The Reelin Signaling Pathway: Some Recent Developments

The Reelin signaling pathway plays a key role in the architectonic development of the central nervous system. Extracellular Reelin binds to receptors of the lipoprotein receptor family and induces tyrosine phosphorylation of the adaptor Dab1. In this paper, we discuss three recent developments. First, we show that the central part of Reelin is involved in receptor binding and signal activation as reflected in Dab1 phosphorylation. Second, we examine the genomic organization, alternative splicing and promoter use of the Dab1 gene, which hint at a particularly complex regulation. Third, we present preliminary studies by in situ hybridization that demonstrate regulated expression of Reelin receptors and Dab1 by radial precursors in the ventricular zone.

Introduction

The mammalian cerebral cortex develops by following a finely tuned sequence of events that regulate cell proliferation, neuronal migration and architectonic pattern formation, differentiation, synaptogenesis and myelination. Neuronal cell precursor proliferation takes place in germinative zones located along the cerebral ventricles, from which neurons migrate over variable distances to settle in the cortical plate. Like other developmental events, neuronal migration and architectonic pattern formation are under genetic control. Among the control mechanisms that have been identified, the Reelin signaling pathway plays a key role in cortical development in man, mouse and presumably all mammals.

Reelin is a large (>400 kDa) extracellular glycoprotein that is secreted by several neurons, particularly, in the embryonic cortex, by Cajal–Retzius cells. Defective Reelin is the cause of the reeler brain malformation in mice (Lambert de Rouvroit and Goffinet, 1998; Rice and Curran, 2001) and of a peculiar type of lissencephaly in man (Hong et al., 2000). In reeler mice, neurons are generated in ventricular zones (VZ) and migrate initially as in normal animals. However, unlike normal cells, reeler neurons form defective architectonic patterns. Whereas normal cortical neurons form a dense, radially and laminarily organized cortical plate (CP) in which maturation proceeds from inside to outside, reeler neurons form a loose CP in which neurons are oriented obliquely and the gradient of maturation is almost inverted.

The organization of the Reelin protein is as follows (D’Arcangelo et al., 1995). The sequence begins with a signal peptide of 25–27 residues, followed by a region with similarity to F-spondin (amino acids 27–190). A unique region between amino acids 191 and 500 is followed by a succession of eight repeats (1–8) of 300–350 amino acids (1, residues 501–860; 2, 861–1220; 3, 1221–1596; 4, 1597–1947; 5, 1948–2314; 6, 2315–2661; 7, 2662–3051; 8, 3052–3428). Each Reelin repeat contains an EGF motif at its center, which divides the repeats into two sub-repeats, A and B, that show weak similarity to each other. The protein terminates with a basic stretch of 33 amino acids (3429–3461). Furthermore, Reelin is cleaved in vivo at two sites approximately located between repeats 2 and 3 and between repeats 6 and 7, as schematized in Figure 1 (Lambert de Rouvroit et al., 1999).

Reelin is not expressed in CP cells, but may act via the extracellular milieu, instructing them to assume their position and orientation. This view is supported by studies of three other genes, Disabled-1 (Dab1), VLDLR and ApoER2, mutations of which generate a reeler-like phenotype. The response of CP neurons to Reelin requires expression of at least one of two receptors, the Very Low Density Lipoprotein Receptor (VLDLR) and the Apolipoprotein-E Receptor Type 2 (ApoER2). Reelin does not bind to the closely related Low Density Lipoprotein Receptor (LDLR) nor to more distantly related members of the family such as LRP (Hiesberger et al., 1999; Trommsdorff et al., 1999). Whereas mutations of either VLDLR or ApoER2 generate subtle neurological phenotypes, mice deficient in both genes have a reeler-like phenotype, demonstrating a redundancy. VLDLR and ApoER2 are expressed in Reelin target neurons. Both have short cytoplasmic tails that contain an NPXY motif to bind Dab1, an intracellular adapter protein expressed in cells that respond to the Reelin signal. Dab1 exists as several predicted isoforms, as explained below. The Dab1 N-terminal region contains a PI-PTB domain to interact with non-phosphorylated tyrosine residues in the NPXY sequence (Howell et al., 1997b, 1999b, 2000). Dab1 contains five tyrosine residues and phosphorylation of these residue, particularly Y198 and Y220 (Keshvara et al., 2001), in response to the binding of Reelin to

Figure 1. Proteolytic processing of Reelin. Lanes on the left are immunoblots of PAGE gels loaded with embryonic brain extracts immunoprecipitated (IP) with antibody G10 directed against the N-terminal region of Reelin (left lane) and with antibody 17 directed against the C-terminal region (right lane). Blots were revealed (WB) with G10 (left lane) and with a mix of antibodies 12 + 14 directed against the C-terminal region (right lane) (de Bergeyck et al., 1998), respectively. The result shows that the Reelin protein (>400 kDa) is cleaved at two main sites (arrowheads), generating fragments of –300, 220, 180 and 100 kDa, as indicated in the schema. The CR50 epitope is approximately located as indicated.

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lipoprotein receptors is necessary for Dab1 function. Most but not all features of the Dab1−/− mutant phenotype can be rescued by replacing the Dab1 gene by a partial Dab1 cDNA encoding the PTB domain and the region with the five tyrosine residues (Herrick and Cooper, 2002). The rest of the Dab1 protein serves some unidentified function and this may be related to the presence of consensus S/T phosphorylation sites, some of which can be phosphorylated by the Cdk5/p35 kinase in a Reelin-independent manner (Keshvaria et al., 2002).

In the present paper, we focus on some recent results from our laboratories that concern: (i) the interaction between Reelin and its receptors and its relation to Dab1 phosphorylation; (ii) the presence and putative functional implications of alternative splicing and promoter utilization events in the Dab1 gene; (iii) mRNA expression data that point to a possible role of Reelin signaling in cortical ventricular zones.

Reelin Binding to Lipoprotein Receptors and Dab1 Phosphorylation

Previous work showed that Reelin binding to the VLDLR and ApoER2 receptors does not require the N-terminal region of Reelin from the N-terminus up to the end of the second repeat (Hiesberger et al., 1999). However, the CR50 antibody, directed against an epitope in the N-terminal region, interferes with binding of full-length Reelin (D’Arcangelo et al., 1999), possibly through a steric mechanism. Other studies show that Reelin has the capacity to aggregate and that this aggregation and the capacity to induce Dab1 phosphorylation are both inhibited by antibody CR50 (Utsunomiya-Tate et al., 2000; Kubo et al., 2002). Together, these data indicate that the N-terminus may contribute to, but is not sufficient for, Reelin signaling.

To better understand the interaction of Reelin with VLDLR and ApoER2, we studied binding of different partial Reelin recombinant proteins to extracellular regions of receptors, fused to an Fc immunoglobulin fragment (provided by J. Herz). The reelin cDNA construct pCrL (provided by T. Curran) was used to express Reelin and as a template to derive different parts of the reelin cDNA, by nuclease restriction or PCR amplification. In the terminology of the Reelin constructs, R is used for repeat, N for N-terminus and Del for deletion of a given region. The constructs containing R3–R8, R3–R6, R3–R5, R3–R4, R4–R5, R5–R6, R7–R8, R4 and R6 were cloned in the pSecTag2B vector (Invitrogen), which encodes the polypeptide in phase with a signal peptide and a Myc epitope. Constructs N–R6, DelR3–R5A, N–R5A and N–R2 were obtained from pCrL by nuclease restriction followed by ligation. Partial and full-length recombinant Reelin were produced by transient transfection of HEK293 cells and fresh supernatant was used without further purification. LDLR, VLDLR and ApoER2 receptor constructs were produced in the same manner. Receptor binding was estimated by co-immunoprecipitation. For estimation of Dab1 tyrosine phosphorylation, primary cortical neuronal cultures were incubated with recombinant LDLR–Fc, ApoER2–Fc and VLDLR–Fc fusion proteins bound to protein A–agarose beads. The complexes were immunoblotted and revealed with antibody G10 (Reelin) or anti-Myc antibody (R3–R6). Both Reelin and R3–R6 bind to VLDLR and ApoER2, but not to LDLR. IP, immunoprecipitation with G10 or anti-Myc, to demonstrate that the corresponding protein is well secreted. (d) Stimulation of Dab1 tyrosine phosphorylation by the Reelin protein. Mouse embryonic neurons (E16) were cultured for 3 days and treated with full-length Reelin, with the R3–R6 construct and with a supernatant of mock-transfected cells (–). Dab1 was immunoprecipitated with a polyclonal anti-Dab1 antibody and blots were revealed with another anti-Dab1 antibody (upper panel) and with an anti-phosphotyrosine antibody (αPY). Unlike the control supernatant, both Reelin and R3–R6 are able to generate tyrosine phosphorylation of Dab1.

N–R2, composed of the N-terminal spondin similarity region, the unique segment and repeats 1 and 2, did not bind lipoprotein receptors. Even construct N–R5A, which includes the first four reelin repeats and part of repeat 5, did not bind significantly. This confirmed that the N-terminal moiety of Reelin is not directly involved in receptor binding (Hiesberger et al., 1999). Similarly, all secreted constructs that contain one, two or three Reelin repeats failed to bind detectably to VLDLR and ApoER2. In contrast, constructs R3–R6 and R3–R8 and N–R6 bound to VLDLR and ApoER2 similarly to full-length Reelin (Fig. 2a). Such an increase in binding capacity with increased repeat number may indicate cooperativity between repeats. Repeat number is not the sole factor involved, however. For example, construct DelR3–R5A, in which repeats 3, 4 and the N-terminal part of repeat 5 are deleted, did not bind detectably to either receptor, even though it contains five Reelin repeats. Altogether, these experiments suggest that at least four repeats are necessary to detect significant binding of Reelin to VLDLR and ApoER2. Furthermore, repeats contained in the central region of Reelin are particularly important for receptor binding.

Dab1 Phosphorylation

As a first approach to assess whether receptor binding is sufficient to trigger the Reelin signal or whether additional features are required, the ability of the different Reelin constructs to induce tyrosine phosphorylation of the Dab1

**Figure 2.** Reelin–receptor interactions. (a) Binding of Reelin to lipoprotein receptors in vitro. The Reelin and R3–R6 cDNAs were transfected into 293T cells. The supernatant was incubated with recombinant LDLR–Fc, ApoER2–Fc and VLDLR–Fc fusion proteins bound to protein A–agarose beads. The complexes were immunoblotted and revealed with antibody G10 (Reelin) or anti-Myc antibody (R3–R6). Both Reelin and R3–R6 bind to VLDLR and ApoER2, but not to LDLR. IP, immunoprecipitation with G10 or anti-Myc, to demonstrate that the corresponding protein is well secreted. (b) Stimulation of Dab1 tyrosine phosphorylation by the Reelin protein. Mouse embryonic neurons (E16) were cultured for 3 days and treated with full-length Reelin, with the R3–R6 construct and with a supernatant of mock-transfected cells (–). Dab1 was immunoprecipitated with a polyclonal anti-Dab1 antibody and blots were revealed with another anti-Dab1 antibody (upper panel) and with an anti-phosphotyrosine antibody (αPY). Unlike the control supernatant, both Reelin and R3–R6 are able to generate tyrosine phosphorylation of Dab1.
adapter in neuronal cells was studied. Full-length Reelin and constructs R3–R6, R3–R8 and N–R6 were equally able to induce Dab1 phosphorylation, whereas all other Reelin polypeptide constructs were inactive (Fig. 2B).

The central part of Reelin defined in these experiments is large in comparison with the extracellular domains of VLDLR and ApoER2, which leaves ample room for interaction with several proteins in addition to lipoprotein receptors. However, the correlation between binding to receptors and stimulation of Dab1 tyrosine phosphorylation indicates that the central region of Reelin is sufficient to initiate the Dab1-dependent part of the signal. This central region of Reelin corresponds approximately to the fragment generated by cleavage; whether this is functionally relevant remains to be demonstrated.

These observations are worth discussing in relation to published aspects of Reelin signaling. The protocadherin CNR1 and integrin α3β1 have been proposed as Reelin co-receptors (Senzaki et al., 1999; Dulabon et al., 2000). Whereas the part of Reelin implicated in integrin binding was not defined, binding of CNR1 to the N-terminal region of Reelin was considered critical. Our observations suggest that binding of Reelin to CNR is not necessary for its Dab1-dependent function, but may serve some other independent role that remains to be defined further. Another point is the recent observations that: (i) the N-terminal region of Reelin mediates Reelin aggregation; (ii) homodimerization of Reelin and Dab1 phosphorylation are both inhibited by the function blocking antibody CR50; (iii) Reelin devoid of its N-terminal region fails to trigger Dab1 phosphorylation, even though it is still able to bind to the VLDLR and ApoER2 receptors (Usutomiya-Tate et al., 2000; Kubo et al., 2002). These findings suggested that Reelin homodimerization is necessary for activation of the signal as reflected by Dab1 phosphorylation. For reasons that we do not understand, our observations that constructs containing Reelin R3–R8 and R3–R6 are able to induce Dab1 phosphorylation are at variance with that evidence. Our results, however, do not necessarily argue against a functional role of Reelin aggregation in vivo. Aggregation could increase the effective local concentration of Reelin, for example by anchoring the protein to the extracellular matrix. Due to the use of transfected cell supernatants and neuronal cultures, our binding and phosphorylation assays could not be quantified using pure components and provide at best an estimate of the relative affinity of the different ligands. Aggregation of full-length Reelin may increase its affinity to an extent that we could not measure.

**Alternative Forms of Dab1**

As Dab1 is a key component of the Reelin signaling pathway, a detailed study of the genomic organization and transcriptional regulation of the Dab1 gene was carried out in man and mouse. Here, we would like to emphasize some elements that concern the regulation of Dab1 expression and may be relevant to its function.

**Alternative First Exons and Organization of the 5′-Region (Figs 3 and 4)**

Comparison of the different Dab1 sequences revealed extensive variation in the 5′-untranslated regions (5′-UTR), as schematized in Figure 3. In mouse embryonic brain RNA, four different products named 1A, 1B, 1C and 1D were obtained. UTR 1B does not correspond to a single exon but is composed of 10 exons, 1B1–1B10. The Dab1 5′-UTR spreads over 850 kb of genomic DNA, pointing to a vast complexity. The human DAB1 gene has an almost identical organization, further suggesting that this evolutionarily conserved, complex organization may be functionally important.

In order to assess whether the different 5′ exons have different expression patterns, RT–PCR reactions were carried out on mouse brain cDNA at different stages from E11 to adult. As shown in Figure 4A, exons 1A and 1D are expressed at all stages tested. The complex UTR 1B is barely detectable at embryonic stages E11 and E12, whereas two main bands are amplified in RNA isolated from brain at E15 and later, including adult. We tested the expression of the alternative first exons in P19 cells, which differentiate into neurons in the presence of retinoic acid (RA) (Fig. 4B). Exons 1A and 1D are detected similarly in undifferentiated and differentiated P19 cells. In contrast, the expression of UTR 1B is complex. Multiple bands are amplified in undifferentiated cells and up to 4 days after RA induction. When neural induction is complete, only two bands are visible. This developmental regulation was confirmed in vivo: a complex pattern of multiple bands is amplified from mouse E11 RNA and becomes restricted to two main amplicons at P0 and in adult (Fig. 4C). These two amplicons contain fragments 1B1, 1B2 and 1B4, with alternative inclusion of 1B8. The more complex amplicons correspond to different combinations of exons 1B1–1B10 and contain variable numbers of ATG codons, suggesting that some ‘upstream open reading frames’ (uORF) are excluded from segment 1B in parallel with neuronal differentiation. The ability of uORF to down-regulate translation has been documented, and our observations suggest that this phenomenon may play a role in the regulation of Dab1 expression.

**Internal Alternative Splicing Events (Fig. 5)**

Four different Dab1 cdna forms were initially described in the mouse (Howell et al., 1997a), as schematized in Figure 5A,B. The main form encodes a protein of 555 residues and is named Dab1-555. Another, Dab1-217, is due to recognition of an alternative polyadenylation signal in intron 7 and is predicted to encode a 217 amino acid polypeptide. A third form, Dab1-271, is due to insertion of an additional exon between exons 9 and 10 and results in the production of a 271 residue protein. Finally, another segment, named 555+, is included in some Dab1 cdna forms between exons 9 and 10. We showed that fragment 555+ corresponds to two small exons of 51 and 48 bp separated by an intron of 91 bp and that both exons are consistently co-amplified. In undifferentiated P19 cells, the Dab1 cdna included fragment 555+. However, when differentiation of P19 cells was induced with RA, a proportion of Dab1 cdna without fragment 555+ appeared at day 2 and increased progressively to become the major form at day 9 (Fig. 5C). In early embryonic mouse brain (E11 and E12), the Dab1 isoform with fragment 555+ was predominant, but RNA from later developmental stages, E12 and later, and from primary neuronal cultures did not contain this fragment (Fig. 5D). In non-neuronal tissues such as liver and kidney, the Dab1 mRNA contained fragment 555+. A similar pattern was found in chick, with inclusion of a small 57 nt exon in RNA from E6 embryo, but exclusion of that small exon from brain RNA at E20 (Fig. 4B). In situ hybridization with a cdna probe covering fragment 555+ and adjacent segments confirmed expression in ventricular zones (Fig. 5F). Altogether, these observations suggest strongly that the exclusion of exon 555+ occurs in parallel with neural differentiation.

In summary, Dab1 mRNA is expressed at high level in post-migratory neurons, but also at a more modest level in ventricular zones. Two main first exons and thus two main promoters are used for Dab1 translation in the brain. Dab1
mRNA in VZ includes the small exon 555*, whereas Dab1 mRNA in post-migratory neurons does not.

Expression of VLDLR and ApoER2 in the Developing Telencephalon

The presence of some Dab1 mRNA expression in the telencephalic VZ prompted us to study expression of Reelin receptors in more detail, using in situ hybridization. Preliminary results in early embryonic brain revealed expression of ApoER2 and VLDLR mRNA in the VZ in addition to Dab1. At E12, the preplate stage (Fig. 6), profuse reelin mRNA expression was found in CR cells that are abundant all around the telencephalic vesicles, as well as in neurons in the basal forebrain. Expression of VLDLR and ApoER2 is also evident in precursor cells in the VZ in almost all sectors of the cortex as well as in post-mitotic, differentiating neurons. Dab1 expression is also concentrated in the ventricular zones, but is restricted to the cortical VZ and is not found, for example, in the VZ of the ganglionic eminences. Somewhat unexpectedly, the expression canvas of Dab1 and lipoprotein receptors are not identical. The expression pattern...
Figure 5. Alternative exon 555* is excluded from neurons. (A) Genomic localization of alternative exons 271 and 555* in the mouse Dab1 gene. (B) The various forms of the mouse Dab1 protein produced by alternative splicing. Form 217 results from the utilization of an alternative polyadenylation signal in intron 7. Three alternative exons are present between exons 9 and 10. Exon 271 contains a STOP codon. Form 555* is composed of two small exons of 51 (555*1) and 48 (555*2) bp. (C) Alternative splicing of exon 555* during P19 cell differentiation induced by RA from day 0 to 9 and P0 brain. Two products, with and without exon 555*, are amplified. During neuronal maturation, the larger product containing exon 555* is progressively replaced by a smaller amplicon lacking exon 555*. (D) Alternative exon 555* is expressed during early mouse brain development, but not in E19, P0 or adult brain. The Dab1 form containing the two exons 555* is expressed in the kidney. (E) An alternative exon corresponding to 555* is also included in chick at early embryonic stages (E6) and in embryonic eye but not in more mature brain (E20). (F) Analysis of the expression of alternative form 555* by in situ hybridization. The signal corresponding to 555* plus some surrounding sequence (c, d) is compared with that of a PTB probe (a, b) at E15. Both signals are detected in the cortex (simple arrow), cerebellum (arrowhead) and tectum (double arrow), but expression of the alternative exons 555* is relatively higher in the VZ at E15. Direct photography of autoradiographic film. Bar 1 mm.
of Dab1 seems better correlated with the pattern of response to Reelin (as indicated by anomalies in reeler mutant mice) than the expression of VLDLR and ApoER2, which is more widespread. This result emphasizes the critical role of the Dab1 adaptor as the main switch in the Reelin signaling pathway. As a corollary, it raises the question of the function of VLDLR and ApoER2 in the sectors of the VZ that are Dab1-negative. In early post-natal brain, ApoER2 mRNA expression remained high in all strata of the CP as well as in the VZ, whereas VLDLR mRNA expression became restricted to the upper cortical plate where late generated neurons settle, but was no longer present in the VZ (not shown).

The co-expression of lipoprotein receptors and Dab1 in the VZ suggests that Reelin may fulfill a novel, hitherto unknown, function at this level, for example in relation to neuron and glial cell precursor proliferation or to initiation of cell migration (Meyer et al., 2002). The late VZ contains progenitor cells destined for the olfactory bulb, and Reelin may play a role in migration of olfactory granule cells, as recently suggested (Hack et al., 2002). As far as we know, there is no expression of Reelin in the vicinity of the mammalian VZ. Some reelin mRNA is present in subventricular zones in non-mammalian species (Bernier et al., 2000; Tissir et al., unpublished results), but not in mammals. A possibility could be that Reelin secreted in the external telencephalic field by CR cells may act locally and signal to the radial extensions from precursor cells in the VZ. Another possibility is that Reelin or Reelin fragments are able to diffuse from the MZ and reach cell bodies in the VZ. Further studies are needed, particularly to better define the expression and localization of the Dab1, ApoER2 and VLDLR proteins in radial precursor cells.

Conclusions
In conclusion, the recent data summarized above suggest that central Reelin repeats bind to VLDLR and ApoER2 receptors and this results in the organization of a supramolecular complex and in signal activation, reflected by Dab1 phosphorylation. Other components of this complex remain unknown but are likely to include tyrosine kinase(s). However, no mice defective in tyrosine kinase genes have a reeler-like phenotype, and the role of Fyn and related kinases in Dab1 phosphorylation remains open to question. Another possibility is that unidentified components of the Reelin receptor complex might not be Reelin co-receptors in that they do not bind Reelin directly, but could be recruited to the complex via ‘cis’ interaction with lipoprotein receptors. The central role of Dab1 in transduction of the Reelin signal is further emphasized by the complexity of its genomic organization and transcriptional regulation. Alternatively, spliced forms of Dab1 appear specifically expressed in precursor cells in the VZ, which also express VLDLR and ApoER2, pointing to a new role of the Reelin signal in precursor proliferation and/or rostral migration in the olfactory system. Several questions remain, particularly concerning the final action of Reelin on target cells and the identification of the downstream effectors of the Reelin signal.

Notes
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References
Howell BW, Hawkes R, Soriano P, Cooper JA (1997b) Neuronal position in...
the developing brain is regulated by mouse disabled-1. Nature 389:733–737.