Binding of Netrin-4 to Laminin Short Arms Regulates Basement Membrane Assembly

Fiona I. Schneiders†, Barbara Maertens‡, Kerstin Böse‡, Yong Li§, William J. Brunken*, Mats Paulsson†¶, Neil Smyth** and, Manuel Koch††

From the †Center for Biochemistry, ‡†Department of Dermatology, ††Center for Molecular Medicine Cologne, Medical Faculty, University of Cologne, Joseph-Stelzmann-Strasse 52, D-50931 Cologne, Germany, §Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts, 02111, **School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, United Kingdom, and †Department of Anatomy and Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York 11203

Netrins were first identified as neural guidance molecules, acting through receptors that are members of the DCC and UNC-5 family. All netrins share structural homology to the laminin N-terminal domains and the laminin epidermal growth factor-like domains of laminin short arms. Laminins use these domains to self-assemble into complex networks. Here we demonstrate that netrin-4 is a component of basement membranes and is integrated into the laminin polymer via interactions with the laminin γ1 and γ3 short arms. The binding is mediated through the laminin N-terminal domain of netrin-4. In contrast to netrin-4, other members of the netrin family do not bind to these laminin short arms. Moreover, a truncated form of netrin-4 completely inhibits laminin-111 self-assembly in vitro, and full-length netrin-4 can partially disrupt laminin self-interactions. When added to explant cultures, netrin-4 retards salivary gland branching morphogenesis.

Netrins were first isolated as long range guidance cues, acting in early embryogenesis by regulating the migration of neurons and the axonal growth cone (1). These proteins showed either chemotactic or chemorepellive effects upon distinct sets of cells, hence cells expressing netrin-1 can mimic the ability of the floor plate to repel the growth cones of trochlear motor neurons in vitro, while attracting the axons of spinal commissural neurons (2–4). Consequently, netrin-1 is considered a bifunctional guidance cue. This activity has been shown to relate to expression levels of specific receptors from either the DCC or UNC-5 families (5–9).

Five members of the netrin gene family have been identified in mammals: netrin-1 (2, 10), netrin-3 (11, 12), netrin-4 (13), netrin-G1, and netrin-G2 (14–16). All netrin genes encode secreted proteins, but in contrast to netrin-1, -3, and -4, netrin-G1 and -G2 are membrane-bound via glycosylphosphatidylinositol anchors. All netrins share homology to the N-terminal parts of the laminin short arms (Fig. 2), being formed of a laminin N-terminal (LN)2 module followed by about three LEa laminin parts of the laminin short arms (Fig. 2). Recent studies in vitro have shown that netrin-4 is also a component of basement membranes in a variety of tissues (13). Basement membranes are specialized extracellular matrices underlying all endothelia and epithelia and surrounding many forms of mesenchymal cells (26). They have both physical and signaling functions that alter with both tissue type and stage of development. In addition to forming surfaces to which cells attach, they also transmit force between cells and the surrounding extracellular matrix. Basement membrane components may also regulate many aspects of intracellular activity by signaling via the integrin, dystroglycan, and syndecan receptor families. Furthermore, basement membranes can act as reservoirs for cytokines and growth factors, e.g. members of the fibroblast growth factor family. Finally, signaling by other cytokines may be complemented by basement membrane-induced signals, as seen with neurotrophins. Hence basement membrane-induced signaling plays an important role in cell survival and differentiation as well as in cell migration and as an axonal guidance cue.

Laminins are the major noncollagenous proteins of basement membranes and are crucial in its formation. Laminin-111, the prototype laminin, is a cross-like shaped molecule formed as a multidomain heterotrimer assembled of one α1, one β1, and one γ1 chain (27–29). The laminin trimer has one long and
three short arms, the latter being formed from the three free N-terminal ends of the α1, β1, and γ1 chains (30, 31). These parts of the β1 and γ1 chains each contain two globular domains, designated LN and L4/LF. The globules are interpersed by multiple LE modules, forming rods in domains LEa and LEb. Certain laminin chains have been described as maintaining the domain structure of those in the original laminin-111 (e.g. α2 and β2), whereas others have N-terminal truncations, lacking an LN domain. So far 15 laminin isoforms have been shown to occur (29). Laminins self-assemble into a network through Ca$^{2+}$-dependent interactions between their N-terminal parts (32), and polymerization of laminin-111 may be inhibited by proteolytic laminin fragments that contain LN domains (33).

Netrin-4 is the most recently described soluble netrin family member, and its biological significance is still poorly understood. We have shown that netrin-4 is widely expressed with the protein being concentrated in certain basement membranes and having a spatial expression that broadens during later development. Here we demonstrate the integration of netrin-4 into the basement membrane via the binding to the N-terminal region of the laminin γ1 chain. Furthermore, we show the significance of netrin-4 in basement membrane assembly and its effect upon branching morphogenesis.

**EXPERIMENTAL PROCEDURES**

**Recombinant Expression and Purification of Murine Netrin and Laminin-derived Proteins**—The following full-length or truncated netrin forms were amplified by PCR and subcloned into an episomal expression vector: mouse netrin-1 (U65418), nucleotides 1–1368 with three additional nucleotides after nucleotide 69, which leads to an insertion of an additional glycine and a single amino acid substitution valine to leucine at nucleotides 259–261 (present in all the sequenced clones, all of which were independent PCR products); mouse netrin-4 (AF281278), nucleotides 311–2166; mouse Δnetrin-4 (AF281278), nucleotides 311–1672; and mouse netrin-G1e (AB038663), nucleotides 52–1305. The PCR products were subcloned (rapid DNA ligation kit, Roche Diagnostics) into a modified pCEP-Pu (13) expression vector (an 8-histidine tag and a thrombin cleavage site were introduced either at the N-terminal or the C-terminal end of the protein sequence). On the basis of the Δnetrin-4 and Δnetrin-G1e constructs, the following Δnetrin-4 deletion and Δnetrin-4/netrin-G1 hybrid constructs were cloned: Δnetrin-4 (LN + EGF 1–2), nucleotides 311–1432; Δnetrin-4 (LN + EGF 1 + 3), nucleotides 311–1242 and 1432–1672; netrin-4 (EGF 1–3.5), nucleotides 1031–1672; Δnetrin-4 (LN) + netrin-G1 (EGF 1–3), nucleotides 311–1036 (netrin-4) and 889–1305 (netrin-G1e); netrin-G1 (LN) + netrin-4 (EGF 1–3.5), nucleotides 52–891 (netrin-G1e) and 1040–1672 (netrin-4). In addition, a fragment of the laminin γ1 chain (J02930), nucleotides 292–1668, containing the N-terminal LN domain and four LEa repeats and a fragment of the laminin γ3 chain (NM 011836), nucleotides 148–3133, containing domains LN, LEa, L4, and LEb, were amplified by PCR and subcloned into the modified pCEP-Pu expression vector.

The expression vectors were transfected into 293-EBNA cells with FuGENE 6 transfection reagent (Roche Diagnostics), and selected clones with the highest protein expression were expanded for large scale production. The purification of the secreted proteins was performed as described previously (13).

**Antibody Production**—For antibody KR1, a rabbit was immunized with purified recombinant Δnetrin-4. The antisera was affinity-purified by applying it to a Sepharose column to which mouse Δnetrin-4 protein had been coupled. Bound antibodies were eluted with triethylamine, pH 11.5, immediately neutralized, and dialyzed against PBS. For antibody KR 24, a rabbit was immunized with purified recombinant netrin-1, and the antisera was affinity-purified as described for antibody KR1.

**Immunofluorescence Microscopy**—Newborn (P2) and adult mouse tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura FineTek Europe). 7-μm thick sections were cut with a Leica cryostat and stored at −20 °C. For use, slides were returned to room temperature, fixed, washed in PBS, and blocked with 0.2% Tween 20 in PBS for 1 h at room temperature. Slides were fixed in 4% paraformaldehyde for 5 min, washed, blocked with 5% goat serum, and incubated with antibodies against netrin-4 (KR1, see under “Antibody Production”) and the laminin γ1 chain (rat anti-laminin β2 chain monoclonal antibody, Chemicon). After incubation with secondary antibodies (Cy3-labeled goat anti-rabbit IgG, Jackson ImmunoResearch; or Alexa 488-labeled goat anti-rat IgG, Molecular Probes), mounted sections were observed under a laser scanning confocal microscope (Leica) scanning the sections 16 times.

**ELISA Style Ligand Binding Assay**—Unless otherwise specified, all solutions used contained 2 mM CaCl$_2$. For testing the divalent cation dependence of interactions, no CaCl$_2$, 2 mM CaCl$_2$, or 20 mM EDTA was added to the solutions. Purified proteins were diluted in TBS, pH 7.4, and coated at 10 μg/ml (500 ng/well) overnight at room temperature onto 96-well plates (Nunc Maxisorb). After washing with TBS containing 0.05% Tween 20, plates were blocked for 2 h at room temperature with TBS containing either 5% milk powder or 1% bovine serum albumin. Ligands were diluted to concentrations between 0.001 and 50 nM and incubated in the wells for 1 h at room temperature. After extensive washing with TBS containing 0.05% Tween 20, bound ligands were detected with specific polyclonal rabbit antibodies directed against netrin-1 and netrin-4, respectively, followed by horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO Cytomab) and tetramethylbenzidine as substrate. Absorption was measured at 450 nm after stopping the reaction with 10% sulfuric acid.

**Surface Plasmon Resonance Binding Assays**—Assays were performed using a Biacore 2000 (BIAcore AB). Coupling of proteins to the CM5 chip was performed in 25 mM sodium acetate, pH 5.0, at a flow rate of 5 μl/min. A 7-min pulse of 0.05 mM N-hydroxysuccinimide, 0.2 mM N-ethyl-N’-dimethylaminopropyl carbodiimide was used to activate the surface. The protein was injected until the desired amount was coupled (500–1000 RU), and excess reactive groups were deactivated by a 7-min pulse of 1 mM ethanolamine HCl, pH 8.5. Measurements were carried out in HBS (20 mM Heps, 150 mM NaCl, pH 7.4) containing 2 mM CaCl$_2$ and 0.005% P20 at a flow of 25 μl/min. The injection of 100 μl of protein solution (0.05–1 μM) was followed.
Netrin-4 Interacts with the Laminin γ1 Short Arm

AUGUST 17, 2007

FIGURE 1. Netrin-4 expression in newborn (P2) and adult mouse kidney. Sections of P2 (A–C) and adult (D–F) mouse kidney were incubated with primary antibodies against the laminin γ1 chain (A and D) and netrin-4 (B and E). The netrin-4 expression is distributed differently in adult mice than in newborn mice. The overlay (C and F) demonstrates that netrin-4 co-localizes with the laminin γ1 chain in the glomerular basement membrane and in vasculature in both newborn and adult kidney. In the adult kidney netrin-4 and the laminin γ1 chain co-localize also in the tubules (F). Scale bar, 100 μm.

by a 400-s dissociation. Each analysis was carried out at six different concentrations. The data were analyzed with BLAevaluation software 3.2 according to the Langmuir model for 1 to 1 binding. All binding curves were fitted with an accuracy of χ² <1% of Rmax (maximal RU). The mean kν, kα, and KD values for the six concentrations are given in Table 2.

Cross-linking Assays—Cross-linking assays were carried out using the lysine side chain-reactive cross-linker bis[sulfosuccinimidyl]suberate (Pierce). The reaction was carried out at a protein concentration of 2.8 μM in a final volume of 50 μl of PBS, pH 7.4. The cross-linker was used at concentrations from 0.5 to 2 mM. The reaction was allowed to continue for 1 h on ice and was stopped by the addition of 10 μl of 1 M Tris-HCl, pH 8.0.

Organ Culture—Submandibular glands from embryonic day 13.5 BL/6 mice were placed on a Nucleopore filter (Whatman) and cultured at the air/medium interface. Cultures were carried out in serum-free Dulbecco’s modified Eagle’s medium/F-12 supplemented with 2 mM L-glutamine, 50 μg/ml transferrin, and penicillin/streptomycin (Invitrogen). For testing the effects of full-length netrin-4 protein and full-length netrin-4 (0.19 mg/ml, Fig. 6B) was added. After supplementing all samples with 1 mM CaCl₂, turbidity development was monitored as absorbance at 360 nm. After 300 min EDTA (5 mM) was added to such samples that had not been kept in the presence of EDTA for the duration of the experiment.

In a second independent polymerization assay, laminin-111 (0.6 mg/ml, laminin-111-nidogen complex) in 0.02 M Tris-HCl, 0.15 M NaCl, 0.05 mM EDTA, pH 7.4, was incubated at 37 °C for 3 h after the initiation of polymerization by addition of 1 mM CaCl₂ (35, 36). Aggregation was assayed after centrifugation at 13,000 × g for 10 min by SDS-PAGE under reducing conditions on 8% polyacrylamide gels. When desired 10 mM EDTA or serial dilutions of Δnetrin-4 or Δnetrin-1, giving a 1:1 to 1:125 molar ratio between netrin and laminin, were added prior to incubation.

RESULTS

Netrin-4 Expression in Newborn and Adult Mice—We described previously the expression pattern of netrin-4 in the adult mouse and showed that it is present in basement membranes in a variety of tissues (13). To extend these observations, the netrin-4 expression in kidney was studied during development, initially by indirect immunofluorescence microscopy with a netrin-4-specific polyclonal antibody on kidney sections from day 14.5 (E14.5) and day 16.5 (E16.5) mouse embryos, newborn mice (P2), and 3–4-month-old adult mice (for antibody specificity see supplemental Fig. 1). Surprisingly, expression was not observed until birth (Fig. 1B), with no immunostaining of the embryonic kidney (not shown). Postnatally expression increases, so that while at P2 netrin-4 staining was mainly localized to the glomerular basement membrane and the vascular basement membranes (Fig. 1, B and C), and in the adult kidney the tubular basement membrane and that of the tissue capsule also stained for netrin-4 (Fig. 1, E and F). A strong co-localization of netrin-4 and the laminin γ1 subunit was observed, although netrin-4 was also observed at non-base-
Netrin-4 Interacts with the Laminin γ1 Short Arm

Netrin-4 Interactions with Laminin LN Domains—The basememnet membrane localization of netrin-4 suggests its involvement in protein-protein interactions. Its marked homology to the LN domains of laminin, which participate in laminin self-interactions, raised the possibility that netrin-4, as well as other members of the netrin family, could be a laminin-binding protein. Hence, recombinant proteins were produced that corresponded to the N-terminal part of laminin chains or were full-length or modified forms of netrin-1 or netrin-4 (Fig. 2). Initially, surface plasmon resonance-based assays were carried out to identify any possible binding between short arms of laminins and netrin-1 and -4. This showed a strong binding of the ∆laminin γ1 chain-(LN + LEa) and γ3 chain (LN + LEa + L4 + LEb)-derived proteins to ∆netrin-4 (LN + EGF 1–3.5; Fig. 3) but not to ∆netrin-1 (LN + EGF 1–3; data not shown); the calculated $K_D$ was $2.35 \times 10^{-8}$ M for the γ1 chain fragment (Fig. 3A) and $1.96 \times 10^{-8}$ M for the γ3 chain short arm (Fig. 3B). In addition binding to the ∆laminin β1 chain (LN + LEa) could be observed (not shown). The following experiments focused on the ∆laminin γ1 chain, since this subunit is common to most laminin heterotrimers (29). We also tested the laminin γ1 chain-netrin-4 interaction in an ELISA style ligand-binding assay. After coating the ∆laminin γ1 protein (LN + LEa) onto wells, increasing concentrations of ∆netrin-4 (LN + EGF 1–3.5) were added, and binding with an apparent $K_D$ of $1.38 \times 10^{-11}$ M was detected (not shown).

In addition, we investigated whether ∆netrin-4 also binds to native laminin-111 isolated from mouse EHS tumor. ELISA style assays were carried out with laminin-111 as the immobilized ligand, and increasing concentrations of ∆netrin-1 and ∆netrin-4 were added to the solution (Fig. 4). ∆Netrin-1 failed to bind laminin-111, whereas ∆netrin-4 bound to native laminin-111 with an apparent $K_D$ of $3.70 \times 10^{-11}$ M, similar to the one observed in the experiment with recombinant ∆laminin γ1 (LN + LEa) chains in the same kind of assay. These results suggest that the netrin-4 binding activity for laminin-111 resides in the LN and LE domains of the laminin γ1 chain.

The laminin γ1 chain-netrin-4 interaction was also studied using the water-soluble covalent cross-linking agent BS. As shown previously and in this cross-linking experiment, the N-terminal ∆laminin γ1 chain fragments do not self-interact (Fig. 5A) (37). ∆Netrin-4 on the other hand shows self-interaction as well as an interaction with ∆laminin γ1 chain fragments.
Netrin-4 Interacts with the Laminin γ1 Short Arm

Remarkably, netrin-4 binding to an N-terminal laminin γ1 chain fragment (LN + LEa) results only in the formation of dimers, whereas the netrin-4 self-interaction results in multimers. As expected no interaction between netrin-1 and the laminin γ1 chain was detected (Fig. 5 C).

The Netrin-4 Interaction with Laminin LN and LE Domains Is Independent of Divalent Cations—To analyze whether Ca\(^{2+}\) ions affect the netrin-4-laminin binding, ELISA style ligand binding assays were performed either in the absence of added CaCl\(_2\) or after the addition of either 2 mM CaCl\(_2\) or excess EDTA. Binding was observed under all conditions and had similar apparent \(K_D\) values (1.38 \(\times\) 10\(^{-11}\) M in the absence of added CaCl\(_2\); 1.07 \(\times\) 10\(^{-11}\) M in the presence of 2 mM CaCl\(_2\) and 1.64 \(\times\) 10\(^{-11}\) M in the presence of excess EDTA) (supplemental Fig. 2A).

Some laminin LN domains undergo a conformational change in the presence of Ca\(^{2+}\) (37). To determine whether the conformation of Δnetrin-4 is also influenced by Ca\(^{2+}\), circular dichro-
**Netrin-4 Interacts with the Laminin \( \gamma_1 \) Short Arm**

TABLE 1

| Protein                        | Secondary structure of \( \Delta \)netrin-1 | \( \alpha \)-Helix | \( \beta \)-Strand | \( \beta \)-Turn | Unordered |
|-------------------------------|---------------------------------------------|-------------------|-------------------|---------------|-----------|
|                               |                                             | CONTIN            | CDSSTR           | CONTIN        | CDSSTR    |
| \( \Delta \)netrin-1           |                                             | 6.3               | 10.6             | 8.6           |           |
| \( \Delta \)netrin-1 + 2 mM CaCl\(_2\) |                                             | 6.3               | 10.6             | 8.6           |           |
| \( \Delta \)netrin-1 + 4 mM EDTA |                                             | 6.3               | 10.6             | 8.6           |           |

**Secondary structure of \( \Delta \)netrin-4**

| Protein                        | Secondary structure of \( \Delta \)netrin-4 | \( \alpha \)-Helix | \( \beta \)-Strand | \( \beta \)-Turn | Unordered |
|-------------------------------|---------------------------------------------|-------------------|-------------------|---------------|-----------|
|                               |                                             | 6                 | 10                | 6             |           |
| \( \Delta \)netrin-4          |                                             | 6                 | 10                | 6             |           |
| \( \Delta \)netrin-4 + 2 mM CaCl\(_2\) |                                             | 6                 | 10                | 6             |           |
| \( \Delta \)netrin-4 + 4 mM EDTA |                                             | 6                 | 10                | 6             |           |

**Surface plasmon resonance analysis of netrin-4 binding to laminin \( \gamma_1 \)**

| Ligand on chip | Analyte | \( k_a \) | \( k_d \) | \( K_D \) |
|----------------|---------|-----------|-----------|----------|
| Netrin-4 (LN + EGF 1–3.5) | Laminin \( \gamma_1 \) (LN + EGF 1–4) | 5.70 \times 10^4 | 1.24 \times 10^{-3} | 3.71 \times 10^{-8} |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-4 (LN + EGF 1–3.5) | 1.83 \times 10^6 | 1.29 \times 10^{-4} | 1.69 \times 10^{-9} |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-G1 (LN + EGF 1–3) | NB* | NB | NB |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-4 (LN + EGF 1–2) | 3.88 \times 10^4 | 1.32 \times 10^{-4} | 5.66 \times 10^{-9} |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-4 (LN) + netrin-G1 (EGF 1–3) | 2.60 \times 10^6 | 9.94 \times 10^{-4} | 1.39 \times 10^{-8} |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-4 (EGF 1–3.5) | NB | NB | NB |
| Laminin \( \gamma_1 \) (LN + EGF 1–4) | Netrin-G1 (LN) + netrin-4 (EGF 1–3.5) | NB | NB | NB |

*NB indicates no binding.

The secondary structures of netrin-4 were recorded in the absence of CaCl\(_2\), in the presence of 2 mM CaCl\(_2\), and after the addition of excess EDTA (Table 1; supplemental Fig. 2B). In contrast to the laminin LN domains, no significant conformational changes in \( \Delta \)netrin-4 could be observed upon addition of CaCl\(_2\) or EDTA. Apparently neither the conformation of the netrin-4 LN domain nor its interaction with the \( \Delta \)laminin \( \gamma_1 \) subunit requires the presence of divalent cations, suggesting a different mechanism of interaction than between laminins.

**Localization of the Laminin-binding Site on Netrin-4**—To identify which domain of netrin-4 interacts with the \( \Delta \)laminin \( \gamma_1 \) subunit, surface plasmon resonance binding studies were carried out with the N-terminal fragment of the \( \Delta \)laminin \( \gamma_1 \) subunit coupled to a Biacore CM5 chip and different \( \Delta \)netrin-4 deletion proteins and \( \Delta \)netrin-4–netrin-G1 hybrid proteins as analytes in solution (Fig. 2B; Table 2). In the \( \Delta \)netrin-4 deletion proteins either the second EGF repeat, the third EGF repeat, or the LN domain were deleted, whereas in the hybrid proteins parts of \( \Delta \)netrin-4 were exchanged for the corresponding domains of netrin-G1 (Fig. 2B). The strongest interactions were detected using \( \Delta \)netrin-4 proteins containing the LN domain and all 3.5 EGF repeats \( (K_D, 1.69 \times 10^{-8} \text{ M}) \) or the first two EGF domains \( (K_D, 1.39 \times 10^{-8} \text{ M}) \). Furthermore, the deletion of the second EGF repeat did not appear to alter binding \( (K_D, 5.66 \times 10^{-8} \text{ M}) \). Although a slightly weaker interaction occurred upon injecting a fusion protein of the netrin-4 LN domain and netrin-G1 EGF-repeats \( (K_D, 5.82 \times 10^{-7} \text{ M}) \), no interaction was detected when injecting netrin-G1, a combination of netrin-G1 LN domain and netrin-4 EGF-repeats, or the isolated netrin-4 EGF repeats, suggesting that the presence of the LN domain is a prerequisite for the interaction.

**Influence of Netrin-4 on the Aggregation of Laminin-111**—We then asked whether the interaction of netrin-4 with laminin \( \gamma_1 \) short arms could have an effect upon the ability of laminin to polymerize. Samples of laminin-111 were preincubated at 37°C for 30 min to allow temperature equilibration. Addition of CaCl\(_2\) to a final concentration of 1 mM gave rise to an immediate increase in turbidity reaching a plateau after 300 min, whereas addition of \( \Delta \)netrin-4 and 1 mM CaCl\(_2\) significantly decreased the turbidity development to a level similar to that of samples treated with EDTA (Fig. 6A). Interestingly, addition of full-length netrin-4 and 1 mM CaCl\(_2\) inhibited the turbidity development to a lesser extent than \( \Delta \)netrin-4 (Fig. 6B). Addition of a molar excess of EDTA to the polymerized samples led to depolymerization and a rapid decrease in turbidity to a level approaching that of the samples that had been kept in the presence of EDTA or \( \Delta \)netrin-4 (Fig. 6B). The influence of netrins on laminin polymerization was also analyzed with a second assay. Purified laminin-111 was allowed to polymerize in the presence and absence of 10 mM EDTA or various concentrations of \( \Delta \)netrin-4 and netrin-1 at 37°C for 3 h. In the absence of EDTA, laminin-111 formed a polymer, which could after centrifugation be detected in the pellet fraction, whereas the addition of 10 mM EDTA prevented the polymerization (Fig. 6C). In the presence of \( \Delta \)netrin-4 the laminin-111 polymerization was almost completely abolished (Fig. 6D). Addition of full length netrin-4 also resulted in a decreased polymerization (results not shown). In contrast, the addition of \( \Delta \)netrin-1 had no effect on the ability of laminin-111 to polymerize (Fig. 6E).

**Influence of Netrin-4 on Epithelial Branching Morphogenesis**—To analyze the effect of netrin-4 on epithelial branching, the salivary gland was studied. First the expression of netrin-4 was determined. In newborn mice a weak, disorganized netrin-4 expression was observed in the sublingual part of the salivary gland, with little co-localization of netrin-4 and the laminin \( \gamma_1 \) subunit (supplemental Fig. 3, A–D). With increasing age netrin-4 expression takes on a network-like pattern that co-localizes with staining for the laminin \( \gamma_1 \) subunit (supplemental
Netrin-4 Interacts with the Laminin γ1 Short Arm

FIGURE 6. Self-aggregation of laminin-111. Purified laminin-111 was pre-equilibrated for 30 min at 37 °C in the cuvettes of a spectrophotometer (0.67 mg/ml (A) and 0.5 mg/ml (B)). Laminin-111 (○) was incubated in the presence of 1 mM CaCl₂ and shows the typical time course of turbidity formation, which was monitored as absorbance at 360 nm. After 300 min, EDTA was added to a final concentration of 5 mM. An identical sample (○) was kept in the presence of 5 mM EDTA for the duration of the experiment. In additional samples, (●) netrin-4 (gray triangle) (A, 0.21 mg/ml) or full-length netrin-4 (gray box) (B, 0.19 mg/ml) and CaCl₂ was added at the beginning of the measurements. In a second experiment, aliquots of laminin-111 (0.6 mg/ml) were incubated in reaction vials. After addition of 1 mM CaCl₂, the samples were kept at 37 °C for 3 h and centrifuged. Supernatant (S) and pellet (P) were analyzed by reducing SDS-PAGE and Coomassie staining. Purified laminin-111 was incubated in the presence or absence of 10 mM EDTA (C). Laminin-111 was incubated under the same conditions with increasing concentrations (1:125 to 1:1 netrin-4/laminin-111 molar ratio) of netrin-4 (D). At a 1:1 ratio of Δnetrin-4 to laminin-111, the polymerization was completely inhibited (D, asterisk). In contrast, Δnetrin-1 had no influence on the laminin-111 polymerization (E).

Fig. 2, E–H). Strikingly, in both perinatal and adult salivary gland netrin-4 is only expressed in the sublingual and not in the submandibular part.

Since the basement membrane localization of netrin-4 is seen first after birth in the sublingual gland, embryonal glands can be used to study the effect of exogenously added netrin-4 on gland morphogenesis. Submandibular glands of day 13.5 mouse embryos were incubated with either full-length netrin-4 (Fig. 7, C–F), Δnetrin-4 (not shown), or Δlaminin γ1 for 3 days. Both kinds of netrin-4 protein caused a decrease of epithelial branching as compared with control glands not treated with protein (Fig. 7, A and B). In contrast, addition of Δlaminin γ1, a molecule with the same domain structure as Δnetrin-4, which had been prepared in an identical manner, had no effect on branch-

interactions between netrin-4 and both the laminin γ1 chain and the whole trimeric laminin-111. In contrast, netrin-1 does not bind to either protein. Ca²⁺ ions are not required for the interaction, and circular dichroism spectroscopy showed no detectable conformational change in netrin-4 induced by adding Ca²⁺ or removing divalent cations with EDTA. Since the laminin short arm self-interaction is Ca²⁺-dependent (37), it appears that netrin-4 binding to laminin-111 does not occur through the same interaction site. Biaxial binding studies showed that the netrin-4 LN domain is crucial for interaction with the Δlaminin γ1 subunit. We speculate that netrin-4 binds to laminin-111 via the netrin-4 EGFR domain, which might be folded back onto the first and second EGF repeat, since exchanging the netrin-4 EGF

DISCUSSION

Netrin-4 is structurally related to the laminin β chains. It is widely expressed outside the nervous system, most abundantly in vasculature, kidney, ovary, and heart (13), and is often deposited in basement membranes. Co-localization studies in mouse kidney and salivary gland revealed overlapping spatial expression patterns of the laminin γ1 chain and netrin-4 in basement membranes of post-natal and adult animals. However, the temporal regulation is independent, as early embryonic expression does not show extensive co-localization; indeed co-distribution is not achieved until birth. Netrin-4 expression increases from the newborn to the adult animal, and in parallel, the co-localization with the laminin γ1 chain becomes more pronounced.

Several binding assays based on different principles revealed strong
Netrin-4 Interacts with the Laminin γ1 Short Arm

repeats for netrin-G1 EGF repeats leads to a slightly reduced binding strength.

Most studies on netrins have concentrated on their role in neuronal guidance within the central nervous system. However, they are widely expressed outside the nervous system, and their functions in these tissues have only recently begun to be addressed (for a review see Ref. 38). We demonstrated that netrin-4 is a basement membrane-associated protein (13), and it is therefore possible that netrin-4 and laminins regulate each others’ activities. Recently the role of netrins in lung branching morphogenesis was explored (20). It turned out that netrin-4, as well as netrin-1, is able to suppress lung budding. For netrin-4, inhibition of lung budding was seen already at concentrations of 10–50 μg/ml, and for netrin-1 a higher concentration (50 μg/ml) was needed to achieve the same level of inhibition as with netrin-4.

To study the in vivo function of netrin-4, we now focused on a different organ, the salivary gland, which also depends upon branching morphogenesis for its development. Since netrin-4 is expressed first at late stages of salivary gland development, this is an excellent model to study the effect of exogenous netrin-4. Treatment of submandibular gland explants with netrin-4 (50 μg/ml) leads to a drastic suppression of epithelial branching. Significantly, this effect was achieved using the full-length netrin-4 protein and Δnetrin-4, the truncated form of netrin-4 lacking the C domain. To ensure the specificity of netrin-4 effect, Δlaminin γ1 chain (50 μg/ml) was added to salivary gland in an identical manner. This did not alter the epithelial branching, indicating that similarly isolated proteins do not contain inhibitory substances. Immunofluorescence staining of the treated explants revealed an accumulation of netrin-4 at the basement membrane, co-localizing with the laminin γ1 chain. In the extracellular matrix, where levels of free Ca2+ are about 1.5–2 mM, laminin forms large insoluble complexes. Remarkably, aggregation assays with laminin-111 showed that adding Δnetrin-4 leads to a complete inhibition of laminin polymerization, whereas addition of full-length netrin-4, which forms dimers over its C domain, inhibits laminin polymerization moderately. Thus full-length netrin-4 might partially, and Δnetrin-4 completely, destabilize the basement membrane and thereby suppresses budding of the glands. Since in embryonic lung culture netrin-1 also inhibits branching morphogenesis, even though to a much lesser extent than netrin-4, it is clear that netrin-4 has only a modulating effect on laminin polymerization.

Several additional explanations for this strong inhibiting effect of netrin 4 are possible. Branching morphogenesis involves interactions between different cell types, but also the extracellular matrix, proteases, and growth factors play a role (for review see Ref. 39). Netrin-4 may act through an alteration of the basement membrane,

FIGURE 7. Morphology of submandibular gland explants treated with Δnetrin-4 or Δlaminin γ1 for 3 days. A, B, G, and H, controls incubated with PBS. C–F, explants treated with 50 μg/ml full-length netrin-4. Incubation of the submandibular glands with netrin-4 led to a significant decrease of epithelial branching. C and F, explants treated in an independent experiment with 50 μg/ml Δlaminin γ1 chain. Incubation of the submandibular glands with Δlaminin γ1 had no influence on epithelial branching. Scale bar, 0.5 mm in F and H (also applies to A–J).

FIGURE 8. Distribution of the laminin γ1 subunit and netrin-4 in submandibular gland explants treated with netrin-4. Double immunofluorescence staining using antibodies against netrin-4 (red) and the laminin γ1 chain (green). The control submandibular glands show hardly any netrin-4 expression (A). The laminin γ1 chain is located in the basement membrane around the ducts and does not co-localize with netrin-4 in native embryonic (E13.5) submandibular glands. In the netrin-4-treated submandibular glands (B and C), netrin-4 diffuses into the tissue and co-localizes with the laminin γ1 subunit in the basement membrane around the ducts. Scale bar, 100 μm.
Netrin-4 Interacts with the Laminin γ1 Short Arm

which can then influence the laminin signaling to the epithelial cells. On the other hand, the integration of netrin-4 into the laminin network may therefore enhance the presentation of netrin-4 to a putative receptor. The fact that netrin-4 has an inhibitory function on branching morphogenesis and is increasingly expressed in postnatal development suggests a role for netrin-4 in late stages of embryonic development, maturation, and in tissue homeostasis. Netrin-4 may also play a role in regulating tissue regeneration and contribute to tissue stability by preventing hyperplasia.

Further studies will aim at determining which effects of netrin-4 are mediated by modulation of basement membrane structure and which effects by its potential interactions with cell surface receptors.

REFERENCES

1. Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M., and Tessier-Lavigne, M. (1994) *Cell* 78, 409–424
2. Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C., and Tessier-Lavigne, M. (1996) *Cell* 87, 1001–1014
3. Kennedy, T. E., Serafini, T., de la Torre, J. R., and Tessier-Lavigne, M. (1999) *Nature* 398, 425–435
4. Colamarino, S. A., and Tessier-Lavigne, M. (1995) *Cell* 81, 621–629
5. Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G., and Tessier-Lavigne, M. (1996) *Cell* 87, 175–185
6. Hedgecock, E. M., Culotti, J. G., and Hall, D. H. (1990) *Neuron* 4, 61–85
7. Leung-Hagesteijn, C., Spence, A. M., Stern, B. D., Zhou, Y., Su, M. W., Hedgecock, E. M., and Culotti, J. G. (1992) *Cell* 71, 289–299
8. Chan, S. S., Zheng, H., Su, M. W., Wilk, R., Killeen, M. T., Hedgecock, E. M., and Culotti, J. G. (1996) *Cell* 87, 187–195
9. Leonardo, E. D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S. L., and Tessier-Lavigne, M. (1997) *Nature* 386, 833–838
10. Meyerhardt, J. A., Caca, K., Eckstrand, B. C., Hu, G., Lengauer, C., Navali, S., Look, A. T., and Fearon, E. R. (1999) *Cell Growth & Differentiation* 10, 35–42
11. Van Raay, T. J., Foskett, S. M., Connors, T. D., Klingler, K. W., Lands, G. M., and Burn, T. C. (1997) *Genomics* 41, 279–282
12. Wang, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Tessier-Lavigne, M. (1999) *J. Neurosci.* 19, 4938–4947
13. Koch, M., Murrell, J. R., Hunter, D. D., Olson, P. F., Jin, W., Keene, D. R., Brunken, W. J., and Burgeson, R. E. (2000) *J. Cell Biol.* 151, 221–234
14. Nakashiba, T., Ikeda, T., Nishimura, S., Tashiro, K., Honjo, T., Culotti, J. G., and Itohara, S. (2000) *J. Neurosci.* 20, 6540–6550
15. Nakashiba, T., Nishimura, S., Ikeda, T., and Itohara, S. (2002) *Mech. Dev.* 111, 47–60
16. Yin, Y., Miner, J. H., and Sanes, J. R. (2002) *Mol. Cell. Neurosci.* 19, 344–358
17. Park, K. W., Crouse, D., Lee, M., Karnik, S. K., Sorensen, L. K., Murphy, K. J., Kuo, C. J., and Li, D. Y. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 16210–16215
18. Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Breatn, C., Claes, F., De Smet, F., Thomas, J. L., Autiero, M., Carmeliet, P., Tessier-Lavigne, M., and Eichmann, A. (2004) *Nature* 432, 179–186
19. Nguyen, A., and Cai, H. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 6530–6535
20. Liu, Y., Stein, E., Oliver, T., Li, Y., Brunken, W. J., Koch, M., Tessier-Lavigne, M., and Hogan, B. L. (2004) *Curr. Biol.* 14, 897–905
21. Dalvin, S., Anselmo, M. A., Prodhon, P., Komatsuzaki, K., Schnitzer, J. J., and Kinane, T. B. (2003) *Gene Expr. Patterns* 3, 279–283
22. De Breuck, S., Lardon, J., Rooman, I., and Bouwens, L. (2003) *Diabetologia* 46, 926–933
23. Srinivasan, K., Strickland, P., Valdes, A., Shin, G. C., and Hinck, L. (2003) *Dev. Cell* 4, 371–382
24. Jiang, Y., Liu, M. T., and Gershon, M. D. (2003) *Dev. Biol.* 258, 364–384
25. Yebra, M., Montgomery, A. M., Diaferia, G. R., Kaido, T., Silletti, S., Perez, B., Just, M. L., Hildbrand, S., Hurford, R., Florkiewicz, E., Tessier-Lavigne, M., and Cirulli, V. (2003) *Dev. Cell* 5, 695–707
26. Yurchenco, P. D., and Wadsworth, W. G. (2004) *Curr. Opin. Cell Biol.* 16, 572–579
27. Chung, A. E., Jaffe, R., Freeman, I. L., Vergnes, I. P., Braginski, J. E., and Carlin, B. (1979) *Cell* 16, 277–287
28. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., and Martin, G. R. (1979) *J. Biol. Chem.* 254, 9933–9937
29. Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C., Kleinman, H. K., Marinkovich, M. P., Martin, G. R., Mayer, U., Meneguzzi, G., Miner, J. H., Miyazaki, K., Patarroyo, M., Paulsson, M., Quaranta, V., Sanes, J. R., Sasaki, T., Sekiguchi, K., Sorokin, L. M., Talts, J. F., Tryggvason, K., Uitto, J., Virtanen, I., von der Mark, K., Wewer, U. M., Yamada, Y., and Yurchenco, P. D. (2005) *Matrix Biol.* 24, 326–332
30. Beck, K., Dixon, T. W., Engel, J., and Parry, D. A. (1993) *J. Mol. Biol.* 231, 311–323
31. Beck, K., Hunter, I., and Engel, J. (1990) *FASEB J.* 4, 148–160
32. Yurchenco, P. D., Tsilivary, E. C., Charonis, A. S., and Furthmayr, H. (1985) *J. Biol. Chem.* 260, 7636–7644
33. Yurchenco, P. D., and Cheng, Y. S. (1993) *J. Biol. Chem.* 268, 17286–17299
34. Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., and Engel, J. (1987) *Eur. J. Biochem.* 166, 11–19
35. Paulsson, M. (1988) *J. Biol. Chem.* 263, 5425–5430
36. Cheng, Y. S., Champlaud, M. F., Burgeson, R. E., Marinkovich, M. P., and Yurchenco, P. D. (1997) *J. Biol. Chem.* 272, 31525–31532
37. Odenthal, U., Haehn, S., Tunggal, P., Merkl, B., Schomburg, D., Frie, C., Paulsson, M., and Smyth, N. (2004) *J. Biol. Chem.* 279, 44504–44512
38. Hinck, L. (2004) *Dev. Cell* 7, 783–793
39. Patel, V. N., Rebustini, I. T., and Hoffman, M. P. (2006) *Differentiation* 74, 349–364