Scaffold-forming and Adhesive Contributions of Synthetic Laminin-binding Proteins to Basement Membrane Assembly*†

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Laminins that possess three short arms contribute to basement membrane assembly by anchoring to cell surfaces, polymerizing, and binding to nidogen and collagen IV. Although laminins containing the α4 and α5 subunits are expressed in α2-deficient congenital muscular dystrophy, they may be ineffective substitutes because they bind weakly to cell surfaces and/or because they lack the third arm needed for polymerization. We asked whether linker proteins engineered to bind to deficient laminins that provide such missing activities would promote basement membrane assembly in a Schwann cell model. A chimeric fusion protein (αLNNd) that adds a short arm terminus to laminin through the nidogen binding locus was generated and compared with the dystrophy-ameliorating protein miniagrin (mAgrin) that binds to the laminin coiled-coil dystroglycan and sulfatides. αLNNd was found to mediate laminin binding to collagen IV, to bind to galactosyl sulfatide, and to selectively convert α-short arm deletion-mutant laminins LmΔαLN and LmΔαLN-L4b into polymerizing laminins. This protein enabled polymerization-deficient laminin but not an adhesion-deficient laminin lacking LG domains (LmΔLG) to assemble an extracellular matrix on Schwann cell surfaces. mAgrin, on the other hand, enabled LmΔLG to form an extracellular matrix on cell surfaces without increasing accumulation of non-polymerizing laminins. These gain-of-function studies reveal distinct polymerization and anchorage contributions to basement membrane assembly in which the three different LN domains mediate the former, and the LG domains provide primary anchorage with secondary contributions from the αLN domain. These findings may be relevant for an understanding of the pathogenesis and treatment of laminin deficiency states.

Basement membranes are specialized cell-adherent extracellular matrices consisting primarily of laminins, collagen IV, nidogens, and the heparan sulfate proteoglycans agrin and perlecan (for review, see Ref. 1). Among these, the laminins constitute a family of heterotrimeric glycoproteins that are essential for the assembly of basement membrane scaffolds (2, 3). One property of laminin thought to be critical for basement membrane assembly is that of its anchorage to cell surfaces, a process that appears to be mediated through the LG domains of the α-subunit. Deletion of the five laminin-111 LG domains or of LG domains 4–5 that contain dystroglycan and sulfatide binding loci or excess inhibiting LG4–5 fragment was found to result in a failure of basement membrane assembly in an experimental Schwann cell model (4–6). These studies further suggested that the reason laminin anchorage is crucial is that it provides the key linkage between the cell surface and the extracellular matrix scaffolding such that the other basement membrane components become tethered through laminin.

A second property of laminin is its polymerization into a network-like scaffolding (7, 8). Laminin-111 (α1β1γ1), the most extensively studied in this regard, self-assembles in a thermally reversible manner with an initial oligomer-forming step followed by a calcium-dependent multimer-forming step (7). Laminin fragment and domain loss-of-function analyses have provided evidence that polymerization requires the participation of all three (α, β, and γ) LN domains located at the N termini of the short arms (6, 9) such that laminins that possess fewer domains (as seen with truncated α3 and α4-laminins) lack the ability to polymerize (6, 10).

A third property of laminin found to contribute to basement membrane assembly and stability is that of the binding of nidogen-1 and nidogen-2 (11–13). The nidogen-1 interaction is mediated between the laminin γ1-LEb3 domain and the nidogen G3 domain. Nidogen G2 and G3 domains, in turn, bind to collagen IV. Although many basement membranes do not exhibit an absolute requirement of this bridging interaction, it appears likely that the interaction increases basement membrane stability (14–16).

The principal laminins of Schwann cell endoneurial and skeletal muscle sarcolemmal basement membranes contain the α2-subunit (17). The absence of this subunit found in laminins 211 and 221 has been shown to cause a congenital muscular dystrophy and peripheral neuropathy in humans (classified as type MDC1A) and in mice (for review, see Ref. 18). Both defects have been corrected by transgenic expression of full-length laminin α1 subunit, indicating interchangeability of the α1 and α2 chains (19, 20). A characteristic of α2-laminin-deficient congenital muscular dystrophy is a compensatory increase in the laminin α4 subunit both in nerve and muscle. The assembly and functions of α4-laminin in basement membrane are not well understood. The protein is thought to be non-polymerizing with low affinity binding for α-dystroglycan, sulfatides, and α6β1 and α7β1 integrins (21, 22). Improved muscle function in

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laminin-deficient dystrophic mice, but not improved nerve function, was observed with transgenic expression of a internal domain-truncated muscle agrin (23, 24) that binds to laminin and to α2-dystroglycan (Denzel et al. 43 and Gesemann et al. 35). Although it is likely that the benefit of effect depends on these interactions, it is less clear whether amelioration of the muscle phenotype is due primarily to the enhancement of α4-laminin adhesion, to alterations of sarcosomal α5-laminin, or to some other effect.

Cultured Schwann cells have provided a useful model with which to study basement membrane assembly (4–6). Studies revealed that the galactosyl sulfatide present on the surface of these cells plays an important role in basement membrane anchorage through their binding to laminins, enabling laminin-dependent signaling through dystroglycan and β1-integrins (5, 25). Furthermore, both laminin LN and LG domains were found to be required for laminin assembly of Schwann cell surfaces either in the absence or presence of nidogen and collagen IV (6). Interestingly, neither β1-integrins nor dystroglycan was required for laminin anchorage during initial basement membrane assembly on these cells (5). These receptors may instead act to link (and hence stabilize) the basement membrane to the underlying cell cytoskeleton (26).

In the current study we asked whether laminin deficits of polymerization resulting from LN domain deletions and/or deficits of cell surface binding resulting from LG-domain deletions could be corrected with laminin-binding proteins that add back missing domain activities. Such synthetic protein reagents could provide analytical tools to help understand the role of different domains in basement membrane assembly with the potential for the development of therapeutic approaches. Because there is a nidogen-binding site on laminin γ1 chain near the intersection of the 3 short arms, we designed a chimeric protein (αLNNd) containing the N-terminal α1 LN-Lea domains attached to nidogen-1 G2-rod-G3 domains. We evaluated the capacity of this synthetic short arm to bind laminin and provide type IV collagen binding in lieu of the nidogen it replaces and compared its behavior in a Schwann cell model of basement membrane assembly with that of muscle (non-neural) miniagrin. The fusion protein was found to specifically facilitate polymer formation and basement membrane accumulation of N-terminal-truncated α1-laminins on cultured Schwann cells, whereas miniagrin corrected adhesion deficits.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**Expression vectors for the mouse laminin α1, human β1, and human γ1 subunits, for deletions of α1LN, α1LN-L4b, α1LG1–5, β1LN and γ1LN, and for chick non-neural miniagrin (mAgrin) have been previously described (6, 27–30). The DNA for α1LN-Nd was generated from α1-wtNm (McKee et al. 6) and NdIIIPEC-Pu (a gift of Takako Sasaki). The 5′ section containing the LN-Lea of α1 laminin, the bm40 signal peptide, c-Myc epitope tag, and enterokinase cleavage site was generated with primers 1F, 5′-ctgtaacagtggccacct-3′, and 2r, 5′-cacaagtctgtgcagcacaggag-3′. The G2-rod-G3 portion of nidogen for the C-terminal part of α1LN-Nd was generated with primers 2f 5′-ctcgtgtgcagcagctttggtg- and 1r 5′-tagaggacagcagtctc-3′. Both fragments were used between the 1f and 1r primers, digested with HindIII-SfiI, and ligated into NdIIIPEC-Pu. The intact open reading frame of α1LN-Nd was moved via a Spel-NotI digest to a pcDNA3.1Zeo vector (Invitrogen). The cytomegalovirus promoter and 5′-untranslated region was replaced by a Clal-HindIII insert from the α1-wtNm vector. To generate the α1LG1–5Nm construct, α1-wtNm was digested with SapI-BplI and ligated with a PCR product using primers SapI 1f, 5′-gcgtcagcaacccactacag-3′, and BplI 1r, 5′-gctagctgctcaggttcgtgg-3′, from α1-wtNm. α1LN-LG1–5Nm was generated by replacing a Nhel-BsrGI fragment of α1LN-G1–5Nm with a Nhel-BsrGI fragment from rLN1-wtN. PCR products were carried out using Jumpstart Taq (Sigma P2893) or Roche Applied Science extend long PCR in an Eppendorf Mastercycler. Restriction enzymes (Fermentase; fastdigest), SV gel PCR clean up (Promega), T4 DNA HC ligase (Invitrogen), calf intestinal alkaline phosphatase (New England Biolabs), and XL10 gold Esherichia coli cells (Stratagene) were used according to the manufacturer's instructions.

**Recombinant and Native Proteins—**Plasmids containing laminin subunits were stably transfected into HEK293 cells followed by selection of stable clones as described (6). Plasmids containing α1LN-Nd, α1LN-LG1–5Nm (designated Δ(α1LN&LG)), α1LG1–5Nm (here shortened to ALG), and mAgrin (N25C9500, a gift of Markus Ruegg) were stably transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All other laminin cell lines (LmαLN-L4b, LmαLN, LmΔ8LN, LmΔγLN, and the laminins in which the γ1LN domain was replaced with α1LN or β1LN, i.e. Lmγ1α1LN, Lmγ1β1LN) and mouse nidogen-1 (pCisNid; gift of Rupert Timpl) were previously described (6).

A stable cell line expressing α1LN-Nd was supplemented with zeocin at 100 μg/ml, whereas recombinant laminin lines were supplemented with puromycin, zeocin, and G418 at a final concentration of 1, 100, and 500 μg/ml, respectively. Immunoprecipitation, SDS-PAGE, and Western blot analysis of secreted protein was used to confirm expression of trimeric laminin, α1LNNd, nidogen-1, and mAgrin in the stable cell lines.

The α1, β1, and γ1 laminin chains were detected with antibodies specific for myc (Roche Applied Science), hemagglutinin (Roche Applied Science), and FLAG (Sigma) epitopes, respectively. Nidogen-1 and α1LN-Nd were confirmed with anti-entactin (Chemicon MAB1946). The α1LN-Nd protein was initially purified on heparin-agarose (Sigma H6508), eluted with 0.5 M NaCl in 50 mm Tris–HCl, pH 7.4, 1 mM EDTA, concentrated in an Amicon Ultra-15 filter (Millipore, 100,000 molecular weight cutoff), and dialyzed in a 20 mM phosphate, 1 mM NaCl buffer. α1LN-Nd as well as nidogen were finally purified by metal chelating chromatography as described (Fox et al. 11) and dialyzed in TBS-50 (50 mm Tris, 90 mM NaCl, pH 7.4, 0.125 EDTA).

mAgrin was purified on His-select nickel affinity gel (Sigma P6611) with a 250 mM imidazole, 300 mM NaCl, 50 mM sodium
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phosphate buffer, then concentrated and dialyzed as described above. Recombinant laminin was purified from media using heparin-agarose (Sigma) and FLAG M2-agarose as previously described (McKee et al. (6)).

Co-purified laminin and αLN-Nd were isolated directly on FLAG M2-agarose and prepared as above. Type IV collagen and laminin-111 were extracted from lathyritic mouse EHS tumor and purified as described (31).

Protein Concentrations—EHS-laminin concentrations were determined by absorbance (280 nm) as described (9) and compared to standard laminins. Molar concentrations were determined based on the protein mass of 710 kDa.

Absorbance was also used to measure the concentration of αLNNd and mAgrin with masses of 156 and 125 kDa, respectively. Protein mass and molar concentrations of laminins containing domain deletions (LmΔLG, 605 kDa; LmΔαLN, 682 kDa; LmΔαLN-L4b, 558 kDa; LmΔαLN&LG, 577 kDa) were determined by gel densitometry of their Coomassie Blue-stained bands compared with those of EHS-laminin with corrections as needed for decreased mass.

Laminin Polymerization Assay—Aliquots (50 μl) of laminin without or with αLN Nd in polymerization buffer were incubated at 37 °C, sedimented to separate polymerized protein, and analyzed by SDS-PAGE as previously described in detail (6). The apparent critical concentration was calculated from the product of x intercept and slope (10).

Assay of αLN Nd Binding to Laminin and Collagen-IV—One μg of recombinant laminin or collagen-IV was bound to a 96-well flat-bottomed dish (Nunc) in 40 mm sodium carbonate buffer overnight at 4 °C. Plates were blocked with 1 mg/ml BSA in phosphate-buffered saline and 0.06% Triton X-100, then incubated with 2-fold increasing amounts of αLN Nd or nidogen (0.013–16 μg/ml). Protein was detected by entactin-specific (i.e. nidogen-1) monoclonal antibody (Chemicon MAB1946), protein A-horseradish peroxidase (Sigma P8651), and o-phenylenediamine (Sigma P3888). To determine collagen binding to αLN Nd- or nidogen-bound laminin, one μg of recombinant laminin was coated onto a 96-well flat-bottomed dish following by blocking with BSA and 5 μg/ml nidogen or αLN Nd incubation for 1 h at room temperature. After 3 washes with phosphate-buffered saline and 0.06% Triton X-100, increasing amounts of collagen-IV (0.01–10 μg/ml) were added for 1 h at room temperature. Bound collagen was detected with collagen-IV-specific antibody (Chemicon AB 756P), protein A-horseradish peroxidase (Sigma P8651), and o-phenylenediamine (Sigma P3888) with absorbance measured at 492 nm with a TECAN Spectrafluor plate reader.

Sulfatide Binding Assay—HSO₄₋-3Galβ1-1°Ceramide (brain galactosyl sulfatides) and galactosyl ceramide (Sigma C4905) were dissolved in methanol, and 0.1 μg was added per immunoblot 1B microtiter well (ThermoLab systems). The plate was dried overnight at room temperature, and the wells were washed and blocked with enzyme-linked immunosorbent assay blocking buffer (1% BSA in TBS-50/ Ca²⁺). Proteins in varying concentrations in enzyme-linked immunosorbent assay blocking buffer were added to each well and incubated for 1 h at room temperature. Protein binding was detected with a horseradish peroxidase-linked monoclonal FLAG antibody (Sigma) and o-phenylenediamine (Sigma) with absorbance at 492 nm on a TECAN Spectrafluor.

Cell Culturing—Schwann cells isolated from sciatic nerves from newborn Sprague-Dawley rats were the kind gift of Dr. James Salzer (New York University). These cells were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum (Gemini Bio Products), neuregulin (0.5 μg/ml, Sigma), forskolin (0.2 μg/ml, Sigma), 1% glutamine, and penicillin-streptomycin. Cells at passages 11−17 were plated onto 24-well dishes (Denville) and treated with the indicated proteins for 1 h at 37 °C followed by washing and fixation. For electron microscopy (see the supplemental data) cells were plated in 60-mm Permanox dishes (Nalgene, Nunc) 2 days before the addition of proteins.

Immunofluorescence Microscopy—Schwann cells were rinsed with phosphate-buffered saline and fixed in 3% paraformaldehyde for 30 min. Immunofluorescence analysis was conducted as previously described (6). Briefly, cultures were blocked with goat serum and then stained with primary and secondary antibodies conjugated with fluorescent probes. Rabbit polyclonal antibodies specific for laminin-111-B1LN-LEa (anti-E4) and αLG4–5 (anti-E3, 1/500) were used as described (4, 6, 32). Nidogen epitopes were stained with entactin monoclonal reagent (1/100). mAgrin and Myc-tagged laminins were stained with chick agrin (1/1000;30) and Myc (1/100) antibodies, respectively. Detection of bound primary antibodies was accomplished with Alexa Fluor 488 and 647 goat anti-rabbit IgG secondary antibodies (Molecular Probes) at 1:500 and 1:100, respectively, and fluorescein isothiocyanate-conjugated donkey anti-mouse IgM at 1:100 (Jackson ImmunoResearch) and counterstained with 4’,6-diamidino-2-phenylindole (32). Laminin, αLN Nd, mAgrin, collagen IV, and nidogen-1 immunofluorescence levels were quantitated from digital images recorded with IPLab 3.7 software (Scanalytics) as described (6). A segmentation range was chosen to subtract background and acellular immunofluorescence. The sum of pixels and their intensities in highlighted cellular areas of fluorescence were measured and normalized by dividing by the number of cells for each image. Data were expressed as the mean and S.D. of normalized summed intensities SigmaPlot and SigmaStat (Jandel).

Rotary-shadowed Pt/C Replicas—Rotary shadow laminins (25–50 μg/ml in 0.15 m ammonium bicarbonate, 60% glycerol) were sprayed onto mica discs, evacuated in a BAF500K unit (Balzers), rotary-shadowed with 0.9 nm Pt/C at an 8° angle, and backed with 8-nm carbon at a 90° angle as otherwise described (9).

RESULTS

The domain and subunit composition of proteins used in this study are shown in Fig. 1. The proteins mAgrin, αLN Nd, and LmΔαLN&LG were characterized and compared with LmΔαLN and wt Lm-111 by SDS-PAGE after purification.

Molecular Morphology—αLN Nd, mAgrin, LmΔαLN-L4b, and wild-type (wt) laminin-111 alone and in complexes were visualized in electron micrographs after Pt/C rotary shadowing (Fig. 2). αLN Nd molecules had the appearance of three globular domains separated by two short rods in either an extended or
bent configuration. Lm\_LN-L4b molecules had the appearance of a laminin with two rather than three short arms. After incubation of this laminin with Lm\_HN, a third short arm-like structure could be seen attached to the laminin from one of the two short arms near the junction of the other arms (Fig. 2, arrows), rendering laminin complexes not unlike (wt) laminin-111. mAgrin Pt/C replicas had the appearance of a flexible rod and globular protein in which four sphere-like structures could often be appreciated. After incubation of laminin-111 with mAgrin, the long arm (coiled-coil domain) was often seen to have a short projecting stub at about the mid-point along its length (arrows).

### Binding Interactions of \alpha LNNd—

The chimeric protein was designed to possess three activities, i.e. binding of the C-terminal G3 domain to the laminin \gamma domain LE3b, binding of domains G2 and G3 to type IV collagen, and the laminin \alpha1-short arm component of polymerization that resides in the LN domain. Purified chimeric protein was evaluated for its ability to bind to laminin-111 and to type IV collagen (Fig. 3) in solid phase assays and found to bind to both. Chimeric \alpha LNNd and nidogen-1 bound in an almost identical manner (apparent \textit{Kd} of 0.29 and 0.26 nM, respectively). \alpha LNNd also bound to type IV collagen with an apparent \textit{Kd} of 4.0 nM (compared with 1.6 nM for nidogen-1). The ability of \alpha LNNd was compared with that of nidogen-1 to mediate attachment of laminin to nidogen (ternary complex). \alpha LNNd did this (\textit{Kd} of 1.4 nM) similar to nidogen-1 (1.4 nM).

The next question addressed was whether \alpha LNNd, when bound to a laminin with two short arms, would mediate laminin polymerization (Fig. 4). This was evaluated in a standard self-assembly assay (37 °C) in which the products are separated by sedimentation and evaluated by SDS-PAGE. Lm\_LN-L4b did not form a polymer when incubated alone. However, when Lm\_LN-L4b and \alpha LNNd were incubated in increasing equimolar concentrations, they co-sedimented in the polymer fraction in a concentration-dependent fashion with apparent critical concentrations (0.06 and 0.08 M) similar to that observed with laminin-111 (0.07 and 0.08 M). \alpha LNNd did not appear to adversely affect the polymerization of (wt) laminin.
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FIGURE 3. Binding of αLNNd to laminin-111 and type IV collagen. Panel A shows plots of the binding of αLNNd (closed circles) and nidogen-1 (Nd, open circles) to immobilized laminin-111 with bound protein detected at 492 nm after treatment with nidogen-specific antibodies (average and S.D., n = 3). No binding (single measurements) was detected with laminin-111 (closed triangles) or nidogen-1 (open triangles) on albumin (BSA)-coated wells. Data fitted for single-ligand binding (fitted half-maximal binding of 0.3 nM for αLNNd and nidogen-1). Panel B shows plots of the binding of αLNNd (closed circles) and nidogen-1 (open circles) to immobilized type IV collagen (average and S.D., n = 3). Half-maximal binding was fitted to 0.3 nM for αLNNd and 1.6 nM for nidogen-1. No binding (single measurements) was detected on BSA-coated wells. Panel C shows plots of type IV collagen binding to immobilized laminin-111 (through ternary complexes) mediated by the presence of either αLNNd (closed circles) or nidogen-1 (open circles) applied at constant concentration (33 nM). Coated wells were incubated with type IV collagen at the indicated concentrations followed by determination of bound protein (average and S.D., n = 3). Half-maximal binding was fitted and found to be 1.4 nM for αLNNd and 1.5 nM for nidogen-1. Chimeric αLNNd, like nidogen-1, showed binding activity for both laminin and type IV collagen and was able to mediate formation of ternary complexes.

111 (critical concentration of 0.06 μM) despite the addition of a fourth short arm creating a laminin complex with two αLN domains. Incubation of LmΔΔLN or LmΔLN-L4b with αLNNd did not enable polymerization. This was interpreted as evidence that αLNNd is only able to rescue a polymerization deficit arising from deletion resulting in loss of the LN domain (LmΔLN and LmΔLN-L4b).

Binding of wt Laminin, LmΔΔLN-L4b, and mAgrin to Sulfatides—The binding of the agrin NtA domain to the coiled-coil domain of laminin-111 through the 1-subunit (KD 2 nM) and of the non-neural agrin LG domains (KD 2 nM) to dystroglycan has been previously described (33–35). Schwann cells contain galactosyl sulfatide that provides cell surface binding to laminins (5). To determine whether mAgrin and αLNNd bind to sulfatides, these proteins and different recombinant laminins were evaluated with a solid-phase assay (Fig. 5). Fitted half-maximal binding values (apparent KD) were determined for mAgrin (0.06 μM), αLNNd (0.02 μM), (wt) laminin-111 (critical concentration of 0.06 μM) despite the addition of a fourth short arm creating a laminin complex with two αLN domains.
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Laminin and Miniagrin have been found to be decreased when sulfated-galactosyl ceramide was diluted into non-sulfated galactosyl ceramide. Structural analysis of the laminin α1 LG4 domain has revealed several lysines and arginines that interact with the small sulfatides and is consistent with the hypothesis that each LG domain engages several glycolipid sulfates (38). Therefore, the decreases in binding in the assay may arise from the loss of sulfate charge density available to bind to the LG4 protein patch. Although the sulfatide composition of Schwann cell surfaces has not been determined, it is likely that it much less than 100%, and therefore, the effective laminin and agrin affinities may be lower than that detected with pure lipid. A caveat is that if the sulfatides are organized into compact rafts, the higher affinities could be preserved.

Laminin Accumulation on the Surface of Cultured Schwann Cells—It was previously found that Schwann cells treated with exogenous laminins assemble a thin basement membrane on the free cell surface in a process dependent upon interactions of the LG domains with cell surface sulfatides and upon the ability of laminins to polymerize (5, 6). Laminin assembly was found in turn to enable the incorporation of collagen IV in the presence of nidogen-1. To determine whether αLNNd and mAgrin were capable of affecting laminin assembly on cells through their respective capacities to alter polymerization and adhesive interactions, Schwann cells were incubated with laminins bearing deletions of different domains in either the presence or absence of the above laminin-binding proteins (Figs. 6–8).

Cells near confluency were incubated for an hour with proteins diluted into the culture medium, washed, fixed, and treated with antibody to laminin followed by detection with a fluorescent secondary reagent. The average cell fluorescent intensities (average and S.D. of sums of pixel intensities/cell) were determined from the antibody-stained images and plotted (Figs. 7 and 8). Treatment of cells with laminin-111 (wt) resulted in the accumulation of laminin (Figs. 6, A and B, and 7A) as reported previously (6). In contrast, treatment with the non-polymerizing laminins LmΔαLN-L4b, LmΔαLN, or the chimeric fusion protein αLNNd (detected with nidogen-specific antibody) resulted in almost no detectable protein (Fig. 6, E and J). However, when LmΔαLN-L4b or LmΔαLN was mixed with αLNNd in equimolar concentrations (14 nM), laminin fluorescence was substantially increased (Fig. 6, F and J, and Fig. 7, A–C) to levels approaching (~60–70% in different experiments) of (wt) laminin. Laminin accumulation on cells increased as a function of increasing concentration (Fig. 7A) and was accompanied by a corresponding increase in nidogen epitope (B) located on αLNNd. αLNNd was specific in its ability to improve laminin accumulation on cells as it had no appreciable effect when incubated with laminins bearing deletions of the β-LN or γ-LN domains or bearing an incomplete complement of α, β, and γ-LN domains or an incomplete set (α-α-β and α-β-β) of domains when combined with αLNNd (Fig. 7C). αLNNd caused a slight increase in wt laminin fluorescence that could be related to the small increase seen in the polymerization slope (Fig. 7D).
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FIGURE 6. Laminin and type IV collagen assembly on cell surfaces. Schwann cells were incubated with the indicated components (14 nm each unless otherwise indicated) for 1 h, washed, fixed, and immunostained for laminin, agrin, nidogen/entactin, and/or collagen IV and counterstained with 4′,6-diamidino-2-phenylindole (blue). Panels A, B, E, F, I, and J. Cells were untreated (NT), treated with (wt) laminin, LmA(LN-L4b), LmA(LN-L4b + αLNNd), LmαLN, and LmαLN + αLNNd and immunostained with anti-Lmγ (green) antibody. Increased laminin immunofluorescence was detected when αLNNd was coincubated with laminins lacking the αLN domain. Panels M and N, αLNNd was not detected on cells when incubated alone (anti-entactin) but was detected colocalized with laminin when co-incubated with LmαLN. Panels C, D, G, and H, cells were treated with LmαLG, LmαLG + mAgrin (mA) and stained with antibodies for laminin (anti-Lmγ, green) or agrin (red). Increased laminin and mA immunofluorescence was detected when mA was incubated with LmαLG. Panels K and L, cells were incubated with Lmα(αLNNdLG) + mA or with Lmα(αLNNdLG) + mA + αLNNd and stained with antibody for Lm. Increased laminin immunofluorescence was detected when Lmα(αLN&LG) was co-incubated with mA and αLNNd but not when incubated only with mA. Panels M, and N, αLNNd (M; 14 nm) incubated alone (M, anti-entactin, red; anti-Lmγ, green) or with LmαLN (N; 14 nm). Little αLNNd accumulated on cell surface in the absence of the laminin. Panels O and P, when Myc-tagged laminin (56 nm) was incubated, both Myc and LG4–5 (E3) epitopes were detected. When Myc-tagged LmαLG (28 nm) was incubated with Myc-free laminin (28 nm), only the LG4–5 epitope (compared with Myc) was detected on the cell surface. Panels Q–V, cells were immunostained for collagen IV (red) after treatment with LmαLN-L4b + collagen IV (Col-IV), laminin + Col-IV, LmαLN-L4b + Col-IV + nidogen (Nd), laminin + Col-IV + Nd, LmαLN-L4b + Col-IV + αLNNd, and laminin + Col-IV + αLNNd. Increased collagen IV was detected with either αLNNd with non-polymerizing laminin or nidogen with either wt or non-polymerizing laminin.

The contribution of mAgrin to the accumulation of laminins on Schwann cells was also examined (Figs. 6, C, D, G, and H, and 8). LmαLG failed to accumulate on cells, even at concentrations as high as 28 nm (Fig. 8A). Similarly, very little mAgrin was detected on cell surfaces when added without laminin (Fig. 6G). In contrast, coincubation of LmαLG with mAgrin resulted in the accumulation of both laminin and mAgrin (Fig. 6, D and H) in a concentration-dependent fashion (Fig. 8, A and B). The addition of mAgrin to (wt) laminin caused only a slight increase over that seen with the laminin alone (Fig. 8C). On the other hand, the addition of mAgrin to a non-polymerizing laminin did not lead to increased laminin accumulation (Fig. 8D).

The ultrastructure of cells treated with wt laminin, LmαLN-L4b, LmαLG, LmαLN-L4b + αLNNd, and LmαLG + mAgrin was evaluated after incubation of the components (14 nm) for one hour (supplemental Fig. S1). Thin basement membrane-like linear electron-dense matrices (lamina densa), separated from the cell surface by an electron lucid zone (lamina lucida), were detected in lengths of several μm or more after treatment with αLNNd + LmαLN-L4b or with mA + LmαLG that was similar to the matrix formed with (wt) laminin and that was absent after treatment with the defective laminins in the absence of the synthetic linker proteins.

Because there appear to be separate polymerization and adhesive contributions required for laminin assembly on Schwann cell surfaces, it seemed reasonable to expect that a laminin that lacked both an LN domain and LG domains would be able to accumulate on cells only in the presence of both αLNNd and mAgrin. The recombinant laminin LmΔ(αLN&LG) was generated to evaluate this possibility. When LmΔ(αLN&LG), co-purified with αLNNd, was incubated with cells, the laminin failed to accumulate at different concentrations (Figs. 6K and 8D). However, when αLNNd-LmΔ(αLN&LG) was also mixed with mAgrin, laminin accumulation was observed on cells (Fig. 6L) in a concentration-dependent fashion (Fig. 8, D and E).

We asked whether laminins lacking LG domains can accumulate on cells by co-polymerization with intact laminins. To address this possibility, LmαLG (20 μg/ml, Myc-tagged) was mixed with (wt) laminin (20 μg/ml and compared with Myc-tagged...
detected on cell surfaces after treatment with collagen IV in the presence of either wt laminin with nidogen-1 or nonpolymerizing laminin with αLNNd. The collagen levels detected with the latter were substantial (½ to ¾ of wt laminin plus nidogen). When mAgrin was incubated with LmΔLG, nidogen, and collagen, near-normal (i.e. wt) levels were detected (Fig. 9C). In agreement with earlier observations (6), non-polymerizing laminin, itself retained at very low levels relative to wt laminin, can maintain substantial accumulation when mAgrin was incubated with LmΔLG (14 nM) and detected with antibody for laminin. LmΔLG alone (open circles) is shown. mAgrin co-accumulated with LmΔLG in a concentration-dependent manner. Panel C, left, plot (n = 3) of the indicated laminins (14 nM) incubated alone, with αLN (14 nM), or with mAgrin (14 nM). Increased laminin accumulation occurred when non-polymerizing laminin was incubated with αLNNd or when LmΔLG was incubated with mAgrin but not when LmΔLN was incubated with mAgrin or when LmΔLG was incubated with αLNNd. Right, plot of laminin-111 (14 nM, n = 6) incubated without or with mAgrin (13 nM). Panel D, cell surface accumulation of LmΔLG (closed inverted triangles) and LmΔLN (open circles). The cell accumulation of nonpolymerizing/non-adhesive laminin treated with either αLNNd alone (closed inverted triangles), co-purified with equimolar LmΔLG or with mAgrin + αLNNd (open triangles) was compared with that of LmΔLG treated with mAgrin (open circles, constant 26 nM). Panel E, laminin (open circles) and mAgrin (closed circles) immunofluorescence were compared for both mAgrin + LmΔLG (left) and mAgrin + αLNNd + LmΔLG (right). The laminin with combined deletions inactivating polymerization and adhesion could only be rescued with a mixture of αLNNd and mAgrin. The laminin and mAgrin epitopes accumulated together with a near-constant ratio. Panel F, laminin and LmΔLG were varied with respect to each other (summed concentration maintained at 14 nM) and detected with antibody for laminin. LmΔLG was unable to accumulate on cell surfaces even in the presence of wt laminin.}

**FIGURE 7. Contribution of αLNNd to laminin accumulation on Schwann cell (SC) surfaces.** Cells were cultured with the indicated proteins for 1 h and immunostained for the laminin B1 subunit (E4-specific antibody). Panels A and B, quantitation of laminin (A) and corresponding entactin antibody (B) immunofluorescence summed cell intensities for laminin (Lm, closed circles), αLNNd (constant 26 nM) with increasing LmΔLN (closed inverted triangles), nidogen-1 (26 nM) with increasing LmΔLN (open circles), and LmΔLN alone (open circles) (average and S.D., n = 5). The addition of αLNNd, but not nidogen-1, to the non-polymerizing LmΔLN enabled self-assembly to a degree approaching that of intact laminin. Panels C and D, cells were incubated with the indicated proteins (constant 14 nM laminin and 14 nM αLNNd). αLNNd enabled the cell surface assembly of laminins bearing deletions of the αL domain but not deletions of the βLN or γLN domains.

**FIGURE 8. Contribution of mAgrin to laminin accumulation on Schwann cell (SC) surfaces.** Cells were incubated followed by detection of adherent protein as in the Fig. 7. Laminin (panel A) and mAgrin (panel B) immunofluorescence (average and S.D., n = 8) of cells incubated with increasing concentrations of LmΔLG with mAgrin (mA constant 26 nM; closed inverted triangles), (wild-type) laminin-111 (closed circles), or LmΔLG alone (open circles) is shown. mAgrin co-accumulated with LmΔLG in a concentration-dependent manner. Panel C, left, plot (n = 3) of the indicated laminins (14 nM) incubated alone, with αLN (14 nM), or with mAgrin (14 nM). Increased laminin accumulation occurred when non-polymerizing laminin was incubated with αLNNd or when LmΔLG was incubated with mAgrin but not when LmΔLN was incubated with mAgrin or when LmΔLG was incubated with αLNNd. Right, plot of laminin-111 (14 nM, n = 6) incubated without or with mAgrin (13 nM). Panel D, cell surface accumulation of LmΔLG (closed inverted triangles) and LmΔLN (open circles). The cell accumulation of nonpolymerizing/non-adhesive laminin treated with either αLNNd alone (closed inverted triangles), co-purified with equimolar LmΔLG or with mAgrin + αLNNd (open triangles) was compared with that of LmΔLG treated with mAgrin (open circles, constant 26 nM). Panel E, laminin (open circles) and mAgrin (closed circles) immunofluorescence were compared for both mAgrin + LmΔLG (left) and mAgrin + αLNNd + LmΔLG (right). The laminin with combined deletions inactivating polymerization and adhesion could only be rescued with a mixture of αLNNd and mAgrin. The laminin and mAgrin epitopes accumulated together with a near-constant ratio. Panel F, laminin and LmΔLG were varied with respect to each other (summed concentration maintained at 14 nM) and detected with antibody for laminin. LmΔLG was unable to accumulate on cell surfaces even in the presence of wt laminin.
A study of two synthetic linker proteins that add functional activities to laminins and restore the ability of deficient laminins to assemble a basement membrane-like extracellular matrix on Schwann cells has provided insights into the mechanisms of basement membrane assembly. A model (Fig. 10) consistent with these findings and supported by our earlier studies is that a laminin initiates assembly by attaching to the cell surface through sulfatides (and also to \(\alpha\)-dystroglycan to the degree to which it is present) and by forming linkages among adjacent laminins through polymerization. Nidogen-1 binds to laminin and also to type IV collagen, increasing the surface concentration of the latter and thereby promoting its own polymerization. Agrins further stabilize the laminins by binding to them and to the cell surface.

The study adds several elements to our understanding of laminin interactions and assembly. First, the data support, through a gain-of-function analysis, that the laminin polymer is formed by the binding of \(\alpha\), \(\beta\) and \(\gamma\) LN domains into a ternary domain complex. An absent \(\alpha\) LN domain, but not an absent \(\beta\) LN or \(\gamma\) LN, could be replaced with the missing \(\alpha\) LN domain with restoration of self-assembly. It is interesting that placement of the synthetic linker arm at the nidogen-binding site located in domain ELe3 of the \(\gamma\) subunit near the intersection of the three short arms created a third arm sufficiently similar to the defective native arm to provide the activity. Furthermore, the internal L4a, L4b, L4c, and L4c of the \(\alpha\) subunit are largely dispensable for polymerization. These internal domains may serve primarily to add length to the short arm, affecting the spacing of laminins within the polymer but might also contribute to polymer stability. Of note, binding of \(\alpha\)LNNd to wt laminin, which adds a fourth short arm duplicating the \(\alpha\) LN domain, was not deleterious for polymerization and slightly enhanced it. The polymerization slope increase seen with recombinant and to a lesser extent with EHS-laminin may be the consequence of a fractional reduction of activity within the LN domain.
A study of interactions between laminin LN-LEa fragment pairs, in which binding was detected for α1LNLEα-α1LNNEα pairs, led to a less-restricting hypothesis of assembly in which a laminin polymer could form with ternary complexes that lacked an α-β-γ composition (39). However, a subsequent domain loss-of-function analysis conducted with heterotrimeric laminins failed to support this alternative (6). The current study now provides gain-of-function evidence that a strict α-β-γ short arm complex is required. The possibility that the α-α subunit interaction is involved in the attachment between laminin polymer layers rather than polymerization per se presents itself as an interesting alternative hypothesis to explain its self-binding.

The linker protein αLNNd was able to largely, but not completely, restore laminin assembly on cell surfaces when coupled to laminin molecules lacking the αLN domain or entire α-short arm. The incompleteness of the rescue may be a consequence of instability in the recombinant linker protein, incorrectness of the length of the linker placement of αLN to the other LN domains, or a missing contribution from the internal α-short arm domains. Although the lack of change of the critical concentration for the linked laminin compared with wt laminin is more compatible with the first interpretation, further study will be required to resolve the question. Preservation of the type IV collagen binding sites of nidogen G2 and G3 domains within αLNNd allowed for the linked laminin complex to recruit type IV collagen to the cell surface in the absence of nidogen. The ability of nidogen to compete for αLNNd accumulation on cells was considerably reduced in the presence of type IV collagen.

mAgrin, an internally truncated protein that binds strongly to the coiled-coil domain of laminin, to sulfatides, and to α-dystroglycan can also enhance basement membrane assembly. In particular, we found that it enables the poorly adhesive laminin LmΔLG to become anchored to the cell surface, assemble, and recruit nidogen and type IV collagen. Anchorage, however, was found to be sufficient for such assembly on Schwann cells only in the presence of LN-mediated polymerization. This limitation was revealed by the specificities of laminin rescue and with a laminin that lacked both an α-LN domain and LG domains with assembly restoration only if mAgrin and αLNNd were co-cultivated with the doubly truncated LmΔ(αLN&LG).

Earlier analysis of the cultured Schwann cells revealed that sulfatides constitute the principal contribution for laminin anchorage (to be distinguished from signaling contributions) and not α-dystroglycan, β1-integrins, or heparan sulfates (5). In another (breast epithelial) cell line, a greater dystroglycan contribution for laminin accumulation was reported (25). The explanation for the observed differences may lie in the relative surface density of these different molecules, all capable of laminin binding through LG domains, i.e. in Schwann cells there are too few dystroglycan molecules available for laminin binding compared with available sulfatide molecules for dystroglycan to serve as principal anchor. However, it seems a reasonable expectation that in some tissues dystroglycan (notably in muscle) or integrins will be found to serve as the chief anchoring species because of their abundance. The significance of these receptors is that they can link the basement membrane to the underlying cortical cytoskeleton to stabilize the basement membrane-cell interface. mAgrin is an interesting linker protein in that it binds to γ1-laminins, α-dystroglycan, sulfatides, and an integrin and may well be suited to provide an anchorage function for laminins in different cellular environments.

A model refinement to be considered is based on the observations that sulfatide binding contributions also arise from αLN domains (this study and Garbe et al. (36). These domains may provide supplemental adhesion of laminin molecules to the cell surface such that contacts form with both LG and αLN. Nonetheless, adhesion through LG must occur for laminin to accumulate on cell surfaces. Furthermore, polymerization is required in addition to αLN and LG for significant assembly on cell surfaces and cannot be accomplished with laminins that only possess αLN and LG domains.

Both this study and that of McKee et al. (6) have revealed a contribution of type IV collagen and nidogen in which a collagen-rich laminin-poor discontinuous extracellular matrix can form on a cell surface in the absence of laminin polymerization and presence of collagen and nidogen. The collagen levels achieved under these circumstances have been found to be about half that with a polymerizing laminin. In contrast, cell surface of laminin or collagen has not been observed to any appreciable degree with a non-adhesive laminin (without LG domains) on Schwann cells.

In summary, domain-modified laminin-111 proteins were used in this study to identify key self-assembly and anchoring activities that distinguish the two synthetic linker proteins and to analyze their contributions to basement membrane assembly in a model culture system. A question that arises is how predictive are these findings for complex basement membranes of different tissues? Of particular interest, from a human disease standpoint, are the basement membranes of the Schwann cell endoneurium in nerve and the sarcolemma in skeletal muscle. Both of these basement membranes are defective in the MDC1A congenital muscular dystrophies and mouse models that result from null, hypomorphic, and domain-altering mutations of the gene coding for the laminin α2 subunit. Laminin-211 is the principal laminin of these basement membranes. However, α4- and α5-laminins are expressed in the dystrophy (23, 40–42). α4-laminins lack the α-subunit short arm for polymerization and bind less well to α-dystroglycan and sulfatides (21, 22), whereas laminin-511, thought to polymerize, has also reduced binding to these components (43). What then is the basis for a mAgrin rescue of muscle and its failure (so far) to rescue in nerve (23, 24, 40)? One possible explanation for the observation in peripheral nerve is that mAgrin cannot rescue an α2-defect through α4-laminin. The phenotypic rescue in muscle, on the other hand, is substantial. It has been suggested that mAgrin accomplishes this in muscle through its interaction with α4-laminins and dystroglycan (23). This would appear to be a reasonable interpretation if the only defect is in adhesion. However, if laminin polymerization is also important, then the rescue of α4-laminin would be insufficient. An alternative possibility, consistent with the findings of this study, is that the rescue is mediated through α5-laminins, a laminin found to increase in the sarcolemmal basement membrane after mAgrin treatment (23). In nerve, there may be too little α5-laminin available for binding to mAgrin to mediate rescue of radial sort-
ing. A caveat to consider in comparing the effects of mAgrin on nerve and muscle is that these tissues may contain different densities of anchors/receptors that could differentially effect a requirement for polymerization. The model does not rule out the possibility that a sufficiently high density distribution of a high affinity surface component could reduce the requirement for polymerization by enabling binding of a sufficiently dense distribution of laminin molecules to form a stable matrix in the absence of the contribution provided by polymerization (6).

A related question is whether aLNNd, like mAgrin, holds potential to ameliorate the radial sorting defect and muscle pathology seen in laminin-α2 deficiency states. The most obvious situation in which one might expect aLNNd to beneficially affect nerve and muscle is with the dystrophic mouse that arises from an in-frame deletion within the α2LN domain (44). In those variants of the syndrome in which there is little or no laminin-α2 expression, one might predict that such amelioration would be less likely, especially if the repair is mediated by binding of aLNNd to laminin-411. The deficit of anchorage for this laminin would remain uncorrected. On the other hand, the combined action of mAgrin and aLNNd might efficiently convert laminin-411 into a strongly adhesive polymerizing laminin.

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