Molecular Analysis of Laminin N-terminal Domains Mediating Self-interactions*

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The ability of laminins to self-polymerize is crucial for the formation of basement membranes. Development of this polymerized network has profound effects on tissue architecture as well as on the intracellular organization and differentiation of neighboring cells. The laminin N-terminal (LN) domains have been shown to mediate this interaction and studies using proteolytic fragments derived from laminin-1 led to the theory that network assembly depends on the formation of a heterotrimeric complex between LN domains derived from α, β, and γ chains in different laminin molecules with homologous interactions being insignificant. The laminin family consists of 15 known isoforms formed from five α, three β, and three γ chains, of which some are truncated and lack the N-terminal LN domain. To address whether the model of heterotrimeric complex formation is applicable to laminin isoforms other than laminin-1, eight LN domains found in the laminin protein family were re-combinantly expressed and tested in three different assays for homologous and heterologous interactions. The results showed that the lack of homologous interactions is an exception, with such interactions being seen for LN domains derived from all α chains and from the β2 and β3 subunits. The γ chain-derived LN domains showed a far more limited binding repertoire, particularly in the case of the γ3 chain, which is found present in a range of non-basement membrane locations. Further, whereas the interactions depended upon Ca2+ ions, with EDTA reversibly abrogating binding, EDTA-induced conformational changes were not reversible. Together these results demonstrate that the assembly model proposed on the basis of laminin-1 may be a simplification, with the assembly of naturally occurring laminin networks being far more complex and highly dependent upon which laminin isoforms are present.

Basement membranes are specialized extracellular matrices found underlying all epithelia and endothelia as well as surrounding many types of mesenchymal cells. Laminins constitute the major noncollagenous protein component within the basement membrane and are crucial for its formation (1, 2). Through their interactions with specific receptors, especially members of the β1 integrin family and α-dystroglycan, they induce many cellular effects, including differentiation and cellular and axonal migration. The prototype, laminin-1, isolated from embryonic carcinoma cells (3) or the Engelbreth-Holm-Swarm tumor (4) has been shown to belong to a family consisting of 15 members (for nomenclature, see Refs. 5–7). Laminins are multidomain heterotrimers formed by the combination of one α, one β, and one γ chain. Laminin-1 is an 800-kDa glycoprotein composed of a 400-kDa α1 chain and β1 and γ1 chains, each of 200 kDa. It has a cross structure with one long and three short arms, the latter being formed from the three free N-terminal ends of the α, β, and γ chains (8). These parts of the β and γ chains each contain two globular domains, designated IV and VI, whereas there are three, IVα, IVβ, and VI, in the short arm contributed by the α chain. The globules are interspersed by multiple laminin epidermal growth factor-like (LE) domains forming rods. The VI domains are also designated laminin N-terminal (LN) domains, and this nomenclature will be used throughout. As well as being found in full-length laminin chains, LN domains are also present in netrins, secreted molecules acting as guidance cues in neuronal path finding.

The α1, β1, and γ1 subunits have been shown to be representative of three distinct but closely related gene families, which consist of five, three, and three known members, respectively. Certain chains have been described as maintaining the domain structure of those in the original laminin-1 (e.g. α2 and β2) whereas others have N-terminal truncations presumably acquired in the process of evolutionary duplication (e.g. α3 and γ3) (9). The trimeric molecules formed by the combination of these chains may appear to have no short arms (e.g. laminin-5 (α2β3γ2) (10) or have a Y shape on electron microscopy (e.g. laminin-7) (α3β2γ1) (11)). The laminin trimer is formed by assembly of the coiled-coil α-helical regions of the three chains, which result in the formation of the long arm structure (12). The amino acid sequence in these regions must contain additional information, beyond the basic heptad repeat with hydrophobic residues in positions 1 and 4, since many but not all the theoretically possible heterotrimers are observed in vivo. Possibly, partially exclusive ionic bridges between the different chains play a role in increasing the stability in the formation of the coiled-coil α-helix (13).

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§ The on-line version of this article (available at http://www.jbc.org) contains six additional tables.

1 The abbreviations used are: LE, laminin epidermal growth factor-like; LN, laminin N-terminal; xMAP, flexible multianalyte profiling; Bicine, C, N-bis(2-hydroxyethyl)glycine; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; BS3, bis(sulfosuccinimidyl)suberate.

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Upon secretion, the laminin molecules aggregate to form a meshwork (14). In vitro this self-assembly is dependant upon Ca$^{2+}$ (15) and is inhibited by chelating agents (14). At high concentrations and in the presence of Ca$^{2+}$, the laminin molecules align to produce an array through the interaction of the N-terminal globular LN domains (16). Calcium ions are believed to produce a conformational change in the LN domains, allowing aggregation (15, 17). The self-aggregation of laminin-1 is shared by many “full-sized” laminin isoforms but not by laminin-5 and -6, which contain chains with N-terminal truncations and thereby lack one or more LN domains (18).

The polymerization of laminin-1 may be inhibited by proteolytic fragments that contain LN domains (19, 20). Fragment E4, containing the LN domain of the $\beta_1$ chain, binds to a fragment representing the LN domains of the other two laminin-1 short arms with a $K_D$ of 1.4 $\mu$M, whereas homologous self-interactions are either very weak or do not occur (20). More recently, recombinant fragments representing the short arms of the $\alpha$ chains have also been shown able to inhibit laminin-1 polymer formation (21, 22).

Laminin self-aggregation is not the only mechanism needed for correct basement membrane assembly, since self-aggregation preferentially occurs while laminin is bound to cell surface receptors such as $\alpha$-dystroglycan or integrins (23, 24). However, upon binding to the laminin network, these receptors are also redistributed, which leads to a reorganization of the cortical cytoskeleton (25). Thus, the aggregation state of laminin in the basement membrane may have regulatory effects on cells. This is highlighted in organ culture experiments, where polymerization-blocking antibodies against laminin or laminin short arm structures inhibited basement membrane assembly and epithelial cell polarization (26) and smooth muscle cell differentiation (27). Recent studies have also shown that polymerization-deficient laminins fail not only to form a basement membrane-like structure but are also unable to induce differentiation in embryonic stem cells (28). Together these studies suggest that regulated laminin aggregation is highly significant for basement membrane-induced cellular differentiation and organogenesis.

To understand the self-interaction mechanism of the laminin molecules in more detail, we have expressed recombinantly the LN domains from eight of the mouse laminin chains and have used these in binding assays. We could show that Ca$^{2+}$ binding was shared between all of the chains but also that they have differing affinity for one another. This suggests that different laminin trimers may have a highly variable arrangement within the basement membrane.

**MATERIALS AND METHODS**

**Expression and Purification of Murine LN Domains**—Total RNA from adult mouse kidney was used as template for reverse transcription in all cases with the exception of $\beta_2$ and $\gamma_2$ subunits where RNA of skin from 3-day-old mice was chosen. Reverse transcription followed by PCR was carried out using the primers shown (Supplementary Table I). The amplified DNA fragments were digested with SpeI of skin from 3-day-old mice was chosen. Reverse transcription followed by PCR was carried out using the primers shown (Supplementary Table I). The amplified DNA fragments were digested with SpeI and NotI and cloned into the NotI-NotI-digested expression vector pCPEP-pu BM40-eHis or pCPEP-puBM40-eStrep (29). This produces a fusion protein where either a His$_6$ tag or a Strep II tag is placed in frame with the LN coding regions. In the case of the $\gamma_2$ domain, which contains an internal NotI site, the cDNA was cloned Spel-Xhol into a polymer formation (21, 22).

The plasmids were transfected into 293-EBNA cells by electroporation, and the cells were subsequently selected for puromycin resistance. Serum-free supernatants were tested for expression, after SDS-PAGE separation on a 10% gel, by immunoblotting using antibodies specific for either the His$_6$ tag (penta-His horseradish peroxidase-conjugated mouse monoclonal antibody, Qiagen, Germany) or the Strep II tag (rabbit polyclonal serum; IBA, Germany).

For His$_6$ tag purification, supernatants were loaded on an IMAC column (Talon metal affinity resin; Clontech) with a flow rate of 0.5 ml/min. After washing with 5 column volumes of buffer (20 mM HEPES, 100 mM NaCl, pH 8.0) containing 2.5 mM imidazol, the proteins were eluted with a linear gradient between 2.5 and 250 mM imidazol.

In the case of Strep II-tagged proteins, supernatants were loaded on a StrepTactin-Sepharose column (IBA) at a flow of 0.3 ml/min. After washing with 10 column volumes of 100 mM Tris, pH 8.0, the proteins were eluted with the same buffer containing 2.5 mM desthiobiotin.

Native laminin-5, obtained after affinity purification from cell culture media, was a kind gift from Dr. R. Burgesson.

Native PAGE—Nondenaturing polyacrylamide gel electrophoresis was performed on a 6% gel in a buffer containing 40 mM Bis and 15 mM Tris at pH 8.3. Since LN$\alpha_1$ + 2LE and LN$\beta_3$ + 4LE did not run off the anode under these conditions, they were rerun after switching the electrodes.

Deglycosylation—Deglycosylation was performed in 20 mM Tris, pH 8.0, or in denaturing buffer (New England Biolabs) after boiling for 10 min. Three $\mu$g of protein were incubated overnight with 200 units PNGase F (New England Biolabs) at 37 °C.

Circular Dichroism—CD spectra were measured in a Jasco J-715 spectropolarimeter. Proteins were dialyzed against 5 mM Tris, pH 7.5, and had a concentration of 100 $\mu$g/ml. Measurements were performed in the presence of 2 mM CaCl$_2$ or after the subsequent addition of EDTA to give a final concentration of 4 mM.

Surface Plasmon Resonance Binding Assays—Assays were performed using a Biacore 2000 (BIACore AB). Coupling of proteins to the CM5 chip was performed in 10 mM sodium acetate, pH 5.0, at a flow rate of 5 $\mu$l/min. A 7-min pulse of 0.05 mM N-hydroxysuccinimide, 0.2 mM N-ethyl-N-diethylenezyl carbodiimide was used to activate the surface. The protein was injected until the desired amount was coupled (300–800 response units), and excess reactive groups were deactivated by a 7-min pulse of 1 mM ethanolamine hydrochloride, pH 8.5. Measurements were carried out in HBS (20 mM HEPES, 150 mM NaCl, pH 7.4) containing 2 mM CaCl$_2$ or 2 mM EDTA at a flow rate of 30 $\mu$l/min. The injection of 120 $\mu$l of the protein solution (10–100 $\mu$l) was followed by a 500-s dissociation. Each analysis was carried out a minimum of three times. The data were analyzed with BIACore software version 3.0 according to the Langmuir model for 1:1 binding. All binding curves could be fitted with an accuracy of $\chi^2 <$ 0.5 (see Supplementary Tables II–VI).

xMAP-Luminex Binding Assays—Measurements were performed in a final total volume of 60 $\mu$l of 10 mM HEPES, 150 mM NaCl, 2 mM CaCl$_2$, pH 7.4. For each data point 1 $\mu$l of stock nitrotriacetic acid-coupled microspheres (Qiagen) was incubated with an excess of His$_6$-tagged ligand for 4 h at 4 °C. After washing the microspheres, the analyte probe was added to give final concentrations from 0–200 $\mu$M. Analyte and ligand were gently agitated for 2 h at 25 °C before the addition of penta-His-Alexa 532-conjugated monoclonal antibody (Qiagen). The reaction mixture was then incubated for a further 90 min. Assays with microspheres alone and assays in the absence of the analyte were included as negative controls. Samples were analyzed using a LiquiChip-100 work station (Qiagen), and the level of fluorescence at 532 nm was measured. In the absence of analyte, only background signal was observed, indicating no binding of the penta-His antibody to either the microsphere or the coupled ligand.

Cross-linking Assays—Cross-linking assays were carried out using the lysine side chain-reactive cross-linker bis(sulfosuccinimidyl) suberate (BS$_3$) (Pierce). The proteins were added at a concentration of 2.5 mM, and the reaction was carried out in a final volume of 40 $\mu$l in HBS buffer and in the presence of 2 mM CaCl$_2$. For most assays, the cross-linker was used at a 200-fold molar excess over the protein concentration. The reaction was allowed to continue for 30 min at 25 °C and was stopped by the addition of 5 $\mu$l of 5 mM Tris, pH 7.4.

MALDI-TOF Mass Spectrometry—For MALDI-TOF mass spectrometry analysis, the samples were dissolved in 5 $\mu$l of 0.1% aqueous trifluoroacetic acid. MALDI mass spectrometry was carried out in linear mode on a Bruker Reflex IV equipped with a video system, a nitrogen UV laser (Omax = 337 nm), and a HiMass detector. 1 $\mu$l of the sample solution was placed on the target, and 1 $\mu$l of a freshly prepared 1 mM solution of sinapinic acid in acetonitrile/H$_2$O (2:1) with 0.1% trifluoroacetic acid was added. The spot was then crystallized by the addition of another 1 $\mu$l of acetonitrile/H$_2$O (2:1), which resulted in a fine crystalline matrix. For recording of the spectra, an acceleration voltage of 20 kV was used, and the detector voltage was adjusted to 1.9 kV. Approximately 500 single laser shots were summed into an accumulated spectrum. Calibration was carried out using the single and doubly protonated ion signal of bovine serum albumin for external calibration.
Laminin N-terminal Domains

Results

Expression and Purification of Laminin LN Domains—LN domains from eight full-length murine laminin chains were expressed and purified. Initial attempts to produce these domains without adjacent LE modules failed; however, recombinant expression did occur upon introducing one or more of the LE modules from domain V. The expression level was dependent upon the number of LE modules following the LN domain and varied between 2 and 15 mg/liter of conditioned medium, with the highest yields obtained from constructs containing four LE domains (Fig. 1).

The individual domains were purified from the serum-free medium either by immobilized metal ion affinity chromatography for the His₆-tagged forms or by StrepTactin affinity chromatography where the Strep II-tag was used. Single step purification was sufficient to obtain the products at a high degree of purity (Fig. 2). This was carried out at 4 °C, and protease inhibitors were used in all solutions; however, EDTA was omitted as it could possibly affect later divergent cation-dependent assays. SDS-PAGE of the purified recombinant proteins, without reduction of disulfide bonds, revealed distinct but weaker double bands for the β₁- and β₂-derived proteins (Fig. 2). These largely resolved upon reduction, which suggested that the proteins possibly had incomplete or variable disulfide closure. Also in the case of the γ₂ LN domain, two bands were seen. However, these remained after treatment with β-mercaptoethanol, raising the possibility that a proportion of the protein had undergone limited proteolysis or some other post-translational modification. To investigate this, we tested all of the expressed LN domains for evidence of proteolysis. Edman degradation of each of the proteins revealed the expected N-terminal amino acid sequence (APLV), produced through cleavage by the signal peptidase of the BM40 fusion signal peptide used instead of the native signal sequence. Since the proteins were produced with C-terminal tags, immunoreactivity with tag-specific antibodies showed that C-terminal degradation had not occurred.

The LN domains are all potentially N-glycosylated, with the α chains carrying the most asparagine residues suitable for substitution. Deglycosylation with endoglycosidase F resulted in a shift in electrophoretic mobility for all of the proteins, which was most marked in the cases of the α and γ chains (Fig. 2b). For the γ₂ fragment, deglycosylation resulted in a single band showing that the two bands of differing mobility were due to variations in N-linked glycosylation.

Analysis of the purified proteins by native PAGE in the absence of Ca²⁺ (Fig. 3) showed the products to run as single bands, suggesting that under these conditions and concentrations self-aggregation was not a major feature.

LN Domain Interactions—Three independent assays were carried out to show interactions between the LN domains. Surface plasmon resonance was used to show binding and to obtain kₐ and kₑ values. All of the possible interactions were carried out in both directions (i.e. both with the proteins coupled and free in solution). These results were verified using the xMAP suspension binding assays. Finally, certain interactions were confirmed and further studied by covalent cross-linking using the water-soluble cross-linking agent BS₃.

Since laminin-1 polymerization has been the focus of most previous studies, our initial experiments using surface plasmon resonance tested the α₁, β₁, and γ₁ LN domains found present in laminin-1 (Fig. 4, Table I, and Supplementary Tables II–VI). These assays were carried out at 25 °C, the temperature commonly used for such experiments. However, since temperature has been shown important in LN domain interactions, the analysis was repeated at 13 and 37 °C. At 13 °C, no binding was observed, whereas at 37 °C, signals indicative of aggregation occurred with nonreversible binding being seen. As expected from earlier experiments with proteolytic fragments (20), binding between heterologous laminin-1 LN domains could be detected; a typical response curve is given in Fig. 4, showing the interactions of the α₁ and β₁ LN domains with a Kᵢ of 6.2 × 10⁻⁷ M. Similarly, binding assays using the recombinant β₁ LN domain showed that this failed to self-interact, again in agreement with the previous findings for the E₄ fragment, which contains this domain. This lack of self-interaction was also seen for the γ₁ LN domain, but the α₁ domain interacted with a higher affinity (Kᵢ of 1.8 × 10⁻⁷ M) with itself than seen for any heterotypic interactions of the LN domains of this laminin isoform. This clearly shows that homotypic interactions occur and can influence laminin network assembly.

We then studied the possibility of self-interaction for the other laminin LN domains by surface plasmon resonance (Table I and Supplementary Tables II–VI). We found that both the α₂ and α₀ LN domains self-interact in a manner similar to that of the α₁ domain. Whereas the γ₂-derived LN domain, like its γ₁ counterpart in laminin-1, failed to self-interact, both β₂ and β₃ LN domains did, in contrast to the results obtained for β₁ LN domain.

In studies of the interactions between LN domains from the same class of laminin chain, we found evidence of α-α and β-β intragroup heterodimers for all members. However, the two γ chain-derived LN domains showed no interaction. When intergroup binding was analyzed, α-β interactions were seen with all combinations, whereas the γ LN domains again showed a more limited binding repertoire. The γ₁ LN domain could be shown to bind to all β chains; however, it failed to interact with the α₂ LN domain despite binding to those of α₁ and α₀. The γ₃ LN domain is the most highly restricted in its binding partners, failing to bind to any α chain LN domain, and interacting only with those of β₂ and β₃.

Previous studies have shown the importance of Ca²⁺ in laminin gel formation. To study this further, the role of divalent cations was evaluated through the addition of EDTA to the analyte in the surface plasmon resonance experiments. In all cases, binding could be inhibited upon Ca²⁺ removal, a change that was reversible upon the readdition of Ca²⁺ ions. Circular dichroism spectra were measured and showed that there was a...
marked reduction in secondary structure for most but not all domains in the absence of Ca²⁺ ions, since there was no evidence for a change in conformation for the \( \alpha_2 \), \( \alpha_3 \), or \( \gamma_3 \)-derived LN domains (Fig. 5).

Independent results on the LN domain interactions were obtained using a xMAP suspension assay. Here the ligand is bound in an orientated manner to the surface of a fluorescent microbead via its His6 tag. After washing to remove unbound ligand, the beads are incubated with the analyte. The aligned microbeads are passed in single file through two lasers, and any interaction is detected by measuring a second fluorochrome emitting at a separate wavelength, which is coupled to an antibody directed against this analyte. The degree of antibody fluorescence (i.e. the ratio between the background signal and that occurring as the bead passes the lasers) is obtained to give relative fluorescence response units. In our study, antibodies to the His₆ tag were used to detect the interaction. Initial experiments showed that protein binding to the beads resulted in the masking of its His₆ tag from the penta-His antibody, thus allowing binding studies to be carried out of two proteins carrying the same tag. To compare the results of the xMAP and the surface plasmon resonance assays, primary studies with various protein combinations were carried out, bringing the analyte concentration to excess. To obtain quantitative results, high levels of the detecting antibody were required as increased free analyte competes for antibody binding to the bead complex. The observed \( K_D \) values were comparable with those obtained from surface plasmon resonance (Table II). All possible interactions were then analyzed in one direction and showed similar results to those seen in surface plasmon resonance (Table III), with the one exception of the \( \beta_2-\beta_2 \) LN domain interaction, where for unknown reasons high background made it impossible to verify a specific interaction. Typical binding results are shown in Fig. 6 for \( \beta_2 \) LN domains coupled to the bead and the \( \beta_1 \) LN domain in solution.

Both surface plasmon resonance and xMAP assays rely on the use of a solid phase. To ascertain that our results were not affected by artifacts, we further studied some of the interactions in solution using the water-soluble lysine-reactive cross-linker BS₃. Fig. 7 shows an example of \( \alpha_2 \) self-cross-linking,
which does not occur with \( \gamma_1 \). These cross-linked products derived from the \( \alpha_2 \) LN domains were then analyzed by MALDI-TOF mass spectrometry and showed both dimer and trimer formation (Fig. 7b). As in the surface plasmon resonance and xMAP assays, we could not find any evidence for a self-interaction for \( \beta_1 \), \( \gamma_1 \), or \( \gamma_3 \) LN domains, whereas all other LN forms, including the \( \beta_2 \) LN domain, did self-interact (Fig. 8). LN domains derived from the \( \beta_1 \) and \( \gamma_1 \) chains were then incubated together at half the concentration used in the self-interaction studies in the presence of a 200-fold molar excess of BS3. Coomassie Blue staining after SDS-PAGE revealed a distinct \( \beta_1\gamma_1 \) dimer (Fig. 8).

Recombinant LN Domains Interact with Native Laminins—Laminin-5 has the simplest short arm structure with a single LN domain found on the \( \beta_3 \) chain. On the assumption that this would lead to a less complex binding behavior, we looked for interactions between laminin-5 and the \( \gamma \) chain-derived LN domains. To measure such interactions by surface plasmon resonance, native laminin-5 isolated from cultured cells was coupled to a CM5 chip and could be shown to interact with both \( \gamma_1 \) (Fig. 9) and \( \gamma_3 \) (not shown) domains in a similar manner to that seen for the recombinant \( \beta_3 \) LN fragment.

**DISCUSSION**

We present results from eight LN domains found on full-length laminin chains. During the course of this work, a ninth LN domain was identified, present on a newly discovered full-length variant of the \( \alpha_3 \) chain (22). Initial attempts, by us and others (22), to produce LN domains alone without the adjacent LE modules failed, presumably due to a defect in folding, leading to their intracellular degradation. Introducing one or more of the LE modules present in domain V resulted in recombinant protein secretion. Interestingly, the levels of protein production seemed dependent on the number of these modules, so that by far the highest yield was seen when four LE domains were present. The exact reason for this is unclear, although the LE module structure is known, and they have well defined boundaries and express as independently folding domains. Possibly, the correct folding of the LN domain depends upon the presence of LE domains, and perhaps there are interaction surfaces between these two modules. It may also be that the folding of a LE domain is itself in part dependent upon adjacent structures; hence, the greater the number of LE domains following the LN domain, the higher the correct folding of both regions.

All of the LN domains showed a different electrophoretic mobility from that expected based upon the molecular mass of their amino acid sequences. All carry a number of \( N \)-linked sugar residues, and deglycosylation with endoglycosidase F resulted in a shift that was most marked in the case of the \( \alpha \) and \( \gamma \) chains.

Equilibrium gel filtration assays, analytical ultracentrifugation, laminin gelation inhibition studies, and rotary shadowing of polymerized molecules have shown that LN domains are important for network formation. The generally accepted mechanism for laminin self-assembly is based upon results using proteolytic fragments of laminin-1 short arms. Laminin gelation studies showed that three fragments, the \( \beta_1 \) short arm (E4), the \( \alpha_2\gamma_1 \)-containing E1' fragment, and the \( \alpha_1 \) short arm (contained in E1) efficiently inhibited polymerization (20). Equilibrium gel filtration assays showed that the \( \beta_1 \) short arm (E4 fragment) could not self-interact, whereas it did bind strongly to the \( \alpha_2\gamma_1 \)-containing fragment E1', which also weakly self-interacted. E4 (\( \beta_1 \)) interacted only very weakly with the E1 fragment containing only the \( \alpha_1 \) LN domain. Rotary shadowing of these proteolytic fragments showed interact-
FIG. 5. Circular dichroism spectra of laminin LN domains. 100 μg/ml of protein in 5 mM Tris, pH 7.4, was measured in the presence of effective concentrations of 2 mM CaCl₂ and after the addition of 4 mM EDTA (dashed lines). Conformational changes were not observed for α₂, α₅, and γ₃ LN domains.
ing trimeric complexes formed from the LN domains of all three arms (20). From this it was assumed that polymerization occurs with the formation of a heterotrimeric LN complex through the cooperative interaction of the $\alpha\beta\gamma$ arms. Ca$^{2+}$ ions, a requirement for polymerization (14, 15), could be shown to bind to the $\gamma_1$ LN-containing fragment (20). This heterotrimeric interaction would of course place restraints upon the laminin arrangement, resulting in a quasiregular network, the structure of which could have physiological relevance (16). Indeed, network formation by laminin on the surface of striated muscle or Schwann cells has been implicated in the redistribution of the cortical cytoskeleton (25, 30).

![Fig. 6. Interactions between $\beta_1$, LN and $\beta_2$, LN as measured by a Luminex xMAP suspension assay. $\beta_2$, LN was coupled to the beads, and $\beta_2$ was used as analyte in solution at different concentrations. The interaction was monitored by the reporter fluorescence of the penta-His Alexa Fluor 532 conjugate.](image)

![Fig. 7. Cross-linking of $\alpha_2$ and $\gamma_1$, LN domains. $\alpha_2$, protein at a concentration of 2.5 $\mu$M was incubated with 0–2.5 mM of BS$_3$ cross-linker and analyzed by SDS-PAGE on 5–15% gels. The arrows mark the cross-linked products. $b$, the product of the $\alpha_2$, LN domain cross-linked with 2.5 mM of BS$_3$ was analyzed by MALDI-TOF spectroscopy.](image)

We could show that laminin-1 self-interaction is of a more complex nature than that previously foreseen. In agreement with earlier suggestions, $\beta_2$ and $\gamma_1$ self-interactions do not occur; however, the $\alpha_1$ LN domain showed evidence for a relatively strong self-interaction ($K_D$ $\alpha_1\alpha_1 \sim 1.8 \times 10^{-7} \text{M}$; $K_D$ $\alpha_1\gamma_1 \sim 1.8 \times 10^{-6} \text{M}$), suggesting that the proposed regular array may not be as uniform as believed.

The laminin LN domains are most conserved between subfamily members (9). The self-aggregation of laminin-1 is a property shared by many if not all “full-sized” lamin isoforms, but not by lamin-5 and -6, which contain chains with N-terminal truncations and thereby lack one or more LN domains (18). Therefore, it might have been expected that the other LN

| Ligand | Analyte | $K_D$-xMAP ($M$) | $K_D$-SPR ($M$) |
|--------|---------|-----------------|-----------------|
| $\beta_1$ | $\beta_1$ | $1.23 \times 10^{-7}$ | $2.80 \times 10^{-8}$ |
| $\beta_1$ | $\gamma_1$ | $3.34 \times 10^{-8}$ | $6.20 \times 10^{-7}$ |
| $\gamma_1$ | $\beta_2$ | $6.48 \times 10^{-9}$ | $1.70 \times 10^{-7}$ |
| $\gamma_1$ | $\gamma_2$ | $3.47 \times 10^{-9}$ | $1.50 \times 10^{-7}$ |
| $\gamma_2$ | $\beta_3$ | $8.53 \times 10^{-9}$ | $1.00 \times 10^{-7}$ |
| $\beta_1$ | $\gamma_3$ | $2.63 \times 10^{-8}$ | $1.80 \times 10^{-7}$ |
| $\gamma_1$ | $\gamma_3$ | NB | NB |
| $\gamma_2$ | $\gamma_3$ | NB | NB |
| $\beta_1$ | $\beta_3$ | $5.43 \times 10^{-8}$ | $4.20 \times 10^{-7}$ |

*SPR, surface plasmon resonance.

$^a$NB, no specific reaction.

**TABLE III**

| Protein on bead | Protein in solution |
|-----------------|---------------------|
| $\alpha_1$      | $\alpha_2$ $\alpha_3$ $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |
| $\alpha_2$      | $\alpha_2$ $\alpha_3$ $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |
| $\beta_1$       | NB $\alpha_2$ $\alpha_3$ $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |
| $\beta_2$       | $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |
| $\beta_3$       | $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |
| $\gamma_3$      | $\beta_1$ $\beta_2$ $\beta_3$ $\gamma_1$ $\gamma_3$ |

*NB, no binding.

$b$ NR, no specific reaction.
domains act like their counterparts in laminin-1. For α chains, this seems to be the case, with these chains being able to self-interact as well as bind to other α LN domains. A recently published study including the α2 LN domain (22) showed that whereas α3 and α5 LN domains efficiently inhibited laminin-1 polymerization, this was less pronounced for the α1- and α2-derived domains, and indeed no laminin-1 α2 LN binding was observed by surface plasmon resonance. This is in part in agreement with our finding that α2 LN domains fail to interact with either of the γ domains. However, on the basis of our results with the individual domains, we would expect to see some, albeit decreased, binding between α2 LN domains and laminin-1. The importance of the α chain LN domain has been shown in the dystrophic mouse, where a deletion in the α2 laminin LN domain leads to defects in the basement membrane of skeletal muscle (24). Whereas the properties of the α LN domains seem to have been generally conserved, this is not the case for the β LN domains. Unlike β1, β2 and β3 can self-interact, and the LN domains of these laminin chains show the widest range of binding partners, interacting with both of the γ isoforms. This may be of particular relevance for laminin-5. This molecule, which is present in the anchoring filaments of the hemidesmosome, contains a single LN domain on the β3 short arm, and the ability to self-interact through this LN domain and its relative promiscuity for LN binding partners could help stabilize the molecule within the anchoring filaments. Many mutations in the LAMB3 gene, encoding the β3 chain, have been described, some of which result in epidermolysis bullosa. However, since this mutation also appears to alter splicing efficiency, resulting in marked reduction in the full-length transcript, the full importance of the LN domain in laminin-5 is still open to speculation (32, 33). We could show that the native laminin-5, the only laminin known to contain the β3 chain, interacts with the γ isoforms in a manner similar to its recombinantly expressed LN domain.

Our finding that the γ1 and γ3 LN interactions with recombinant β3 LN have $K_D$ values very similar to those for native laminin-5 strengthens the potential application of our model. The significance of the limited choice of binding partners for the γ3 LN domain leads to speculation that it is important for the reported largely non-basement membrane or basement membrane-associated distribution of γ3-containing laminins (34). Further, it is to be noted that both β3 and γ3 are predominantly expressed in the skin (34). The inclusion of a laminin molecule with a more limited interaction potential will lead to a less well organized meshwork within the basement membrane and a more open form of structure. It may be that, for instance, laminin γ3-containing molecules induce a weakened matrix network.

The results from surface plasmon resonance assays were confirmed using an independent system. xMAP-Luminex suspension assays offer the advantages of allowing directed binding to the carrier matrix, and, since all His$_6$ epitopes are blocked by binding of the first protein to the beads, it allows both the ligand and analyte to be tagged with the same peptide. The system offers an alternative to obtain qualitative binding results and can be used to obtain an observed $K_D$. However, it was of interest that this system consistently showed a stronger binding when compared with the surface plasmon resonance results (3–20-fold). The cause of this is unknown, but it is possibly due to the fact that the ligand is presented in an...
orientated fashion upon the xMAP beads. It should also be noted that the presence of free analyte in the reaction mixture introduces a potential source of error. Finally, the method is very sensitive, allowing interactions of relatively low affinity to be observed. Even so, no $\beta_1$ or $\gamma_1$ self-interactions could be observed. We could show that Ca$^{2+}$ ions were required for all of the interactions and could demonstrate by circular dichroism spectroscopy a conformational change in the laminin-1 LN domains induced by Ca$^{2+}$, an effect not seen in earlier studies. This could possibly be due to the method used to derive the proteolytic fragments used in those studies (20) or due to variations in post-translational modifications between the Engelbreth-Holm-Swarm tumor and 293-EBNA cells. Curiously, these conformational changes were not conserved between all of the LN domains in the laminin family.

Gel formation is a property shared by the full-sized laminin isoforms. Whereas the individual interactions between any two LN domains appear to be relatively weak, electron microscopy after rotary shadowing shows that the laminin trimers may form up to six LN-LN interactions (16), suggesting that a network produced by an array of molecules held by such interactions could be relatively stable. Indeed, gene inactivation experiments and natural mutations have often shown that specific laminin chains are required for basement membrane stability (24, 35, 36). Studies are currently under way to examine the potential cooperativity in LN self-interaction.

LN domains are found in the full-length laminin molecules but are also present in netrins, which have homology to the $\beta_3$ and $\gamma_3$ laminin short arms (38), and most recently have been discovered in a two-protein family, the laminets, which are found widely in the central nervous system and share homology to netrins (39). Whereas laminins and netrins are secreted molecules, laminets appear to be associated with the cell surface (39), possibly through a predicted transmembrane C-terminal $\alpha$-helix. Both laminins and netrins play important roles as axonal guidance molecules (40).

Most studies on netrins have concentrated upon neuronal signaling and axonal path finding within the CNS; however, they are widely expressed, and recent results suggest that certain netrins are basement membrane-associated proteins (38). This raises the possibility that they interact with other LN domains within this extracellular matrix. Laminins are found present in specific areas and at specific time points in the CNS and may be present in non-basement membrane localities. Laminin $\beta_2$, for instance, has been shown highly important for CNS development (41), and it is possible that the three protein families, laminets, netrins, and laminins, regulate each other’s actions.

The results presented here strongly suggest that the assembly mechanism leading to the formation of the laminin network in the basement membrane is complex and may vary between different laminin molecules.

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Molecular Analysis of Laminin N-terminal Domains Mediating Self-interactions
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