Primary Structure, Developmental Expression, and Immunolocalization of the Murine Laminin a4 Chain*

(Received for publication, January 15, 1997, and in revised form, June 19, 1997)

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The complete primary structure of the mouse laminin a4 chain was derived from cDNA clones. The translation product contains a 24-residue signal peptide preceding the mature a4 chain of 1,792 residues. Northern analysis on whole mouse embryos revealed that the expression was weak at day 7, but it later increased and peaked at day 15. In adult tissues the strongest expression was observed in lung and cardiac and skeletal muscles. Weak expression was also seen in other adult tissues such as brain, spleen, liver, kidney, and testis. By in situ hybridization of fetal and newborn tissues, expression of the laminin a4 chain was mainly localized to mesenchymal cells. Strong expression was seen in the villi and submucosa of the developing intestine, the mesenchymal stroma surrounding the branching lung epithelia, and the external root sheath of vibrissae follicles, as well as in cardiac and skeletal muscle fibers. In the developing kidney, intense but transient expression was associated with the differentiation of epithelial kidney tubules from the nephrogenic mesenchyme. Immunohistologic staining with affinity-purified IgG localized the laminin a4 chain primarily to lung septa, heart, and skeletal muscle, capillaries, and perineurium.

The laminins are a complex family of trimeric extracellular matrix proteins (1–5). They are composed of three types of chains, which are classified as α, β, and γ. The chains associate with one another to form a triple-helical coiled coil. To date, the primary structure of 10 genetically distinct laminin chains, α1–α5, β1–β3, and γ1 and γ2 have been determined from mammals (6–28). The different laminin chains are composed of specific modules that include domains with globular structures, rod-like domains containing cysteine-rich repeats, and domains participating in the coiled coil of the long arm. Additionally, the α chains contain a large carboxyl-terminal globular domain with five internal repeat motifs that share homology with corresponding motifs in perlecain (29, 30), agrin (31), and Drosophila crumbs protein and the sex hormone-binding globulin (33).

Numerous functions have been proposed for laminins (for review, see Refs. 1, 4, and 34), but the physiological roles of the different isoforms are still largely unknown. However, the laminin subunit chains differ extensively with respect to their distribution suggesting tissue-specific functions. Insight into the functions of individual chains and isoforms has come particularly from studies on genetic diseases and by targeted gene disruption in mice. First, in epidermolysis bullosa junctionalis, a skin blistering disease, mutations have been described in all three human genes encoding the subunit chains of the laminin-5 isoform (α3β3γ2) (35–38). This demonstrates the requirement of this hemidesmosomal protein for the attachment of epithelial cells to the underlying matrix. Secondly, defects involving the gene for the α2 chain (LAMA2) have been described in dy mice (39) and in patients with congenital muscular dystrophy (40). This suggests that this chain, a component of the laminin-2 isoform (α2β1γ1), is important for the differentiation or function of muscle fibers. Thirdly, the requirement of the laminin β2 chain for a functional glomerular filtration barrier has been demonstrated by the development of proteinuria-associated nephropathy in mice with inactivated gene (LAMR2−/−) (41). However, the actual chain composition of the GBM laminin isoform containing the β2 chain is not known.

The development of nephrons, the filtration units of the kidney, involves a specific interplay of signaling between the epithelial cells of the ureter bud and the surrounding mesenchymal cells (42, 43). During this process, the epithelial cells induce condensation and polarization of the mesenchyme at the tip of the ureter bud. This group of mesenchymal cells undergoes transepithelialization to form an S-shaped mass referred to as the S body. Later, the S body fuses to the ureter bud. Eventually, the S body gives rise to the proximal and distal parts of the tubular system, as well as to the epithelial component of the glomeruli, i.e. cells covering the inside of the Bowman’s capsule and the podocytes that cover the outside of the glomerular capillaries. It has been shown earlier that the expression of laminin genes is subject to characteristic changes in the developing kidney. Specifically, α1 chain expression is absent in the undifferentiated mesenchyme, but it is turned on concomitantly with the start of differentiation and polarization of the mesenchyme (44). Furthermore, the α1 chain is expressed in the epithelial cells of the ureter bud and the tubules that form the loops of Henle (44).

The function and expression of the laminin a4 chain is still poorly known. We have previously reported the sequence of the human laminin a4 chain, but thorough studies on developmental expression of this protein are difficult in human tissues. In the present study we have cloned the murine a4 chain, determined its primary structure, and examined its developmental
expression in mouse tissues. Furthermore, polyclonal antibodies were made against the \( \alpha 4 \) chain and used for immunolocalization of the protein in murine tissues. The results showed the \( \alpha 4 \) chain to be located mainly in lung, around heart muscle fibers, and in capillary basement membranes as well as in perineurium.

**EXPERIMENTAL PROCEDURES**

**Isolation and Characterization of cDNA Clones**—A murine lung cDNA library in the Agt11 cloning vector (CLONTECH) was screened using standard techniques (45) with a human laminin \( \alpha 4 \) chain cDNA clone covering about 1.1 kb of the 3'-coding sequence (15). Clone ML1 was sequenced and shown to encode the mouse \( \alpha 4 \) chain. The ML1 clone was subsequently used as a probe to screen a mouse lung cDNA library, and in this manner a series of overlapping cDNA clones were identified. The 5'-end clone, ML89, was obtained using an amplified library sequence. A 50-\( \mu \)l PCR was performed using 0.02 \( \mu \)l of library stock as a template and Agt11 reverse primer as one primer and (5'ttggcagac-ccgtacctac) as the other. One \( \mu \)l of the product was reamplified using the same vector primer and a nested specific primer (5'ctcgggccgag- tctggtc3'). The resulting product was electrophoresed on agarose (SeaPlaque GTG). A pipette tip was stabbed through a 1-kb product and swirled in a PCR mixture. A following round of amplification was carried out using the same specific primer and biotinylated vector primer. Reasonably specific product was obtained and gel-purified, and carried out using the same specific primer and biotinylated vector primer.

**Preparation of Recombinant Laminin \( \alpha 4 \) Chain Antigens and IgG**—For the generation of antibodies, recombinant protein containing base pairs 1221–2342 of the cDNA, corresponding to residues 408–781 in domain I/II of the human \( \alpha 4 \) chain was expressed. The insert was cloned into the pGEX-1AT vector (Pharmacia), expressed according to standard procedures, and the recombinant glutathione S-transferase fusion protein was then purified using glutathione-Sepharose affinity columns (Pharmacia). Two rabbits were immunized five times with standard procedures using 50–100 \( \mu \)g of antigen for each injection.

Laminin \( \alpha 4 \) chain-specific IgGs were affinity-purified as follows. The bacterial fusion protein, eluted from the glutathione-Sepharose matrix was further purified by reversed phase chromatography. Aliquots of the fusion protein (150 \( \mu \)l) were applied onto a Delta Pak RP4 3.9 × 150 mm columns (Waters) and eluted with a CH3CN gradient (45–52% in 28 min, 0.8 ml/min) in 0.1% trifluoroacetic acid. The correct fractions were identified by SDS-polyacrylamide gel electrophoresis, concentrated in a SpeedVac, and redissolved in 2 \( \mu \)l, 50 mM NaHCO3, pH 8.3, 0.5 M NaCl. This material was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The IgG was first preadsorbed with bovine serum albumin-coupled Sepharose, and then the nonbound fraction was incubated with the glutathione S-transferase-antigen fusion protein coupled to Sepharose. Following extensive washing with phosphate-buffered saline the bound IgG was eluted with 0.1 M glycine, pH 3.0, immediately neutralized, pooled, and dialyzed against phosphate-buffered saline. The affinity-purified IgG was used for immunostaining at a 15 \( \mu \)g/ml concentration.

**Western Blotting and Immunocytochemistry**—Specificity of the antisera was examined by Western blotting analyses. Briefly, denaturing 6% polyacrylamide gels were electrophoresed under reducing conditions, after which the proteins were blotted onto nitrocellulose filters using a semidyblotting device (Bio-Rad) according to the manufacturer's instructions. The bound antibodies were detected using horseradish peroxidase-coupled secondary antibodies (Dako) and chemiluminescence (NEN Life Science Products). For immunohistochemical analyses mouse tissues were embedded in paraffin following fixation in 10% formalin. Endogenous peroxidase activity was quenched with 3% \( \text{H}_2\text{O}_2 \) for 30 min, and the samples were boiled for 10 min in 1 \% urea. Skeletal muscle was fixed with 3% acetic acid, 70% ethanol. Staining was performed by indirect techniques using biotinylated secondary antibodies in conjunction with a horseradish peroxidase ABC kit (Dako). Peroxidase activity was detected with diaminobenzidine or aminoethylcarbazole.

**RESULTS**

**Primary and Domain Structure of the Mouse Laminin \( \alpha 4 \) Chain**—Six overlapping cDNA clones isolated and characterized in this study (Fig. 1) together covered a total of 5,824 nucleotides, including 208 bp of a 5'-untranslated region and 168 bp of a 3'-untranslated region, respectively (GenBank\textsuperscript{TM} accession number U59865). The open reading frame of 5,448 nucleotides coded for a 1,816-residue laminin \( \alpha 4 \)-type chain (Fig. 2), characterized by the presence of a carboxyl-terminal G domain with five internal repeat motifs. The predicted sequence of the first 24 residues is characteristic of a hydrophobic signal peptide which is present in all other laminin chains, and the amino acid sequence around position 24 is characteristic for a signal peptidase cleavage site as described by von Hejne and co-workers (50, 51). Thus, the mouse laminin \( \alpha 4 \) chain proper contains 1,792 residues. The calculated mass of the entire translation product is 221,818 and that of the processed \( \alpha 4 \) chain 199,164.

\[^{1}\text{The abbreviations used are: kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction.}\]
FIG. 2. cDNA-derived amino acid sequence of the murine laminin a4 chain and alignment with the human chain. Upper sequence, mouse a4 chain; lower sequence, human chain where different from the mouse sequence. The cysteines are circled, and N-linked glycosylation sites are boxed. A dotted rounded box indicates a nonconserved site for N-linked glycosylation. A two-residue gap in the alignment corresponding to mouse residues 881–882 (see text) is indicated by dashes, and a similar gap in the mouse sequence corresponding to human residues 1418–1419 is depicted by a vertical bar. Potential cleavage site for the signal peptide is indicated by a solid triangle. The approximate domain location in the a4 chain sequence is indicated on the right.
was also observed. In testis a second weak band of about 5 kb of the a chain was seen in all other tissue studies, and in skeletal and heart muscle tissues. Weaker expression was seen in the mesenchymal cells surrounding the branching lung epithelia (Fig. 4, B and C). Expression was also seen in the villi and submucosa of the intestine (Fig. 4, C and D) and in the external root sheath of vibrissae follicles (Fig. 4, E and F). The expression in intestine and vibrissae may be associated with the endothelial cells of capillaries. Distinct expression was also shown to be present surrounding the nuclei of both cardiac and skeletal muscle fibers (Fig. 5). Some of the signals in muscle appeared to be present in endothelial cells of capillaries.

Expression of the Laminin a4 Chain during Mouse Development—In our previous work (14) we showed by Northern hybridization studies that the human laminin a4 chain is expressed in a highly tissue-restricted manner, indicating that this chain is present in a laminin isoform with highly specific, but as yet unknown functions. However, in situ hybridization studies were only possible to perform on a limited number of embryonic brain, lung, and muscle tissues. These studies indicated that the expression of the a4 chain is mainly confined to cells of mesenchymal origin. The present cloning of the mouse a4 chain allowed a more detailed analysis of its expression in mouse tissues.

Expression of the laminin a4 chain was first studied for levels of mRNA using Northern analysis (Fig. 3). Using RNA from whole mouse embryos, the level of expression was low at embryonic day 7, but it later increased being highest at day 15. In adult, strong expression of a 6-kb mRNA was seen in lung and in skeletal and heart muscle tissues. Weaker expression was seen in all other tissue studies, i.e. brain, spleen, liver, kidney, and testis. In testis a second weak band of about 5 kb was also observed.

In situ hybridization of mouse embryos revealed expression of the a4 chain in mesenchymal cells of several tissues (Fig. 4). In the developing lung strong expression was seen in mesenchymal cells surrounding the branching lung epithelia (Fig. 4, A and B). Expression was also seen in the villi and submucosa of the intestine (Fig. 4, C and D) and in the external root sheath of vibrissae follicles (Fig. 4, E and F). The expression in intestine and vibrissae may be associated with the endothelial cells of capillaries. Distinct expression was also shown to be present surrounding the nuclei of both cardiac and skeletal muscle cells of mesenchymal origin. The present cloning of the mouse tissues.

The results revealed a single 6-kb band in fetal and adult tissues, except for testis, where an additional 5-kb band was observed. A, poly(A) RNA from whole embryos; B, poly(A) RNA from adult mouse tissues.

Expression of the Laminin a4 Chain in Mouse Embryos—In situ hybridization of laminin a4 expression in developing organs of mouse embryos. A and B, E12 lung. Expression is seen in the mesenchyme surrounding the bronchial epithelium. The epithelia are negative. C and D, newborn mouse intestine. Expression is seen in the center of intestinal villi and along the submucosa, possibly in endothelial cells of capillaries. E and F, E18 vibrissae. The hair follicle expresses the a4 chain in the external root sheath. No expression above background could be detected in E12 liver. ct, connective tissue; sm, submucosa; v, villi.

Characterization of Polyclonal Antibodies—The antiserum raised against a portion of domain III of the laminin a4 chain was found to be suitable for characterization by Western blotting. To examine for monospecificity of the antiserum against the a4 chain, Western blotting was carried out on extracts from cultured cells and tissues, such as heart and lung. As shown in Fig. 7, the antiserum, used at 1:1000 dilution, detected a distinct band corresponding to slightly over 200 kDa in the cell culture medium of human SK-LMS-1 leiomyosarcoma cells (ATCC HTB 88). The calculated size of the unglycosylated a4 chain is 198,164 daltons and the slightly larger size obtained here is therefore likely to correspond to a glycosylated polypeptide chain. The synthesized polypeptide chain which contains several potential glycosylation sites is therefore likely to correspond to a glycosylated polypeptide chain. The synthesized polypeptide chain which contains several potential glycosylation sites is therefore likely to correspond to a glycosylated polypeptide chain. The synthesized polypeptide chain which contains several potential glycosylation sites is therefore likely to correspond to a glycosylated polypeptide chain.
DISCUSSION

The present work describes the full sequence of the mouse laminin α4 subunit chain. Importantly, the clones isolated in this study facilitated thorough studies on the temporal and spatial expression of this chain, which was shown to have a highly restricted expression pattern during mouse development.

Comparison of the mouse α4 chain sequence with that of human one revealed a sequence identity of 88.1%. Of the 44 cysteines and 19 potential N-linked glycosylation sites in the mature polypeptide, 43 and 18 are conserved, respectively. It is of interest that although the total length of the mouse and human chains is identical, in the alignment there are two balancing 2-residue gaps located in the G-domain. The significance of these gaps is not clear, but it is possible that the structural constraints posed by the folded polypeptide chain allow some variation at these sites. The first gap is close to the boundary between domains I/II and G, and the second gap is located between the third and fourth subdomains of the G domain. In the drosophila α-chain, a spacer sequence of threonines and serines lies between these subdomains (52, 53).

In general, tissue distribution of the laminin α4 chain was shown to extensively differ from that of the other laminin α chains. The α4 chain is predominantly located in lung alveolar septa, around heart muscle fibers, in the perineurium, as well as in subendothelial basement membranes of capillaries. In contrast, the α1 chain is expressed mainly in the central nervous system, and in the developing kidney (44, 54).
of the α1 chain is also seen in developing lung and gut epithelium (54), but the expression is never mesenchymal as was shown to be the case for the α4 chain in the present study. The α2 chain is expressed predominantly by skeletal and heart muscle cells (11, 55, 56). Although the α2 chain is expressed in mesenchymal cells in several tissues during human embryonic development, the expression pattern is different from that of the α4 chain (10). Here, the α4 chain was shown to differ from α2 as α4 is present around adult cardiac but not skeletal muscle fibers. However, the α4 chain is expressed in fetal skeletal muscle fibers as determined by in situ hybridization and is present around these fibers in embryos and newborn mice as shown by immunohistochemical staining. Therefore, the developmentally regulated α4 chain may be of importance for the development of skeletal muscle and for function during early development. The α2 and α4 chains also differ from each other in that the α2 chain is expressed in the developing human kidney in the mesenchyme adjacent to the condensing pretubular epithelia, while α4 is absent in this region (10).

Although the α4 chain is structurally most closely related to the α3 chain, their tissue distribution is very different. The α3 chain is epithelial. It is seen in the epithelium of skin, lung, and

**Fig. 8. Immunolocalization of the laminin α4 chain using peroxidase-labeled second antibodies.** A, low magnification of adult cardiac muscle reveals linear staining of most muscle fibers and intense staining of capillary basement membranes. B, higher magnification of heart muscle emphasizes the intense subendothelial staining of capillaries (straight arrow), as well as the reaction around individual muscle fibers (bent arrow). C, staining of adult skeletal muscle yields staining in what appears to be capillaries, whereas the sarcolemma does not exhibit any strong staining (longitudinal section). D, staining of peripheral nerves shows distinct staining of the perineurium as well as Schwann cell sheaths. E, staining of lung reveals distinct, but discontinuous staining of alveolar septa with anti-α4 chain IgG, whereas preimmune IgG (F) is completely negative.
digestive tract, but never in the mesencephalon (57). In contrast, the a4 chain is mainly mesenchymal and endothelial. In addition, the enamel organ shows strong signals for the a3 chain (57), but we did not see any signals above background for the a4 chain in the developing teeth (not shown). The newly discovered a5 chain is strongly expressed in the adult kidney (16) where the level of a4 chain expression is very low. There is little evidence for the presence of the other a chains in several mesenchymal tissues, i.e. in lung and intestine, where we see the a4 chain expressed (10, 55–58). These tissues do express b1 and y1 chains (54, 56), but not b3 or y2 (57). Furthermore, in the embryonic kidney the mesenchyme adjacent to the ureter bud lacks b2, b3, and y2 chain expression (41, 57, 58), while it gives strong signals for the b1 and y1 chains (44, 54). Therefore, it is possible that the a4 chain associates in these tissues with the b1 and y1 chains to form the eighth laminin isoform.

However, this and the other possible chain combinations involving the a4 chain remain to be determined.

The subendothelial location of the a4 chain suggests that the laminin isoform containing it may serve as an adhesion protein for endothelial cells in a similar manner as the a3 chain containing laminin-5 (59). However, it is apparent that a4 chain containing laminin isoforms have broader physiological functions, as this polypeptide chain is also so prominent in striatic muscle fibers and peripheral nerves. The unique expression pattern of the laminin a4 chain during a special period of nephrogenesis, i.e. the formation of the proximal nephron, implies specific temporal requirements for a laminin isoform containing the a4 chain. The a1 chain may also be important for the formation of the proximal nephron, but it is apparently also important for the formation and maturation of the entire nephron as it has an unique expression pattern in the distal parts of the tubuli (44). Expression of both chains decreases in the kidney with age.

Thus far, no diseases have been linked to the genes for the a1, a4, or o5 chains, as is the case for the o2 and a3 chain genes that are affected in congenital muscular dystrophy and epidermolysis bullosa, respectively (35–40). However, based on the wide distribution of the laminin a4 chain, it can be anticipated that mutations in its gene can lead to severe complications and diseases. The generation of transgenic mice containing an interrupted LAMA4 gene may shed light on the role of this interesting laminin component.

Acknowledgments—We are grateful to Hanna Valtoton, Veterinary Faculty of the University of Helsinki for expert technical assistance.

REFERENCES

1. Engel, J. (1992) Biochemistry 31, 10643–10651
2. Tryggvason, K. (1993) Curr. Opin. Cell Biol. 5, 877–882
3. Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H., Martín, G. R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P. D. (1994) Matrix Biol. 14, 209–211
4. Timpl, R., and Brown, J. (1994) Matrix Biol. 13, 243–252
5. Ibáñez, A., Vueloenehao, S., Sainio, K., Janit-Sait, S., Eddy, R., Shows, T., Sariola, H., and Tryggvason, K. (1994) Matrix Biol. 14, 489–497
6. Noakes, P. G., Gautam, M., Mudd, J., Sanes, R. S., and Merlie, J. P. (1995) Nature 374, 258–262
7. Gerecke, D. R., Wagman, D. W., Chiquet, M.-F., and Tryggvason, R. E. (1996) J. Biol. Chem. 271, 11073–11080
8. Galliano, M. F., Aberdam, D., Aguzzi, A., Ortonne, J. P., and Meneguzzi, G. (1995) J. Biol. Chem. 270, 21820–21826
9. Richards, A. J., Al-Imaina, L., Carter, N. P., Lloyd, J. C., Leversha, M. A., and Pope, F. P. (1994) Genomics 22, 237–239
10. Ibáñez, A., Vueloenehao, S., Sainio, K., Sariola, H., and Tryggvason, K. (1995) FEBS Lett.