Reelin, Integrin and Dab1 Interactions during Embryonic Cerebral Cortical Development

Extracellular matrix-like molecule reelin and cell surface adhesion receptors such as α3β1 integrin can regulate neuronal migration and position in the developing cerebral cortex. Here we show that α3β1 integrin binds to the N-terminal region of reelin, a site distinct from the region of reelin shown to associate with other reelin receptors such as VLDLR/ApoER2. Furthermore, Dab1, a member of the reelin signaling pathway, can complex with the cytoplasmic region of β1 integrin in a reelin-dependent manner. Thus, α3β1 integrin–reelin interactions may contribute to appropriate neuronal placement in the developing cerebral cortex.

Keywords: cerebral cortex, Dab1, integrin, neuronal migration, reelin

Introduction

Coordinated migration of neurons from the ventricular zone to the cortical plate is essential for the emergence of laminar organization of cerebral cortex (Rakic, 1972; O'Rourke et al., 1992; Nadarajah et al., 2001; Hatten, 2002; Nadarajah and Parnavelas, 2002; Marin et al., 2003). The family of integrin cell surface adhesion receptors has been implicated in the signaling mechanisms that contribute to this process (Schmid and Anton, 2003). Integrins are heterodimeric cell surface receptors that serve as structural links between the extracellular matrix (ECM) and the internal cytoskeleton. Different integrin receptors display different adhesive properties, regulate different intracellular signal transduction pathways and thus different modes of adhesion-induced changes in cell physiology (Hynes, 2002). Integrins are also capable of synergizing with other cell surface receptor systems to finely modulate cell's behavior in response to multiple environmental cues (Hynes, 2002). Developmental changes in the cell surface integrin repertoire and function may thus modulate distinct aspects of neuronal migration in the developing cerebral cortex by altering the strength and ligand preferences of cell–cell adhesion during development.

Different α integrin subunits dimerize preferentially or exclusively with β1 integrin, which is ubiquitously expressed in the developing cerebral cortex. The varied, yet distinct, cortical phenotypes of α1, α3, α6, αV and β1 integrin subunit null mice provide striking insights into the distinct roles that cell–cell or cell–ECM interactions play in neuronal laminar organization in cerebral cortex. α1 integrin-deficient cortex appears to develop normally (Gardner et al., 1999). In contrast, substantial disruption of cellular organization in cerebral wall and lateral ganglionic eminence is seen by E11–12 in αv null mice (Bader et al., 1998). Mice homozygous for a targeted mutation in the α3 integrin gene display disrupted neuronal migration and laminar organization (Anton et al., 1999; McCarty et al., 2005; Schmid et al., 2004). α6 integrin null mice die at birth (Georges-Labouesse et al., 1996) with ectopic neuronal distribution and outgrowth in the cortical plate of the cerebral cortex and retina (Georges-Labouesse et al., 1998). Coinciding abnormalities of laminin synthesis and deposition also occur in the α6 integrin mutant brain. Most of the α subunits expressed in the developing cortex dimerize with β1 integrins. Conditional inactivation of β1 integrins in cortical neurons and glia from around E10.5 (Graus-Porta et al., 2001) leads to disrupted cortical layer formation, defective meningeal basement membrane assembly, marginal-zone formation and glial end feet anchoring at the top of the cortex.

α3 integrin appears to dimerize primarily with β1 integrin, and normal cortical neuronal migration is thought to be modulated by interaction of α3β1 integrins with ECM components, such as fibronectin, thrombospondin or reelin, that are present along the migratory route (Anton et al., 1999). Of these ligands, reelin plays an essential role in the generation of appropriate neuronal positioning (D'Arcangelo et al., 1995). Previous studies indicated that reelin can associate with α3β1 integrin during corticogenesis (Dulabon et al., 2000); however, the nature of this interaction and the involvement of Dab1, a downstream signaling molecule in reelin pathway, in this process were not known.

Here we show that α3β1 integrin can associate with the N-terminal region of reelin. Reelin regulated intracellular adaptor protein Dab1 can associate with the α3β1 integrin receptor complex. Thus deficits in the ability to engage positional cues such as reelin, which are present along the migratory route, may in part underlie the misplacement of neurons in the cerebral cortex of α3 integrin mutant mice.

Materials and Methods

Antibodies

The following antibodies were used: Dab1 polyclonal antibodies (a gift from Dr B. Howell, NINDS; Ab5840, Chemicon) and β1 integrin polyclonal antibodies (Ab1952, Chemicon), α3 integrin antibodies (8–4, a gift from Dr DiPersio, Albany Medical College; #611045, BD Transduction Labs; Chemicon), PY20 p-Tyr antibodies (Santa Cruz), Fer polyclonal antibodies (a gift from Dr T. Pawson, U. of Toronto) and anti-reelin monoclonal antibodies (a gift from Dr. A. Goffinet).

Immunoprecipitation and Western Blotting

E16 mouse cortices were dissociated and plated at a density of 2 million cells/60 mm dish (28.57 cm² area) in DMEM+10% FBS. Previous studies indicate that these cultures contain a mix of migratory and post migratory neurons, as well as radial glial cells (Anton et al., 1996, 1997; Schmid et al., 2003). Reelin can influence both neurons and radial glia (Dulabon et al., 2000; Forster et al., 2002; Hartfuss et al., 2003; Jossin et al., 2003; Zhao et al., 2004). In vivo, α3β1 integrin is expressed widely in the developing cerebral wall (Anton et al., 1999; Sanada et al., 2004). Thus, these cells are useful for assessing potential...
Integrin-linked beads were washed and incubated with reelin (full three times with phosphate-buffered saline (PBS) and incubated over-

either a 6, 3- -6, 3- -8) were myc-tagged. reelin repeats 3--6 and 3--8, respectively. R3 and R6 contains only repeat 3 or DelR3- -R5A lacks reelin repeats 3- -5. R3--R6 and R3--R8 contain only repeats 3- -6 and 3- -8, respectively. R3 and R6 contains only repeat 3 or repeat 6, respectively. Reelin fragments from reelin repeat regions (R3, 6, 3- -6, 3- -8) were myc-tagged.

Equal volumes of anti-rabbit IgG beads (Zymed) were incubated with either a3 integrin antibodies (Chemicon) or control antibodies of the same isotype (anti-GFAP; Dako) for 12 h at 4°C. Beads were then washed three times with phosphate-buffered saline (PBS) and incubated overnight at 4°C with purified a3b1 integrin (0.2 mg/ml; Chemicon). Integrin-linked beads were washed and incubated with reelin (full length or fragment) containing media for 12 h. The beads were then washed several times in PBS+ 1% Triton X-100, eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer, analyzed by SDS-poly-

acrylamide gel electrophoresis (PAGE) and immunoblotted with anti-a3 integrin, anti-reelin or anti-myc antibodies to determine if a3b1 integrin associated with reelin.

**Results**

**Recombinant a3b1 Integrin Binds to the N-terminal Region of Reelin**

To determine the nature of reelin-a3b1 integrin interactions in the embryonic cerebral cortex, reelin fragments containing different regions of full-length reelin were incubated with purified a3b1 integrin protein (Fig.1A). Reelin fragment-

containing supernatants from transfected 293T cells were used without additional purification (Supplementary Fig. 1). Reelin fragments that were bound to a3b1 integrin were analyzed following immunoprecipitation with anti-a3 integrin antibodies. Full-length reelin and reelin fragments containing the N-terminal region up to the second reelin repeat bound to a3b1 integrin (Fig. 1B). Reelin fragments containing the rest of the reelin repeats, including the C-terminus, did not associate with a3b1 integrin (Fig. 1B). These results suggest that the N-terminal of reelin binds to a3b1 integrin.

**Dab1 Associates with Integrin b1**

Reelin signaling in the embryonic cerebral cortex is characterized by the activation of Dab1. Dab1 is a cytoplasmic protein expressed in developing cortical neurons, containing a PTB domain that interacts with the NPxY motif in the cytoplasmic domains of the VLDL and ApoER2 receptors (Howell *et al.*, 1997, 1999; Hiesberger *et al.*, 1999). Dab1 is known to be phosphorylated upon interaction of neurons with reelin, and it has been shown that the reelin:ApoER2 or VLDLR interaction triggers this phosphorylation (Hiesberger *et al.*, 1999; Howell *et al.*, 1999). Based on our observations that reelin interacts
with \( \alpha 3 \beta 1 \) integrin, we hypothesized that Dab1 may associate with the NPxY motif in the \( \beta 1 \) integrin subunit cytoplasmic domain. To test for an interaction between \( \beta 1 \) integrins and Dab1, we first determined whether \( \beta 1 \) integrin could be co-immunoprecipitated with Dab1 from embryonic cortical neurons. An anti-Dab1 antibody was used to immunoprecipitate (IP) Dab1 from extracts prepared from untreated or reelin-treated embryonic cortical neurons. Probing of immunoprecipitates with anti-\( \beta 1 \) integrin antibodies revealed that \( \beta 1 \) integrin co-immunoprecipitates with Dab1 (Fig. 2). Interestingly, there was significantly less Dab1 bound to \( \beta 1 \) integrin in neuronal cells treated with reelin. Whether this indicates that reelin-induced phosphorylation of Dab1 causes dissociation of Dab1 from \( \beta 1 \) integrins remains to be determined. Immunoblotting of Dab1 immunoprecipitates with \( \alpha 3 \) integrin antibodies indicate that \( \alpha 3 \) integrin is part of the \( \beta 1 \) integrin–Dab1 complex (Fig. 2). The reverse IP (i.e. IP \( \beta 1 \) or \( \alpha 3 \) integrin and probe with anti-Dab1 antibodies) demonstrated the same \( \alpha 3 \beta 1 \)--Dab1 interactions. As reported before (Howell et al., 1999), Dab1 became robustly phosphorylated when cells were challenged with reelin.

\( \beta 1 \) integrin is also known to associate with other intracellular signaling partners during cortical development. For example, \( \beta 1 \) integrin’s direct interactions with cytoplasmic protein kinase Fer has an important role in integrin-cadherin cross-regulation (Arregui et al., 2000). Thus, as control, we tested for \( \beta 1 \) integrin–Fer association in our immunoprecipitation assays. Fer was found to be associated with \( \beta 1 \) integrin (Fig. 2). Unlike Dab1, the tyrosine phosphorylation status of Fer did not change when cells were exposed to reelin (Fig. 2). Together, these data demonstrate that \( \alpha 3 \beta 1 \) integrin can recruit Dab1, a cytoplasmic adaptor protein essential for downstream effects of reelin.

**Discussion**

\( \alpha 3 \) integrin can modulate neuronal migration and placement in the developing cerebral cortex. In this study, we show that \( \alpha 3 \beta 1 \) integrin binds to the N-terminal region of reelin, a critical determinant of neuronal positioning during cortical development. Furthermore, we demonstrate that Dab1, an essential intracellular component of reelin signaling cascade, can complex with the cytoplasmic region of \( \beta 1 \) integrin in a reelin-dependent manner. These findings implicate a role for reelin–\( \alpha 3 \beta 1 \)--Dab1 interactions in the appropriate placement of neurons in the developing cerebral cortex.

**\( \alpha 3 \) Integrin–Reelin Interactions**

Reelin, an extracellular matrix protein released from the layer I cortical neurons, has been shown to interact with \( \alpha 3 \beta 1 \) integrin during neuronal detachment from radial glial guides (Dulanob et al., 2000). Reelin is also known to bind with high affinity to integrins in synaptoneurosomes from mature cortex (Dong et al., 2003). During neuronal migration to the cortical plate neuronal \( \alpha 3 \) integrin may interact with ECM molecules, such as fibronectin, thrombospondin or laminin-2, that are distributed along the migratory route (O'Shea et al., 1990; Sheppard et al., 1991, 1995; Pearlman and Sheppard, 1996; Yakubova and Komuro, 2002), and at the top of the cortical plate the ligand preference of \( \alpha 3 \beta 1 \) integrins may change to other ECM molecules, such as reelin. The change in ligand preference or concentration in turn can differentially determine the cell surface distribution pattern and level of expression of \( \alpha 3 \beta 1 \) integrins. Though the interaction of \( \alpha 3 \beta 1 \) integrin with fibronectin, laminins 2, 5 and 10/11, entactin/nidogen and collagen is well established, its biochemical association with reelin needed further characterization. Our results with recombinant reelin fragments spanning different segments of reelin indicate that \( \alpha 3 \beta 1 \) integrin associates with the N-terminal region of reelin. This site does not overlap with the region of reelin shown to associate with other reelin receptors, such as VLDLR/ApoER2 (Koch et al., 2002; Benhayon et al., 2003; Jossin et al., 2003). It remains to be determined whether the association of distinct regions of reelin with different receptors imparts different

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**Figure 2.** Reelin-integrin-Dab1 interactions. To determine Dab1–\( \alpha 3 \beta 1 \) integrin association, untreated or reelin-treated cortical cell extracts were immunoprecipitated (IP) with \( \alpha 3 \) or \( \beta 1 \) integrin antibodies and western blotted (WB) with Dab1 antibodies, or vice versa. Dab1 consistently co-immunoprecipitated with \( \alpha 3 \beta 1 \) integrin. As a control, a known interactor of \( \beta 1 \) integrin cytoplasmic region, Fer kinase, was also detected in the \( \beta 1 \) integrin IPs. Conversely, \( \beta 1 \) integrin was detected in Fer kinase IPs. In addition, characteristic Dab1 phosphorylation was noticed in cells exposed to reelin, whereas no changes in Fer kinase phosphorylation was noticed.
functional outcomes. Proteolytic processing of reelin in vivo generates N-terminal (~180 kDa, corresponding to N-R2; Fig. 1A), central (~120 kDa, corresponding to R3-R6; Fig.1A) and C-terminal (~100 kDa, corresponding to R7-C; Fig.1A) fragments (Lambert de Rouvroit et al., 1999). The central fragment containing reelin repeats 3–6 can bind to VLDLR/ApoER2 and partially rescue the preplate developmental deficits of reeler mice (Jossin et al., 2004). Similarly, reelin N-terminus-α3β1 integrins may subserve a distinct function during cortical development. This distinct pattern of reelin domain–receptor interactions may also facilitate the coordinated endocytosis of integrin receptors that are complexed in sufficiently close proximity to the VLDL/Apo receptors. Furthermore, Reelin potentially can induce α3β1 integrin receptor clustering upon binding, as has been demonstrated recently for ApoER2 and VLDLR (Strasser et al., 2004). Such regulation of availability and function of integrins on the cell surface can be critical for modulating changes in specific cell-cell adhesion needed for the final placement of neurons in cerebral cortex.

Recent studies indicate that reelin also can modulate radial glial morphology in a β1 integrin-dependent manner (Hartfuss et al., 2001; Forster et al., 2002). Ablent radial glial differentiation is also evident in α3 integrin mutant cortex (Anton et al., 1999). Thus it is possible reelin-α3β1 mediated effects on radial glial function may also contribute to normal corticogenesis.

The cytoplasmic domain of β1 integrin, containing the NPXY motif, can bind directly with PTB domain containing Dab1, a downstream cytoplasmic target of reelin. A recent screen for integrin β1 cytoplasmic domain interactions with PTB domain containing proteins also demonstrates direct association between β1 integrin and Dab1 (Caldewoord et al., 2003). In spite of its interactions with reelin and Dab1, α3β1-deficient cortical phenotype does not phenocopy the reeler phenotype. Dab1 is phosphorylated in response to reelin even in α3 integrin mutant cortical cells (Dulabon et al., 2000). Intriguingly, recent studies indicate that Dab1 phosphorylation alone is not sufficient to rescue reeler cortical phenotype (Jossin et al., 2004). The functional interactions of α3β1 integrins with other ligands in the cerebral wall during neuronal migration, prior to its association with reelin in the cortical plate at the end of migratory process suggest that α3 integrin mutant phenotype is likely to reflect both the role of α3β1 integrin in normal neuronal migration and in reelin signaling. As such, the α3 integrin mutation is unlikely to recapitulate the phenotype of the reelin mutation. Furthermore, continued migration of neurons in α3β1-deficient mice, albeit aberrantly, is also indicative of the presence of strong compensatory molecular mechanisms capable of functional overlap with α3β1 signaling in the developing cortex.

How could reelin binding to α3β1 integrin assist proper layer formation in the developing cerebral cortex? Reelin interactions with other receptors such as VLDLR/ApoER2 could induce Dab1 phosphorylation. Phosphorylation of Dab1 on Tyr220 and Tyr232 can modulate integrin levels at the cell surface (Sanada et al., 2004). Thus both Dab1 phosphorylation and N-terminal reelin fragment-α3β1 integrin binding-induced events such integrin receptor clustering or endocytosis could influence the pattern and level of cell surface α3β1 integrin receptor expression. The resulting changes in the adhesive properties of migrating neurons may thus facilitate their detachment from the radial glial guides at the top of the developing cortical plate.

**Supplementary Material**

Supplementary Material can be found at: at: http://www.cercor.oupjournals.org/.

**Notes**

This research was supported by NIH grant MH63660 to EA. We thank A-S. LaMantia, A. Goffinet and P. Maness for helpful comments. Address correspondence to E.S. Anton, UNC Neuroscience Center and the Department of Cell and Molecular Physiology, Rm 7109B, 103 Mason Farm Rd, The University of North Carolina School of Medicine, Chapel Hill, NC 27599-7250, USA. Email: anton@med.unc.edu.

**References**


