Structural and functional analysis of the recombinant G domain of the laminin α4 chain and its proteolytic processing in tissues

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Abstract

The C-terminal G domains of laminin α chains have been implicated in various cellular and other interactions. The G domain of the α4 chain was now produced in transfected mammalian cells as two tandem arrays of LG modules, α4LG1-3 and α4LG4-5. The recombinant fragments were shown to fold into globular structures and could be distinguished by specific antibodies. Both fragments were able to bind to heparin, sulfatides and the microfibrillar fibulin-1 and fibulin-2. They were, however, poor substrates for cell adhesion and had only a low affinity for the α-dystroglycan receptor when compared to the G domains of the laminin α1 and α2 chains. Yet antibodies to α4LG1-3 but not to α4LG4-5 clearly inhibited α6β1 integrin-mediated cell adhesion to laminin-8 indicating the participation of α4LG1-3 in cell-adhesive structure of higher complexity. Proteolytic processing within a link region between the α4LG3 and α4LG4 modules was shown to occur during recombinant production and in endothelial and Schwann cell culture. Cleavage could be attributed to three different peptide bonds and is accompanied by the release of the α4LG4-5 segment. Immunohistology demonstrated abundant staining of α4LG1-3 in vessel walls, adipose and perineural tissue. No significant staining was found for α4LG4-5 indicating their loss from tissues. Immunogold staining demonstrated an association of the α4 chain primarily with microfibrillar regions rather than with basement membranes, while laminin α2 chains appear primarily associated with various basement membranes.
Introduction

The protein family of laminins consists of at least 12 different isoforms which are mainly localized in basement membranes. They are involved in major biological functions such as interactions with cellular receptors and the formation of networks that are intermingled with and bound to networks of collagen type IV (1,2). Most of these heterotrimeric isoforms consist of β1/β2 and γ1 chains but differ in their α chains, α1 to α5. The α4 chain (200kDa) is the shortest variant known so far and is present in laminin-8 (α4β1γ1) and laminin-9 (α4β2γ1) (3-5). The existence of such relatively small laminins was originally indicated from biosynthetic studies with endothelial and adipose cells (6,7), but their molecular nature was only understood after the complete human (8,9) and mouse (4,10,11) α4 chain sequences became available. The domain structure of the α4 chain (1816 residues) predicted a small N-terminal region contributing a truncated short arm structure, a coiled-coil domain II/I used for chain association and a large C-terminal G domain. This prediction was confirmed by electron microscopy of laminin-8 and 9, which lacked one of the three short arm structures found in other laminins (3,4).

Northern and in situ hybridization demonstrated a moderate to strong expression of the α4 chain in heart, lung, skeletal muscle and in skin while some other tissues were negative (4,8-11). The chain was also expressed at midgestation stages of mouse development (4,11) and in various endothelial and adipocyte cell lines (3,4). Antibodies raised against fusion proteins of the α4 chain were useful in showing the extracellular deposition of the
corresponding laminins by immunohistology (5,11). This demonstrated a distinct localization in striated muscle, perineurium, capillaries and some mesenchymal regions but only a low abundance in epithelial basement membrane zones. This suggested that α4 chains are a distinct component of subendothelial regions and that they may have an adhesive function for endothelial cells (11). It was also speculated that they may promote angiogenesis (3,7).

Specific binding functions have not yet been examined for the α4 chain G domain, although such functions are shared by the G domains of all other laminin α chains (2). These G domains consist of a tandem array of five LG modules, LG1 to LG5, each of about 200 amino acid residues. Previous data for α1 and α2 chains showed the involvement of their G domains in integrin-mediated cell adhesion and binding to heparin, sulfatides and the α-dystroglycan receptor (2,12-15). Some of the binding epitopes could be mapped by site-directed mutagenesis to the laminin α1LG4 and α2LG5 modules and showed a considerable overlap (13,14). The recent elucidation of the crystal structure of α2LG5 (16) was instrumental in understanding the spatial organization of these epitopes. Furthermore, a recombinant fragment corresponding to laminin α2LG1-5 was shown to promote the attachment of Mycobacteria leprae to Schwann cells (17,18), indicating that LG modules are also likely to be involved in pathological processes.

Based on our previous experience with the recombinant production of LG modules of laminin α1 and α2 chains in mammalian cells (13,19), we have
now prepared the tandem arrays $\alpha 4$LG1-3 and $\alpha 4$LG4-5 for the mouse laminin $\alpha 4$ chain. These fragments had a strong affinity for heparin but no or only little activity in cell adhesion and the binding of $\alpha$-dystroglycan. Furthermore, the data indicated a substantial absence of the $\alpha 4$LG4-5 structure from tissues due to proteolytic processing.

**Materials and methods**

**Sources of protein and carbohydrate ligands**

Recombinant mouse fibulin-1 (20), fibulin-2 (21) and human nidogen-2 (22) were produced as described. Sources of all other extracellular proteins used as ligands have been previously documented (14). Recombinant $\alpha 1$LG (13) and $\alpha 2$LG fragments (19) and laminin fragment E8 containing $\alpha 1$LG1-3 (23) were prepared as described. Pepsin-solubilized collagen IV (23) was obtained from human placenta and human plasma fibronectin was of commercial origin (Behringwerke). $\alpha$-dystroglycan purified from chicken skeletal muscle (24,25) was kindly donated by Andrea Brancaccio. Heparin coupled to bovine serum albumin and bovine brain sulfatides were from a commercial source (Sigma).

**Sources of cells**

The mouse endothelioma cell line eEnd.2 was that used previously (26). A stem-cell-like endothelial cell line from mouse embryos (27) was a kind gift.
of Antonis Hatzopoulos. Several more human endothelial cells (EC) and smooth muscle cells (SMC) obtained from umbilical vein (HUVEC), aorta (HAEC), dermal microvessels (HMVEC-d), bladder microvessels (HMVEC-Bd), cervical microvessels (CRMV-En), lung microvessels (HMVEC lung) pulmonary artery (PASMC) and aorta (AOSMC) were purchased (Clonetics). RN22 rat Schwannoma, HBL100 human mammary epithelia and Rugli rat glioblastoma cells were those used previously (28).

Construction of expression vectors

Mouse laminin α4 chain cDNA clone M16 (10) was used as a template to amplify the sequence encoding the α4LG4-5 modules (residues 1428-1816) by polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs) following the manufacturer’s instruction. The primers used were GTCAGCTAGCGGATGCGCCTTCATGGG for the 5’ end and GTCACTCGAGTCAGGCTGTGGGACAGGA for the 3’ end. In addition to the coding sequences, these primers introduced a stop codon and single Nhel and Xhol restriction sites in order to allow in-frame insertion of the cDNA distal to the BM-40 signal peptide sequence in the episomal expression vector pCEP/pu (29). Clones M47 and M16 (10) were used for the preparation of the laminin α4LG1-3 construct (residues 827-1427) in two steps. The primer pairs GTCAGCTAGCATTCATCATGGG and ACGTGCCGTCTGTGGACAGGA (for M47) and the primer pairs GTGGACAGACGGCAGCAGT and GTCACTCGAGCTACTTACTCTCTCC (for M16) were used for amplification and the two PCR products were then fused by overlap extension. The final PCR-derived
construct contained the same restriction sites and a stop codon as the construct described above. Both were initially ligated into plasmid pUC18 (Pharmacia) for sequence verification on a 373A automated sequencer (Applied Biosystems). They were then released by NheI and XhoI digestion and ligated into plasmid pCEP/Pu (29).

Expression and purification of recombinant proteins

Human embryonic kidney cells which constitutively express the EBNA-1 protein from Epstein-Barr virus (293 EBNA, Invitrogen) were transfected with the episomal expression vectors (29) and transfected cells were selected with 0.5µg/ml puromycin (Sigma) and 250µg/ml G418 (Gibco). They were washed extensively with phosphate-buffered saline (pH 7.2) to remove residual serum proteins, and grown in serum-free DMEM/F12 medium (Gibco) for 2d, after which medium was harvested and new serum-free medium added for another 2d. Conditioned serum-free medium (1l) was dialyzed against 0.05M Tris-HCl, pH 7.4, containing 0.5mM phenylmethylsulfonyl fluoride (Serva) and 0.5mM N-ethylmaleimide (NEM) (Merck). It was then passed over a 2x30cm heparin-Sepharose column which was equilibrated in the same buffer and eluted with a linear NaCl gradient (0-0.6M NaCl, 500ml). Recombinant proteins were further purified on a Superose 12 column (HR16/50, Pharmacia) equilibrated in 0.2M ammonium acetate, pH 6.8, lyophilized and redissolved in 0.2M NH₄HCO₃.

Purification of laminin proteins from culture medium
Conditioned serum-free medium (0.5-1l) was harvested from eEnd.2 and rat Schwannoma RN22 cells. After addition of protease inhibitors (0.05mM Pefabloc, 1mM EDTA, 0.5mM NEM), medium was dialyzed against 0.1M NaCl, 0.05M Tris-HCl, pH 7.4, and passed over a 5ml heparin HiTrap column (Pharmacia) equilibrated in the same buffer. Bound proteins were eluted with a 0.1-0.6M NaCl gradient (60ml). Concentrated pools were subsequently passed over a Superose 12 column (HR 10/30) in 0.2M ammonium acetate, pH 6.8, and analyzed by immunoblotting and by SDS-gel electrophoresis using Coomassie blue staining.

Analytical methods

Protein and hexosamine concentrations were determined on a Biotronik LC3000 analyzer after hydrolysis (16h, 110°C) with 6M or 3M HCl, respectively. Edman degradation was performed with 473A or Procise sequencers, following the manufacturer's instructions. Electrophoresis in SDS-polyacrylamide gradient gels followed standard protocols. Circular dichroism spectra were recorded using a J-175 spectropolarimeter (Jasco Labor) and evaluated as previously described (30). Rotary shadowing electron microscopy was carried out according to established procedures (31).

Ligand binding assays
A 1ml heparin-HiTrap column (Pharmacia) in 0.05M Tris-HCl, pH 7.4, was used to determine the NaCl concentration required to displace bound ligands from the column with a precision of ±0.01M NaCl (13,14). Solid-phase binding assays were carried out with various proteins (5µg/ml) and heparin-albumin conjugate (10µg/ml) adsorbed onto the plastic surface of microtiter wells at 4°C following a previous procedure (32) with some modifications (14). Coating with sulfatides dissolved in methanol (0.2mg/ml; 50µl) was performed by drying overnight at room temperature. 1mM CaCl₂ and MgCl₂ were added to the buffer in the assays with α-dystroglycan. Binding of soluble α4LG1-3 and α4LG4-5 was detected by specific antisera (see below). Surface plasmon resonance assays were performed with BIAcore 1000 instrumentation (BIAcore AB) using proteins coupled through carbodiimide to CM-5 sensor chips (research grade). Binding assays were carried out in neutral buffer containing 2mM CaCl₂ under controlled conditions to prevent mass transport problems (33). Kinetic constants were calculated by non-linear fitting of association and dissociation curves according to a 1:1 model following the manufacturer’s instructions (BIAevaluation software version 3.0).

Cell adhesion assays

Cell attachment to plastic-coated laminin fragments was detected by rigorous washing followed by staining with 0.1% crystal violet and colorimetry according to established protocols (34). Collagen IV, fibronectin and laminin-1 were used as positive controls (26). Adhesion to bovine serum albumin, which was used for the blocking of coated wells, was negligible. Adhesion-
blocking monoclonal antibodies against α6 (GoH3) and β1 (AIIB2) integrin subunits were kindly provided by A. Sonnenberg and C.H. Damsky. They were used together with substrate-specific antibodies in inhibition assays (34).

Immunological assays

Rabbit antisera were generated against the two α4 chain fragments by two injections of 0.2mg in complete Freund’s adjuvant and antibodies were affinity-purified (35). Rabbit antibodies against mouse laminin fragments α2LG1-3 and α2LG4-5 have been previously described (19). ELISA titrations followed standard protocols. Immunoblotting followed a previously used procedure (36).

Immunohistochemistry

Paraffin sections of adult NMRI mice were deparaffinized, rehydrated and incubated (10min) with protease XXIV (Sigma) to block endogenous peroxidase. They were then exposed for 1h at room temperature to affinity-purified rabbit antibodies against α2 chain (19) and α4 chain fragments diluted to 5-7µg/ml. Peroxidase anti-peroxidase staining and counterstaining with hematoxylin followed a previously described procedure (37). Negative controls were carried out with normal rabbit serum diluted 1:100. Frozen tissue sections were used for indirect immunofluorescence (36).
Tissue sections on nickel grids were used for indirect immunogold staining (38). They were incubated for 1h at room temperature with affinity-purified antibodies (10-15µg/ml), rinsed and incubated for 20min with affinity-purified goat anti-rabbit IgG (Medac, Hamburg) coupled to 16nm gold particles diluted 1:300. Sections were rinsed with water, stained with uranyl acetate (15min) and lead citrate (5min) and then examined with a Zeiss EM 109 electron microscope. Controls with antibody-coated or uncoated gold particles were all negative.

Results

Recombinant production of two fragments comprising the G domain of the laminin α4 chain

The G domain of the mouse laminin α4 chain was prepared in the form of two recombinant fragments, α4LG1-3 (residues 827-1427) and α4LG4-5 (residues 1428-1816) following a previous strategy used for the laminin α2 chain (19). The boundaries chosen were outside the predicted β sandwich structure of the LG modules (16) and the border between the two fragments was placed in the center of a long link region (residues 1398-1460). Both fragments were produced and were obtained in good yields (1-2µg/ml) after purification. Because of their strong heparin affinity, they could be readily purified by a two step chromatographic procedure, as shown by electrophoresis (Fig.1).
Fragment α4LG1-3 migrated as a band of 67kDa and showed a single N-terminal sequence APLAVSM, where APLA is derived from the foreign signal peptide region. Fragment α4LG4-5 could be separated by electrophoresis into two bands of 43-44kDa. Edman degradation of the upper band demonstrated the expected APLADAPXWD sequence. Two sequences, XKFLEQKA and XEQKAP, which were identified for the lower band, represent starting positions of 1437 and 1440, respectively, indicating proteolytic trimming within the linker region. Hexosamine analysis of α4LG1-3 demonstrated 7 residues of glucosamine but no galactosamine, in agreement with the presence of four potential N-glycosylation sites in the sequence (10,11). No hexosamine could be detected in fragment α4LG4-5, which lacks N-glycosylation sites.

Both fragments were folded into compact globular structures, as shown by electron microscopy (Fig.2). They thus had the same shape as previously shown for analogous tandem arrays of LG modules derived from laminin α1 and α2 chains (13,19). Circular dichroism spectra of α4LG1-3 and α4LG4-5 (data not shown) were nearly identical to that previously published for the proteolytic fragment E3 of laminin-1, which corresponds to α1LG4-5 (39). They showed a minimum at 210-215nm (θ=-5500 to 8000 deg.cm^2.dmol^-1), indicating a content of 47-60% β strands and β turns. Together, the data demonstrated that both recombinant fragments of the laminin α4 chain were properly folded.

Binding to sulfated ligands and extracellular
Binding of laminin fragment E3 to heparin (39) and sulfatides (40) were the first activities assigned to laminin LG modules and subsequently confirmed with various other recombinant LG fragments (13,14). Fragments $\alpha_4$LG1-3 and $\alpha_4$LG4-5 were similar in this context (Table 1). They bound quantitatively to an analytical heparin affinity column and needed 0.27M and 0.34M NaCl, respectively, for displacement. This indicated that they are potential ligands for heparin/heparan sulfate at physiological ionic strength, as found before for recombinant LG fragments of the laminin $\alpha_1$ and $\alpha_2$ chains (13,14). Their binding activities for a heparin-albumin conjugate and for sulfatides in solid phase assays were, however, distinctly lower than $\alpha_2$ chain fragments (Table 1). As shown previously (13), recombinant fragment $\alpha_1$LG4-5 is also a stronger ligand in both solid-phase assays (half maximal binding at 4-6nM).

The basement membrane proteins fibulin-1, fibulin-2 and nidogen-2 were used as ligands for $\alpha_4$LG1-3 and $\alpha_4$LG4-5 in surface plasmon resonance assays in order to compare their binding activities with those previously determined for similar $\alpha_2$ chain fragments (Table 2). The $\alpha_4$ fragments bound to both fibulins, although the affinities differed 2- to 10-fold from those of the $\alpha_2$ chain fragments. Nidogen-2 was a poor ligand for $\alpha_4$LG4-5 and did not bind to $\alpha_4$LG1-3. Several other proteins (nidogen-1, perlecan, BM-40, collagens I and IV) were also tested with $\alpha_4$LG1-3 and $\alpha_4$LG4-5 in solid-phase assays, but showed no or only marginal binding which did not reach plateau levels, up to a concentration of 1µM for the soluble ligands.
LG modules have previously been shown to be good candidates for binding to $\alpha$-dystroglycan, which is an important receptor in many cell types (41). Immobilized $\alpha$-dystroglycan was therefore used in solid-phase assays to compare the binding of $\alpha$4LG1-3, $\alpha$4LG4-5 and $\alpha$2LG1-3 fragments (Fig.3). This demonstrated a strong binding of $\alpha$2LG1-3 as shown before (14). The two $\alpha$4 chain fragments, however, were only poor ligands which did not reach plateau levels up to a concentration of 1µM. This indicated a 30- to 100-fold lower binding activity than $\alpha$2LG1-3.

Previous studies have shown that $\alpha$2LG1-3 but not $\alpha$2LG4-5 strongly promotes $\beta$1 integrin-mediated attachment and spreading of several cell lines (15). Three of these cell lines, Rugli glioma (Fig.4), RN22 Schwannoma and epithelial HBL100 cells, showed no distinct binding to fragment $\alpha$4LG1-3. Because of the localization of laminin $\alpha$4 chain in various vessel walls (5,11), it was of particular interest to examine endothelial and smooth muscle cells in these assays. Pulmonary artery smooth muscle cells (Fig.4) and aortic endothelial cells attached rather weakly to $\alpha$4LG1-3 and $\alpha$2LG1-3 and not at all to $\alpha$4LG4-5. Four further endothelial cell lines (see Materials) showed no significant binding to the three substrates tested. By contrast, an embryonic endothelial cell line (27) bound strongly to $\alpha$2LG1-3, exceeding the level of binding of Rugli cells, but did not attach to $\alpha$4LG1-3 or $\alpha$4LG4-5 substrates (data not shown). Fibronectin and collagen IV were used as positive controls.
in the assays and were strongly adhesive for all cells examined, in agreement with previous observations (26).

Recombinant laminin-8 (α4β1γ1), however, was recently shown to promote adhesion of HT1080 cells by binding to α6β1 integrin (42). We could now show the same for laminin-8 from RN22 cells by using blocking monoclonal antibodies. This interaction could also be inhibited in a dose-dependent manner by incubating the substrate with affinity-purified antibodies (see below) against α4LG1-3 but not against α4LG4-5 (Table 3). Together, the data indicate contributions of α4LG1-3 to cell adhesion but only in the context of an entire laminin structure.

Immunological analyses of cells and tissues

Since the recombinant data indicated a possible proteolytic processing of the G domain of the laminin α4 chain in situ, we generated rabbit antisera against fragments α4LG1-3 and α4LG4-5. Antibodies were purified by affinity chromatography on the antigen used for immunization. As shown in (Fig.5), the antibodies against α4LG1-3 did not cross-react substantially with α4LG4-5, α1LG1-3 and α2LG1-3. A similar high specificity was also observed for the antibodies against α4LG4-5. This specificity was confirmed by immunoblotting of the α4 chain fragments (Fig.6A,B, lanes 1 and 5).

The antibodies against the two different α4 chain epitopes both showed distinct reactions with various cultured cells and their conditioned
media by immunofluorescence or immunoblots. The two antibodies showed quite different staining patterns in reduced immunoblots of culture medium from the mouse endothelial cell line eEnd.2, the rat Schwannoma RN22 cells and endothelial cells (HUVEC) from the human umbilical vein (Fig.6A,B lanes 2-4). Antibodies against α4LG4-5 reacted mainly with two to three bands of about 43-45kDa but also with bands of about 210kDa. Antibodies against α4LG1-3, however, primarily bound to bands in the range 180-210kDa with only little reaction with smaller bands. Together, the data indicated a substantial release of the α4LG4-5 structure by proteolytic processing but also a certain variability in the cleavage sites.

RN22 cell medium was used to separate individual α4 chain components by heparin affinity and molecular sieve chromatography, which was monitored by immunoblotting. This allowed a partial separation of the 200kDa components from the 45kDa bands, which eluted later from the heparin column. A final separation of the 45kDa fragments was then achieved on a Superose 12 column equilibrated in neutral buffer. Electrophoresis of this material demonstrated a 43/45kDa doublet band which was, however, still contaminated with some other proteins (Fig.1, lane 5). Edman degradation after blotting the doublet demonstrated the sequences XEKSKDAPSW (upper band) starting at position 1423 of the α4 chain and LKFLEXKAP (lower band) starting at position 1437. A similar separation could be achieved for eEnd.2 medium, but the yields were insufficient for sequencing. Since all separations were performed under non-
dissociating conditions, it indicates that, once released, the α4LG4-5 structure does not stay associated with the remaining laminin.

Immunohistology was used to determine the mouse tissue localization of α4LG1-3 and α4LG4-5 at the light and electron microscopical level and to compare it with that of corresponding laminin α2 chain fragments. Affinity-purified antibodies against α4LG1-3 showed a distinct staining (peroxidase technique) of capillary walls in heart (Fig. 7A) and skeletal muscle (Fig. 7F) but failed to react significantly with basement membrane zones (endomysium) around the muscle cells. No staining was observed with antibodies against α4LG4-5 as shown for heart muscle (Fig. 7B). Comparable staining patterns of both endomysium and capillaries could be obtained with antibodies against α2LG1-3 (Fig. 7C, E) and α2LG4-5 (Fig. 7D). Further strong reactions for α4LG1-3 were detected in basement membrane zones around smooth muscle cells of skin blood vessels, bronchial regions, in alveolar septa, around the perineurium, and in the tunica media and around adventitial adipocytes of aorta. None of these regions reacted with antibodies against α4LG4-5, in contrast to α2LG1-3 and α2LG4-5-specific antibodies, which produced indistinguishable staining patterns in all tissues examined. Staining for α4LG1-3 but not for α4LG4-5 was also confirmed by indirect immunofluorescence on various frozen tissue sections (data not shown).

Immunogold staining with antibodies to α2LG1-3 and α4LG1-3 was used in order to distinguish laminin α2 and α4 chains at the ultrastructural level. The α2 chain could be clearly detected within basement membranes around
skeletal muscle cells and adjacent to endothelial cells and pericytes of capillaries and small arterioles (Fig. 8A,B). In heart muscle, however, no basement membrane staining was found around cardiomyocytes but staining occurred in deeper microfibrillar layers of the endomysium (Fig. 8C). By contrast, the α4 chain was not a basement membrane component of either endothelial or muscle cells but was instead located in the adjacent interstitial region of skeletal muscle (Fig. 8D) and heart muscle (Fig. 8E,F).

**Discussion**

The recombinant production of the mouse laminin α4 chain domain G in the form of two tandem arrays, α4LG1-3 and αLG4-5, as described here, has set the stage for several functional and biological studies. Electron microscopy and circular dichroism spectroscopy demonstrated that they were properly folded, as shown before for analogous fragments from the laminin α2 chain (19), which made the α4 modules suitable for ligand binding studies. A limited proteolytic processing of recombinant α4LG4-5 led us to investigate whether similar processing may occur in cell cultures and tissues. Proteolytic processing has been previously identified within the α2LG3 module of the laminin α2 chain (19) and was predicted to occur between the α3LG3 and α3LG4 modules of the laminin α3 chain (43). No cleavage has yet been reported for the G domain of the laminin α1 chain.

When compared to the laminin α2 chain fragments, both recombinant α4 fragments bound with a similar strength in heparin affinity chromatography but showed a more moderate interaction in solid phase assays with heparin
and sulfatides. Such binding could be important for cellular interactions and, as shown for the laminin α1LG4 module (44,45), also for binding to the heparan sulfate chains of the extracellular proteoglycan perlecan. The heparin/sulfatide binding epitopes have been mapped by site-directed mutagenesis to a few basic residues in the laminin α1LG4 (13) and α2LG5 (14) modules. Furthermore, the crystal structure of α2LG5 (16) demonstrated that they are localized in short loops between β strands F/G and H/I for α1LG4 and between β strands H/I and L/M for α2LG5, which are in close proximity to the surface of LG modules. The basic character of these loops is maintained in all five of the LG modules of mouse and human laminin α4 chains (4,8-11). Their role in binding can now be examined by appropriate mutants of recombinant α4LG1-3 and α4LG4-5 fragments.

The LG modules of the laminin α2 chain were previously shown to bind to fibulin-1, fibulin-2 and nidogen-2 (14), interactions which could be important for the supramolecular organization of extracellular structures. Similar interactions, with some differences in binding affinities, could now be demonstrated for α4LG1-3 and α4LG4-5, suggesting that this property could also be shared by other laminin α chains. This is supported by previous studies, which showed binding of a laminin fragment E3 (α1LG4-5) to fibulin-1 (46) and recent observations on the binding of α1LG1-3 to fibulin-2. 1 The fibulins and nidogen-2 are known to occur in basement membranes but are also associated with fibrillin and fibronectin microfibrils and elastic sheets (36,47-

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1 Footnote 1
Their possible interaction with LG modules in situ now needs to be examined by immunogold colocalization studies.

Laminin LG modules are also important ligands for cellular receptors, including several integrins and α-dystroglycan (12,41). Here we show a rather low binding of α4LG1-3 and α4LG4-5 to α-dystroglycan when compared to LG modules derived from perlecan and laminin α1 and α2 chains (13,14). Studies with laminin α1LG4 demonstrated that α-dystroglycan binding depends on residues involved in heparin/sulfatide binding as well as several other basic amino acids (13) that are located more distantly in loops between β strands J/K, K/L and M/N (16). These latter regions are not very well conserved in the LG modules of the laminin α4 chains, which may explain the low binding activity. The laminin α2LG1-3 but not the α2LG4-5 fragment was a strongly cell-adhesive substrate, mediated by interactions with α3β1 and α6β1 integrins (15). Fragment α4LG1-3, however, was a poor adhesive substrate for several standard tumor cells and endothelial cells. However, the laminin α1LG1-3 structure needs to be associated with the adjacent rod domain of the long arm to express strong binding activity for α6β1 integrin (50,51). Laminin-8 was in fact recently shown to bind cells via the α6β1 integrin (42,52) which could be confirmed in the present study. This interaction was furthermore specifically inhibited by antibodies against α4LG1-3 but not by antibodies against α4LG4-5 (Table 3). This suggests, like for α1LG1-3, that interactions between α4LG1-3 and the rod are required for the expression of a strong cell-adhesive epitope.
Proteolytic processing of the LG region of the laminin α4 chain was confirmed with cultured endothelial and Schwannoma cells. It occurred in a 65-residue link region between the α4LG3 and α4LG4 modules and included three different cleavage sites (Fig.9). The principal fragments released showed a limited size heterogeneity and included C-terminal fragments of 43-45kDa and N-terminal fragments of 180-210kDa. The latter correspond to the 180-200kDa α4 chains previously detected in embryo extracts (5), leiomyosarcoma cells (11) and platelet laminin-8 (52). This indicates at least a partial release of α4LG4-5 which, however, was not identified in the previous studies. It is also noteworthy that the identified cleavage sites are not entirely conserved in the mouse and human α4 chains (Fig.9). Together with the multiple cleavage sites, this suggests that several types of proteases could be involved in processing. As a consequence, the α4LG4-5 entity no longer remains associated with the parental laminin. This is different from the processing of the laminin α2 chain, where proteolysis occurs at a single R-Q bond at the C-terminal end of a furin-type cleavage sequence of the α2LG3 module (15,19). This cleavage is not accompanied by dissociation, probably due to the fact that cleavage occurs in a longer insert in the loop between β strands D and E of the LG module and should therefore not disrupt the β sandwich (16).

A conserved feature of the link region is an odd cysteine close to its C-terminal end (Fig.9). A recent crystal structure of the α2LG4-5 tandem array (53) demonstrated that this cysteine forms an intermodular disulfide bridge to a cysteine in the short α-helix of the α2LG5 module. Furthermore, about 15 C-
terminal residues of the link form an extended interface contact region which forces a distinct and stable topological orientation of the modules relative to each other. Based on sequence comparisons and the fact that processing occurs on the N-terminal side of the interacting link region, it is likely that the same tertiary structure should be present in α4LG4-5 and in all other laminin α chains. This may also be the case for other LG modules present in protein S and the receptor kinase ligand Gas6 (53).

Immunolocalizations with antibodies against the α4LG1-3 fragment demonstrated the expression and extracellular deposition of laminin α4 chain particularly in heart and skeletal muscle, lung tissues, around fat and peripheral nerve cells and in various vessel walls. They agree with previous expression data obtained by either in situ hybridization or by staining with antibodies generated against fusion proteins encoding domains II/I or the LG2-3 modules of the α4 chain (4,5,8,11). Surprisingly, no distinct staining could be obtained with antibodies against α4LG4-5 in tissues which were otherwise strongly stained for α4LG1-3, α2LG1-3 and α2LG4-5. This probably indicates loss of α4LG4-5 from tissues after proteolytic release or, less likely, masking of its antigenic epitopes by interactions with other tissue components. Ultrastructural localizations by immunogold staining demonstrated the restriction of laminin α2 chains to basement membranes of skeletal muscle cells, pericytes and endothelial cells. In heart, however, the α2 chains were not detected in the basement membrane around cardiomyocytes but instead in the interstitial microfibrillar matrix adjacent to them. The laminin α4 chain was not a constituent of endothelial or other basement membranes,
but was deposited in adjacent extracellular regions. Since endothelial cells produce α4 chain containing laminin, as shown here and previously (4), these proteins presumably diffuse away and contribute primarily to the connection of the outer regions of vessel walls to the extracellular matrix. It could also indicate the association of these laminins with microfibrils, which are known to contain fibulins (36,47,48).

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**Footnotes**

1 H. Wizemann, R. Timpl, unpublished
Table 1

Binding of laminin LG modules to heparin and sulfatides. In heparin affinity chromatography, the NaCl concentrations required for displacement are recorded. Solid phase assays with immobilized heparin-albumin conjugate and sulfatides were used to determine the concentrations (nM) required for half maximal binding. Values for the corresponding laminin α2 chain fragments were taken from (14).

| soluble ligands | heparin (M NaCl) | sulfatides (nM) |
|----------------|-----------------|-----------------|
| α4LG1-3        | 0.27            | 150             |
| α4LG4-5        | 0.34            | 200             |
| α2LG1-3        | 0.36            | 20              |
| α2LG4-5        | 0.23            | 45              |
Table 2

Binding of fragments $\alpha$4LG1-3 and $\alpha$4LG4-5 to immobilized fibulins and nidogen-2 in surface plasmon resonance assay. Soluble ligands were examined at various concentrations and kinetic and thermodynamic constants are average values of 3-4 independent determinations. The last column records the $K_d$ values determined previously for the corresponding LG fragments from the laminin $\alpha$2 chain (14) as immobilized ligands.

| pairs of ligands | $k_\text{d} \times 10^3$ (s$^{-1}$) | $k_\text{a} \times 10^3$ (M$^{-1}$s$^{-1}$) | $K_\text{d}$ (nM) | $K_\text{d}$ of $\alpha$2 (nM) |
|------------------|-------------------------------|---------------------------------|----------------|-----------------|
| fibulin-1C $\alpha$4LG1-3 | 1.5                           | 30                             | 50            | 13              |
|                   | $\alpha$4LG4-5               | 1.5                           | 20            | 75              | 148             |
| fibulin-2 $\alpha$4LG1-3 | 1.4                           | 31                             | 45            | 13              |
|                   | $\alpha$4LG4-5               | 0.34                          | 27            | 12              | 117             |
| nidogen-2 $\alpha$4LG1-3 | no binding                   |                                 |               | 59              |
|                   | $\alpha$4LG4-5               | 7.0                           | 19            | 368             | 51              |
Table 3

Inhibition of HT1080 cell adhesion to laminin-8 by affinity-purified antibodies against LG modules and monoclonal antibodies against integrin β1 and α6 subunits. The latter were used as hybridoma medium diluted 1:10.

| Inhibitor      | Concentration (µg/ml) | Inhibition (%) |
|----------------|-----------------------|----------------|
| anti-β1        | 100                   |                |
| anti-α6        | 96                    |                |
| anti-α4LG1-3   |                       | 200            |
|                | 50                    | 46             |
|                | 20                    | 20             |
|                | 5                     | -3             |
| anti-α4LG4-5   |                       | 200            |
|                | 50                    | -16            |
|                | 20                    | 4              |
|                | 5                     | -2             |
Legends to the Figures

Fig.1: SDS gel electrophoresis of purified recombinant fragments $\alpha4LG1\text{-}3$ and $\alpha4LG4\text{-}5$ from the mouse laminin $\alpha4$ chain and of an $\alpha4LG4\text{-}5$ analogue obtained from rat Schwannoma RN22 cells. Samples used were $\alpha4LG1\text{-}3$ (lanes 1,3), $\alpha4LG4\text{-}5$ (lanes 2,4) and a heparin-binding fraction from serum-free culture medium of RN22 cells (lane 5). The latter eluted at about 0.28 M NaCl and the doublet band of 43-45kDa was shown to correspond to $\alpha4LG4\text{-}5$ by immunoblotting and Edman degradation. The major 28kDa band showed no blot reaction and was identified as the chromosomal protein HMG-1 by sequencing. Lanes 1, 2 were run under non-reducing and lanes 3-5 under reducing conditions. Staining was with Coomassie blue.

Fig.2: Electron microscopical images of recombinant fragments $\alpha4LG1\text{-}3$ (A) and $\alpha4LG4\text{-}5$ (B) after rotary shadowing. The bar indicates 100nm for both parts.

Fig.3: Binding of soluble $\alpha4LG1\text{-}3$ and $\alpha4LG4\text{-}5$ fragments to immobilized $\alpha$-dystroglycan in solid-phase binding assays. The ligands $\alpha4LG1\text{-}3$ (●) and $\alpha4LG4\text{-}5$ (■) were compared with $\alpha2LG1\text{-}3$ (○).

Fig.4: Adhesion of Rugli glioma cells (black symbols) and of pulmonary artery smooth muscle cells (open symbols) to plastic-immobilized LG fragments. Wells were coated with $\alpha4LG1\text{-}3$ (▲,△), $\alpha2LG1\text{-}3$ (●,○) and $\alpha4LG4\text{-}5$ (□).
Relative adhesion was determined by colorimetry after crystal violet staining (34).

Fig.5: Titration of affinity-purified antibodies against $\alpha$4LG1-3 in EUSA. Antigens used were $\alpha$4LG1-3 ($\circ$), $\alpha$4LG4-5 ($\square$), $\alpha$1LG1-3 ($\triangledown$) and $\alpha$2LG1-3 ($\Delta$).

Fig.6: Immunoblotting of serum-free culture medium from $\alpha$4 chain laminin producing cells with antibodies specific for $\alpha$4LG1-3 (A) and $\alpha$4LG4-5 (B) epitopes. Samples used were recombinant $\alpha$4LG1-3 (0.5ng, lane 1), medium from mouse endothelial eEnd.2 cells (20µl, lane 2), from rat Schwannoma RN22 cells (10µl, lane 3) and from human umbilical vein endothelial cells (20µl, lane 4) and recombinant $\alpha$4LG4-5 (0.5ng, lane 5). Electrophoresis was performed under reducing conditions.

Fig.7: Peroxidase staining of cross-sections of adult mouse heart (A-D) and skeletal muscle (E,F) with antibodies against LG modules of the laminin $\alpha$2 and $\alpha$4 chains. A: Anti-$\alpha$4LG1-3 stains basement membrane zones of capillaries (arrows) but only poorly stains those around cardiomyocytes (cmc), bar=40µm. B: Lack of staining of heart by anti-$\alpha$4LG4-5, bar=40µm. C,D: Staining of heart by anti-$\alpha$2LG1-3 (C) and anti-$\alpha$2LG4-5 (D) reveals reactions of basement membrane zones of cardiomyocytes (cmc) and capillaries (arrows), bar=30µm. E: Skeletal muscle (M.soleus) staining by anti-$\alpha$2LG4-5 shows depositions around basement membrane zones of skeletal muscle cells (smc) and capillaries (arrow), bar=40µm. F: Staining of a longitudinal section of skeletal muscle (M.soleus) by anti-$\alpha$4LG1-3 shows an exclusive reaction with
capillary walls (arrows). Note that there is no staining of the basement membrane zones of skeletal muscle cells (smc), bar=40µm.

Fig.8: Immunogold localization of \(\alpha_2\)LG1-3 (A-C) and \(\alpha_4\)LG1-3 (D-F) structures in adult mouse skeletal muscle and heart tissues. A,B: Staining of soleus muscle shows localization of laminin \(\alpha_2\) chain in basement membranes (asterisks) around a myocyte (my) and along endothelial cells (en, arrows) and along endothelial cells (en, asterisks) and around a pericyte (pe, arrows) of a small arteriole. l=vessel lumen. C: Heart muscle laminin \(\alpha_2\) chain is found in the interstitial matrix of the endomysium but not in the basement membrane (asterisks) around the cardiomyocyte (my). D: Staining of soleus muscle shows laminin \(\alpha_4\) chain in the interstitial matrix adjacent to a capillary but not in the endothelial cell (en) basement membrane (arrows). E,F: A similar staining of heart muscle reveals labeling of the interstitial matrix next to a small arteriole (E) and a capillary (F) but not in the basement membranes (asterisks) adjacent to endothelial cells (en) and cardiomyocytes (my). Pictures D, F and E also show an erythrocyte (ery) within the capillary lumen (l) and a pericyte (pe). Bars 0.32µm (A,C-F), 0.43µm (B).

Fig.9: Amino acid sequence of the link region between \(\alpha_4\)LG3 and \(\alpha_4\)LG4 modules of mouse (top) and human (bottom) laminin \(\alpha_4\) chain. The latter shows only the amino acids which differ. Major (\(\nabla\)) and minor (\(\triangledown\)) proteolytic cleavage sites and the start of the recombinant \(\alpha_4\)LG4 fragment (\(\bullet\)) are indicated. The sequence includes the last Cys of \(\alpha_4\)LG3 and the first His in the
A strand of α4LG4. An extra Cys probably involved in an intermodular disulfide bridge to the LG5 module is circled (53).
Structural and functional analysis of the recombinant G domain of the laminin α4 chain and its proteolytic processing in tissues
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