Contributions of the LG Modules and Furin Processing to Laminin-2 Functions*

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The a2-laminin subunit contributes to basement membrane functions in muscle, nerve, and other tissues, and mutations in its gene are causes of congenital muscular dystrophy. The a2 G-domain modules, mutated in several of these disorders, are thought to mediate different cellular interactions. To analyze these contributions, we expressed recombinant laminin-2 (a2β1γ1) with LG4–5, LG1–3, and LG1–5 modular deletions. Wild-type and LG4–5 deleted-laminins were isolated from medium intact and cleaved within LG3 by a furin-like convertase. Myoblasts adhered predominantly through LG1–3 while α-dystroglycan bound to both LG1–3 and LG4–5. Recombinant laminin stimulated acetylcholine receptor (AChR) clustering; however, clustering was induced only by the proteolytic processed form, even in the absence of LG4–5. Furthermore, clustering required αβ1 integrin and α-dystroglycan binding activities available on LG1–3, acting in concert with laminin polymerization. The ability of the modified laminins to mediate basement membrane assembly was also evaluated in embryonic bodies where it was found that both LG1–3 and LG4–5, but not processing, were required. In conclusion, there is a division of labor among LG-modules in which (i) LG4–5 is required for basement membrane assembly but not for AChR clustering, and (ii) laminin-induced AChR clustering requires furin cleavage of LG3 as well as α-dystroglycan and αβ1 integrin binding.

The laminin a2-chain, a subunit of laminin-2 (a2β1γ1) and laminin-4 (α2β2γ1), is expressed in the basement membranes of skeletal muscle, peripheral nerve, brain, and placenta (1–3). a2-Laminins, similar in molecular morphology and functional attributes to a1-laminin, are thought to play important roles in basement membrane assembly and the maintenance of the neuromuscular axis (reviewed in Ref. 4). Mutations in the laminin a2-subunit are a cause of a human congenital muscular dystrophy typically characterized by early onset, severity, patchy peripheral nerve dysmyelination, and decreased complexity of infoldings and post-junctional membrane lengths of the NMJ motor endplate (6, 8–12).

Studies on cultured myotubes have revealed that a1- and a2-laminin polymerization and G-domain contributions drive laminin assembly on the cell surface and direct a redistribution of interacting cytoskeletal components (13). The ability of laminin to induce such cytoskeletal reorganizations is thought to reflect an important receptor-dependent property of laminin during early steps in assembly of a basement membrane. It has also been shown that laminins can induce the clustering of the acetylcholine receptor (AChR), a property shared with agrin (14). However, while agrin is secreted by terminal neurons and triggers this differentiation in a MuSK-dependent and topographically site-specific manner, the activity of laminins is MuSK-independent and topographically diffuse (8, 14–17). The laminin activity has been found to depend on binding contributions for αβ1 integrin and dystroglycan and appears to synergistically enhance the role of agrin (16, 17).

Although the laminin a2 chain is closely related to the embryonic-type laminin-a1 chain, and although both laminins are capable of polymerization mediated through short-arm domains (18), it is different in that the a2-chain becomes proteolytically cleaved within G-domain and has a different distribution of dystroglycan- and heparin-binding sites. Laminin’s biologically relevant functions are thought to derive from coordinated contributions from different domains of all three subunits (4). To analyze the LG subdomain dependence of these functions in a2-lamins, we generated recombinant laminins of the subunit composition α2β1γ1 bearing three deletions of the LG modules. These laminins were proteolytically processed in LG-3 by a furin-like convertase, and both unprocessed and processed forms were isolated for study. Using these reagents, we investigated their effects on cultured myotubes and laminin γ1-null embryoid bodies, the latter a model system with which to evaluate basement membrane assembly. We report that laminin-2 binds to cell surfaces, mediates basement membrane assembly, and induces AChR clustering. While all activities depend on LG1–3, AChR clustering requires only LG1–3. Furthermore, furin processing is needed for AChR clustering but not for basement membrane assembly.

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**EXPERIMENTAL PROCEDURES**

**Human DNA Constructions**

Lm-β1/pCEP4—A 5.5-kb BamHI fragment containing the full-length human β1 cDNA (19, 20) was isolated from a plasmid and inserted into the pCEP4 (puromycin) vector. Laminin β1 cDNA with a 3′-terminal FLAG sequence preceded the stop codon. PCR was accomplished with the following primers:

- 5′-CTCCTACTGCTGGG-3′
- 5′-GGGATCCTACTTGTCATCGTCGTCCTTGTAGTCGGGCTTTTCAATCGTCACTGCTGACTACGCCCAGCAAAGAGGTTTATTCCCT-3′

Lam-β1FLAG-pReCMV—The γ1-chain cDNA (21) in pVL941 was modified to contain a nucleotide sequence encoding the FLAG epitope. Laminin γ1 cDNA with a 3′-terminal FLAG sequence was prepared from plasmid pVL941 by replacing a Sau3A-SnaBI fragment containing the γ1 cDNA with the latter digested PCR product synthesized using primers 5′-CACTGCTCTGGCAGCA-3′ and 5′-GGGGCTCTGGA-CTGGTACACTGCTGTTGACCAAGAGATTGTCTTCC-3′ containing an HA-TAG and BM40 (SPARC) signal peptide and 5′/H11032.

PCR was accomplished with cDNAs coding for the full protein sequence (2, 22). For this purpose a 5′-CTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCTTACCCTTACGATCATGAGGGCCTGGATCTTCTTTCTCCTTTG-3′ cDNA sequences was used together with a second primer 5′-CTCCTACTGCTGGG-3′. cDNA fragments were isolated and expanded. Laminin-2/4 and E4 specific antibodies, the latter recognizing epitopes from the pVL941 plasmid by replacing a BamHI/SnaBI fragment with 0.1 mg/ml solution of FLAG peptide (Sigma, 25 ml). The eluates were purified by HPLC affinity chromatography using a heparin-SPW (0.8 × 7.5 cm; Tosoh-Haas) equilibrated in 50 mM Tris-HCl, pH 7.4, 1 ml EDTA. The column was eluted with a shallow NaCl gradient and fractions from the separated peaks were collected.

**Proteins and Antibodies**

Laminin-1 (DEAE-unbound fraction) and laminin-2/4 were purified from mouse EHS tumor and human placenta as described (18). Laminin-1 fragment E1′ (short arm complex) was purified from EHS tumor (26) and AEBSF-E1′ (nonpolymerization inhibition control) was prepared by treatment of E1′ with 5 mM aminoethylbenzenesulfonfluoride (13). Polyclonal antibodies specific for laminin-1, laminin-2/4, and laminin α2-G domain have been previously described (18, 24). Immunoprecipitation was accomplished by incubating ~5 ml of conditioned medium with 10 μg/ml primary antibody followed by precipitation with 50 μl of a 50% suspension of either protein A-Sepharose or anti-FLAG agarose beads. Mouse monoclonal antibodies specific for HA (clone 12CA5) and the C-terminal region of β-dystroglycan (used at 1:25 dilution) were purchased from Roche Molecular Biochemicals and Novocea. Hamster monoclonal IgG specific for the β1 integrin subunit was used at ~20 μg/ml for blocking experiments, and at 5 μg/ml for indirect immunofluorescence (Ha2/5, PharMingen, San Diego, CA). Monoclonal antibodies used to inhibit integrin-αv (mouse), integrin-αt (mouse), integrin-β1 (hamster), and α-dystroglycan (mouse) binding to laminin were, respectively, GoH3 (BD PharMingen, CA), Ha2/5 (BD PharMingen), O26 (used at 30 μg/ml; gift of Steven Kaufman, University of Illinois; Ref. 27), and IIH6 (hybridoma conditioned medium used at a 1:10 dilution, gift of Kevin Campbell, University of Iowa).

**Biochemical Assays and Rotary Shadowing**

Dystroglycan Binding Assay—96-Well polystyrene plates were incubated overnight at 4 °C with 0.5 μg/well of either purified laminins or bovine serum albumin (Sigma) diluted in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA. Laminin-coated wells were blocked for 2 h at room temperature with 1% bovine serum albumin in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, followed by a 2-h incubation with varying concentrations of iodinated α-dystroglycan dilitated in blocking solution. Wells were washed with blocking solution, removed, and bound α-dystroglycan radioactivity was measured. Non-specific binding of labeled α-dystroglycan to bovine serum albumin-coated wells was subtracted (28).

Co-polymerization—This assay, used to detect laminin-specific self-assembly with small amounts of test laminin, depends on the ability of the two proteins to co-aggregate in a concentration-dependent manner with polymerization driven by the EHS-laminin (18). Briefly, aliquots of EHS-laminin in polymerization buffer were incubated with a fixed concentration of recombinant laminin at 37 °C. Samples were centrifuged and the supernatant and pelleted fractions analyzed by SDS-PAGE under reducing conditions. SDS-PAGE was carried out in 3.5–12% linear gradient gels (26) and stained with Coomassie Blue R-250 or transferred to nitrocellulose membranes for immunoblotting. Immunoblotting of proteins was performed as described (26).

Rotary Shadowing—Laminin (25–50 μg/ml in 0.15 x ammonium bicarbonate, 60% glycerol) was sprayed onto mica discs, evacuated in a Balzers BAF500K unit, rotary shadowed with 0.9 nm Pt/C at an 8° angle, backed with 8 nm carbon at a 90° angle, and viewed in an electron microscope as otherwise described (26). Electron micrograph images are shown contrast-reversed.

**Cell Culturing and Analysis**

Mouse C2C12 and rat L6/E63 myoblasts were cultured in medium containing 10% fetal calf serum. The former were converted to myotubes in 5% horse serum, and analyzed 3–5 days post-fusion as described (13). For quantitation of myoblast adhesion, cells labeled with [3H]thymidine were incubated (70 mCi) in tissue culture wells (3 × 104 cells/well) pre-treated with nitrocellulose and coated with laminins as

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described (29) followed by determination of bound radioactivity. Fused myotubes were incubated at 37 °C with laminins (10 μg/ml) suspended in Dulbecco’s modified Eagle’s medium F-12 containing 0.5% bovine serum albumin. Unattached protein was removed by washing with PBS containing 1 mM CaCl2. Blocking antibodies and fragments in 100-fold molar excess, when used, were added to the culture medium prior to addition of laminin at the following final concentrations: IIH6 (1/10 dilution), HA5/2 and GoH3 (10 μg/ml), and E1’ (500 μg/ml). Wild type R1 and laminin-γ1 null ES cells (30) were grown on feeder layers of mitomycin-treated (100 μg/ml, 2 h) SNL STO cells in ES medium (Invitrogen) supplemented with 15% ES-grade fetal calf serum (Invitrogen). 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 100 μg of penicillin/ml, 100 μg/ml streptomycin, and 1000 units/ml leukemia inhibitory factor (LIF, Invitrogen). To culture EBs, subconfluent ES cells were dispersed with 0.25% trypsin, 0.53 mM EDTA and plated onto gelatin-coated dishes (3 h) to allow feeder cells to attach. Nonadherent ES aggregates were dispersed and cultured on bacteriological Petri dishes in ES medium without LIF.

**Microscopy**

Following incubation, myotubes were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, blocked in PBS containing 0.5% bovine serum albumin and 5% normal goat serum, and incubated with primary antibodies diluted in the same buffer. Following several washes, the cells were incubated with FITC- or Cy3-conjugated secondary antibodies for 1 h at room temperature, washed, and overlaid with DABCO, mounted, and examined with an Olympus IX-70 inverted fluorescent microscope fitted with a Princeton Instruments 5 mHz Micromax cooled CCD camera. Myotube surface coverage by laminins was determined from digital images with IPLab version 3.0 (Scanning) using segmentation parameters adjusted to detect laminin-covered areas from background. The automatically marked areas were quantitated and the average area and S.E. were determined (n = 6–10 fields). Rat monoclonal anti-laminin γ1 (clone A5; Upstate Biotechnology, Lake Placid, NY), rabbit anti-mouse type IV collagen antibody (Rockland Immunocompounds, Gilbertville, PA), and rat anti-mouse perlecann monoclonal antibody (Chemicon, Temecula, CA) were used for immunostaining of EBs at 1, 2.5, 2 and 2.5 μg/ml, respectively.

**Acetylcholine Receptor Clustering**—C2C12 myotubes were incubated for 18 h in the presence of either different concentrations of rLm2 recombinant or other laminins, without or with blocking reagents. Myotubes were then incubated for 1 h with 5 μg/ml FITC-a-bungarotoxin (Molecular Probes) to label clusters of AChR’s, washed 4 times with PBS, 1 mM CaCl2 to remove unattached protein, and fixed. Myotubes were incubated with antibody IIH6 (1/10 dilution from conditioned medium), washed, and then treated with goat anti-mouse Cy3 (1:100). Confocal images of fixed cells were acquired using a laser confocal microscope (Zeiss LSM 510) to determine the number of clusters per field. Only AChR clusters that co-localized with dystroglycan were scored.

Myotubes were collected by gravity sedimentation, washed, and fixed with 3% paraformaldehyde in PBS followed by incubation in 7.5% sucrose-PBS (3 h) and 15% sucrose (4°C, overnight). Frozen sections were prepared with nonspecific binding sites blocked with 5% goat serum, 100 mM NaCl, and 0.1% Triton X-100. Sections were incubated with primary antibodies diluted in the same buffer. Following several washes, the sections were incubated with FITC- or Cy3-conjugated antibodies. For immunostaining, Cy3-conjugated goat anti-mouse IgG was added to determine the number of clusters per field. Only AChR clusters that colocalized with dystroglycan were scored.

**Electron Microscopy**—Electron microscopy of thin sections was carried out as described (31).

**RESULTS**

Expression of Recombinant Laminin Subunits in 293 Cells—We followed a two-step transfection protocol (24) to express recombinant heterotrimeric laminin. 293 cells stably expressing laminin γ1 and selected with the antibiotic G418 were transfected with the β1-expression vector and α2-expression vectors coding for wild-type protein or protein bearing deletions within the LG modules (Fig. 1). Clones expressing protein with epitope tag were generated in parallel with highly efficient expression of γ1 bearing a C-terminal FLAG tag. The latter proved to be more successful for the preparation of heterotrimeric laminins and furthermore, permitted purification from serum-containing medium. For the α2 deletion mutants and a wild-type control, a hemagglutinin (HA) tag was placed N-terminal to the mature sequence. Wild-type (WT) recombinant protein and all protein bearing LG module deletions (ΔG1–5, ΔG1–3, and ΔG4–5) were secreted into the culture medium.

Heterotrimeric laminin expression was initially detected by the ability of α2 chain and β1 chain-specific antibodies to react with the respective laminin subunits first immunoprecipitated with FLAG antibody (data not shown). The α2 chain bands reacted with both antibodies specific for laminin-2/4 and laminin-α2-G domain and the β1 chain was identified under nonreducing conditions (necessary for antibody reactivity) with E4-specific antibody.

**Heparin Interactions and Identification of Unprocessed and Processed α2 Chains**—For purification (Fig. 2), recombinant laminins in conditioned medium were initially bound to anti-FLAG antibody beads and eluted with FLAG peptide, facilitating subsequent steps by eliminating contaminating serum and secreted proteins. The eluted fraction was bound through its α-subunit to a heparin-5PW column and eluted with a salt gradient (Fig. 2a). Two wild-type and rLm2-G4–5 protein peaks each were eluted at 0.16 and 0.33 M NaCl, with relative peak sizes varying among different media collections. The different fractions were analyzed by reducing SDS-PAGE (Fig. 2, b–d). The more weakly bound wild-type laminin fraction was found to consist of laminin-2 similar to that obtained from placenta and consisting of a -275-kDa (processed, "p") α2 band (previously designated α2m in Ref. 18) and ~75-kDa fragment band (known as a C-terminal moiety) with the β1 and γ1 chains (Fig. 2b), while the more strongly bound fraction consisted of a larger α2-subunit (α2, unprocessed “u”) with no observed additional α-chain component. It has previously been shown in recombinantly expressed mouse laminin α2-LG1–3 fragment that two closely overlapping furin-type recognition sites exist in the LG3 module with cleavage between Arg-2575 and Gln-2576 generating a blocked N terminus (32, 33). We therefore predicted that deletion of LG modules 4 and 5 should leave a smaller cleavable C-terminal fragment of ~19 kDa plus carbohydrate. A fragment of ~22 kDa was identified in the low salt, but not the high salt, fractions of rLm2-G4–5 (Fig. 2c), in good agreement with the fragment generated from α2-LG1–3 by Taltis et al. (33) and paralleling the salt-elution behavior seen with wild type recombinant protein. The main α2 band of this high salt fraction appeared to migrate marginally slower than...
When the FLAG-containing fractions of rLm2-H9004 G1 and the low salt fraction (predicted mass difference is 7%) corresponded to laminin-2 bearing an unprocessed N-terminal HA-tag by immunoprecipitation, further supporting the conclusion that the high salt fraction corresponded to laminin-2 bearing the HA epitope by immunoblotting with laminin-2/4 antibody (reduced SDS-PAGE). WT (N-terminal HA-tagged) and LG deletion laminins detected by stained polyacrylamide gels.

PAGE (reducing) analysis of purified rLm2 proteins in Coomassie Blue-glassine engineered laminin proteins from a heparin-5PW column. Performance liquid chromatography heparin-affinity elution profiles of engineered laminin-2 LG Modules. When conditioned media were subjected to heparin-affinity chromatography, each yielded a single low salt-eluting peak detected by reducing SDS-PAGE (Fig. 2, a and d). When N-terminal HA-tagged wild-type protein was evaluated (Fig. 2c), the more slowly and faster migrating a2 bands were found to possess HA epitope by immunoprecipitation, further supporting the conclusion that the high salt fraction corresponded to laminin-2 bearing an unprocessed a2 chain while the low salt fraction corresponded to laminin-2 bearing the typical-sized G-domain processed a2 domain. Finally, the ~75-kDa species reacted with a2-G-specific antibody (blot not shown). Thus heparin affinity chromatography resolved the different laminin species and revealed that high-affinity heparin binding is lost upon LG3 cleavage.

Molecular Shape of Recombinant Laminins—The different recombinant fractions were analyzed by electron microscopy following rotary Pt/C replication of glyceraldehyde on mica (Fig. 3). Wild-type processed recombinant laminin-2 had a typical laminin shape consisting of three short arms with small terminal and mid-arm globules, and one long arm ending in the large elliptical structure corresponding to the LG modules (arrows, upper left panel). The long axis of the LG modules varied with respect to that of the lower portion of the long arm coiled coil domain. The most striking difference in the deletion mutants was observed with rLm2ΔG1–5 (upper middle panel and extreme right lower two panels) which completely lacked the globular domain of the long arm. The long arm globules of rLm2-ΔG1–3 and rLm2-ΔG4–5 were similar in appearance, characterized by a more spherical shape, on average smaller than the elliptical globule of wild-type protein. Processed and unprocessed forms of wild-type laminin-2 could not be distinguished from each other in the electron micrographs.

Furin Processing—While both unprocessed and processed a2-subunit forms were secreted by WT-transfected 293 cells, only unprocessed laminin subunit was detected in the cell lysates, either in Western blots of transiently transfected cells (Fig. 4a) or FLAG-epitope protein from stably transfected cells (data not shown). When stably transfected cells expressing rLm2-WT protein were treated with different concentrations of the furin protease-inhibitor d-RVKR-cmk, proteolytic cleavage of the laminin was prevented (Fig. 4b). When rLm2-WTp was added to the medium of nontransfected 293 cells, C2C12 myotubes, and rat Schwann cells, laminin in the conditioned media (collected after 2 days) was partially processed from the 293 and Schwann cell cultures unless treated with d-RVKR-cmk, but minimally if at all from the C2C12 myotube cultures (Fig. 4c). Thus cultured 293 and Schwann cells can process extracellular laminin in a furin-dependent fashion while myotubes do not to any appreciable degree.

Cell Adhesion, Dystroglycan Binding, and Polymerization Activities—Mouse myoblasts adhered to and spread on both processed and unprocessed wild-type protein with 50% maximal coat concentrations of about twice that of laminin-1 (Fig. 5). Cells also adhered to and spread on both processed and unprocessed recombinant laminin bearing deletions of LG4–5 with only a small reduction of coat concentration dependence. However, cells adhered poorly to, and did not spread on, recombinant laminin bearing deletion of LG1–3. Deletion of all LG modules abolished cell adhesion over the concentration range studied, indicating that G-domain is required for myoblast cell adhesion. Adhesion contributions were detected from α2β1 and α2β3 integrins for the myoblasts. Furthermore, this interaction was largely dependent upon LG1–3 but not LG4–5 and was independent of LG processing.

A solid-phase assay was used to evaluate α2-dystroglycan binding (Fig. 6). Radiiodinated rabbit skeletal muscle α2-dystroglycan was incubated at different concentrations in wells coated with equimolar coatings of different recombinant laminins. Dystroglycan bound to wild-type rLm2, rLm2ΔG4–5, and rLm2-ΔG1–3, but not to rLm2-ΔG1–5, in a concentration-dependent manner. Binding to all active laminin-2 proteins was salt-sensitive but heparin-insensitive. The data were fitted by nonlinear regression analysis with single- and two-class binding algorithms, and the dystroglycan concentrations at half-maximal binding (indicated in parentheses) were determined typical-sized G-domain processed α2 domain. Finally, the ~75-kDa species reacted with α2-G-specific antibody (blot not shown). Thus heparin affinity chromatography resolved the different laminin species and revealed that high-affinity heparin binding is lost upon LG3 cleavage.

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by inspection from the fitted curves. rLm2-ΔG4–5p (18 nM) and rLm2-ΔG1–3 (15 nM) could be well fit by the single-class algorithm while rLm2-WTp (average 43 nM), WTu (38 nM), and rLm2-ΔG4–5u (62 nM) were better fit by the two-class algorithm. The levels of binding to wild-type protein at a given concentration was greater than twice that observed for either rLm2-ΔG4–5p or rLm2-ΔG1–3 alone, suggesting that at least two dystroglycan-binding sites are present in the full complement of α2-LG modules. Dystroglycan bound to rLm2-WTu and rLm2-ΔG4–5u, both unprocessed proteins, as well. The rLm2-WTu binding plot was similar to that of rLm2-WTp while the rLm2-ΔG4–5u binding was at least 2-fold higher compared with that of rLm2-ΔG45p, suggesting that there may be two sites within LG1–3 and that cleavage of G-domain decreases dystroglycan binding. However, rLm2-WTu was not observed to achieve higher binding levels compared with rLm2-ΔG45u as would be expected from the summation of rLm2-ΔG1–3 and rLm2-ΔG4–5u contributions. Both the apparent inaccessibility of more than three sites and two-class binding behavior may be due to steric hindrance occurring at adjacent G-domain-binding sites occurring at high dystroglycan concentrations. Since dystroglycan binding was not reduced in the unprocessed state, and since alanine mutagenesis of the furin-recognition site caused a substantial decrease of dystroglycan affinity for recombinant α2LG1–3 (32), its seems likely that the furin-recognition site overlaps with the cleavage site.

Laminin polymerization is a property of full-sized laminins including laminins-1, -2, and -4 that is dependent upon interactions among the short arms (18, 26). A laminin co-polymerization assay, developed to measure the polymerization potential of test laminins in small amounts, was employed to evaluate the recombinant proteins (18). Wild-type processed protein was found to co-precipitate with laminin-1 in a concentration-dependent manner (Fig. 7). The fraction of polymeric recombinant laminin was lower than (lagged behind) that of laminin-1 as observed previously for placental laminin-2 (18). The co-polymerization of

![Rotary shadowing](http://www.jbc.org/)

Fig. 3. Rotary shadowing. Contrast-reversed electron microscopic images of Pt/C replicas of the different purified recombinant laminin-2 proteins are shown. Note the reduction of size and ellipticity of G-domain (arrows) for rLm2-ΔG4–5, rLm2-ΔG1–3, and its complete absence in rLm2-ΔG1–5.

![Proteolytic processing](http://www.jbc.org/)

Fig. 4. Proteolytic processing. Panel a, conditioned media and cell lysates from 293 cells transiently transfected with r-α2-HA-tag cDNA were probed with anti-HA antibody (mk, mock-transfected control). b, laminin-2/4 specific antibody immunoblot (reduced) of conditioned media obtained from 293 cells stably expressing WT rLm2 and maintained for 40 h in the presence of the furin protease-inhibitor d-RVKR-cmk. c, laminin-2/4 antibody immunoblot of culture media immunoprecipitated with FLAG-specific antibody and recovered from 40-h incubations of exogenous purified rLm2-WTu in presence of Schwann cell, C2C12 myotubes, or 293 cells maintained either without (L) or with d-RVKR-cmk inhibitor (L+I).
Laminin-2 LG Modules

rLm2ΔG1–5, which possessed an N-terminal α2 HA epitope tag was compared with that of wild-type protein lacking an HA tag and was found to possess similar activity, i.e. neither the HA tag nor loss of G interfered with polymerization.

Laminin-2 Binding to Myotube Surfaces and Co-localization with Dystroglycan and Integrin—Recombinant wild-type and deleted rLm2-WTp,u and rLm2-WTu protein in the absence and presence of blocking antibodies for α₁-integrin (GoH3, with mouse cells), β₁-integrin (Ha2/5, both cells), and α₇-integrin (O26, rat cells).

Fig. 5. Myoblast adhesion. C2C12 myoblasts were incubated in wells coated with different concentrations of rLm2-WTp (closed circles), rLm2-WTu (open circles), rLm2-ΔG45p (open triangles), rLm2-ΔG45u (closed triangles), rLm2-ΔG13 (closed squares), and rLm2-ΔG15 (closed diamonds) and the degree of adhesion was determined (average ± S.E., n = 3). b, mouse C2C12 and rat L863 myoblasts were incubated in wells coated with rLm2-WTp protein in the absence and presence of blocking antibodies for α₂-integrin (GoH3, 3).

Fig. 6. Dystroglycan binding. Radiiodinated α-dystroglycan was incubated at the indicated concentrations with wells coated with different recombinant laminins (rLm2-WTp, closed circles; rLm2-WTu, open circles; rLm2-ΔG45p, closed squares; rLm2-ΔG45u, open triangles; and rLm2-ΔG13, closed triangles; average of S ± S.E.). a–c, direct binding data shown in three panels corresponding to three different experiments with data normalized to the maximal binding of WTp. d, effects of heparin, high salt, and EDTA on α-dystroglycan binding to different recombinant laminins.

Fig. 7. Polymerization. rLm2-WTp was incubated with laminin-1 (0–0.4 mg/ml, 37 °C), separated into polymer (P) and supernatant (S) fractions, and analyzed by SDS-PAGE (g). Quantitation of scans of Coomassie Blue-stained laminin-1 and FLAG-specific immunoblot are shown in plot for rLm2-WTp and rLm2-ΔG15 (h).
FIG. 8. Laminin and receptors on myotubes. Recombinant Lm2-WTp (a and c), WTu (b and d), and laminins bearing deletions were incubated (10 μg/ml) with C2C12 myotubes, washed, and detected with antibody for laminin-2/4 (a–d) and either α-dystroglycan (IIH6, α' and b', after 2 h incubation) or β1 integrin (Ha2/5, c' and d', after 4 h incubation). Relative laminin surface areas measured from the immunofluorescence images are shown in panel e (n = 6). rLm2-WTp, rLm2-WTu, rLm2-ΔG45p, and rLm2-ΔG45u (2 h) were also treated with β1 integrin (Ha2/5) and α-dystroglycan (IIH6) blocking antibodies, and heparin, as shown in panel f (n = 10). The inhibitory effects on laminin surface coverage following incubation with rLm2-ΔG45p,u, the minimally active recombinant laminin, are also shown (g, n = 7).

significantly less inhibition was observed following treatment of the unprocessed deletion mutant.

Contribution of Laminin α2-LG Modules to AChR Clustering—When recombinant laminin-2 was incubated with myotubes for longer periods, a substantial (5–10-fold) increase in AChR clusters (detected with bungarotoxin) was observed over that detected in the absence of exogenous laminin (Fig. 9). These clusters co-localized with α-dystroglycan that was concentrated at these sites. Maximal induction was achieved by about 10 μg/ml (Fig. 9h). Surface laminin co-localized with the bungarotoxin as well but was also present in areas away from the clusters (Fig. 9a, inset). Of all the recombinant laminins tested, only rLm2-WTp and ΔG4–5p induced AChR clustering above background levels (Fig. 9, b–g and i). Both antibodies IIH6 and Ha2/5 blocked AChR clustering induced by either of the laminins (Fig. 9, k and n). Since GoH3, an α6-integrin-specific blocking reagent, also inhibited clustering of these two proteins (l and n), α6β1 was implicated in mediating clustering. Heparin (1 mg/ml) inhibited clustering induced by rLm2-ΔG4–5p (n), but not that induced by WTp (j). This observation seems likely to be related to the ability of heparin to nearly completely inhibit 2–4 h accumulation of WTp. In contrast, laminin-1-induced AChR clustering was substantially inhibited with heparin as well as anti-α-dystroglycan (m). Laminin polymerization, a process mediated by the three short arms, was previously found to be selectively inactivated following treatment with AEBSF (13). To determine whether polymerization contributes to laminin-induced AChR clustering, myotubes were incubated overnight with 10 μg/ml recombinant laminin-2 (WTp) either alone or in the presence of laminin-1 fragment E1' which inhibits polymerization unless treated with AEBSF (o). rLm2-WTp induced AChR clustering was inhibited by 1 μM (0.5 mg/ml) E1', but not by AEBSF-E1'.

Laminin Mediation of Basement Membrane Assembly—The greatly increased coverage of intact laminin on myotubes compared with all deletion mutants suggested that deletion of LG modules would have an adverse effect on the efficiency of basement membrane assembly. The myotubes produce little or no type IV collagen or other basement membrane components and we find they do not form stable basement membranes. Therefore, to examine the role of the LG modules in basement membrane assembly, we evaluated the ability of the recombinant laminins to mediate basement membrane assembly in embryoid bodies (EBs) derived from ES cells that are null for the laminin-γ1 subunit. These EBs, when treated with exogenous laminin-1, form basement membranes (34) that contain type IV collagen, nidogen, and perlecan in addition to the added laminin. rLm2-WTp, WTu (in the presence of 10 μg/ml furin inhibitor), ΔG4–5p, ΔG1–3, and ΔG1–5 laminins were incubated (50 μg/ml) with laminin-γ1-null EBs and examined by immunofluorescence microscopy (Fig. 10). Wild-type laminin-2, regardless of processing (immunoblots of a cell lysate at the end of the incubation revealed that essentially all of the α2 chain was still...
in the unprocessed state, Fig. 10
mediated formation of a
subendodermal linear co-staining for laminin, type IV collagen,
nidogen, and perlecan. In contrast, all deletion mutants were
unable to mediate basement membrane formation. The forma-
tion of basement membrane following treatment with rLm2-
WTp was confirmed by electron microscopy.

**DISCUSSION**

The C-terminal G-domain, composed of five LG modules,
appears to be the principal cell-interactive domain of laminin.
The structure deduced from the
/L92512-LG45 crystal is that each
LG module is a
/L9252-sandwich with the first three modules sepa-
rated from the last two by a hinge disulfide-linked to LG5 (35).
Although the
/L92512-laminin subunit has fairly high homology with
the
/L92511-subunit, it exhibits several G-domain differences. First,
the
/L92512-subunit becomes proteolytically processed in LG-3 by a
furin-like convertase that cleaves at the end of the sequence
RRKRR in laminin-2 (32). Second,
/L92512-laminin G-domain pos-
sesses dystroglycan sites in both LG-3 and LG4–5, compared
with a limitation of binding to LG module-4 in laminin-1. Each
dystroglycan-binding site within
/L92512 LG1–5 is insensitive to
heparin treatment (unlike laminin-1), accounting for the near-
complete lack of inhibition of dystroglycan binding reported for
intact laminin-2/4 (28).

Previously we found the short and long arm domains of
laminin-1, through polymerization and cell binding, act in con-
cert to assemble ECM and alter cytoskeleton (13). To dissect
LG-modular functions in a laminin isoform yet preserve the
potential for such cooperativity, we introduced LG1–3, and
LG4−5 deletions into the laminin
/L92512 subunit DNA and
expressed the respective heterotrimeric laminins. 293 cells, by
virtue of a furin-like convertase, were found to cleave much,
but not all, of the wild-type and LG4–5 deleted laminin within
LG3, providing us with two additional laminin states for study.
Through our analysis we found that myoblast adhesion and
spreading are mediated by LG1−3 through
/L92516
/L92521 and
/L92517
/L92521 inte-
grins (similar to laminin-1), that
/L92512-dystroglycan binds sepa-
rately to LG1–3 and LG4–5 (unlike laminin-1), and confirmed
that polymerization is independent of G-contributions. In our
evaluation of myotubes and embryoid bodies, we found that: (a)
LG1–3 is required for basement membrane and for clustering
of AChR, (b) LG4−5 is required for basement membrane as-
sembly but not for AChR clustering, (c) furin-processing of
LG-3 is required for AChR clustering, but not for basement
membrane assembly, (d) αβ6 integrin as well as dystroglycan
are required for AChR clustering, and (e) polymerization of
laminin is required for AChR clustering (providing the evi-
dence for a hypothesis as described in Ref. 36). The laminin
contribution to AChR clustering can be regulated by furin in
α2-laminins but is constitutively active in α1-laminin.

It is conceptually useful to distinguish *anchorage*, interac-

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**FIG. 9. Induction of AChR clustering.** C2C12 myotubes were incubated with recombinant laminins for 18 h. Panels a–g, fluorescence images shown stained for AChR (bungarotoxin, green) and α-dystroglycan (IIH6, red). Separate and merged images shown for rLm2-WTp (a, a’, and a’’) and merged images for the other laminins. *Inset* in a’ shows co-localization with laminin (blue). Panels h–o, quantitation of AChR clustering (n = 20): h, dependence of AChR receptor clustering on rLm2-WTp protein concentration; i, comparison of different recombinant laminins, 10 μg/ml; j, effect of heparin (1 mg/ml) on rLm2-WTp; k, effect of Ha2/5 and IIH6 on WTp. Hamster and mouse IgM controls were 6.5 ± 0.7 and 7.8 ± 0.7, respectively; l, AChR clustering inhibition by GoH3; m, comparison of different recombinant laminins, 10 μg/ml; n, inhibition of rLm2-3G45p induction of clustering by antibodies. α, the laminin short arm complex E1’, which inhibits laminin polymerization unless inactivated with AEBSF (A-E1’), blocked AChR clustering induction by rLm2-WTp.
Laminin-2 LG Modules

Fig. 10. Basement membrane assembly in embryoid bodies. Laminin γ1-null ES cells were cultured for 7 days in suspension as embryoid bodies with 50 μg/ml rLm2-WTp (α–γ), ΔG45p (g and h), ΔG13 (i and j), or rLm2-WTqs with d-RVKR-cmk (k and l). Immunoblot (Lm1/4 antibody) of EB conditioned media from WTu-treated EBs (7 days) without (lane 1) and with (lane 2) d-RVKR-cmk. Cryosections were prepared and immunostained for laminin-γ1 (FITC, a, g, i, and k), type IV collagen (Cy3, b, h, j, and l), perlecan (FITC, d), or nidogen (Cy3, e). Untreated (a) and rLm2-WTq-treated (o) embryoid bodies were also fixed with gluteraldehyde and embedded in plastic for electron microscopy (endodermal/inner cell mass interface shown; paired arrows indicate no basement membrane in untreated and thick basement membrane in rLm2-WTp-treated EBs; n = nucleus).

The previously identified anchorages partially provided through heparin-type interactions, the latter minimally dependent upon α-dystroglycan and αβ3-integrin binding in the presence of anchorage partially provided through heparin-type interactions. The previously identified αβ3-integrin contribution (27) and complete inhibition of clustering achieved with GoH3 suggests that αβ3 works in concert with αβ3 to cluster AChR.

To compare the basement membrane requirements to those of AChR induction, we employed the recently described laminin γ1-null embryoid bodies that generate all required basement membrane components except laminin and that when treated with laminin assemble a basement membrane and then differentiate to form epiblast (34, 40, 41). The necessity of both LG1–3 and LG4–5 for basement membrane assembly revealed important G-domain contributions emanating from both sides of the hinge region. However, unlike AChR clustering, several studies reveal that β3-integrin and dystroglycan are not, at least separately, essential for basement membrane assembly. First, Schwann cell basement membranes, which contain laminin-2, do not require β3-integrin (42). Second, basement membrane is detected in dystroglycan-free muscle of genetic chimera mice (43, 44). Third, we have found that basement membranes can assemble in dystroglycan-null and polymerizing laminin-treated β3 integrin-null embryoid bodies. A conclusion suggested by the above and data described herein is that G-domain anchorage with high surface accumulation of laminin, rather than ligation of a particular integrin or dystroglycan, is critical for basement membrane assembly, while specific integrin and dystroglycan receptor ligation, rather than high accumulation, is essential for AChR clustering. This characteristic, along with the presence of an appropriate integrin-binding site and dystroglycan-binding site in α2-laminin LG1–3 (the latter is missing in α1-laminin LG1–3) can explain why LG4–5 is superfluous for clustering. A general hypothesis is that the relative LG-module contributions for different basement membrane functions differ among laminin isoforms.

All laminins with the exception of α1-laminins become proteolytically cleaved, often in G-domain (see Ref. 4). These modifications may have evolved to allow for post-depositional regulation of laminin function during development and tissue remodeling. Unprocessed laminin-2 can support cell adhesion/spreading, bind to dystroglycan, accumulate on myotube surfaces, and form basement membrane, yet strikingly cannot cluster the AChR. The only binding interaction we found to be

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markedly different in unprocessed laminin is increased heparin binding affinity. However, it is not known if this difference is causal in preventing AChR clustering activity in the unprocessed state. Furin, a serine protease, is a membrane-bound enzyme that is found in the trans-Golgi stacks and plasma membrane and that can cleave a number of ECM components (45). In an examination of its distribution in muscle, we have detected furin in relevant locations, i.e. at the neuromuscular junction, and, to a lesser extent, in embryonic muscle.3 We have found that extracellular laminin is processed by Schwann and 293 cells but much less efficiently by EBs and (particularly) cultured myotubes, revealing that furin can cleave laminin-2 following its secretion and that this activity can vary. The existence of laminin processing in G-domain argues for an important functional dependence of a number of isoforms on controlled proteolysis through the LG modules and leads to the attractive possibility that furin regulates laminin-2 activity.

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Contributions of the LG Modules and Furin Processing to Laminin-2 Functions
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