# Modification of the laminin α4 chain by chondroitin sulfate attachment to its N-terminal domain

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**Abstract The N-terminal domain of laminin α4 chains corresponds to a short rod-like structure which after recombinant production was found to be modified by chondroitin sulfate. Substitution occurred mainly to a single serine in its N-terminal ASGDG sequence. A similar yet partial modification was also demonstrated for the α4 chain present in extracts of adult mouse tissues. Antibodies to the fragment were useful to demonstrate a relatively high content of α4 in several tissues and for the immunolocalization in various blood vessels, some basement membranes and interstitial regions.** 

*Key words:* Basement membrane; Laminin; Proteoglycan; Tissue expression

## 1. Introduction

Laminins represent a large family of basement membrane proteins and are crucial elements for the structural integrity of these extracellular matrices as well as for mediating cell–matrix interactions. They are composed of disulfide-linked α,β,γ chains and five α, three β and three γ chains have been identified so far [1,2]. The α chains consist of a central coiled-coil domain II,I important for chain assembly, and have a unique set of five globular LG modules (LG1–LG5) at the C-terminus implicated in cell adhesion. Their N-terminal short arms are composed of tandem arrays of LE modules which form tight rod-like segments [3] and some other globular domains. This region is particularly short in the α4 chain and consists mainly of three LE modules [4–8]. As a consequence, laminin-8 (α4β1γ1) and laminin-9 (α4β2γ1) [9] lack one of the three short arms when visualized by rotary shadowing [7,10]. A further truncation may occur through proteolytic processing which releases the most C-terminal α4 LG4–5 modules. Corresponding fragments could still be detected in cell cultures but no longer in tissues by immunostaining [11]. This could in part explain a considerable electrophoretic heterogeneity of α4 with bands of 190, 200, 220 and 230 kDa detected in platelet extracts and endothelial cells by immunoblotting with α4-specific antibodies [11,12].

The laminin α4 chain is strongly expressed in a large variety of tissues including heart, lung, muscle, embryonic kidney, peripheral nerves and adipose tissue [4–9,11,13] and is considered to be a major component of endothelial cells and vessel walls. Recent studies of mouse lines with α4 chain deficiency underscored this interpretation by showing massive but transient hemorrhages at neonatal stages and impaired angiogenesis in adult mice [14]. This deficiency caused in addition an improper localization of motoneuron synapses [15]. Our understanding of the various functions of the α4 chain at the molecular level, however, is still at the beginning. Laminins containing this chain were shown to be cell-adhesive through the binding of α6β1 and α6β4 integrins [10,12]. This very likely involves the α4LG1–3 modules and part of the long arm rod as shown by antibody inhibition [11]. Such or related interactions could be involved in the recruitment of T cells across the blood–brain barrier [15]. The affinity for α-dystroglycan receptors seems, however, to be relatively low compared to other laminin α chains [11]. The α4LG modules also showed binding to heparin, sulfatides and fibulins [11] and a particularly strong heparin binding site has been mapped to the α4LG4 module [17]. No binding functions have so far been identified for the short arm region of the α4 chain. However, this region contains an SGD and several more SG sequences which could be modified by glycosaminoglycan attachment as shown before for perlecan [18,19].

In the present study we have prepared the N-terminal region (fragment α4IIIa) by recombinant production in mammalian cells and show its nearly complete substitution by chondroitin sulfate. A partial substitution of α4 chains could be demonstrated in tissue extracts by antibodies against α4IIIa. These antibodies were also useful to quantitate the α4 chain in tissue extracts and to examine tissue localizations.

## 2. Materials and methods

### 2.1. Production of recombinant proteins

An expression vector for the N-terminal region of mouse laminin α4 chain was constructed by overlap extension PCR using the 5′ and 3′ primers GTCAGCTAGCCCTGTTGAATATCTGCAG and GTGCGGAAGAATCCAGCGTC (nt 363–607) and e.end2 cell RNA as template and GACGCTGGATTCTTCCGCAC and GTCACTCGAGTCAGCCTGTAGGGACTTCAAATC (nt 588–1181) and cDNA clone M-47 [5] following standard protocols. The final product was then inserted through *Nhe*I/*Xho*I restriction sites into the episomal expression vector pCEP/Pu and used for the transfection of human kidney 293-EBNA cells [20]. Serum-free culture medium (1 l) was passed over a DEAE cellulose column (2.5×8 cm) equilibrated in 0.05 M Tris–HCl, pH 8.6, and eluted with a linear gradient of 0–0.6 M NaCl (800 ml). This was followed by chromatography on a Superose 12 column (HR16/50; Amersham) equilibrated in 0.2 M ammonium acetate, pH 6.8. A carbazole assay for uronic acids [21] was used to identify the position of the recombinant fragment. Recombinant

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N-terminal fragments α3AIIIa (position 1–212) and α2VI/V (1–513) from mouse laminin α3A and α2 chains were prepared and purified by similar procedures used in previous studies [11,19]. All vectors used were verified by sequencing.

*2.2. Protein characterization*

Protein samples were hydrolyzed (16 h, 110°C) with 6 M or 3 M HCl for the determination of protein concentrations and hexosamine compositions, respectively, on a Biotronic LC3000 analyzer. Edman degradation was performed on 473A or Procise sequencers following the manufacturer's instruction and the extent of serine substitution estimated from the relative yield of the PTH derivative [18]. Digestions with heparitinase, chondroitinase ABC (Seikagaku) and chondroitinase B (Sigma) were carried out as previously described [18]. Limited digestion with trypsin (4 h, 37°C) was achieved at an enzyme–substrate ratio of 1:100. Complete reduction and alkylation of proteins was performed in 6 M guanidine HCl [22]. Electrophoresis was performed in calibrated SDS gels followed by Coomassie blue staining.

*2.3. Immunological assays*

Immunization of rabbits, affinity purification of antibodies, ELISA and radioimmunoassays followed standard protocols [23]. Rabbit antibodies against mouse laminin α4LG1–3 [11] and a rat monoclonal antibody BE1/4 against mouse nidogen-1 [24] have been described previously. Extraction of mouse tissues was performed by neutral buffer containing 10 mM EDTA followed by the same buffer containing detergents and protease inhibitors [25]; the extracts were used for radioimmunoinhibition assays specific for α4IIIa. Similar inhibition assays for mouse laminin γ1III3–5 [26] and α2VI/V (T. Sasaki, R. Giltay, U. Talts, R. Timpl, J.F. Talts, submitted) have been described. Detergent extracts (100 μl) and 5 μg purified anti-α4LG1–3 were used for immunoprecipitation with protein A-Sepharose [27]. These precipitates were used without or after treatment with chondroitinase ABC for SDS gel electrophoresis under reducing conditions followed by immunoblotting with anti-α4LG1–3 [11]. Double immunofluorescence staining of tissue cryo-sections with purified rabbit anti-α4IIIa (5 μg/ml) and BE1/4 (10 μg/ml) was followed by secondary Cy3 goat anti-rabbit (Jackson ImmunoResearch) and Alexa 488 goat anti-rat IgG (Molecular Probes). Control incubations with normal IgG gave negative results. Some double stainings were also performed with α-bungarotoxin labeled with Texas red (Molecular Probes).

## 3. Results

The N-terminal short arm region of the mouse laminin α4 chain [5,7,8] consists of 265 amino acids including a 24-residue signal peptide. This segment contains three typical disulfide-bonded laminin LE modules [3] (position 82–238) flanked on each side by regions with a low cysteine content. The sequence contains in addition five Ser-Gly pairs (26-SGD, 53-SGV, 76-SGE, 95-SGF, 190-SGN) known from other proteins [28] to be potential candidates for glycosaminoglycan attachment. We have therefore prepared the entire segment as a recombinant fragment α4IIIa in transfected human 293 cells which, as shown before for perlecan domains [18,19], makes it possible to identify glycosaminoglycan substitutions. Examination of serum-free culture medium by SDS gel electrophoresis failed to demonstrate a protein band of the size expected from the sequence (30–40 kDa) even though the medium contained 4–6 μg/ml α4IIIa as shown by radioimmunoassay (see below).

The modified form of the recombinant fragment was therefore initially purified on DEAE cellulose (not shown) and eluted as a strong uronic acid-positive peak at the end of the gradient (0.4–0.56 M NaCl). Subsequent molecular sieve chromatography showed a considerably size heterogeneity (Fig. 1a) and the fragment was divided into four pools for separate examination by SDS gel electrophoresis (Fig. 1b). This demonstrated broad and diffusely stained protein bands

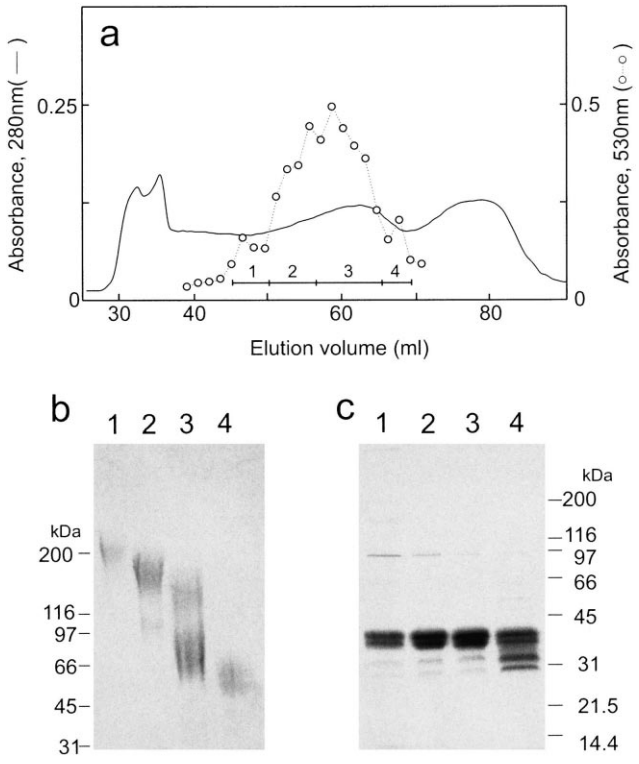


Fig. 1. Molecular sieve chromatography of recombinant fragment α4IIIa (a) and analysis by SDS gel electrophoresis under reducing conditions (b,c). Purification on a Superose 12 column was monitored for protein absorbance (solid line) and by carbazole assay for uronic acids (dashed line) and pools 1–4 were collected as indicated. Electrophoreses were performed prior to (b) and after (c) digestion of pools 1–4 with chondroitinase ABC and the runs were calibrated with reduced marker proteins (in kDa).

of 200–220 kDa (pool 1), 150–200 kDa (pool 2), 60–80 kDa (pool 3) and 50–60 kDa (pool 4) which are typical for proteoglycans [18,19]. This heterogeneity was confirmed by determination of galactosamine contents which varied from about 80 residues (pools 1, 2) to 50 (pool 3) and 35 residues (pool 4). All pools showed in addition the same glucosamine content (six residues) indicating the partial modification of Asn-104 and Asn-215 which are in a typical consensus sequence for *N*-glycosylation.

Digestion of the α4IIIa pools by chondroitinase ABC released a uniform set of smaller fragments consisting of a 38/40-kDa doublet band and smaller amounts of 30/33-kDa bands (Fig. 1c). No release was observed after digestion with chondroitinase B or heparitinase (not shown) demonstrating that α4IIIa is exclusively modified by chondroitin sulfate side chains. As based on the galactosamine content their molecular mass can vary between 15 and 40 kDa, Edman degradation of chondroitinase ABC-treated α4IIIa pools was used to localize potential glycosaminoglycan acceptor sites since the corresponding serines still contain some carbohydrate and can therefore not be identified as such [18]. This showed for the 38- and 40-kDa bands the same single sequence 25-AXGDGNAFP confirming the predicted signal peptide cleavage site [4–8]. Ser-26 could not be identified indicating its full occupation by chondroitin sulfate. The N-terminal sequences of the 31- and 33-kDa bands started with 55-VTLGRL and two more fragments generated by brief trypsinization started at positions 45 and 60, respectively. This demonstrated the pres-

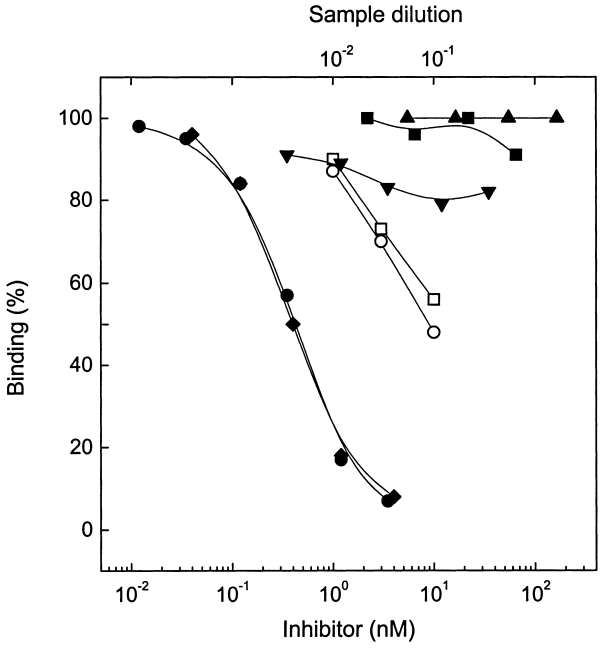


Fig. 2. Radioimmunoinhibition assay for laminin fragment α4IIIa. The assay consisted of 1 ng $^{125}$I-labeled α4IIIa and a constant amount of a rabbit antiserum against α4IIIa. Inhibitors used were mouse laminin fragments α4IIIa, prior to (●) and after chondroitinase treatment (♦) or reduction and alkylation (▼), α3AIIIa (■) and α2VI/V (▲). EDTA extracts from adult mouse heart (○) and intestine (□) were used at the dilution shown on top.

ence of Ser-53 and Ser-76 indicating the lack or only minimal occupation by chondroitin sulfate.

In order to identify similar modifications in tissue forms of α4 chain-containing laminins and to analyze their tissue contents and distribution we prepared a rabbit antiserum against the glycosaminoglycan form of α4IIIa. This antiserum showed high titers in ELISA and radioimmunoassay and only minimal cross-reaction with similar fragments from related α chains. We also established a sensitive radioimmunoinhibition assay which could be inhibited by α4IIIa (half-maximal at 0.4 nM) while the related N-terminal fragments of α2 and α3 chains (α2VI/V, α3AIIIa) showed no effects at more than 100-fold higher concentrations (Fig. 2). Digestion of α4IIIa with chondroitinase ABC did not change its inhibitory capacity but a large loss was observed after reduction and alkylation indicating that the antibodies recognize protein epitopes.

The radioimmunoassay could be also inhibited by mouse

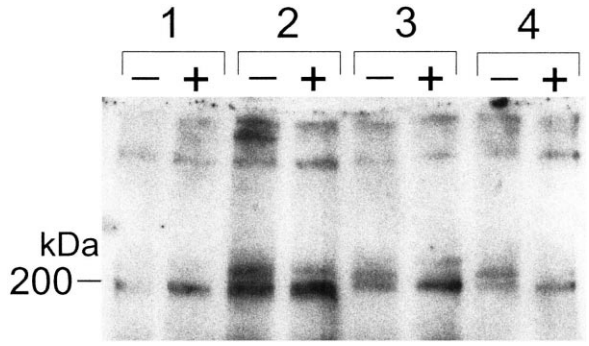


Fig. 3. Immunoblot detection of laminin α4 chain in tissue extracts and effects of chondroitinase digestion. Laminins containing α4 chains were immunoprecipitated from detergent extracts of mouse skeletal muscle (1), placenta (2), heart (3) and lung (4) and examined prior to (−) and after (+) digestion with chondroitinase ABC.

tissue extracts showing a very similar dose–response profile compared to α4IIIa (Fig. 2). This demonstrated that the assay will be useful for quantitation and was applied to various adult mouse tissues which were sequentially extracted with a neutral EDTA-containing buffer followed by detergents [25]. This protocol was separately shown to solubilize 70–90% of the laminins from tissues (T. Sasaki, R. Timpl, unpublished). The extracts were in addition examined for their content of laminin γ1 chain (γ1III3–5 assay), which is shared by most laminin isoforms, and α2 chain (α2VI/V assay), which is the most prevalent laminin α chain in most tissues [2]. The data demonstrated a variable content of α4 chain (10–65 pmol/g wet tissue) comprising 5% (placenta) to 30% (lung, skin, stomach) of the γ1 chain content (Table 1). Interestingly, some tissues (lung, skin) contained significantly more α4 than α2 chain while in most other tissues, especially in heart and skeletal muscle, the α2 chain predominated.

Several tissue extracts were then used to examine the possible modification of α4 chains by chondroitin sulfate substitution. The corresponding laminins were first immunoprecipitated with antibodies to α4LG1–3 and then examined prior to and after treatment with chondroitinase ABC by reducing electrophoresis followed by immunoblotting (Fig. 3). This demonstrated a major α4 chain band of 190–200 kDa and one to three additional bands in the range of 210–230 kDa. Several of these additional bands were lost or reduced in intensity after digestion indicating their substitution by chondroitin sulfate. The blot also demonstrated some larger α4 bands of about 600 kDa or higher molecular mass suggesting covalent cross-linking of the laminin chains by non-reducible

Table 1
Contents of laminin γ1, α2 and α4 chains in tissue extracts from adult mice determined by specific radioimmunoinhibition assays

| Tissue | Assays for | | |
|---|---|---|---|
| | γ1III3–5 (pmol/g wet tissue) | α2VI/V (pmol/g wet tissue) | α4IIIa (pmol/g wet tissue) |
| Heart | 567 | 281 | 47 |
| Lung | 225 | 27 | 65 |
| Skeletal muscle | 133 | 84 | 13 |
| Skin | 31 | 11 | 11 |
| Placenta | 510 | 10 | 23 |
| Intestine | 97 | 18 | 33 |
| Stomach | 168 | 98 | 45 |
| Kidney | 64 | 21 | 10 |

Tissues were extracted sequentially with neutral buffer containing EDTA followed by detergents and the sum of both extracts is recorded. Placenta is particularly rich in α1 chain (484 pmol/g).

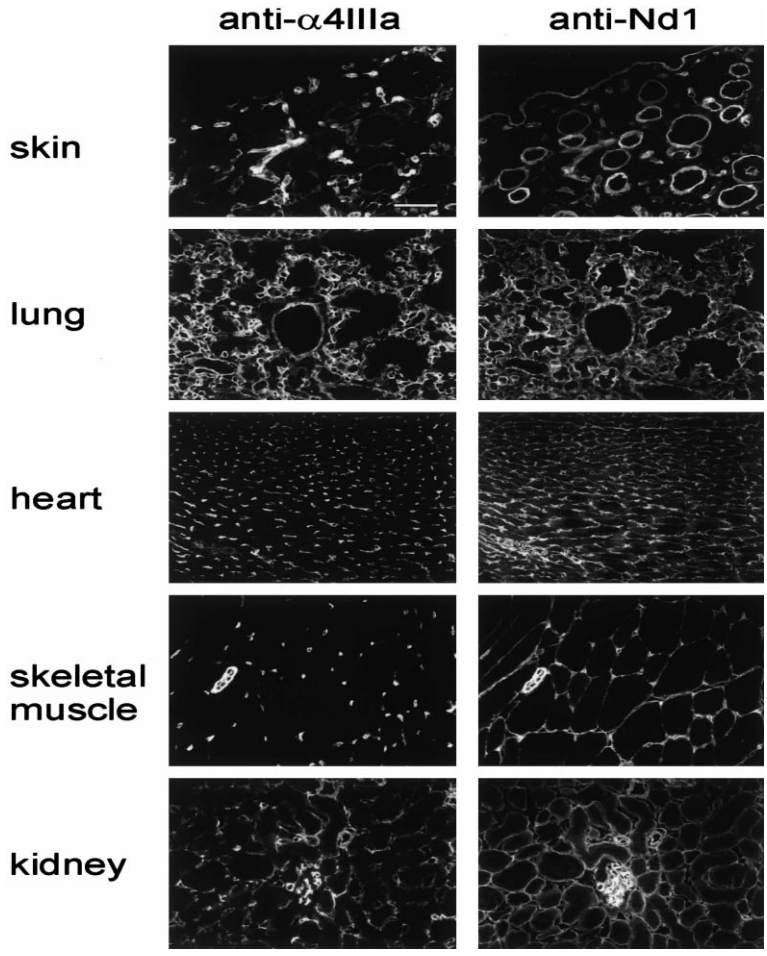


Fig. 4. Double immunofluorescence staining of mouse tissues with rabbit antibodies against α4IIIa and rat monoclonal antibody against nidogen-1 (Nd1). Tissues used were neonatal skin, embryonic lung (day 18) and adult heart, muscle and kidney. The bar (skin) indicates 50 μm for all tissues.

bonds. A few of these bands were also changed after chondroitinase ABC digestion indicating their substitution.

Previous studies with antibodies against recombinant C-terminal fragments of laminin α4 showed a clear immunostaining for the α4LG1–3 structure while the most C-terminal α4LG4–5 could not be detected in tissues [11]. We have therefore also used affinity-purified antibodies against α4IIIa for tissue immunofluorescence and compared them in double staining with the ubiquitous basement membrane protein nidogen-1 (Fig. 4). This showed that in neonatal skin nidogen staining was restricted to the basement membrane zones at the dermal–epidermal junction and around hair follicles and to some capillaries and larger vessels while the reaction with dermal tissue was rather weak. The α4 chain could, however, be primarily detected around these vessels and as large dermal deposits between the hair follicles with only little overlap with their basement membranes. A restriction of the α4 chain to small and some larger vessels and bronchial stroma was also found in embryonic lung and only little staining was observed for alveolar basement membranes. The latter could be clearly stained for nidogen which was also present in most of the vessel walls. In heart and muscle the α4 chain was also a typical component of capillaries and of the perineurium. In skeletal muscle it included a few synaptic regions as demonstrated by double staining with α-bungarotoxin (not shown). Nidogen was also found in these locations but in addition in the basement membranes around myocytes and cardiomyocytes. A similar difference was detected in kidney with nidogen being present in tubular and glomerular basement membranes and some vessel walls. A much stronger staining of various vessels was again observed with antibodies against α4IIIa which also reacted with some glomerular regions and tubular basement membranes in a segmental fashion (Fig. 4).

## 4. Discussion

A mature form of the laminin α4 chain has recently been shown to have lost the most C-terminal α4LG4–5 modules which could no longer be detected in adult tissues [11]. A similar processing has so far been known only for the laminin α3A chain and included as a second step also the release of the N-terminal segment α3AIIIa [29]. This segment has a similar size and modular structure as the N-terminal domain (α4IIIa) of the laminin α4 chain [1,2], raising the interesting question whether it is also removed by proteolytic cleavage. We therefore prepared recombinant α4IIIa in order to obtain antibodies for these analyses and to examine in addition its posttranslational modifications as suggested from some sequence peculiarities. The data showed the persistence of the α4IIIa segment in tissues and its efficient substitution by

chondroitin sulfate, a modification not known before for other laminin chains.

The nearly complete modification of recombinant α4IIIa facilitated its purification and demonstrated a distinct size heterogeneity (15–40 kDa) for the chondroitin sulfate chains attached. A single SGD sequence close to the N-terminus represents the major acceptor site while another candidate SGE sequence seems to be not or only minimally modified. Three SGD sequences and a single SGG were shown to be the major glycosaminoglycan attachment sites in the basement membrane proteoglycan perlecan but are usually modified by heparan sulfate [18,19,30]. The lack of modification of SGE in the recombinant α4 chain fragment may reflect the lack of adjacent controlling sequences as shown for perlecan [30]. Alternatively, since this SGE is located in an LE module-type disulfide-bridged d-loop known to make tight intermodular contacts in tandem arrays of such modules [3], this could impose strong constraints on accessibility. A similar yet partial modification was also shown for the α4 chains obtained from several tissues indicating a biological function but also tissue variability. Laminins containing α4 chains were also obtained from platelets [12], some cultured cells [7,11] and as a recombinant product [10], and showed in part a similar electrophoretic heterogeneity. It will be of interest to examine to which extent chondroitin sulfate modification accounts for this.

Chondroitin sulfate proteoglycans are prominent components of cartilage and many other interstitial matrices including in particular the families of lecticans/hyalectans and some leucine-rich repeat proteoglycans [31,32]. They have also been identified as cellular receptors including syndecans and NG2 [33], as a minor perlecan variant and another basement membrane proteoglycan bamacan [18,34]. Our data now add laminins-8 and -9 as further members to these components. Compared to heparan sulfates [31,33] very little is known about the molecular functions of chondroitin sulfates. They certainly endow their core proteins with a strong polyanionic character which could compartmentalize and influence supramolecular organization in extracellular matrices [35]. They also play a major role in the extracellular brain matrix and could be involved in nerve guidance [32]. This now remains to be examined by the identification of potential ligands for the α4IIIa segment.

Rabbit antisera against C-terminal LG modules of the α4 chain were previously shown to be useful for immunoblotting and immunostaining of cells and tissues [11]. These antigenic fragments could, however, not be used for radioimmunoassays apparently due to a high sensitivity for radiation damage (unpublished). This was not the case with fragment α4IIIa, which made it possible to establish a sensitive and reproducible inhibition assay for the quantitation of the α4 chain in tissue extracts. This revealed particularly high levels in lung, heart, stomach, placenta, intestine, muscle and skin and accounted for 5–30% of the laminin γ1 chain contents in these extracts. The data are consistent with a comparably high expression of α4 mRNA in these tissues described previously [4–9,13]. The antibodies against α4IIIa were also suitable for immunofluorescence analysis of tissues with staining patterns similar to those described for anti-α4LG1–3 [11] and also with some differences, with antibodies against α4 domain I,II fusion proteins [8,9]. In the heart and skeletal muscle α4 staining was restricted to capillary walls and perineural basement membranes and some synapses while basement membrane zones where positive for nidogen-1 but not for α4. Capillary staining for α4 chain was also found in skin, lung and kidney (Fig. 4) and has been described for brain [16]. The corresponding laminins are very likely produced by endothelial cells as shown by in situ hybridization [7] and in cultured cells [7,11]. They are, however, not necessarily deposited in the endothelial basement membranes but in some adjacent regions as indicated by immunogold staining [11].

The deposition of α4 chain laminins to interstitial regions close to basement membranes was also suggested by the staining patterns of some other tissues. This was clearly evident for skin which showed a distinct staining of the dermis between hair follicles but not around their basement membranes which is in agreement with α4 in situ hybridization of skin mesenchymal cells [7]. There was also a lack of staining of alveolar basement membranes which are, however, surrounded by many α4 chain-positive capillaries. Typical basement membranes such as in renal glomeruli and tubules appeared also not or only segmentally stained. A definitive proof of these indications will now require immunogold staining with the antibodies described here and previously. This may reflect the peculiar structure of the N-terminal region of the α4 chain which lacks an LN module known to be important in other α chains for the polymerization of laminins into non-covalent networks within basement membranes [36,37]. It also suggests that laminins-8 and -9 participate in different supramolecular assemblies when deposited in non-basement membrane regions which also, as shown here for skin, are stained only weakly for nidogen-1, a major ligand of the laminin γ1 chain [26], which is needed for the stabilization of such networks.

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