which bind laminin and interact with ILK through the β1 cytoplasmic domain. Both ex vivo deletion of ilk and a small molecule inhibitor of ILK kinase activity decreased laminin-Shh-induced BrdU labeling in cultured GCPs. Together, these results implicate ILK as a critical effector in a signaling pathway necessary for granule cell proliferation and cerebellar development.

Key words: granule cell precursor; integrin; integrin-linked kinase; laminin; proliferation; sonic hedgehog

Introduction

In the developing early postnatal cerebellum, β1 integrins are important in supporting the proliferation of granule cells, thereby promoting the postnatal expansion of the cerebellum. During development, granule cell precursors (GCPs) form the external granule layer (EGL), a postnatal proliferative zone that lies between the meningeal layer and the Purkinje cell layer (PCL) (Goldowitz and Hamre, 1998; Wang and Zoghbi, 2001). GCPs express laminin receptors within the β1 integrin family (α6β1 and α7β1), and proliferating cells are in close association with laminin isoforms contained within the basement membrane (Blas et al., 2004). Within the EGL, laminin strongly enhances the mitogenic effects of sonic hedgehog (Shh), a potent inducer of GCP proliferation (Pons et al., 2001; Lewis et al., 2004). In vitro, β1 integrins appear to act cell autonomously to regulate laminin-Shh-induced GCP proliferation. Presently, the downstream effectors in this proliferative pathway remain essentially unknown.

Functions of integrins are mediated through the recruitment of cytoplasmic proteins that connect integrins to the actin cytoskeleton (Grashoff et al., 2004; Hannigan et al., 2005). Integrin-linked kinase (ILK) is one of these proteins, first discovered as a protein that interacts with the cytoplasmic tails of the β1- and β3-integrin subunits (Hannigan et al., 1996). ILK is a key effector of integrin function, regulating cell adhesion and anchorage-dependent growth (Grashoff et al., 2004; Hannigan et al., 2005). ILK functions as both an adaptor protein and serine/threonine kinase: the former important for the adhesive functions of integrins and the latter critical for cell cycle progression (Grashoff et al., 2004; Hannigan et al., 2005). Loss-of-function studies in Cae norhabditis elegans and Drosophila have revealed an important role for ILK as a scaffold protein, recruiting integrins and stabilizing actin at adhesion sites (Zervas et al., 2001; Mackinnon et al., 2002). In addition, genetic or pharmacological inhibition of ILK suppresses proliferation in vitro and in vivo (D’Amico et al., 2000; Persad et al., 2000; Grashoff et al., 2003; Terpstra et al., 2003; Tan et al., 2004). In the majority of cases, these effects can be explained by inhibition of ILK kinase activity (Grashoff et al., 2004; Hannigan et al., 2005). ILK phosphorylates glycogen synthase kinase 3β (GSK-3β) and protein kinase B (PKB)/AKT, two enzymes known to be involved in cell proliferation (Delcommenne et al., 1998; Persad et al., 2000, 2001a,b). These phosphorylation events ultimately induce the expression of cell-cycle-promoting genes such as cyclins and c-myc (Grashoff et al., 2004; Hannigan et al., 2005).

Defects in integrin function have profound effects on development and may underlie some of the genetic diseases that lead to
cortical malformations (Georges-Labouesse et al., 1998; Miner et al., 1998; Walsh, 1999; Halfter et al., 2002; Moore et al., 2002; Beggs et al., 2003). Lack of integrin β1 gene expression in mice leads to peri-implantation lethality (Fassler and Meyer, 1995). In nervous system-restricted integrin β1 knock-out mice, aberrant formation of lamina and folia in the cerebral and cerebellar cortices have been observed (Graus-Porta et al., 2001). These developmental abnormalities are primarily attributable to altered glia and basement membrane interactions, which result in marked instability of the basal lamina. In the cerebellum, the absence of β1 integrins also results in reduced GCP proliferation (Blaess et al., 2004). Similar to the absence of β1 integrins, the absence of ILK expression in mice also leads to peri-implantation lethality (Sakai et al., 2003). Therefore, to investigate ILK function in the brain, we selectively knocked-out ilk using Cre recombinase (Cre)–loxP technology. In the cerebellum, we found that ILK deficiency caused phenotypic changes similar to those observed in mice lacking β1 integrins, including granule cell ectopia, defective laminin deposition, glial network changes, and reduced GCP proliferation.

Materials and Methods
Transgenic mouse strains
We used previously described mouse lines, namely, nestin-Cre (Tronche et al., 1999), glial fibrillary acidic protein (gfap)–Cre (Kwon et al., 2001), and ilkloxP (Terpstra et al., 2003). IlkloxP/gfap-Cre and ilkloxP/nestin-Cre mice were generated by crossing IlkloxPloxP mice with IlkloxP/+; gfap-Cre and IlkloxPloxP/nestin-Cre, respectively. IlkloxPloxP/gfap-Cre and IlkloxPloxP/nestin-Cre were compared with littermates. The animals examined were of mixed background. Specifically, the background of the nestin-Cre, gfap-Cre, and IlkloxPloxP mice were C57BL/6/J, FVB/C57BL/6/J, and C57BL/6J, respectively.

Cerebellar cultures and drug exposure
 Cultures of cerebellar granule cells were prepared from postnatal day 4 (P4) to P5 mice and plated onto coverslips coated with poly-L-lysine (100 μg/ml) and laminin (5 μg/ml) as described previously (Cohen-Coey et al., 1991; Mills et al., 2003). Primary cerebellar neuronal cultures from IlkloxPloxP or wild-type mice were infected with Cre recombinase-expressing adenovirus (AdCre) at 2 d in vitro as described previously (Trousse et al., 2003). Conditioned media was removed, and cells were incubated with virus in OptiMEM for 4 h at 37°C. The conditioned media was then added back, and cells were incubated for 4 d. For proliferation assays, Shh (1–3 μg/ml; R & D Systems, Minneapolis, MN) was added to Neurobasal media at 6 d in vitro, and cultures were incubated for 3 d. For experiments using the ILK kinase inhibitor KP-392 (formerly known as KP-SD-1 (Persad et al., 2001b)) Shh (1 μg/ml) was added to cultures at 6 d in vitro with or without KP-392 (50 or 100 μM) or vehicle control. Four hours before fixation, cultures were treated with 20 μM 6-bromodeoxyuridine (BrdU). Cells were fixed with methanol, treated with 2 M HCl for 1 h, neutralized in 0.1 M sodium borate buffer, pH 8.5, rinsed in 70% dH2O/95% ethanol and chloroform, and differentiated for 10 min before the addition of primary antibody (see below). The presence of iron was detected in paraffin sections using Perl’s Prussian blue method (Carleton, 1980). For Nissl staining, brains were submerged in 30% sucrose overnight at 4°C and sectioned directly at 40 μm using a frozen sliding microtome. Sections were then dehydrated overnight in 70% ET0H, stained with 0.1% cresyl violet/0.5% acetic acid, rinsed in 70% dH2O/95% ethanol and chloroform, and differentiated with 1.7% acetic acid in 95% ET0H. Frozen and paraffin-embedded sections were used for immunohistochemistry (see below).

Immunohistochemistry
Sections were blocked in 3% milk or 5% horse serum and solubilized in 0.2% Triton X-100 in PBS. We performed immunohistochemistry with primary antibodies to BrdU (Roche Diagnostics, Indianapolis, IN), proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA), Englebreth Holm-Swarm laminin (Sigma, St. Louis, MO), monoclonal ILK (Santa Cruz Biotechnology), polyclonal ILK (Cell Signaling Technology, Beverly, MA), neuronal-specific nuclear protein (NeuN) (Chemicon, Temecula, CA), and GFAP (Chemicon or DakoCytonomation, Carpenteria, CA). Sections were incubated in primary antibodies for 2 h at room temperature or overnight at 4°C. For immunofluorescence microscopy of nestin-Cre adult animals, we performed detection of primary antibodies with Alexa Fluor 488 or 594 secondary antibodies (Invitrogen, Carlsbad, CA). For all other immunofluorescence staining, unconjugated primary antibodies were detected with biotinylated secondary antibodies, followed by amplification with streptavidin–FITC or streptavidin–Texas Red. For costaining by immunofluorescence, ILK was visualized using a polyclonal ILK antibody, whereas BrdU and GFAP were visualized using conjugated monoclonal antibodies anti-BrdU–fluorescein (Roche Diagnostics), and anti-GFAP–cyanine 3 (Sigma, respectively). For light microscopy, we performed detection of primary antibodies with biotinylated secondary antibodies, followed by amplification with a streptavidin–peroxidase conjugate, treatment with Nova Red substrate, and hematoxylin coverstain (when stated).

Western blotting
Lyssates from primary cultures were prepared in Tris-Cl buffer, pH 7.6, containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 3.8 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 2 mM NaN3, and 1 mM NaN3V04, as described previously (Mills et al., 2003). ILK was detected using a monoclonal anti-ILK antibody (BD Transduction Laboratories, Lexington, KY). All other antibodies are as described previously (Mills et al., 2003).

Histology
For BrdU labeling, mice were injected intraperitoneally with 100 μg of BrdU per gram of body weight and perfused 2 h later. Mice were perfused cardially with 4% paraformaldehyde in PBS. Brains were either processed for paraffin sectioning or submerged in 30% sucrose, embedded, and sectioned using a cryostat. Ten micrometer sections were treated with 4N HCl for 10 min before the addition of primary antibody (see below). The detection of iron was performed in paraffin sections using Perl’s Prussian blue method (Carleton, 1980). For Nissl staining, brains were submerged in 30% sucrose overnight at 4°C and sectioned directly at 40 μm using a frozen sliding microtome. Sections were then dehydrated overnight in 70% ET0H, stained with 0.1% cresyl violet/0.5% acetic acid, rinsed in 70% dH2O/95% ethanol and chloroform, and differentiated with 1.7% acetic acid in 95% ET0H. Frozen and paraffin-embedded sections were used for immunohistochemistry (see below).

Results
Cre recombinase activity and ILK expression in brain
To generate mice with postnatal, CNS-restricted knock-out of the ILK gene, we crossed gfap–Cre mice or nestin-Cre mice with IlkloxPloxP mice. IlkloxPloxP/gfap-Cre mice were born at the expected Mendelian ratios and were indistinguishable from littermate controls. However, death increased at 21 weeks in IlkloxPloxP/gfap-Cre mice (4%), increasing to 50% mortality at 30 weeks of age. Premature death did not occur in IlkloxPloxP/+; gfap-Cre or IlkloxPloxP/+ mice (4%), increasing to 50% mortality at 30 weeks of age. Cre-mediated biotinylated UTP nick end labeling and DAPI staining. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) of cerebellar neuronal cells was measured using the In Situ Cell Death Detection kit (fluorescein; Boehringer Mannheim, Indianapolis, IN) according to the methods of Miller et al. (1997). The number of TUNEL-positive cells was calculated as a percentage of total cell number (DAPI-stained cells). TUNEL of cerebellar slices was measured with the same In Situ Cell Death Detection kit according to the instructions of the manufacturer.

Statistical analysis
Data depicted in the graphs represent the mean ± SEM of results. The minimum level of statistical significance was set at α = 0.05. Intergroup comparisons were made with a one-way ANOVA, followed by either Tukey’s multiple comparison post hoc test or Scheffe’s correction unless otherwise stated.
The pattern of Cre recombination in the nestin-Cre mouse line has been characterized previously by crossing nestin-Cre mice with ROSA R28R reporter mice in which Cre-mediated recombination is required for expression of the lacZ reporter gene. Nestin is an intermediate filament protein expressed in neural precursors in the developing embryo (Lendahl et al., 1990). In the nestin-Cre mouse line, Cre-mediated recombination occurs in the precursors of neurons and glia in the CNS. Therefore, LacZ expression was evident in embryonic day 15.5 (E15.5) embryos throughout the cerebellar anlage (Blaess et al., 2004). At P7, all cells in the EGL, internal granule cell layer (IGL), and PCL expressed LacZ. A similar breeding strategy was used to characterize the pattern of Cre-mediated recombination in a gfap-Cre transgenic mouse model in which Cre recombination was under the control of a modified glial fibrillary acidic protein (GFAP) promoter (Kwon et al., 2001). Although traditionally considered a glial promoter, Cre recombination in neuronal cells has been reported for a number of gfap-Cre transgenic mice and is likely attributable to Cre being transiently expressed in neuronal precursors during development (Zhuo et al., 2001). In the cerebellum of these gfap-Cre reporter mice, Cre recombination had occurred in the majority of granule neurons in the EGL and IGL at P14 (Kwon et al., 2001). Cre-mediated recombination was variable in granule cells in the EGL at earlier developmental time points (at P5, Cre-mediated recombination had occurred in ~20% of granule cells in some folia, whereas in other folia this number was less). Surprisingly, Cre activity in cells immunopositive for S100β (a glial marker) was only occasionally observed (Kwon et al., 2001).

We analyzed the expression of ILK in gfap-Cre and nestin-Cre mice immunohistochemically using two different ILK antibodies. At P10, ILK immunoreactivity was relatively strong in the EGL of gfap-Cre littermate control mice (Fig. 1a). Although a sizeable reduction in ILK immunoreactivity was observed in some folia of ilkloxP/loxP, gfap-Cre mice at P10, ILK excision was not complete at this developmental time (Fig. 1b). A more dramatic reduction in ILK expression was observed in adult ilkloxP/loxP, gfap-Cre mice (Fig. 1d,f) compared with littermate controls (Fig. 1a,c,e). Loss of ILK expression was evident in the granule cell layer (gcl; containing granule cell somas) and molecular layer (ml; containing granule cell axons) of the cerebellum (Fig. 1d,f). The reduction of ILK staining was consistent with a loss of ILK expression in both neuronal as well as glial cells (Fig. 1d,f). In contrast to gfap-Cre mutant mice, a dramatic loss of ILK immunoreactivity was observed in nestin-Cre mutant mice at P10. Loss of ILK staining was visible at low magnification throughout the cerebellum, consistent with a dramatic reduction of ILK expression in neuronal and glial cells (Fig. 1h).

We have previously characterized ILK expression in various tissues and found ILK to be relatively highly expressed in the adult mammalian brain in neuronal cells (Mills et al., 2003). To further characterize the cell type in which ILK is normally found (and thereby exerting its effect), we costained cerebellar sections at P10 with antibodies to ILK and GFAP. Costaining revealed a pattern of ILK immunoreactivity that only partially colocalized with GFAP-positive cells (Fig. 2a–c). ILK staining that was GFAP negative was consistent with GCP staining (Fig. 2b,c). Costaining of sagittal sections of P10 mice (that had been previously injected with BrdU) with antibodies to ILK and BrdU revealed ILK-positive cells within the proliferative zone of the EGL (Fig. 2d–f), suggesting that ILK is expressed in GCPs.

**Developmental changes in the mutant mouse brain**

The cerebellar architecture of CNS-restricted ilk knock-out mice was markedly different between ilkloxP/loxP, gfap-Cre and ilkloxP/loxP, nestin-Cre. The cerebellar architecture of ilkloxP/loxP, nestin-Cre was similar to that seen for IlkBloxP/loxP, nestin-Cre mice (Graus-Porta et al., 2001; Blaess et al., 2004). At P10, nestin-Cre mice born at the expected Mendelian ratio. As with ilkloxP/loxP, gfap-Cre mice, increased death was not observed in young adult nestin-Cre mutant mice. Although the life expectancy, beyond early adulthood, was not formally studied, an increase in death was not observed in older nestin-Cre mice colonies housed for 24 weeks. Locomotor abilities were not studied in ilkloxP/loxP, nestin-Cre mice.
or entirely absent from the cerebellar surface (Fig. 6b, see area within arrows) and did not always penetrate into the cerebellar folia (Fig. 6b, see folia with arrowhead). Even in areas of the cerebellum in which laminin was present, assembly may have been altered because staining appeared thicker and disorganized (Fig. 6d). In the adult, laminin deficits were observed in the meningeal basement membrane of both ilkloxP/loxP;nestin-Cre (Fig. 6e–h) and ilkloxP/loxP;gfp-Cre mice (data not shown). Laminin staining was either markedly reduced or absent from the cerebellar surface (Fig. 6, compare h with g, a littermate control) of ilkloxP/loxP;nestin-Cre mice and from between the folia (Fig. 6, compare f with e, a littermate control), especially when there was fusion of folia. Loss of basal lamina matrix integrity has been reported to result in microvascular permeability and erythrocyte leakage. Microvascular abnormalities were also observed in CNS-restricted ILK knock-out mice (Figs. 5i,j, 6i,j). Laminin staining appeared fragmented in large blood vessels within the molecular layer of ilkloxP/loxP;nestin-Cre mice (Fig. 6j). In P15 ilkloxP/loxP;gfp-Cre mice, remote (Fig. 5j, black arrow) and recent hemorrhage (Fig. 5j, white arrow) in the form of focal parenchymal and perivascular collections of erythrocytes, serum proteins, and blood breakdown products were observed. Distinct clusters of cells resembling erythrocytes appeared within the granule cell layer and molecular layer (data not shown). Positive iron staining associated with these cells in ilkloxP/loxP;gfp-Cre mutant mice was also evident (Fig. 5j) but was absent from littermate controls (Fig. 5i).

Abnormal glial fibers resulting from altered glia–basement membrane interactions have been observed in nervous system-specific integrin β1-deficient mice (Graus-Porta et al., 2001). To investigate the integrity of the glial network in ILK knock-out mice, glial fibers were stained for GFAP. In wild-type mice, glial processes formed a regular network, extending across the molecular layer (Fig. 7a,c). Processes terminated with end feet at the basement membrane, forming a continuous layer (Fig. 7a,c). In ilkloxP/loxP;nestin-Cre mice, glial processes extended across the molecular layer, but end-feet formation was disrupted at the cerebellar surface and between the folia (Fig. 7b,d). Glial network defects were much more pronounced in nestin-Cre mutant mice at P10 and occurred throughout the cerebellum, even in areas in which the basal lamina appeared intact. Glial processes in ilkloxP/loxP;nestin-Cre mice were highly irregular, meandering within the molecular layer, and lacked end feet (Fig. 7, compare GFAP staining in nestin-Cre mutant mice in f with littermate controls in e). nestin-Cre mice at P10 were pulse labeled with BrdU and costained for GFAP (red) and BrdU (green). Compared with the controls, BrdU immunoreactivity indicates that a proliferative zone in the EGL was still present but reduced within the folia, despite severe glial network abnormalities (see g and h depicting the same folia, except with both BrdU and GFAP costaining). In adult nestin-Cre mutant mice, glial processes also

Figure 2. ILK expression occurs in granule precursor cells and Bergmann glial cells of the EGL. Sagittal sections of the cerebellum of P10 mice were stained with antibodies to ILK (green) and GFAP (red) (a–d). Although ILK immunoreactivity (b) appears to colocalize with some GFAP-positive cells (c), a large number of ILK-positive cells are GFAP negative, appearing as a honeycomb staining pattern within the cerebellar folium (b). c, Inset, A high-magnification view of glia end feet is shown, illustrating colocalization of the same folium (b). c, Inset, A high-magnification view of glia end feet is shown, illustrating colocalization of the proliferating zone (b) of P10 wild-type mice (that were previously injected with BrdU) were costained with antibodies to ILK (red) and BrdU (green). ILK-immunoreactive cells (d) occurring near the basement membrane were also BrdU positive (e, f). Inset, A high-magnification view of cells within the proliferating zone (BrdU-positive cells) that costain for ILK. Scale bars, 50 μm.
lacked end feet at the cerebellar outer surface (Fig. 7j) and between the folia (data not shown).

**ILK deletion causes abnormalities in proliferation**

In *integrin β1* CNS-restricted knock-out mice, defects in postnatal proliferation of GCPs were observed. BrdU pulse labeling revealed that, at P0, the EGL contained normal numbers of granule cells (indicating that generation of the initial GCP pool was normal in these mutant mice). However, a proliferative defect in the EGL by P2 was observed, particularly in developing folia (Blaess et al., 2004). In addition, survival of cells within the EGL was not different from that in controls (Blaess et al., 2004). Because ILK is a downstream effector of β1, we wanted to determine whether loss of ILK expression also inhibits the postnatal proliferation of GCPs. To examine this possibility, we analyzed BrdU pulse labeling and the expression of PCNA in * ilkloxP/loxP;nestin-Cre* adult mice (Fig. 8f). TUNEL was performed on the cerebellar surface below the pial surface (b, d). A high-magnification view illustrating inappropriate positioning of granule cells within the molecular layer and ectopias between folia (arrow) was shown at higher magnification in a and c, respectively. e, f. Sagittal sections of P10 animals were stained for hematoxylin–eosin. The folia in the mutant mice lacked fissures, and adjacent EGLs were fused (a–d). Similar areas of littermate controls are provided for comparison (a, c). Areas of the folia labeled by asterisks in a and b are shown at higher magnification in c and d, respectively. e, f. Sagittal sections of P10 animals were stained for the postmitotic neuronal marker NeuN. Ectopic granule neurons staining positively for NeuN invade the molecular layer of * nestin-Cre* mutant mice, indicating that these cells are no longer dividing. Scalloping of the IGL of mutant mice was also observed at this developmental time. Scale bars: a, 500 μm; c, 50 μm; e, 200 μm.

Compared with controls (Fig. 8g), loss of BrdU pulse labeling in * ilkloxP/loxP;nestin-Cre* mice at P10 (Fig. 8r) was much more profound than that observed for * ilkloxP/loxP;gfap-Cre*. This was in part attributable to the loss of folia. Although some fissures were often absent in * nestin-Cre* mutant mice, the primary and secondary fissures were consistently present. Therefore, GCP proliferation was measured along the cerebellar surface adjacent to these fissures by counting the number of cells having incorporated BrdU. Because depths of folia differ between *Cre* mutant animals and littermate controls, the length of the cerebellar surface along each fissure was measured. Proliferation was then
quantified per millimeter of cerebellar surface, and this was expressed as a percentage of littermate control animals (Fig. 8s). BrDU pulse labeling in ilk1loxP/loxP; nestin-Cre mice indicate that proliferation was <65% (of that in littermate controls) within the cerebellar surface adjacent to the secondary fissure of both nestin-Cre and gfp-Cre mutant animals. A similar reduction in BrDU labeling was seen within the cerebellar surface adjacent to the primary fissure of nestin-Cre mutant mice. In contrast, in gfp-Cre mutant mice, proliferation was not reduced (Fig. 8s). The length of the cerebellar surface adjacent to the primary and secondary fissures (expressed as a percentage of control) for nestin-Cre mutant animals was 63.9 ± 3.4 and 75.2 ± 6.8%, respectively, whereas the length of the cerebellar surface adjacent to the primary fissure and secondary fissures for gfp-Cre mutant animals was 59.3 ± 2.5 and 84.8 ± 2.1%, respectively.

Serial sections comparing hematoxylin–eosin staining (Fig. 9b) with BrDU staining (Fig. 9d) revealed that, in nestin-Cre mice at P10, both the width of the external granule cell layer and BrDU labeling were reduced in areas of the cerebellum lacking fissures (Fig. 9, compare regions marked by white arrows in b and d with comparable regions in control animals marked in a and c). In P10 nestin-Cre littermate control mice, laminin staining is continuous and intimately associated with the proliferative zone in the EGL (Fig. 9c,e). Although in nestin-Cre mutant mice at P10, dual staining for BrDU and laminin revealed that, even in areas in which laminin is present, it was dissociated from proliferating GCPs (Fig. 9df). Moreover, areas of the cerebellum in which...
GFAP staining indicates that the glial fiber network is severely disrupted in birth, and sagittal sections of the cerebellum were costained for GFAP (red) and BrdU (green) (Fig. 7b). GCPs derived from wild-type or ilkloxP/loxP mice were plated on laminin and infected with AdCre virus. After AdCre infection, the number of BrdU-incorporating cells after a 3 d exposure to Shh (1 and 3 μg/ml) was significantly reduced in ilkloxP/loxP cultures compared with ILK wild-type cultures (Fig. 10a). Granule cell survival after AdCre infection was determined by the percentage of TUNEL-negative cells. Survival of AdCre-infected cells was not different from mock-infected sister cultures, indicating that viral infection did not reduce proliferation by inhibiting survival (Fig. 10c). Proliferation was also quantitated in GCP cultures plated onto laminin and exposed to Shh (1 μg/ml) in the presence or absence of KP-392, a small molecule inhibitor of ILK kinase activity. In a previous study, we have shown that, although KP-392 (at concentrations of 50 and 100 μM) inhibited phosphorylation of the ILK substrates AKT and GSK-3, survival of neuronal cell lines in the presence of trophic support was not compromised (Mills et al., 2003). Similar results were found in a subsequent study using cultured primary neurons (Zhou et al., 2004). The number of BrdU-incorporating granule cells after exposure to Shh was markedly decreased by KP-392 (50 and 100 μM) compared with numbers in cultures treated with vehicle control (Fig. 10d).

Discussion

We have selectively excised the ilk gene from the CNS by Cre-loxP technology and have shown abnormal brain development and early lethality in mutant mice. Some developmental changes in ilk-excised brain regions are reminiscent of integrin β1 CNS knock-out mice, including granule cell ectopia, defective laminin deposition, and glial network changes (Graus-Porta et al., 2001). A follow-up study on integrin β1 CNS knock-out mice indicated that Shh induced GCP proliferation is severely reduced (Blaess et al., 2004). In the present paper, we show that ILK is also required for normal GCP proliferation. In vivo, deletion of ilk from the cerebellar anlage decreased laminin deposition and proliferation of granule cells in the EGL. Ex vivo deletion of ilk from purified cerebellar granule cells decreased laminin-Shh-induced BrdU labeling as did a small molecule inhibitor of ILK kinase activity. Overall, our data demonstrate that ILK is a critical effector in a signaling pathway necessary for granule cell proliferation and cerebellar development.

In the developing CNS, the initial deposition of extracellular matrix components into the basement membrane is dependent on meningeal cells, whereas subsequent remodeling is dependent on glial cells. Specifically, meningeal fibroblasts secrete components of the basal lamina that bind receptors on glial end feet of the glia limitans, allowing assembly of a functional basal lamina (Sievers et al., 1994; Shearer and Fawcett, 2001; Beggs et al., 2003; Blaess et al., 2004). Results from β1loxP/loxP;nestin-Cre knock-out mice support the idea that interactions between β1-class integrins in glial cells with the ECM are important for proper glial network development and maintenance of the basement membrane (Graus-Porta et al., 2001; Blaess et al., 2004). If β1 and ILK
Figure 8. Granule cell precursor proliferation is impaired in the ilk

- -labeled secondary antibody in combination with Nova Red substrate (reddish brown). Sections have been counter-

Stained with hematoxylin (blue). Proliferative defects occur in the EGL of the folia of mutant mice (Bláez et al., 2004). Similarly, in both ILK mutant models, decreased BrdU labeling in the prolif-

ative zone of the EGL correlated with defects in the basal lamina. Shh is a powerful mitogen of postnatal GCP proliferation in the cerebellum (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). A previous study has shown that Shh-induced granule cell proliferation is potentiated by the integrin ligand laminin (Bláez et al., 2004). In cerebellar granule cells cultured from β1loxP/loxP;nestin-Cre mice and plated onto laminin, Shh-induced prolif-

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dependent assembly of the meningeal basement membrane. Glial cells may be the pri-

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growth and differentiation, glial network defects in ilk mutant mice would in turn lead to abnormalities in basement mem-

brane assembly. Glial cells may be the primary cell type responsible for the basal lamina defects in mutant mice because excision of ILK from postmitotic neurons did not result in laminination defects (Niewmierzycka et al., 2005). Indeed, glial network defects in ilk mutant mice were much less dra-

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thesis that ILK is an important effector in glia development, mediating integrin-

dependent assembly of the meningeal basement membrane.
els exhibit defects in proliferation and the basal lamina, clear differences exist between the two mouse models. The \( \text{ilk}^{\text{loxP/loxP}}/\text{nestin-Cre} \) mice were most similar to \( \beta 1^{\text{loxP/loxP}}/\text{nestin-Cre} \) mice, exhibiting gross changes in cerebellar architecture (fused and irregular folia) and extreme disorganization of the superficial glial limitans (changes that were not observed in \( \text{ilk}^{\text{loxP/loxP}}/\text{gfap-Cre} \) mutant mice). These differences are likely attributable to the pattern of Cre-mediated ilk excision. For example, in \( \text{nestin-Cre} \) mice, it has been reported that Cre recombination activity is present in E15.5 embryos throughout the cerebellar anlage. In contrast to \( \text{nestin-Cre} \) mice, Cre activity in \( \text{gfap-Cre} \) transgenic mice has been reported to occur postnatally in granule neurons of the cerebellum: only by P14 had Cre recombination occurred in the majority of granule cells in the EGL and IGL (Kwon et al., 2001). Although both \( \text{nestin-Cre} \) and \( \text{gfap-Cre} \) mouse lines are suitable in vivo models to study a role for ILK in neuronal cell precursor proliferation, each come with limitations. For example, in the \( \text{nestin-Cre} \) mouse model, migration defects and resulting ectopies confound the proliferative defect. Specifically, it is difficult to discern whether or not a proliferation or migration defect underlies ectopic granule cells that are no longer proliferating (but rather express NeuN, a marker associated with differentiated neurons). Furthermore, early excision of ilk might cause secondary defects that exacerbate cerebellar changes. For example, the fused folia that were observed in \( \text{nestin-Cre} \) mutant mice would prevent meningeal cell penetration into fissures. This would likely contribute, in part, to cerebellar foliation defects because meningeal cells have been shown to be important in neuronal cell migration and radial glial cell differentiation (Hartmann et al., 1998). Conversely, although changes in cerebellar architecture were not observed in \( \text{gfap-Cre} \) mutants, Cre recombination activity occurs in the majority of granule cells in the EGL at P14, a developmental time near the end of the EGL proliferative period. Indeed, at P10, ILK immunoreactivity was observed in some cells of the EGL, and pulse labeling revealed that proliferative defects occurred in only some of the folia (likely reflecting incomplete and somewhat variable ILK excision at this developmental time point). Furthermore, folia depth was reduced in both Cre mutants (which might lead to a decrease in BrdU pulse labeling because of a decrease in the number of precursor cells). Therefore, BrdU pulse labeling was expressed per millimeter of cerebellar surface in both Cre mutant models. Controlling for the decrease in cerebellar surface, proliferative defects were observed in both CNS-restricted ilk knock-

Figure 9. Dual staining for BrdU and laminin in \( \text{nestin-Cre} \) postnatal mice. Costaining for BrdU (green) and laminin (red) in \( \text{nestin-Cre} \) mice at 10 d postnatal (c, d) and corresponding area stained for hematoxylin–eosin to reveal general cytoarchitecture (a, b). A reduction in the thickness of the external granule cell layer is observed in areas of the cerebellum lacking fissures (white arrows, a–d). Dual staining for BrdU and laminin in an adjacent section revealed that laminin staining is intimately associated with the proliferating zone in the EGL of wild-type (mock) control (e). High-magnification view of fissures labeled by asterisks (f) compared with littermate control (e). Scale bars: a, c, 200 \( \mu \)m; e, 50 \( \mu \)m.
out models, providing evidence that ILK is an important regulator of neuronal precursor proliferation. Indeed, in vitro results indicate that Shh-induced proliferation in GCP cells is reduced in the absence of ILK (in which the initial number of precursor cells is the same), further supporting a role for ILK in neuronal precursor proliferation.

ILK phosphorylates GSK-3β and PKB/AKT, two enzymes known to be involved in cell proliferation (Delcommenne et al., 1998; Persad et al., 2000, 2001a,b). Phosphorylation of these targets had been shown to occur in vivo (Edwards et al., 2005; Younes et al., 2005) and in vitro (Delcommenne et al., 1998; Persad et al., 2000, 2001a,b). In a recent publication characterizing the role of ILK in the dorsal forebrain, no difference in phosphorylation levels of GSK-3β or Akt was observed in E14.5 dorsal forebrain extracts of Emsx1-Cre; ILKh20/h2 mutants (Niewmierzyczka et al., 2005). ILK was not deleted in the cerebellum in this study. To determine whether GSK-3β phosphorylation was important in GCP proliferation in the cerebellum, we stained sagittal sections of wild-type mice using a phosphospecific antibody to GSK-3β. We could not detect phosphospecific GSK-3β immunoreactivity in the EGL, although the molecular layer stained modestly for this phosphorylated protein (J.M., unpublished observations). Nevertheless, a role for ILK kinase activity in GCP proliferation is suggested by the fact that the ILK kinase inhibitor KP-392 reduced Shh-induced proliferation in vitro. Previously, ILK has been shown to act downstream of phosphatidylinositol 3 (PI3)-kinase in mouse DRG neurons to regulate localized GSK-3β phosphorylation and axon elongation (Mills et al., 2003; Zhou et al., 2004). Therefore, assessment of ILK kinase activity by this substrate may be more relevant in other in vivo neuronal mouse models.

The precise mechanism of ILK-mediated regulation of GCP proliferation remains unknown. At least two possibilities exist, involving its signaling or scaffolding functions. If Shh-β1 complex bind to laminin, then ILK, by binding to the integrin β1-cytoskeletal tail, may link the Shh-β1 complex to the actin cytoskeleton, thereby regulating the cytoskeletal changes required for proliferation. However, the kinase activity of ILK also plays an important role in integrin-mediated cell–matrix interactions and cell proliferation. In vitro, constitutive ILK activation or overexpression has been shown to stimulate cyclin D1 expression (Radeva et al., 1997; D'Amico et al., 2000; Persad et al., 2001a). Proliferative defects were observed in mice having ILK-null growth-plate chondrocytes, whereas ex vivo excision of ILK resulted in reduced cyclin D1 expression (Grashoff et al., 2003; Terpstra et al., 2003). In GCPs, Shh-induced proliferation and regulation of D-type cyclin expression occurs via N-myc (Kenney et al., 2003, 2004). Moreover, Shh and PI3-kinase signaling pathways converge on N-myc to regulate neuronal cell-cycle progression (Kenney et al., 2003, 2004). Therefore, ILK may be a key effector in a PI3-kinase-dependent proliferative pathway in neuronal precursor cells. Of course, ILK scaffolding function and kinase activity are not entirely distinct because the kinase and adapter properties of ILK appear to function together in a PI3-kinase-dependent manner, regulating integrin-mediated cell attachment and signal transduction (Attwell et al., 2003). Regardless of the precise mechanism, the fact that ILK regulates proliferation in a variety of tissues and is an important effector in Shh signaling suggests that ILK plays a fundamental role in regulating the proliferative capacity of precursor cells throughout the body.

References


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