Integrin-Linked Kinase Deletion from Mouse Cortex Results in Cortical Lamination Defects Resembling Cobblestone Lissencephaly

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Abstract

Integrin-linked kinase (Ilk) is a scaffold and kinase that links integrin receptors to the actin cytoskeleton and to signaling pathways involved in cell adhesion, migration, and extracellular matrix deposition. Targeted deletion of Ilk from embryonic mouse dorsal forebrain neuroepithelium results in severe cortical lamination defects resembling cobblestone (type II) lissencephaly. Defects in adult mutants include neuronal invasion of the marginal zone, downward displacement of marginal zone components, fusion of the cerebral hemispheres, and scalloping of the dentate gyrus. These lesions are associated with abundant astrogliosis and widespread fragmentation of the basal lamina at the cortical surface. During cortical development, neuronal ectopias are associated with severe disorganization of radial glial processes and displacement of Cajal-Retzius cells. Lesions are not seen when Ilk is specifically deleted from embryonic neurons. Interestingly, targeted Ilk deletion has no effect on proliferation or survival of cortical cells or on phosphorylation of two Ilk substrates, Pkb/Akt and Gsk-3β, suggesting that Ilk does not regulate cortical lamination via these enzymes. Instead, Ilk acts in vivo as a major intracellular mediator of integrin-dependent basal lamina formation. This study demonstrates a critical role for Ilk in cortical lamination and suggests that Ilk-associated pathways are involved in the pathogenesis of cobblestone lissencephalies.

Key words: integrin-linked kinase; extracellular matrix; cortical development; cobblestone lissencephaly; radial glia; reelin

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Introduction

Mammalian cortical development proceeds in a highly orchestrated manner in which neurons migrate along radial glial fibers toward the surface of the brain to form a multilayered “inside-out” cortex. Integrin receptors are critical for several aspects of this process. The interaction between migrating neurons and radial glia, and the timely detachment of neurons at the end of migration involves α3β1 integrin (Anton et al., 1999; Sanada et al., 2004; Schmid et al., 2004). In contrast, integrin subunits α6 and β1 are involved in basal lamina assembly and radial glial end-feet anchorage at the cortical surface, and their absence results in abnormalities of cortical lamination in mutant mice (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001). The mechanisms by which the various functions of integrins are coordinated inside cells during corticogenesis are not known.

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Integrin-linked kinase (Ilk), a scaffolding protein and serine/threonine protein kinase, is a key effector of integrin function. Ilk exists as a multiprotein complex, binding the cytoplasmic portion of β1 and β3 integrins as well as adaptor proteins such as PINCH and α-parvins that regulate the actin cytoskeleton (Grashoff et al., 2004; Wu, 2004; Hannigan et al., 2005). Loss-of-function studies in Caenorhabditis elegans indicate that PAT-4/Ilk recruits integrins to adhesion complexes (Mackinnon et al., 2002). In Drosophila, loss of Ilk function leads to detachment of filamentous actin from the plasma membrane (Zervas et al., 2001), revealing an important role for Ilk in actin stabilization at integrin attachment sites. In vitro, localization of the multiprotein Ilk complex to focal adhesions regulates extracellular matrix deposition and provides an important physical connection between integrins and the actin cytoskeleton (Guo and Wu, 2002).

Ilk also has kinase activity in vitro and is able to induce phosphorylation of proteins such as Pkb/Akt, a downstream effector in a phosphatidylinositol 3 kinase-dependent Rac1 signaling pathway that regulates both integrin-associated rearrangements of actin filaments and cell migration (Qian et al., 2005). In addition, inhibition of Gsk-3β by Ilk was suggested as a major mechanism for neurite extension and axonal growth in vitro (Mills et al., 2003; Zhou et al., 2004). Although Ilk has been implicated in cell adhesion, migration, and extracellular matrix modification, its role in the development of the CNS has not been studied.

To determine the essential function of Ilk in brain develop-
ment, we targeted Ilk for deletion in the mouse dorsal forebrain using cre/lox technology. The resulting mutant has severe cortical laminar defects with overmigration of neurons into the marginal zone, breakdown of the basilar lamina, disorganization of radial glia, and displacement of Cajal-Retzius (CR) cells. The defects resemble those seen in mouse mutants of integrins, dystroglycan, and other genes involved in basal lamina assembly and suggest that Ilk is a major intracellular mediator of integrin and/or dystroglycan-mediated basal lamina assembly during brain development. Strikingly, absence of Ilk does not appear to perturb the proliferation, survival, or differentiation of neuronal precursors, although Ilk has been shown previously to regulate these functions in other cell types in vitro.

Materials and Methods

Mouse lines. The Ilk floxed mice (Ilkflo/flo) have been described previously (Terpstra et al., 2003). The Emx1crefl/fl mice were obtained from Jessica Gorski and Kevin Jones (University of Colorado, Boulder, CO), and the Nex-cre mice were obtained from Sandra Goebels and Klaus-Armin Nave (Max-Planck-Institute, Göttingen, Germany). The pattern of recombination promoted by the Emx1crefl/fl allele has been described previously (Gorski et al., 2002). Emx1creIlkfl/fl mutants were compared in all experiments with sex-matched Emx1creIlkfl/+ and Ilkfl/+ littermates. Similarly, Nex-creIlkfl/fl mutants were compared with Nex-cre Ilkfl/+ and Ilkfl/+ sex-matched littermates. The animals examined were of a mixed background. All animals were handled in accordance with protocols approved by the University of California, San Francisco Committee on Animal Research.

Antibodies. Ilk monoclonal antibody (mAb) (BD Transduction Laboratories, Lexington, KY), neuronal-specific nuclear protein (NeuN) mAb (Chemicon, Temecula, CA), calbindin polyclonal antibody (pAb) (Swant, Bellinzona, Switzerland), Englebreth-Holm-Swarm laminin pAb (Sigma, St. Louis, MO), GFAP mAb (Chemicon), dystrophin MAN- DRA1 mAb (Sigma), chondroitin sulfate proteoglycan (CSPG) (CS-56) mAb (Sigma), RC2 mAb and Otx1 mAb (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), calretinin pAb (Chemicon), reelin mAb G10 (André Goffinet, University of Louvain, Brussels, Belgium), phospho-histone pAb (Upstate Biotechnology, Lake Placid, NY), Ki67 mAb (Novocastra, Newcastle upon Tyne, UK), Akt[pS473] pAb (Cell Signaling Technology, Beverly, MA), Akt mAb (Cell Signaling Technology), Gsk-3β[pS9] pAb (Cell Signaling Technology), and Gsk-3β mAb (BD Transduction Laboratories).

Histological analysis. Mice were deeply anesthetized with 2.5% avertin and perfused with 4% paraformaldehyde in PBS. Embryos and neonates were decapitated, and heads (embryos) or brains (neonates) were suband perfused with 4% paraformaldehyde in PBS. Embryos and neonates were then rinsed in dH2O, 70% ethanol, 95% ethanol, and chloroform. Sections for Nissl staining were dehydrated overnight in solutions D and E. Sections were dehydrated through descending alcohol, and chloroform. All animals were handled in accordance with protocols approved by the University of California, San Francisco Committee on Animal Research.

Western blotting. Cortices from embryonic day 14.5 (E14.5) mice were dissected and lysed in modified radioimmunoprecipitation assay buffer [10 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM NaVO4, 10% glycerol, 1 mM DTT, 100 mM NaCl, 0.05% NP-40, and 0.1% SDS]. The cell lysate was then centrifuged at 14,000 rpm for 15 minutes to pellet debris and cellular organelles. The supernatant was collected and stored at -80°C. The protein concentration was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL).

Statistical analysis. Statistical significance was determined using the Student’s t test in all studies.

Results

Generation of conditional knock-out mice with dorsal forebrain-restricted Ilk deletion

To gain insight into the role of Ilk in the CNS, we deleted Ilk from the vast majority of cells in the dorsal forebrain by crossing mice expressing cre recombinase driven by the Emx1 promoter to mice expressing a floxed Ilk allele (Gorski et al., 2002; Terpstra et al., 2003). Emx1cre recombinase activity is first observed in precur-

sors of neurons and glia at embryonic day 9 (Gorski et al., 2002), before initiation of cortical neurogenesis (Kubo and Nakajima, 2003). Deletion of Ilk from the dorsal forebrain was confirmed by immunofluorescence and Western blot analysis, which showed a significant decrease of Ilk in the targeted regions (Fig. 1). Residual Ilk staining was attributable to its presence in cells not derived from the dorsal forebrain neuroepithelium, such as inhibitory interneurons, vascular endothelial cells, and meningeal cells.

Mutant mice were born at the expected Mendelian ratios and were indistinguishable from littermates at the time of weaning. However, at ~4–5 months of age, homozygous mutants began

Figure 1. Expression pattern of Ilk in control and forebrain-specific Emx1-cre;Ilkfl/fl mutant littermates. A, B, Coronal sections (10 μm) from an E14.5 littermate control (A) and Ilk mutant (B) immunostained with an Ilk antibody showed selective absence of Ilk from the cortex, but not the midbrain, of the Ilk mutant. Residual Ilk staining was attributable to the presence of Ilk in meninges, interneurons, and blood vessels, which are not targeted by the Emx1 promoter-driven cre. C, Western blot analysis of E14.5 forebrain extracts (2.5 μg) showed a significant decrease of Ilk expression in an Ilk mutant compared with a littermate control. β-Tubulin was used as a loading control in separately loaded lanes. The Ilk protein blot was also stripped and reprobed with Akt, showing comparable protein levels in the two samples (see the same ex-

tracts in Fig. 9). Mutant genotypes were Emx1-cre, Ilkfl/fl. Littermate control genotypes were Ilkfl/fl. Con, Control; Mut, mutant. Scale bar, 100 μm.

Golgi staining. Modified Golgi–Cox impregnation of neurons was performed using the FD Rapid Golgi stain kit (FD NeuroTechnologies, Ellicott City, MD) according to the instructions of the manufacturer. Briefly, 2-month-old nonperfused mouse brains were immersed in impregna-

tion solution for 2 weeks, transferred to “solution C” for 2 d, and cut at 100 μm on the cryostat. Sections were mounted on 3% gelatin-coated slides and allowed to dry before staining with silver nitrate solution, “solution D and E.” Sections were dehydrated through descending alco-

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showing progressive weight loss and premature death. In addition, mutant animals were sterile.

**Targeted deletion of Ilk results in severe cortical lamination defects**

Analysis of brains from adult Ilk mutants showed severe defects of cortical lamination. Coronal sections of mutant brains showed multiple regions of cell invasion into the marginal zone and downward expansion of hypocellular regions into the underlying cortex (Fig. 2). There was extensive midline fusion of the cerebral hemispheres (Fig. 2F), and the dentate gyrus of the hippocampus showed an abnormal, scalloped appearance with displacement of granule cells toward the pial surface (Fig. 2I). Staining with anti-NeuN antibody demonstrated that neurons were the major constituent of the ectopias, which were most prominent in medial and caudal regions of the cortex (Fig. 3A–D). Dense accumulations of neurons were found just below hypocellular downward expansions of marginal zone components (Fig. 3B, D). Calbindin-positive interneurons were found within these dense accumulations (Fig. 3F, H), suggesting misplacement of these cells from their usual location beneath the marginal zone (Fig. 3E, G). Otx1-expressing neurons that are localized normally to cortical layers V and VI were misplaced within ectopic outgrowths toward the surface of the cortex (Fig. 3F). Analysis of neuronal dendritic processes using microtubule-associated protein 2 (MAP2) immunostaining (Fig. 3K, L) showed that, although in control brains there was an orderly arrangement of dendritic bundles coursing through the cortex and terminating at the marginal zone (Fig. 3K), in mutant brains dendritic processes were severely disorganized, veering toward and terminating at misplaced hypocellular marginal zone-like regions within the cortex (Fig. 3L). A similar pattern was observed with Goji impregnation in which apical dendrites in mutant brains lacked the orderly, parallel arrangement of dendrites seen in control brains (Fig. 3M, N). There were no obvious defects in spine formation between control and mutant brains (Fig. 3O, P).

**Absence of Ilk results in fragmentation of the basal lamina**

Fragmentation of the basallamina is frequently observed after deletion of extracellular matrix constituents or receptors in mice (Georges-Labouesse et al., 1998; Costell et al., 1999; Graus-Porta et al., 2001; Halfter et al., 2002; Moore et al., 2002; Beggs et al., 2003; Poschl et al., 2004). We therefore examined the basallamina in Ilk mutant mice. Using anti-laminin, we observed extensive fragmentation of the basallamina throughout the entire dorsomedial cortical surface of adult Ilk mutants (Fig. 4A, B). Fragmentation was also prominent around large blood vessels penetrating into the cortex from the pia-meninges but was not seen in smaller cortical blood vessels (Fig. 4A, B). Mutant brains also showed marked astrogliosis, as assessed using anti-GFAP, that extended far from the pial surface into deep layers of the cortex (Fig. 4C, D). Midline regions of mutant brains showed complete loss of basallamina accompanied by fusion of the hemispheres, as highlighted by dystrophin immunostaining (Fig. 4E, F).

**Ectopias and basallamina fragmentation originate during embryonic cortical development**

To examine early events that lead to the observed defects in brain development, we examined Ilk mutants during embryonic cortical development. Mutant E14.5 embryos showed numerous small neuronal ectopias that invaginated the marginal zone and were associated with irregularities of the cortical plate (Fig. 5B). However, as assessed by chondroitin sulfate proteoglycan expression, the preplate split normally into the marginal zone and subplate (Fig. 5C, D). Basal lamina fragmentation was also readily detectable at this stage of cortical development. Large gaps in the basallamina that were associated with neuronal ectopias and contained small scattered fragments of laminin were evident in these embryos, especially in mediocaudal regions of the developing brain (Fig. 5E, F). Fusion of the hemispheres with splitting and fragmentation of the basallamina were observed in mediocaudal regions of the mutant cortex (data not shown). In more lateral regions, fragmentation of the basallamina was less frequent, although small scattered fragments of laminin could be found in these regions throughout the thickness of the cortical plate (Fig.
5H). Ectopias were also rare in lateral regions. These studies showed that fragmentation of the basal lamina coincides temporally and spatially with formation of neuronal ectopias early in embryonic development. Additional studies at postnatal day 0 (P0) showed that the cortical lamination defects, including neuronal ectopias and basal lamina breakdown, were well developed by birth and resembled defects seen in the adult (data not shown).

Radial glial morphology is disrupted in the absence of Ilk

Previous studies have shown that proper anchorage of radial glial end feet is dependent on the presence of an intact basal lamina and a functioning adhesion system (Hartmann et al., 1998; Graus-Porta et al., 2001; Hafetter et al., 2002; Beggs et al., 2003). To determine whether Ilk is important for radial glial development and function, we examined radial glia in Ilk mutant E14.5 embryos. Mutants showed severe morphologic abnormalities of radial glia that occurred beyond sites of surface basal lamina disruption (Fig. 6). Whereas in control animals radial glia were aligned in parallel arrays throughout the cortical plate, terminating in well defined end feet at the pial surface, in mutant embryos, radial glial fibers were severely disorganized, with excessive crossing and branching seen throughout the targeted region (Fig. 6A, B). At the pial surface of mutants, radial glia often terminated in tangled heaps of fibers (Fig. 6B), and, occasionally, radial glial end feet were retracted from the pial surface (Fig. 6F). Radial glia were abnormal even in lateral regions in which the basal lamina

Figure 3. Analysis of the cellular composition of cortical ectopias in Emx1-cre; Ilkfl/fl adult mutants. Coronal (A, B, E–P) and sagittal (C, D) sections (40 μm, A–L; 100 μm, M–P) from adult littersmate controls (A, C, E, G, I, K, M, O) and Ilk mutants (B, D, F, H, J, L, N, P). A–D. In control animals, NeuN immunostaining highlighted the laminar organization of the cortex in which neurons were excluded from hypocellular marginal zone regions at the surface of the brain (A, C). In Ilk mutants, ectopic neurons invaded and obliterated the marginal zone (arrow, B). Hypocellular regions were found deep within the mutant cortex (asterisk, B), and neurons piled up underneath these hypocellular regions (arrow, D). The granule cell layer of the dentate gyrus had an abnormal, undulating appearance in the mutant (arrowhead, B). E–H. In control animals, calbindin staining highlighted interneurons that were widely scattered but also formed a distinct layer beneath the marginal zone (arrows, E, G). In midline regions of mutant brains, this calbindin-positive layer was absent (arrow, F). Instead, interneurons were found at the periphery of misplaced hypocellular marginal zone regions (asterisk, F, H). In lateral regions of mutant brains, interneurons were distributed in a similar manner to controls, although the calbindin-positive layer below the marginal zone had an abnormal, wavy appearance in the mutant (arrows, H). I, J, Otx1 antibody identifies a subset of neurons localized to layers V and VI of controls (I) (Frantz et al., 1994). In mutant brains, many of the Otx1-expressing neurons were mislocalized to more superficial layers of the cortex within ectopic outgrowths (arrows, J). K, L, MAP2 staining in control brains highlighted linear bundles of dendrites coursing radially through the cortex and filling the marginal zone (K). In the mutant, the arrangement of dendrites was severely disorganized, with many dendrites seen veering away from the surface of the cortex to terminate and fill misplaced hypocellular marginal-zone like regions (asterisk, L, M, N, Golgi) staining in control brains highlighted the orderly arrangement of cortical neurons and their processes (M). The orderly, radial arrangement of apical dendrites was disturbed in mutant sections, especially near the midline (N, right side of image). There were no apparent differences between mutant and control brains in the distribution and branching of basal dendrites (O, P) or in the density or morphology of dendritic spines (O, P, insets). Mutant genotypes were Emx1-cre; Ilkfl/fl. Control littersmate genotypes were Ilkfl/fl (A, E, G, I) or Emx1-cre; Ilkfl/fl (C, K, M, O). cc, Corpus callosum; gcl, granule cell layer; mb, midbrain; mz, marginal zone; lv, lateral ventricle. Scale bars: A–F, M, N, 500 μm; G–J, 200 μm; K, L, O, P, 100 μm; insets in O, P, 20 μm.
Positioning of Cajal-Retzius cells is defective in Ilk mutants

Cajal-Retzius cells secrete reelin, a signaling molecule that is critical for proper positioning of neurons (Tissir and Goffinet, 2003). A defect in localization of CR cells may lead to aberrant neuronal migration caused by mislocalized release of reelin. Previous studies have suggested that integrin adhesion is important for CR cell localization (Graus-Porta et al., 2001). Because CR cells are targeted by Emx1 promoter-driven cre (Gorski et al., 2002), we asked whether any defects of CR cell localization or reelin secretion are associated with the absence of Ilk in these cells. Using both reelin and calretinin to identify CR cells, we found that, in control E14.5 embryos and P0 neonates, there was a relatively continuous single layer of CR cells at the marginal zone (Fig. 7A, I). In contrast, mutant embryos showed multiple gaps in the CR cell layer, especially in medio-caudal regions (Fig. 7B, F). These gaps were associated with large neuronal ectopias (Fig. 7B, H) and corresponded to breaks in the basal lamina (Fig. 7D). The distribution of CR cells changed dramatically in mutant neonates, in which CR cells were found in ectopic clusters deep within the cortex, mostly in midline regions (Fig. 7K, L). Cortical areas containing the CR cell clusters were hypocellular but were underlain by dense bands of neurons (Fig. 7K, L) as also seen with anti-NeuN (Fig. 3B, D). These findings suggest that, although CR cells form and migrate normally in the absence of Ilk during embryonic cortical development, sporadic breakdown of the basal lamina results in loss of CR cells from these regions. Loss and displacement of CR cells during the course of cortical development may contribute to the abnormal positioning of neurons seen in the adult mutant cortex.
Deletion of Ilk from postmitotic neurons does not result in lamination defects

Our initial targeting strategy resulted in ablation of Ilk from both neuronal and glial precursors. To determine whether Ilk functions in neurons to control cortical lamination, we generated conditional mutant mice in which Ilk was specifically deleted from postmitotic neurons using a transgenic mouse line with cre recombinase driven by the Nex promoter. The Nex-cre transgene is expressed as early as E11, and, by E12.5, expression is robust throughout the forebrain (Shimizu et al., 1995) (S. Goebbels and K.-A. Nave, unpublished results). The resulting mutants showed no obvious behavioral difference from controls. Nissl staining did not reveal any obvious cortical lamination defects in mutants (Fig. 8A–D). No differences in GFAP or synaptophysin staining were seen between the mutant and control animals (data not shown). Golgi staining revealed a normal distribution and morphology of neurons in the mutants with no significant abnormalities of dendritic projection, branching, or spine formation (Fig. 8E–H). These results suggest that glia-derived Ilk, and not neuronal Ilk, is the major contributor to the lamination defects observed in Emx1-cre;IlkΔ/Δ mutants.

Discussion

In this study, we demonstrated a critical role for Ilk in cortical development. Mice lacking Ilk showed cortical lamination defects with breakdown of the basal lamina and abnormalities of radial glia and CR cell positioning. In contrast to in vitro studies, we found that cell proliferation, survival, and differentiation were not affected by the absence of Ilk, and that phosphorylation levels of two major Ilk targets, Pkb/Akt and Gsk-3β, were not reduced in the embryonic cortex of Ilk mutants.

Role of Ilk in formation and maintenance of the basal lamina

Ilk most likely regulates cortical lamination by affecting formation or maintenance of the basal lamina. Basal lamina assembly takes place at the interface between the glia limitans and the meninges and depends on both of these neighboring structures for proper execution (Sievers et al., 1994; Shearer and Fawcett, 2001; Beggs et al., 2003). Meningeal fibroblasts secrete components of the basal lamina, which then bind to receptors on radial glial end feet of the glia limitans to be assembled into a functioning basal lamina (Sievers et al., 1994; Shearer and Fawcett, 2001; Beggs et al., 2003). Defects of several proteins involved in basal lamina formation and maintenance have been implicated in control of proliferation, cell migration, and actin rearrangement (Mills et al., 2003; Zhou et al., 2004). To determine whether phosphorylation of the two most prominent substrates of Ilk is reduced in the absence of this kinase in vivo, we examined the phosphorylation levels of Pkb/Akt and Gsk-3β in control and Emx1-cre;IlkΔ/Δ mutant E14.5 dorsal forebrain extracts (Fig. 9A). We used phospho-specific antibodies against Akt-[pS473] and Gsk-3β-[pS3], which represent the reported sites of Ilk phosphorylation (Persad et al., 2001a,b). There were no significant differences in protein levels or phosphorylation levels of either of these two targets between the mutant and control extracts (Fig. 9A). Similar studies at P0 also showed no differences in protein levels or phosphorylation of these two Ilk targets (data not shown). Consistent with the above findings, mutant embryos showed no significant changes in proliferation of cortical cells, as assessed by phospho-histone H3 staining (Fig. 9B–D). There were also no differences in proliferation as assessed by Ki67 staining (data not shown). In addition, there was no significant increase in the number of apoptotic cells in mutant brains compared with controls, as assessed by TUNEL assays (Fig. 9E,F).
assembly, including laminin, perlecan, collagen, dystroglycan, and integrins, cause basal lamina abnormalities associated with neuronal migration defects (Georges-Labouesse et al., 1998; Miner et al., 1998; Costell et al., 1999; Graus-Porta et al., 2001; Halfter et al., 2002; Moore et al., 2002; Beggs et al., 2003; Poschl et al., 2004). The Ilk mutant shares many of the cortical phenotypes with these mutants. Our studies showed extensive breakdown of the basal lamina and displacement of neurons toward the cortical surface. In regions in which the basal lamina was intact, cortical lamination was normal, although neurons and glia lacked Ilk. In addition, glial processes appeared disorganized throughout the cortical plate, and occasional radial glial end feet were seen retracted from the pial surface. These abnormal radial glia may provide inadequate support for matrix assembly at the pial surface. In fact, glia appear to be the primary cell type responsible for the defect in the Ilk mutant because ablation of Ilk specifically from postmitotic neurons did not result in lamination defects. These findings support a role for Ilk within radial glia to promote basal lamina assembly.
Mechanism of Ilk action during cortical development

Ilk is probably involved in matrix assembly through its interaction with β1 integrin cytoplasmic domain (Hannigan et al., 1996). Integrin β1 heterodimers bind several basal lamina constituents and play key roles in basal lamina assembly (Henry et al., 2001; Lohikangas et al., 2001; Li et al., 2002). As in other cell types, Ilk may act at radial glial end feet as a scaffold to link cell surface integrin receptors to the actin cytoskeleton. Rearrangement of the actin cytoskeleton is required for laminin assembly after engagement of integrin and dystroglycan receptors (Colognato et al., 1999), and Ilk is likely involved in this process. Interestingly, although phosphorylation of Pkb/Akt and Gsk-3β by Ilk was shown to be important in vitro for actin reorganization, cell migration, and neurite extension (Mills et al., 2003; Zhou et al., 2004; Qian et al., 2005), phosphorylation of these proteins was not reduced by the absence of Ilk in vivo during cortical development. It remains to be determined whether the kinase activity of Ilk is required for its role in basal lamina assembly in the forebrain in vivo.

Our studies did not reveal major defects of proliferation or cell survival in the absence of Ilk. In contrast, several studies have shown Ilk to be important for proliferation of cells such as chondrocytes and granule cell precursors (Grashoff et al., 2004; Terpstra et al., 2003) (J. Mills, A. Niewmierzycka, A. Oulumi, B. Rico, R. St-Arnaud, I. Mackenzie, N. Majwi, L. F. Reichardt, and S. Dedhar, unpublished observations) and in survival of cells, such as endothelial cells and hippocampal neurons (Gary et al., 2003; Friedrich et al., 2004). The conflicting results most likely reflect differences of in vivo versus in vitro conditions plus tissue-specific differences of Ilk function. For example, cerebellar granule cells, which migrate in an outside-in manner, may rely much more heavily than cortical cells on interactions with surface laminin for proliferation (Blaess et al., 2004). In contrast, proliferation of cortical neuronal precursors, which begin their migration at the subventricular zone, may depend on mechanisms that do not rely on integrin signaling, such as calcium wave propagation (Weissman et al., 2004).

Role of Cajal-Retzius cells in cortical lamination defects of Ilk mutants

A major feature observed in Ilk mutants was mislocalization of CR cells during embryonic cortical development. Our results indicate that retention of CR cells at the marginal zone may rely on Ilk and integrin-dependent adhesion to an intact basal lamina. Indeed, gaps in the CR cell layer of Ilk mutant embryos were consistently associated with gaps in the basal lamina. Although the Emx promoter-driven Cre targets CR cells (Gorski et al., 2002), it is unlikely that deletion of Ilk from CR cells contributed directly to their mislocalization. CR cells migrated normally and were evenly distributed at the marginal zone in all regions except those with breakdown of the basal lamina.

Sporadic loss of CR cells and reelin from regions of basal lamina breakdown may contribute to the formation of neuronal ectopias. We found that ectopic outgrowths were consistently associated with gaps in the CR cell layer. Reelin has been shown to direct neuronal migration (Dulabon et al., 2000), and its loss in Ilk mutants may allow neurons to overmigrate. Later in development, displacement of the remaining CR cells to deep regions of the cortex may further contribute to the severe, scalloped appearance of the Ilk mutant brain. Indeed, in the Ilk mutant neonate, dense aggregates of neurons appear below displaced clusters of CR cells, suggesting that mislocalized reelin secretion results in retention of large subsets of neurons in deep regions of the cortex.

Finally, loss of CR cells may contribute to the abnormal radial glial scaffold observed in the Ilk mutants. However, radial glia abnormalities were widespread, whereas CR cell loss was limited to small, sporadic regions, suggesting that radial glial defects precede the loss of CR cells.
Comparison of the Ilk mutant to other basal lamina-related mutants

The lamination defects in the Ilk mutant were similar to those described in several other mouse mutants deficient in various gene products involved in basal lamina assembly. For example, the Ilk mutant shares many cortical features with the β1 integrin conditional mutant (Graus-Porta et al., 2001), indicating that Ilk may be a major cytoplasmic mediator of β1 integrin function during cortical development. In contrast, a conditional mutant of another integrin-associated protein kinase, focal adhesion kinase (Fak) (Beggs et al., 2003), had a milder phenotype than either the β1 integrin or Ilk mutants and did not show loss or displacement of CR cells. The apparently normal localization of CR cells in the Fak mutant indicates that the degree of laminin breakdown in the absence of Fak may be insufficient to eliminate or displace CR cells from their normal positions. Additional differences were also observed between the Ilk and Fak mutants. The Ilk mutant exhibited very early and severe morphologic radial glial abnormalities, whereas the Fak mutant showed partial agenesis of the corpus callosum that was not present in the Ilk mutant. The divergent phenotypes indicate that these cytoplasmic proteins are involved in distinct signaling pathways. Interestingly, the Ilk mutant also shared many features with the conditional dystroglycan mutant (Moore et al., 2002), indicating that Ilk may be involved in cytoplasmic pathways, including regulation of actin dynamics, common to both the integrins and the dystrophin–glycoprotein complex.

Role of Ilk in the pathogenesis of congenital muscular dystrophies

The Ilk mutant cortex resembles cobblestone (type II) lissencephaly seen in brains of congenital muscular dystrophy patients. These conditions include Muscle-Eye-Brain disease, Walker-Warburg syndrome, and Fukuyama congenital muscular dystrophy (Fukuyama et al., 1981; Haltia et al., 1997; Kobayashi et al., 1998; Cormand et al., 2001). Aﬀected patients present with a myriad of abnormalities, including cobblestone cortex, severe mental retardation, seizures, muscular dystrophy, and cerebellar and ocular abnormalities (Muntoni and Voit, 2004). Several genes responsible for these diseases were found to code for glycosyltransferases, with α-dystroglycan being a major target of glycosylation (Kobayashi et al., 1998; Yoshida et al., 2001; Michele et al., 2002; Moore et al., 2002). However, the genes mutated in many patients remain unidentified. Integrins and dystroglycans are laminin receptors that play synergistic roles in laminin matrix assembly (Henry et al., 2001; Li et al., 2002). In addition, both surface receptor systems connect to the actin cytoskeleton through several adaptor molecules. The disorganized cortex and basal lamina fragmentation seen in forebrains of Ilk mutants suggest that Ilk signaling may be perturbed in congenital muscular dystrophies and, consequently, may play a role in their pathogenesis. Interestingly, additional mouse mutants with targeted deletion of Ilk recently generated in our laboratory show eye, cerebral, and skeletal muscle abnormalities similar to those seen in congenital muscular dystrophy patients (A. Niewmierzycka and L. F. Reichardt, unpublished observations). Future studies will clarify the role played by Ilk and related proteins in this group of diseases.

References


