ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

DEVELOPMENTAL BIOLOGY

Developmental Biology 263 (2003) 153–164

www.elsevier.com/locate/ydbio

Role for laminin-α5 chain LG4 module in epithelial branching morphogenesis

Yuichi Kadoya,[a,*] Mayumi Mochizuki,[b] Motoyoshi Nomizu,[b] Lydia Sorokin,[c] and Shohei Yamashina[a]

[a] *Department of Anatomy, Kitasato University School of Medicine, Sagamihara 228-8555,Japan*
[b] *Graduate School of Environmental Earth Science, Hokkaido University Graduate School, Sapporo 060-0810, Japan*
[c] *Department of Experimental Pathology, Lund University, SE 22185 Lund, Sweden*

Received for publication 13 December 2002, revised 8 July 2003, accepted 11 July 2003

Abstract

Laminin-α5 chain was localized in all epithelial basement membranes (BMs) of mouse submandibular gland (SMG) from the onset of branching morphogenesis and became restricted to BMs of epithelial ducts in the adult. To investigate whether the laminin-α5 chain plays a role in branching morphogenesis, a set of cell-adhesive peptides from the C-terminal globular domains (LG1-5) was tested for their effects in SMG organ cultures. One peptide, LVLFLNHGH (A5G77f), which represents a sequence located in the connecting loop between strands *E* and *F* of LG4, perturbed branching morphogenesis and resulted in irregularities in the contours of epithelial structures, with formation of deep clefts. The data suggest a role for the laminin-α5 LG4 module in the development of the duct system, rather than in the bifurcation of epithelial clusters. The epithelial BM of A5G77f-peptide-treated explants was continuous, which was in contrast to our previous finding of impaired epithelial BM assembly in explants treated with the laminin-α1 LG4 module peptide, or with a monoclonal antibody against this domain. A5G77f also perturbed in vitro development of lung and kidney. These results suggest a crucial role for the LG4 module of laminin-α5 in epithelial morphogenesis that is distinct from that of the laminin-α1 LG4.

Keywords: Basement membrane; Branching morphogenesis; Immunohistochemistry; Laminin-α chain; Laminin-α5; Organ culture; Peptide; Submandibular gland

Introduction

Submandibular glands (SMG) of developing mouse and rat are classic and well-described systems for studying branching morphogenesis (Borghese, 1950; Grobstein, 1953). The earliest rudimentary SMG epithelium consists of a large cell cluster connected distally to a stalk. As a result of repetitive bifurcations of the cluster, a tree-like duct system forms, the distal ends of which terminate in end buds. The duct system further differentiates along the proximal–distal axis, resulting in the excretory duct, striated duct, granular convoluted tubule (GCT) (Gresik, 1994), and intercalated duct (Pinkstaff, 1979). The end buds differentiate into acini, comprised of well-polarized secretory cells.

The developing SMG exhibits a spaciotemporal expression of various growth factors (Jaskoll and Melnick, 1999), including EGF and FGF, which have been shown in organ culture studies (Kashimata et al., 2000; Morita and Nogawa, 1999; Nogawa and Takahashi, 1991) and in investigations of genetically mutated mice (Celli et al., 1998; De Moerlooze et al., 2000; Jaskoll and Melnick, 1999; Ohuchi et al., 2000) to be essential for development. In addition to these soluble factors, direct involvement of extracellular matrix (ECM) molecules (Nakanishi et al., 1986), in particular those of basement membranes (Bernfield et al., 1984; Takahashi and Nogawa, 1991), has been implicated in SMG development. However, studies on the molecular basis of the effects of individual basement membrane molecules on SMG development are limited.

The laminin family of basement membrane proteins consists of 15 isoforms (Colognato and Yurchenco, 2000;

* Corresponding author. Fax: +81-427-778-8441.
E-mail address: kadoya@kitasato-u.ac.jp (Y. Kadoya).

0012-1606/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0012-1606(03)00446-9

Libby et al., 2000). Specific antibodies against various laminin isoforms have been established and have been employed in immunohistochemistry to define the in vivo localization of several laminins during mouse development (Lefebvre et al., 1999; Miner et al., 1997; Salmivirta et al., 1997; Sorokin et al., 1992, 1997a,b), including SMG (Kadoya et al., 1995, 1998; Kadoya and Yamashina 1999). Such studies have revealed a developmentally regulated expression patterns and suggest roles for laminin isoforms in the development of several organs (Ekblom et al., 1998; Sorokin and Ekblom, 1992).

Laminins are large cross- or Y-shaped glycoproteins composed of an α, β, and γ chain (Sasaki and Timpl, 1999). Three or two short arms and one long arm have been identified by electron microscopy. Various biological activities of laminins, such as cell adhesion, spreading, migration, and cell growth (Colognato and Yurchenco, 2000), have been mapped to distinct structural domains (Martin et al., 1996) or amino acid sequences (Makino et al., 1999). The short arms are composed of the amino-terminal ends of α, β, and γ chains, and have been shown to mediate laminin polymerization (Garbe et al., 2002; Yurchenco and Cheng, 1993). In contrast, the long arm is composed of the carboxyl-terminal end of the α chain and is involved in the binding to cellular receptors. The best analyzed domain of the laminins is the most distal end of the long arm, the G domain (Timpl et al., 2000). Sequence analysis indicates that the G domain consists of a tandem repeat of five LG modules (LG1-5). LG modules of some laminin α chains, if not all, have been found to bind cell surface molecules, such as integrins (Aumailley et al., 1996), syndecans (Salmivirta et al., 1994), α-dystroglycan (Ervasti and Campbell, 1993; Talts et al., 1999), and the Lutheran blood group glycoprotein (Kikkawa et al., 2002; Moulson et al., 2001). Recently, we identified more than 20 cell-adhesive peptide sequences by systematic screening of an overlapping synthetic peptide library covering the entire G domain sequences of laminin-α1, -α4, and -α5 (Makino et al., 2002; Nomizu et al., 1995; Okazaki et al., 2002), and LG4 of laminin-α3 (Utani et al., 2001). Of these sequences, the laminin-α1 peptides have been systematically screened for their ability to interfere with morphogenesis of SMG and hair follicles in organ cultures. A synthetic peptide, RKRLQVQLSIRT (residues 2719–2730 of laminin-α1 chain LG4 module and designated AG73), was found to inhibit epithelial morphogenesis (Hayashi et al., 2002; Hoffman et al., 2001; Hosokawa et al., 1999; Kadoya et al., 1998).

In the present study, we have examined the distribution of the laminin-α5 chain in the developing SMG. Comparisons have been made with other laminin α chains (Kadoya et al., 1995, 1998; Kadoya and Yamashina, 1999), revealing colocalization of laminin-α1 and -α5 in epithelial basement membranes from the onset of SMG formation. Laminin-α5 G domain peptides were tested for their ability to interfere with epithelial cell development in the SMG organ culture system. Sixteen different peptides were tested, resulting in the identification of one peptide sequence, LVLFLNHGHFVA (residues 3307–3318 and designated A5G77), which strongly inhibited in vitro organ development. The activity was restricted to LVLFLNHGH, a 9-amino-acid carboxyl-terminal deletion sequence (designated A5G77f). The morphology of A5G77f-treated SMG explants was compared with that of explants treated with AG73 (Kadoya et al., 1998), anti-α-dystroglycan antibody (Durbeej et al., 2001), or a monoclonal antibody against the laminin-α1 LG4 module (Kadoya et al., 1995).

Materials and methods

Tissues and organ culture

Pregnant ICR mice were purchased from SLC (Hamamatsu, Japan). The morning of the discovery of a vaginal plug was designated day 0 (E0). Tissues were dissected from embryonic, newborn, and adult mice under ether anesthesia. For organ cultures, rudiments of E13 SMG, E12 kidney (metanephros), or E11 lung were placed on a polycarbonate filtration membrane (pore size 0.05 μm; Nucleopore, Whatman, Clifton, NJ) at the air-medium interface. Cultures were carried out in Improved MEM Zinc Option or D-MEM/F12 (both from GIBCO, Grand Island, NY), supplemented with 50 μg/ml transferrin but without serum as described previously (Kadoya et al., 1995, 1998). Laminin-α5 LG module peptides were dissolved in distilled water at 5 mg/ml and added to the culture medium at a final concentration of 0.19 mM, unless otherwise stated. After 3 days without a medium change, explants were photographed under the microscope. To compare the degree of epithelial branching, the numbers of terminal end buds, air way sacs, and metanephric vesicles were counted for SMG, lung, and kidney, respectively. Six to 20 explants were utilized for each peptide concentration.

Histology and electron microscopy

Explants were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed in 1% OsO4 in the same buffer, and processed as described previously (Kadoya and Yamashina, 1991). Sections of 1 μm were cut and stained with 1% toluidine blue aqueous solution. Ultrathin sections were cut and stained with both lead citrate and uranyl acetate, and examined under an electron microscope (1200EX; JEOL, Tokyo, Japan).

Immunofluorescence

Tissues were embedded in OCT compound (Miles, Elkhart, IN) and immediately frozen in liquid nitrogen. Frozen sections of 5 μm were cut and fixed with methanol. After blocking with 1% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS) for 1 h, sections

Table 1
Synthetic G domain peptides

Peptide	Sequence[a]	Chain	Modules
A5G3	GKNTGDHFVLYM	α5	LG1
A5G26	RFDQELRLVSYN	α5	LG2
A5G27	RLVSYNGIIFFLK	α5	LG2
A5G33	ASKAIQVFLLAG	α5	LG2
A5G35	VLVRVERATVFS	α5	LG2
A5G64	VFDLHQNMGSVN	α5	LG3
A5G65	HQNMGSVNVSVG	α5	LG3
A5G71	GPLPSYLQFVGI	α5	LG4
A5G73	RNRLHLSMLVRP	α5	LG4
A5G76	APMSGRSPSLVLF	α5	LG4
A5G77	LVLFLNHGHFVA	α5	LG4
A5G81	AGQWHRVSVRWG	α5	LG4
A5G82	VRWGMQQIQLVV	α5	LG4
A5G99	VLLQANDGAGEF	α5	LG5
A5G101	DGRWHRVAVIMG	α5	LG5
A5G112	KQGKALTQRHAK	α5	LG5
AG76.5	ATLQLQEGRLHF[b]	α1	LG4
A3G75	KNSFMALYLSKG[c]	α3	LG4
A4G82	TLFLAHGRLVFM[d]	α4	LG4

[a] Sequences of the synthetic peptides are given in the single-letter code. All peptides have carboxyl-terminal amides. See Makino et al. (2002) for residues.
[b] Mouse laminin-α1 chain, residues 2749–2760 (Sasaki et al., 1988).
[c] Human laminin-α3 chain, residues 1411–1422 (Ryan et al., 1994).
[d] Mouse laminin-α4 chain, residues 1514–1525 (Iivanainen et al., 1997).

were incubated with culture supernatant of the hybridoma producing anti-laminin-α5 rat IgG (4G6; Sorokin et al., 1997b). Bound antibodies were visualized by using DTAF-labeled anti-rat IgG antibody (Chemicon, Temecula, CA), diluted 1:100 in 1% BSA in PBS. For whole-mount immunofluorescence staining for nidogen, explants were fixed with Zamboni's fixative (4% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2), blocked for 1 h with PBS containing 1% BSA and 0.05% Tween 20, incubated overnight with a rat monoclonal antibody against nidogen (ELM-1; Upstate Biotechnology, Lake Placid, NY), diluted 1:100 in 1% BSA in PBS, washed with PBS containing 0.05% Tween 20 (30 min × 3 times), and again incubated overnight with DTAF-labeled secondary antibody diluted 1:100 in 1% BSA in PBS. After washing with PBS containing 0.05% Tween 20 (30 min × 3 times), specimens were mounted in Permafluore (Shandon, Pittsburgh, PA). All specimens were examined under the confocal scanning microscope (MRC 1024; Bio-Rad, Hertfordshire, England).

Adhesion assay

HT1080, a human fibrosarcoma cell line, was cultured in DMEM (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. For cell attachment assays, 10 μg of each peptide was coated onto 96-well plates and air dried. HT1080 cells were plated in the peptide-coated wells under serum-free conditions, and the number of attached cells after 1 h incubation at 37°C was determined as described previously (Makino et al., 2002). In all cases, peptide activities were evaluated relative to the activity observed with C16, a laminin-γ1 chain N-terminal domain VI peptide, previously shown to support HT1080 cell adhesion (Nomizu et al., 1997).

Peptides

Tables 1 and 2 show laminin peptides tested in the present study. All sequences were of mouse origin (Makino et al., 2002; Okazaki et al., 2002), with the exception of A3G75, which was derived from the human laminin-α3 chain sequence (Utani et al., 2001). All peptides were manually synthesized by the 9-fluorenyl-methoxycarbonyl (Fmoc)-based solid-phase strategy and prepared as the carboxyl-terminal amide form; subsequent purification involved reverse phase high performance liquid chromatography (Nomizu et al., 1995). Purity and identity of the peptides were confirmed by using analytical, reverse-phase high performance liquid chromatography, and mass spectroscopy, respectively. Throughout the text, amino acids are given in the single letter code.

Results

Localization of laminin-α5 chain in epithelial basement membranes of SMG rudiments at the onset of branching morphogenesis

In E13 embryos, SMG rudiments consist of several large epithelial clusters connected to an epithelial stalk and surrounded by mesenchyme. The laminin-α5 chain was clearly detected throughout basement membranes of both the epithelial clusters and the stalk (Fig. 1A). No expression was seen in capillary basement membranes or in the mesen-

Table 2
Cell binding activity of amino- and carboxyl-terminal, truncated peptides of the A5G77

Peptide	Sequence[a]	HT-1080 cells attachment activity[b]
A5G77	LVLFLNHGHFVA	+++
A5G77a	VLFLNHGHFVA	−
A5G77b	LFLNHGHFVA	−
A5G77c	FLNHGHFVA	+
A5G77d	LVLFLNHGHFV	++
A5G77e	LVLFLNHGHF	+
A5G77f	LVLFLNHGH	++
A5G77fT	LNGVLHHLF	−
C16	KAFDITYVRLKF	+++

[a] Sequences of the synthetic peptides are given in the single-letter code. All peptides have carboxyl-terminal amides.
[b] Activity was scored on the following subjective scale: +++, adhesion comparable to that of C16; ++, weak adhesion compared with that on C16; +, very weak adhesion compared with that on C16; −, no adhesion. Triplicate experiments gave similar results.

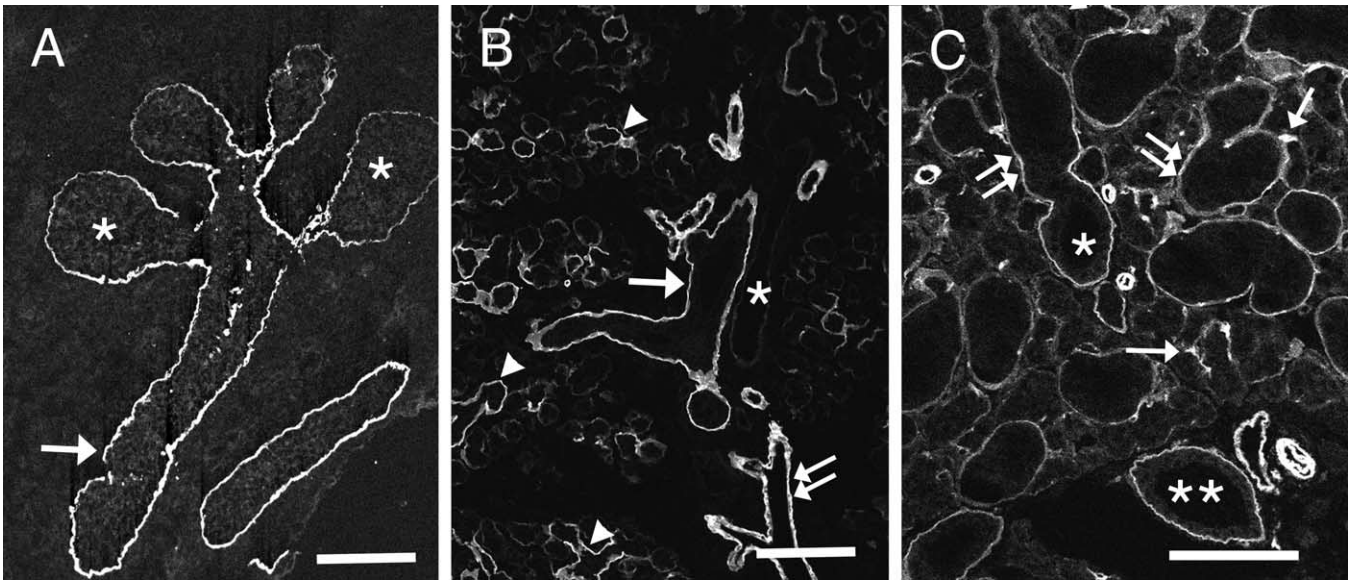

Fig. 1. Expression of laminin-α5 in the developing and adult mouse SMG as shown by immunofluorescence. Frozen sections were stained with monoclonal anti-laminin-α5-chain antibody (4G6). (A) On E13, the epithelium of the rudimentary SMG consists of several cell clusters connected to a stalk. A continuous distribution of the laminin-α5 chain can be seen in the basement membranes of both the terminal cell clusters (asterisks) and a stalk (arrow). Capillaries are negative. (B) In newborn SMG, the expression of laminin-α5 is seen in the basement membranes surrounding the interlobular (arrow) and intralobular duct (arrowheads) epithelia. Weak staining is also seen in basement membranes of some acini. Interlobular arteries (double allows) but not veins (asterisk) show positive staining. (C) In adult mice, localization of laminin-α5 is evident in basement membranes of the interlobular excretory duct (double asterisks), intralobular striated duct (single asterisk), and granular convoluted tubules (GCT, double arrows). The expression of laminin-α5 chain is also apparent in star- or crescent-shaped structures surrounding the acinus, GCT, and striated duct (arrows). Bars, 100 μm.

chyme. In newborn mice, a well-branched duct system consisting of polarized epithelial cells and ending in the acini was apparent. Laminin-α5 occurred in basement membranes of interlobular and intralobular ducts (Fig. 1B). In adult mice (Fig. 1C), the SMG epithelium has differentiated into distinct segments: acinus, intercalated duct, granular convoluted tubule (GCT), striated duct, and excretory duct (Pinkstaff, 1979). Laminin-α5 chain was detected in basement membranes of inter- and intralobular excretory ducts, and of the GCT. Scattered staining for laminin-α5 in spindle- or crescent-shaped structures surrounding the acini, GCT, or striated duct suggested the presence of laminin-α5 chain in myoepithelium (Redman, 1994). Clear staining for the laminin-α5 chain was also seen in the walls of small arteries and nerves in the parenchyma, but not in the capillary basement membranes.

Systematic screening for the effects of laminin-α5 G domain-derived cell-adhesive peptides for in vitro growth and differentiation of SMG

The localization of laminin-α5 in the SMG is consistent with a role in early epithelial branching morphogenesis. We, therefore, tested whether laminin-α5 G domain peptides, which supported HT1080 cell adhesion (Table 1) (Makino et al., 2002), could affect in vitro branching morphogenesis using the SMG organ culture system. Using a stereoscopic microscope, the gross morphology of the submandibular epithelium was assessed in the explants cultured for 3 days in the presence or absence of 0.19 mM laminin-α5 chain G domain peptides. A clear inhibition of epithelial branching was apparent in the explants treated with the A5G77 peptide (Figs. 2B and 3), while other adhesive laminin-α5 G domain peptides failed to show any effect (data not shown). Peptides corresponding to the A5G77 sequence but derived from laminin-α1, -α3, or -α4 chains (AG76.5, A3G75, and A4G82; see Table 1) were also tested. Although the cortical mesenchyme of the explants treated with A4G82 appeared slightly expanded after 3 days of culture (Fig. 2C), none of the peptides had an effect on epithelial branching (data not shown), even at concentrations of 0.53 mM. A5G19 and A5G109 (Makino et al., 2002) were not soluble in the medium and were, therefore, not tested.

Active sequence of A5G77

To determine the active sequence within A5G77, amino- and carboxyl-terminal deletion peptides and a scrambled peptide of A5G77 were synthesized and initially screened for their ability to support adhesion of HT1080 cells. As shown in Table 2, carboxyl-terminal deletion peptides, A5G77d-f, retained most of the cell adhesion activity. In contrast, amino-terminal deletion peptides, A5G77a-c, were less adhesive. A shortened carboxyl-terminal deletion peptide, A5G77f, was used in the SMG organ culture assay at different concentrations. A similar degree of inhibition of epithelial branching was apparent at

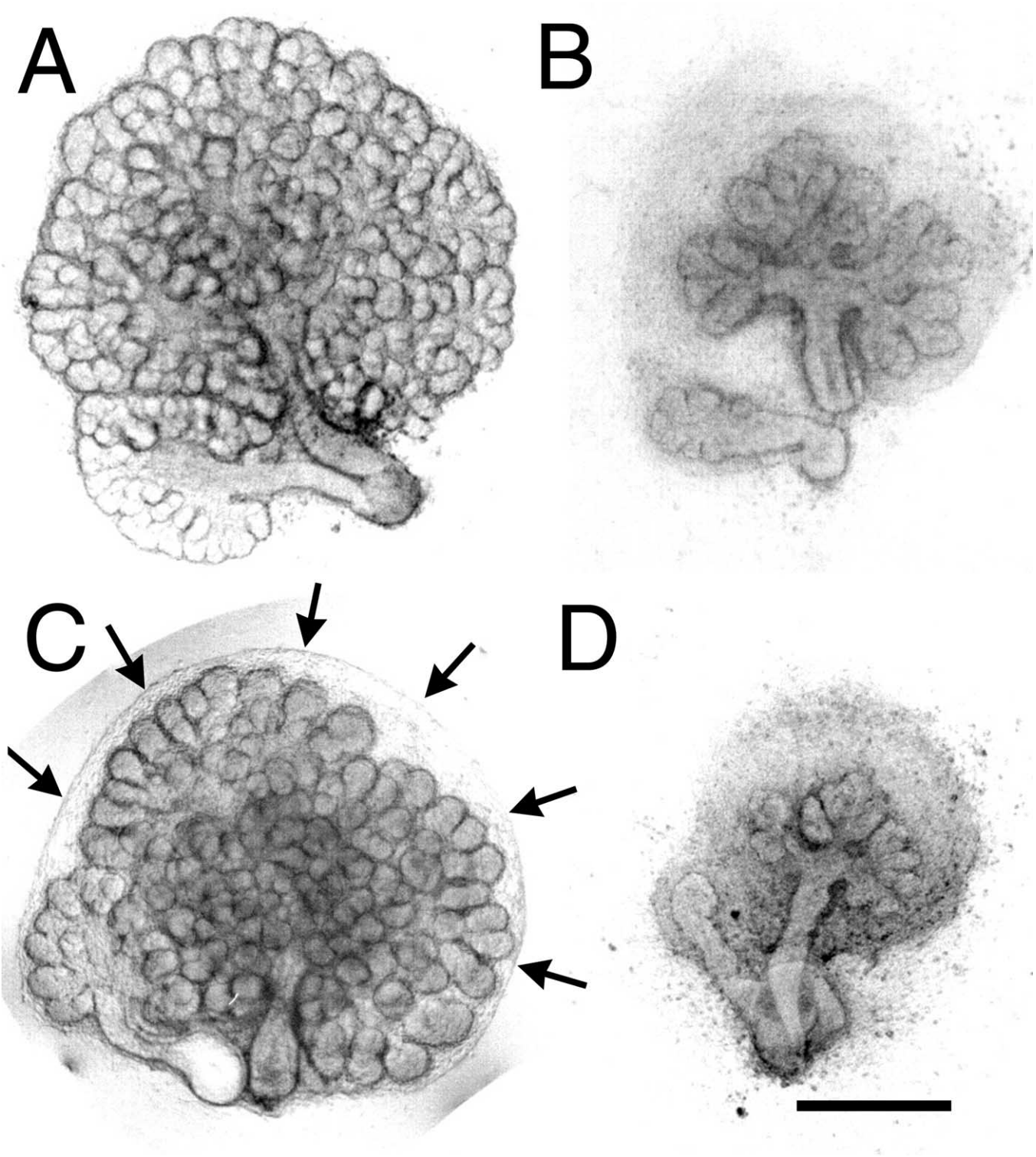

Fig. 2. Effects of synthetic laminin peptides on in vitro epithelial branching morphogenesis in SMG explants. Rudiments from E13 mice were cultured for 3 days in the absence of peptide (A), and in the presence of 0.19 mM A5G77 (LVLFLNHGHFVA) (B), 0.19 mM A4G82 (TLFLAHGRLVFM) (C), or 0.53 mM A5G77f (LVLFLNHGH) (D). Clear inhibition of epithelial branching is evident in (B) and (D). In (C), no apparent inhibition of epithelial branching is noted; however, the outer margin of the mesenchyme is extended (C, arrows). Bar, 0.5 mm.

concentrations of 0.19, 0.25, and 0.53 mM of A5G77f (Figs. 2D and 3). A5G77c was also tested in the organ culture system at 0.25 mM but failed to interfere with SMG branching. A scrambled sequence (A5G77fT, Table 2) did not support HT1080 cell binding or interfere with in vitro SMG morphogenesis even at 0.53 mM (Fig. 3).

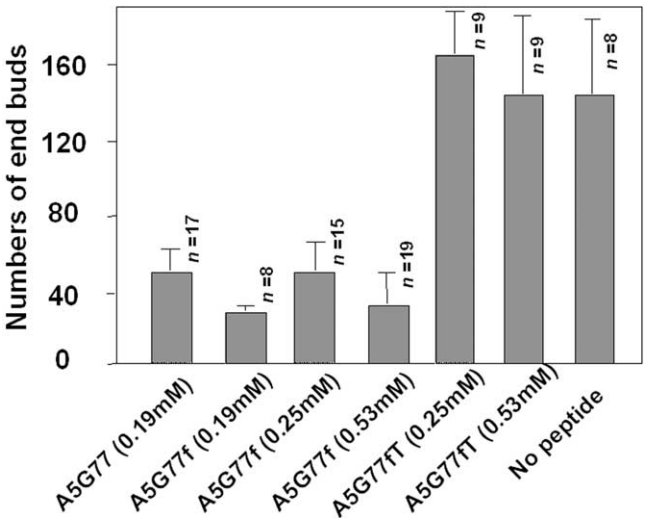

Fig. 3. Numbers of terminal end buds in organ cultures of embryonic SMG. Rudiments from E13 mice were cultured in serum-free medium with or without various concentrations of laminin-α5 chain-derived peptide A5G77 (LVLFLNHGHFVA), its carboxyl-terminal deletion peptide A5G77f (LVLFLNHGH), or a scrambled peptide A5G77fT (LNGVLHHLF). The numbers of terminal end buds were counted from photographs after 3 days in organ culture and expressed as means + S.D.

Morphology of A5G77f-treated SMG rudiment

As shown in Fig. 4A, the terminal end buds of SMG rudiments treated with 0.25 mM A5G77f for 3 days lost the smooth contour of normal terminal end buds and were partially separated by deep clefts. The mesenchyme appeared condensed compared to controls. In the untreated cultures and cultures grown in the presence of a control scrambled A5G77fT peptide (0.25 mM), terminal end buds appeared as rounded structures with only shallow indentations (Fig. 4B). Immunofluorescence staining for nidogen revealed continuous basement membrane staining adjacent to the entire epithelial surface in 3-day-cultured rudiments treated with 0.53 mM A5G77f peptide (Fig. 4C), with an amino-terminal deletion peptide A5G77c (Fig. 4D), and without peptide (data not shown). Transmission electron microscopy confirmed the presence of intact basement membranes in the explants treated with 0.19 mM A5G77f peptide or the same concentration of the control A5G77fT peptide (Fig. 5).

Effects of A5G77f peptide for kidney and lung organ cultures

E12 kidney rudiments treated for 3 days with 0.25 mM of a control scrambled peptide, A5G77fT, grew well and showed extensive branching of the ureteric bud and normal tubule formation (Fig. 6A). No difference was observed between A5G77fT-treated and untreated cultures (data not shown). In contrast, the rudiments treated with same concentration of A5G77f peptide exhibited dramatic perturbation of ureteric bud branching and reduced nephrogenesis (Fig. 6B and E). Similarly, E11 lung rudiments cultured in the presence of 0.25 mM A5G77f showed reduced epithelial branching morphogenesis (Fig. 6D and E), while the control peptide, A5G77fT, had no effect (Fig. 6C).

Discussion

Localization of laminin-α5 in the developing and mature mouse SMG

We have examined the localization of laminin-α5 during morphogenesis of the mouse SMG using a well-characterized antibody, 4G6, revealing its occurrence in all epithelial basement membranes from the onset of branching morphogenesis (E13), and a broad distribution in basement membranes of various epithelial segments of the duct system of the mature gland. This is in accordance with previous in situ hybridization studies, which demonstrated strong expression of laminin-α5 mRNA in the developing SMG epithelium (Durbeej et al., 1996; Miner et al., 1997). At these early stages of development, the laminin-α1 also occurs in epithelial basement membranes of the SMG (Kadoya et al., 1995). Moreover, a crucial role for laminin-α1 and its receptors in branching morphogenesis was established through the use of specific antibodies (Durbeej et al., 2001; Kadoya et al., 1995; Kashimata and Gresik, 1997) or inhibitory peptides (Hoffman et al., 2001; Hosokawa et al., 1999; Kadoya et al., 1998) in the SMG organ culture system. The question, therefore, arises whether the laminin-α5 chain also plays a role in epithelial branching morphogenesis and, if so, whether such a role is distinct from that of the laminin-α1 chain. However, no blocking antibody for the laminin-α5 chain for use in the in vitro organ cultures has been described to date. To investigate whether laminin-α5 plays a role in epithelial branching morphogenesis in the SMG, we tested laminin-α5 G domain peptides, known to support HT1080 cell adhesion (Makino et al., 2002), in the SMG organ culture system. A similar approach was successful in identifying a functionally significant peptide sequence (AG73) in laminin-α1 involved in epithelial morphogenesis in both the SMG (Kadoya et al., 1998, Hoffman 2001) and in hair follicles (Hayashi et al., 2002).

Laminin-α5 chain G domain peptide influences epithelial branching morphogenesis

A 12-amino-acid peptide sequence, LVLFLNHGHFVA (A5G77), derived from the LG4 module of the G domain of laminin-α5, was found to inhibit epithelial branching morphogenesis. The activity was restricted to LVLFLNHGH, a 9-amino-acid carboxyl-terminal deletion sequence (A5G77f), as established through the use of various truncated peptides in SMG organ cultures. A5G77f reduced the number of terminal end buds to 33% of control values (i.e., 67%

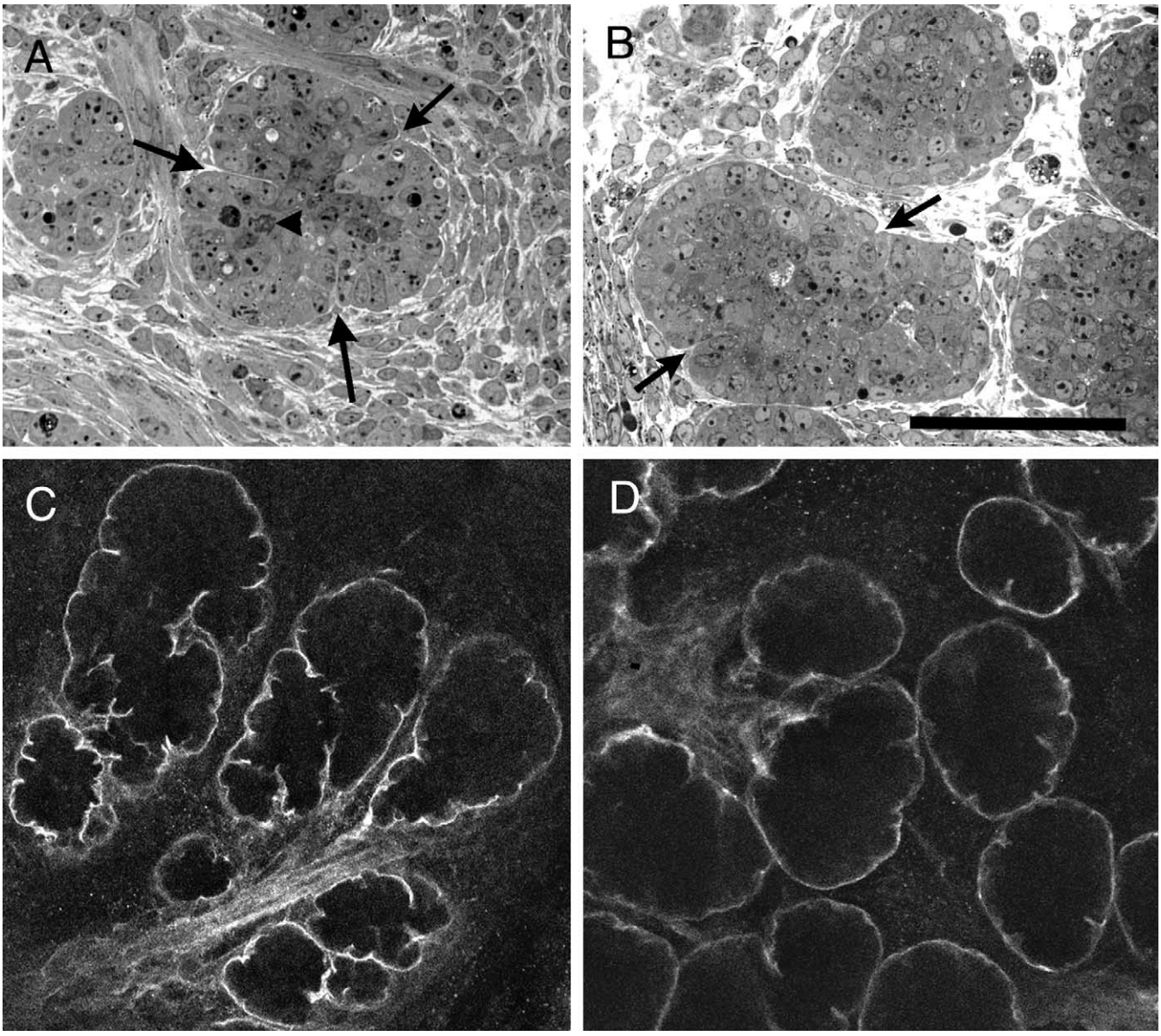

Fig. 4. Histology showing epithelia of the SMG rudiments treated with peptides. (A, B) Toluidine-blue-stained, 1-μm plastic sections of the SMG rudiments cultured for 3 days in the presence of A5G77f (LVLFLNHGH) at 0.25 mM (A) or with the same concentration of control scrambled peptide, A5G77fT (LNGVLHHLF) (B). Terminal end buds of A5G77f-treated rudiment are partially separated by deep clefts (A, arrows). Arrowhead in (A) indicates a mitotic cell. In contrast, terminal end buds of the control peptide-treated rudiments are round and only shallow indentations (B, arrows) are seen. Note the tightly packed mesenchymal cells in (A). (C, D) Immunofluorescence staining for nidogen in the rudiments cultured for 3 days with A5G77f peptide at 0.53 mM (C) or with the same concentration of an amino-terminal truncated peptide A5G77c (FLNHGHFVA) (D) as shown by confocal laser scanning micrographs. A continuous basement membrane staining surrounding the entire epithelial surfaces of both A5G77f-treated (C) and control (D) rudiments. Bar, 100 μm.

inhibition), while the same concentration of AG73, a previously identified laminin-α1 chain LG4-derived peptide, reduced terminal end buds to 17% of controls (i.e., 83% inhibition) (Kadoya et al., 1998). Similar reductions in terminal end bud formation in SMG organ cultures have been reported to occur in the presence of blocking antibodies against the laminin-α1 LG4 module (Mab 200) (Kadoya et al., 1995), or against laminin receptors, such as integrin-α6 subunit (GoH3) (Kadoya et al., 1995) and α-dystroglycan (Durbeej et al., 2001). However, morphological analyses revealed that A5G77f not only reduced the numbers of developing terminal end buds, but also altered their morphology in a manner distinct from that observed in the previous studies. Treatment of SMG rudiments with AG73, GoH3, Mab 200, or antibody against α-dystroglycan, resulted in less branching and terminal end buds with smooth contours. In contrast, epithelium of SMG organ cultures treated with A5G77f showed extensive irregularities in their contour, with formation of deep clefts, indicating inhibition of outgrowth or elongation of end buds, rather than the

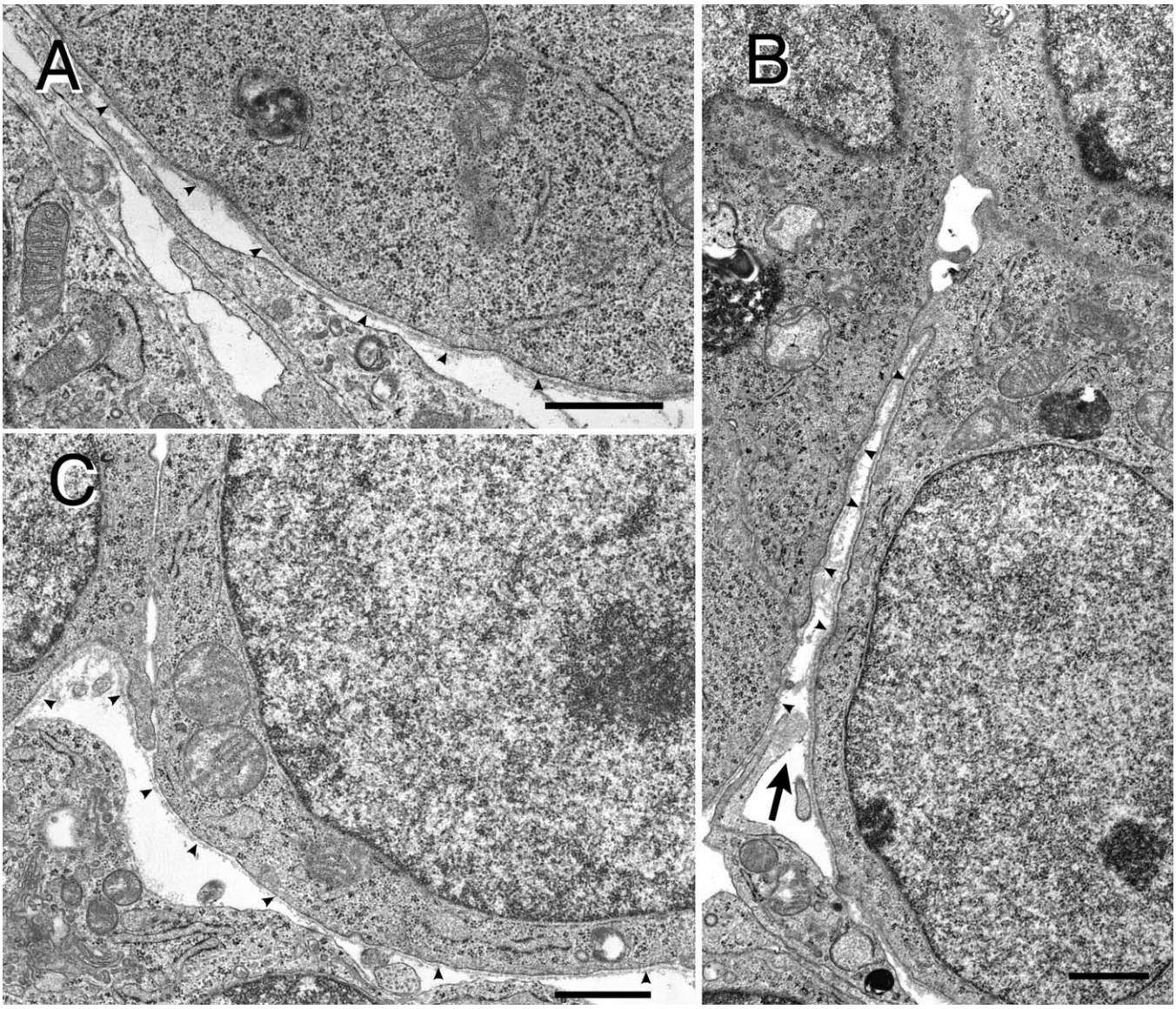

Fig. 5. Transmission electron micrographs showing basal surfaces of epithelia of SMG rudiments treated with peptides. Periphery (A) and cleft (B, arrow) of epithelial end bud of SMG rudiment cultured for 3 days in the presence of 0.19 mM A5G77f (LVLFLNHGH). (C) The outer surface of SMG rudiment cultured for 3 days with the same concentration of a scrambled peptide, A5G77fT (LNGVLHHLF). Continuous basement membranes along the basal surface of epithelial tip and cleft are seen in A5G77f-treated (A, B; arrowheads) and in the control (C; arrowheads) explants. Bars, 1 μm.

failure of bifurcation of epithelial clusters. This may indicate a role for laminin-α5 in the development of epithelial duct system of the SMG. Among the laminin-α5 peptides tested, A5G77 was not the most adhesive in HT1080 cell binding assays (Makino et al., 2002). Moreover, A5G81, which was formerly called F4 and defines a major heparin and cell binding site within the laminin-α5 LG4 module (Nielsen et al., 2000), failed to interfere with in vitro SMG development. Taken together, this suggests that A5G77f interferes with a specific process that is relevant for SMG epithelial development, possibly a specific receptor-mediated process.

The crystal structure of the laminin-α2 LG5 module has been resolved (Hohenester et al., 1999). The LG module consists of a 14-stranded β sheet (*A* to *N*) sandwich structure. A calcium-binding site is mapped to one edge of the LG module, surrounded by the residues implicated in α-dystroglycan binding (Tisi et al., 2000). When we accommodate the A5G77f sequence within this structural model (Timpl et al., 2000), it forms part of the *E* strand and the connecting loop between *E* and *F* strands (EF loop), which is located opposite to the calcium binding site. Recently, two independent investigations identified two biologically active peptides, A3G75 and A4G82, which include the EF loop sequences of LG4 modules of laminin-α3 and -α4 chains. Interestingly, A3G75 inhibited syndecan-mediated cell-binding to recombinant laminin-α3 LG4 module (Utani et al., 2001), while A4G82 inhibited heparin- and cell-

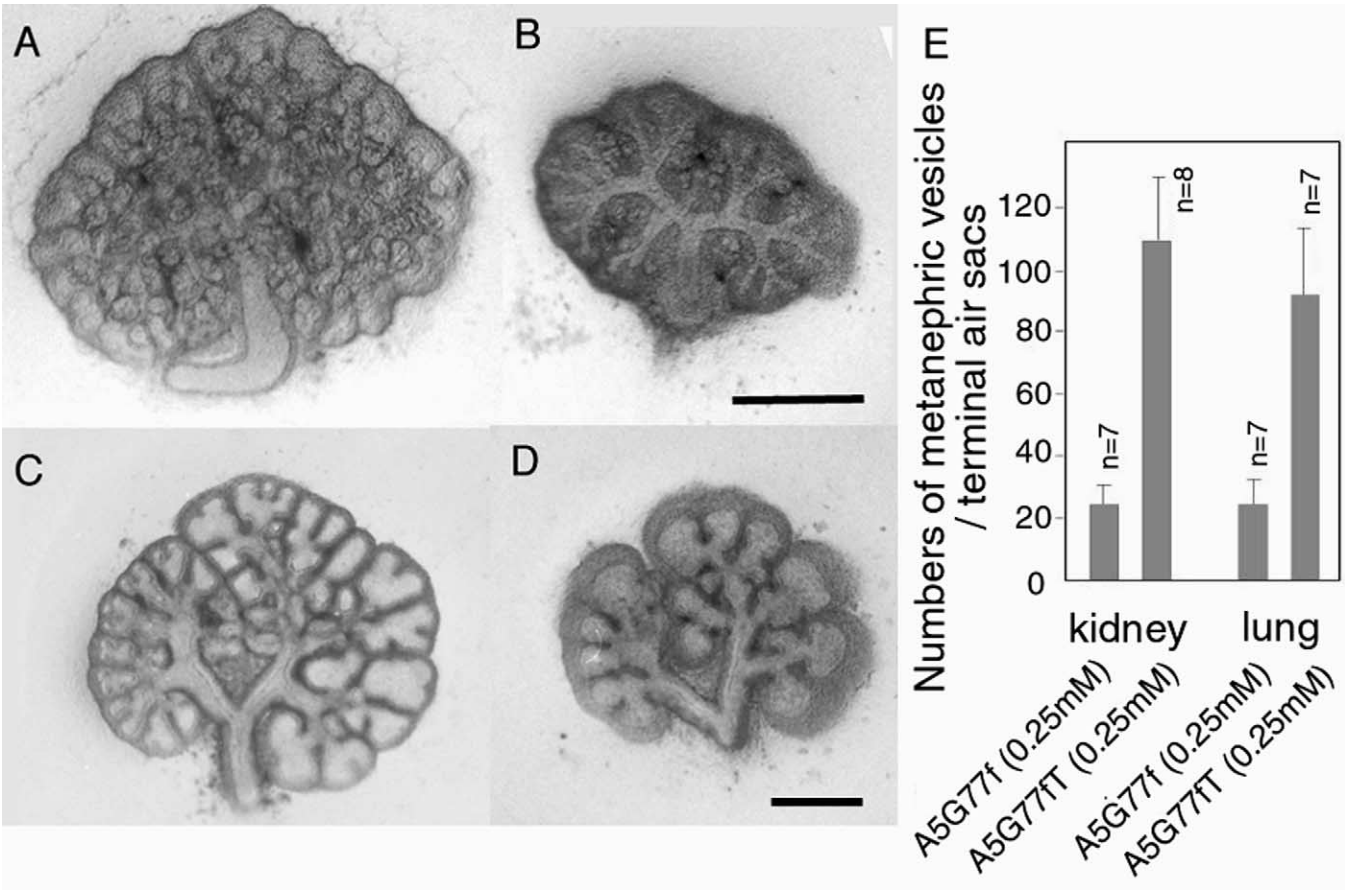

Fig. 6. Effects of synthetic A5G77f peptide (LVLFLNHGH) on in vitro morphogenesis of E12 kidney and E11 lung. Kidney and lung rudiments were cultured for 3 days in the presence of control A5G77fT (LNGVLHHLF) (0.25 mM) (A, C) or 0.25 mM A5G77f peptide (B, D). In (E), the numbers of terminal air sacs or metanephric vesicles were counted and expressed as means + S.D. Clear inhibition of epithelial branching is evident in kidney (C) and lung (D) organ cultures treated with A5G77f. Bars, 0.5 mm.

binding to recombinant laminin-α4 LG1-5 (Okazaki et al., 2002; Yamaguchi et al., 2000). These findings support the concept that the EF loop of the LG4 module plays a role in cellular recognition of laminins, via cell surface molecules with heparin-like activity, such as the syndecans.

Besides syndecans, several different cell surface molecules, including integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ (Feretta and Ekblom, 1999; Kikkawa et al., 1998), and Lutheran blood group glycoprotein (Lu) (Kikkawa et al., 2002; Moulson et al., 2001), can bind to the laminin-α5. α-Dystroglycan is also reported to bind to the G domain of laminin-α5 (Shimizu et al., 1999), but this binding is contested (Hohenester et al., 1999; Timpl et al., 2000). In epithelial–mesenchymal organs, the expression of all these receptors are essentially found on all epithelial cells (Durbeej et al., 1995; Kadoya and Yamashina, 1993; Moulson et al., 2001). It is unlikely that A5G77f primarily interferes with the interaction between laminin-α5 and α-dystroglycan, or between laminin-α5 and α6-integrin, due to the morphological differences in explants treated with the laminin-α5 chain peptide and with anti-α-dystroglycan (Durbeej et al., 2001) or anti-α6-integrin antibodies (Kadoya et al., 1995). The binding site for Lu has recently been mapped to LG3 module; thus, it is also unlikely that A5G77f impaired cell recognition of laminin-α5 via Lu receptor (Kikkawa et al., 2002).

Immunoblot experiments of Miner et al. (1997) suggest that the tissue form of laminin-α5 is a mixture of both intact (450 kDa) and several truncated polypeptides. The specific binding of monoclonal antibody 4G6 to laminin-α5 chain has been tested only with the intact laminin-α5 chain (Sorokin et al., 1997b) and it is not clear whether it recognizes truncated forms of the chain. Hence, the present immunofluorescence does not distinguish between the intact and the truncated forms of the laminin-α5 chain. It may be that the truncation of laminin-α5 chain occurs by posttranslational cleavage within the G domain, as has been shown for the laminin-α2 chain which is cleaved within LG3 module (Talts et al., 1998), and for laminin-α3 (Burgeson, 1996) and -α4 (Talts et al., 2000) chains which are cleaved between LG3 and LG4 modules. Upon cleavage, the release of such LG4-5 modules or fragments thereof, carrying peptides such as A5G77 and/or A5G77f, may either remain associated with the main body of the chain, as has been shown for laminin-α2 (Talts et al., 1998) or as reported for

laminin-α4 (Timpl et al., 2000) become released from the basement membrane and potentially interact with mesenchymal cells. As we also noticed some abnormality in the mesenchyme of the explants treated with A5G77f, we cannot rule out the possibility that the A5G77f affects the mesenchymal cells.

In the present study, A5G77f inhibited the in vitro morphogenesis of SMG, kidney, and lung, suggesting that the region containing the EF loop of laminin-α5 LG4 module may be involved in the development of various organs. This is consistent with the multiorgan defects found in *Lama5*$^{-/-}$ mice (Miner et al., 1998), which show a dramatic reduction in branching of ureteric duct in the kidney (Miner and Li, 2000) and abnormalities in the salivary glands (Miner et al., 1998). However, no defect in epithelial branching of lung of *Lama5*$^{-/-}$ mice has been described (Nguyen et al., 2002), which is probably due to compensation by ectopic expression of laminin-α4 chain in the lung epithelial basement membranes.

Even though the absence of laminin-α5 underlying some epithelia of *Lama5*$^{-/-}$ mouse embryos results in severe defects in basement membrane assembly (Miner et al., 1998), epithelial basement membranes remained continuous in the A5G77f-treated SMG explants. This may indicate that the A5G77f portion of the laminin-α5 chain is not important for basement membrane assembly in the SMG, and/or that the expression of laminin-α1 in the embryonic SMG epithelial basement membranes (Kadoya et al., 1995) is sufficient for the structural organization of the embryonic basement membrane. This view is supported by the fact that perturbation of basement membrane formation in *Lama5*$^{-/-}$ mice was found mainly in basement membranes in which laminin-α1 chain was absent in the normal mice, such as glomerular basement membrane of capillary loop stage (Miner and Li, 2000) and visceral pleural basement membrane (Nguyen et al., 2002). It should be pointed out that impaired basement membrane formation was seen in the SMG explants treated with a laminin-α1 LG4 module peptide AG73 (Kadoya et al., 1998), and a monoclonal antibody Mab 200 (Kadoya et al., 1995), which has been shown to interfere α-dystroglycan binding to laminin-α1 LG4 module (Durbeej et al., 2001). This suggests a predominant role for laminin-α1, rather than laminin-α5, in embryonic basement membrane assembly in the SMG.

In summary, our data showed that the laminin-α5 chain was deposited in the SMG epithelial basement membrane from the onset of branching morphogenesis, suggesting a role in epithelial branching morphogenesis. Laminin-α5 chain G domain peptide, LVLFLNHGHFVA (A5G77), and its carboxyl-terminal deletion 9-amino-acid peptide, A5G77f, containing a sequence located in the connecting loop between strands *E* and *F* of LG4 module, perturbed epithelial branching morphogenesis. The epithelial basement membranes of A5G77f-treated explants were intact, which was in contrast to the impaired basement membrane formation of the explants treated with a laminin-α1 LG4 module peptide, AG73 (Kadoya et al., 1998), and monoclonal antibody against laminin-α1 LG4 (Kadoya et al., 1995). This suggests crucial but distinct roles for the LG4 module of laminin-α1 and -α5 chains for branching morphogenesis. The identity of cell surface molecule that binds to the A5G77 sequence remains to be determined.

Acknowledgments

Part of this work was done at the Electron Microscope Laboratory, Kitasato University School of Medicine. This work is supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (nos. 10670028, 13670023).

References

Aumailley, M., Gimond, C., Rousselle, P., 1996. Integrin-mediated cellular interactions with laminins, in: Ekblum, P., Timpl, R. (Eds.), The Laminins, Harwood Academic Publisher, Amsterdam, pp. 127–158.

Bernfield, M., Banerjee, S.D., Koda, J.E., Rapraeger, A.C., 1984. Remodeling of the basement membrane as a mechanism of morphogenetic tissue interaction, in: Trelstad, R.L. (Ed.), The Role of Extracellular Matrix in Development. Alan R. Liss, New York, pp. 545–572.

Borghese, E., 1950. The development in vitro of the submandibular and sublingual glands of *Mus musculus*. J. Anat. 84, 287–302.

Burgeson, R.E., 1996. Laminins in epidermal structures, in: Ekblom, P., Timpl, R. (Eds.), The Laminins. Harwood Academic Publishers, Amsterdam, pp. 65–96.

Celli, G., LaRochelle, W.J., Machem, S., Sharp, R., Merlino, G., 1998. Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. EMBO J. 17, 1642–1655.

Colognato, H., Yurchenco, P.D., 2000. Form and function: the laminin family of heterotrimers. Dev. Dyn. 218, 213–234.

De Moerlooze, L., Spencer-Dene, B., Revest, J., Hajihosseini, M., Rosewell, I., Dickson, C., 2000. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signaling during mouse organogenesis. Development 127, 483–492.

Durbeej, M., Larsson, E., Ibraghimov-Beskrovnaya, O., Roberds, S.L., Campbell, K.P., Ekblom, P., 1995. Non-muscle α-dystroglycan is involved in epithelial development. J. Cell Biol. 130, 79–91.

Durbeej, M., Fecker, L., Hjalt, T., Zhang, H.-Y., Salmivirta, K., Klein, G., Timpl, R., Sorokin, L., Ebendal, T., Ekblom, P., Ekblom, M., 1996. Expression of laminin α1, α5 and β2 chains during embryogenesis of the kidney and vasculature. Matrix Biol. 15, 397–413.

Durbeej, M., Talts, J.F., Henry, M.D., Yurchenco, P.D., Campbell, K.P., Ekblom, P., 2001. Dystroglycan binding to laminin α1LG4 module influences epithelial morphogenesis of salivary gland and lung in vitro. Differentiation 69, 121–134.

Ekblom, M., Falk, M., Salmivirta, K., Durbeej, M., Ekblom, P., 1998. Laminin isoforms and epithelial development. Ann. N. Y. Acad. Sci. 857, 194–211.

Ervasti, J.M., Campbell, K.P., 1993. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J. Cell Biol. 122, 809–823.

Ferletta, M., Ekblom, P., 1999. Identification of laminin-10/11 as a strong cell adhesive complex for a normal and a malignant human epithelial cell line. J. Cell Sci. 112, 1–10.

Garbe, J.H.O., Göhring, W., Mann, K., Timpl, R., Sasaki, T., 2002. Complete sequence, recombinant analysis and binding to laminins and

sulphated ligands of the N-terminal domains of laminin α3B and α5 chains. Biochem. J. 362, 213–221.

Gresik, E.W., 1994. The granular convoluted tubule (GCT) cell of rodent submandibular glands. Microsc. Res. Tech. 27, 1–24.

Grobstein, C., 1953. Epithelio-mesenchymal specificity in the morphogenesis of mouse sub-mandibular rudiments in vitro. J. Exp. Zool. 124, 383–413.

Hayashi, K., Mochizuki, M., Nomizu, M., Uchinuma, E., Yamashina, S., Kadoya, Y., 2002. Inhibition of hair follicle growth by a laminin-1 G-domain peptide, RKRLQVQLSIRT, in an organ culture of isolated vibrissa rudiment. J. Invest. Dermatol. 118, 712–718.

Hoffman, M.P., Engbring, J.A., Nielsen, P.K., Vargas, J., Steinberg, Z., Karmand, A.J., Nomizu, M., Yamada, Y., Kleinman, H.K., 2001. Cell type-specific differences in glycosaminoglycans modulate the biological activity of a heparin-binding peptide (RKRLQVQLSIRT) from the G domain of the laminin α1 chain. J. Biol. Chem. 276, 22077–22085.

Hohenester, E., Tisi, D., Talts, J.F., Timpl, R., 1999. The crystal structure of a laminin G-like module reveals the molecular basis of α-dystroglycan binding to laminins, perlecan, and agrin. Mol. Cell 4, 783–792.

Hosokawa, Y., Takahashi, Y., Kadoya, Y., Yamashina, S., Nomizu, M., Yamada, Y., Nogawa, H., 1999. Significant role of laminin-1 in branching morphogenesis of mouse salivary epithelium cultured in basement membrane matrix. Dev. Growth Differ. 41, 207–216.

Iivanainen, A., Kortesmaa, J., Sahlberg, C., Morita, T., Bergmann, U., Thesleff, I., Tryggvason, K., 1997. Primary structure, developmental expression, and immuinolocalization of the murine laminin α4 chain. J. Biol. Chem. 272, 27862–27868.

Jaskoll, T., Melnick, M., 1999. Submandibular gland morphogenesis: stage-specific expression of TGF-α/EGF, IGF, TGF-β, TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF-β2, TGF-β3, and EGF-R null mutations. Anat. Rec. 256, 252–268.

Kadoya, Y., Yamashina, S., 1991. Reconstruction of the basement membrane in a cultured submandibular gland. Anat. Embryol. 183, 491–499.

Kadoya, Y., Yamashina, S., 1993. Distribution of α6 integrin subunit in developing mouse submandibular gland. J. Histochem. Cytochem. 41, 1707–1714.

Kadoya, Y., Kadoya, K., Durbeej, M., Holmvall, K., Sorokin, L., Ekblom, P., 1995. Antibodies against domain E3 of laminin-1 and integrin α6 subunit perturb branching epithelial morphogenesis of submandibular gland, but by different modes. J. Cell Biol. 129, 521–534.

Kadoya, Y., Nomizu, M., Sorokin, L.M., Yamashina, S., Yamada, Y., 1998. Laminin α1 chain G domain peptide, RKRLQVQLSIRT, inhibits epithelial branching morphogenesis of cultured embryonic mouse submandibular gland. Dev. Dyn. 212, 394–402.

Kadoya, Y., Yamashina, S., 1999. Localization of laminin-5, HD1/plectin, and BP230 in the submandibular glands of developing and adult mice. Histochem. Cell Biol. 112, 417–425.

Kashimata, M., Gresik, E.W., 1997. Epidermal growth factor system is a physiological regulator of development of the mouse fetal submandibular gland and regulates expression of the α6-integrin subunit. Dev. Dyn. 208, 149–161.

Kashimata, M., Sayeed, S., Ka, A., Onetti-Muda, A., Sakagami, H., Faraggiana, T., Gresik, E.W., 2000. The ERK-1/2 signaling pathway is involved in the stimulation of branching morphogenesis of fetal mouse submandibular glands by EGF. Dev. Biol. 220, 183–196.

Kikkawa, Y., Sanzen, N., Sekiguchi, K., 1998. Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells. Laminin-10/11 mediates cell adhesion through integrin α3β1. J. Biol. Chem. 273, 15854–15859.

Kikkawa, Y., Moulson, C.L., Virtanen, I., Miner, J.H., 2002. Identification of the binding site for the Lutheran blood group glycoprotein on laminin α5 through expression of chimeric laminin chains in vivo. J. Biol. Chem. 277, 44864–44869.

Lefebvre, O., Sorokin, L., Kedinger, M., Simon-Assmann, P., 1999. Developmental expression and cellular origin of the laminin α2, α4, and α5 chains in the intestine. Dev. Biol. 210, 135–150.

Libby, R.T., Champliaud, M.-F., Claudepierre, T., Xu, Y., Gibbons, E.P., Koch, M., Burgeson, R.E., Hunter, D.D., Brunken, W.J., 2000. Laminin expression in adult and developing retinae: evidence of two novel CNS laminins. J. Neurosci. 20, 6517–6528.

Makino, M., Okazaki, I., Nishi, N., Nomizu, M., 1999. Biologically active sequences in laminin, a multifunctional extracellular matrix protein. Connect. Tissue 31, 277–234.

Makino, M., Okazaki, I., Kasai, S., Nishi, N., Bougaeva, M., Weeks, B.S., Otaka, A., Nielsen, P.K., Yamada, Y., Nomizu, M., 2002. Identification of cell binding sites in the laminin α5 chain G domain. Exp. Cell Res. 227, 95–106.

Martin, G.R., Liotta, L.A., Kleinman, H.K., 1996. Interaction of tumor cells with basement membranes and laminin-1, in: Ekblom, P., Timpl, R. (Eds.), The Laminins. Harwood Academic Publishers, Amsterdam, pp. 277–290.

Miner, J.H., Patton, B.L., Lentz, S.I., Gilbert, D.J., Snider, W.D., Jenkins, N.A., Copeland, N.G., Sanes, J.R., 1997. The laminin-α chains: expression, developmental transitions, and chromosomal locations of α1-5, identification of heterotrimeric laminin 8–11, and cloning of a novel α3 isoform. J. Cell Biol. 137, 685–701.

Miner, J.H., Cunningham, J., Sanes, J.R., 1998. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin α5 chain. J. Cell Biol. 143, 1713–1723.

Miner, J.H., Li, C., 2000. Defective glomerulogenesis in the absence of laminin α5 demonstrates a developmental role for the kidney glomerular basement membrane. Dev. Biol. 217, 278–289.

Morita, K., Nogawa, H., 1999. EGF-dependent lobule formation and FGF7-dependent stalk elongation in branching morphogenesis of mouse salivary epithelium in vitro. Dev. Dyn. 215, 148–154.

Moulson, C.L., Li, C., Miner, J.H., 2001. Localization of Lutheran, a novel laminin receptor, in normal, knockout, and transgenic mice suggests an interaction with laminin α5 in vivo. Dev. Dyn. 222, 101–114.

Nakanishi, Y., Sugiura, F., Kishi, J., Hayakawa, T., 1986. Collagenase inhibitor stimulates cleft formation during early morphogenesis of mouse salivary gland. Dev. Biol. 113, 201–206.

Nguyen, N.M., Miner, J.H., Pierce, R.A., Senior, R.M., 2002. Laminin α5 is required for lobar septation and visceral pleural basement membrane formation in the developing mouse lung. Dev. Biol. 246, 231–244.

Nielsen, P.K., Gho, Y.S., Hoffman, M.P., Watanabe, H., Makino, M., Nomizu, M., Yamada, Y., 2000. Identification of a major heparin and cell binding site in the LG4 module of the laminin α5 chain. J. Biol. Chem. 275, 14517–14523.

Nogawa, H., Takahashi, Y., 1991. Substitution for mesenchyme by basement-membrane-like substratum and epidermal growth factor in inducing branching morphogenesis of mouse salivary epithelium. Development 112, 855–861.

Nomizu, M., Kim, W.H., Yamamura, K., Utani, A., Song, S.-Y., Otaka, A., Roller, P.P., Kleinman, H.K., Yamada, Y., 1995. Identification of cell binding sites in the laminin-α1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. J. Biol. Chem. 270, 20583–20590.

Nomizu, M., Kuratomi, Y., Song, S.-Y., Ponce, M.L., Hoffman, M.P., Powell, S.K., Miyoshi, K., Otaka, A., Kleinman, H.K., Yamada, Y., 1997. Identification of cell binding sequences in mouse laminin γ1 chain by systematic peptide screening. J. Biol. Chem. 272, 32198–32205.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S., Itoh, N., 2000. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. Biochem. Biophys. Res. Commun. 277, 643–649.

Okazaki, I., Suzuki, N., Nishi, N., Utani, A., Matsuura, H., Shinkai, H., Yamashita, H., Kitagawa, Y., Nomizu, M., 2002. Identification of biologically active sequences in the laminin α4 chain G domain. J. Biol. Chem. 277, 37070–37078.

Pinkstaff, C.A., 1979. The cytology of salivary glands. Int. Rev. Cytol. 63, 141–261.

Ryan, M.C., Tizard, R., VanDevanter, D.R., Carter, W.G., 1994. Cloning of the LamA3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair. J. Biol. Chem. 269, 22779–22787.

Redman, R.S., 1994. Myoepithelium of salivary glands. Micros. Res. Tech. 27, 25–45.

Salmivirta, K., Sorokin, L.M., Ekblom, P., 1997. Differential expression of laminin α chains during murine tooth development. Dev. Dyn. 210, 206–215.

Salmivirta, M., Mali, M., Heino, J., Hermonen, J., Jalkanen, M., 1994. A novel laminin-binding form of syndecan-1 (cell surface proteoglycan) produced by syndecan-1 cDNA-transfected NIH-3T3 cells. Exp. Cell Res. 215, 180–188.

Sasaki, M., Kleinman, H.K., Huber, H., Deutzmann, R., Yamada, Y., 1988. Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. J. Biol. Chem. 263, 16536–16544.

Sasaki, T., Timpl, R., 1999. Laminins, in: Kreis, T., Vale, R. (Eds.), Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins. Oxford University Press, Oxford, pp. 434–443.

Shimizu, H., Hosokawa, H., Ninomiya, H., Miner, J.H., Masaki, T., 1999. Adhesion of cultured bovine aortic endothelial cells to laminin-1 mediated by dystroglycan. J. Biol. Chem. 274, 11995–12000.

Sorokin, L., Ekblom, P., 1992. Development of tubular and glomerular cells of the kidney. Kidney Int. 41, 657–664.

Sorokin, L.M., Pausch, F., Durbeej, M., Ekblom, P., 1997a. Differential expression of five laminin α(1–5) chains in developing and adult mouse kidney. Dev. Dyn. 210, 446–462.

Sorokin, L.M., Pausch, F., Frieser, M., Kröger, S., Ohage, E., Deutzmann, R., 1997b. Developmental regulation of the laminin α5 chain suggests a role in epithelial and endothelial cell maturation. Dev. Biol. 189, 285–300.

Takahashi, Y., Nogawa, H., 1991. Branching morphogenesis of mouse salivary epithelium in basement membrane-like substratum separated from mesenchyme by the membrane filter. Development 111, 327–335.

Talts, J.F., Mann, K., Yamada, Y., Timpl, R., 1998. Structural analysis and proteolytic processing of recombinant G domain of mouse laminin α2 chain. FEBS Lett. 426, 71–76.

Talts, J.F., Andac, Z., Göhring, W., Brancaccio, A., Timpl, R., 1999. Binding of the G domains of laminin α1 and α2 chains and perlecan to heparin, sulfatides, α-dystroglycan and several extracellular matrix proteins. EMBO J. 18, 863–870.

Talts, J.F., Sasaki, T., Miosge, N., Göhring, W., Mann, K., Mayne, R., Timpl, R., 2000. Structural and functional analysis of the recombinant G domain of laminin α4 chain and its proteolytic processing in tissues. J. Biol. Chem. 275, 35192–35199.

Timpl, R., Tisi, D., Talts, J.F., Andac, Z., Sasaki, T., Hohenester, E., 2000. Structure and function of laminin LG modules. Matrix Biol. 19, 309–317.

Tisi, D., Talts, J.F., Timpl, R., Hohenester, E., 2000. Structure of the C-terminal laminin G-like domain pair of the laminin α2 chain harbouring binding sites for α-dystroglycan and heparin. EMBO J. 19, 1432–1440.

Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P.K., Shinkai, H., 2001. A unique sequence of the laminin α3 G domain binds to heparin and promotes cell adhesion through syndecan-2 and -4. J. Biol. Chem. 276, 28779–28788.

Yamaguchi, H., Yamashita, H., Mori, H., Okazaki, I., Nomizu, M., Beck, K., Kitagawa, Y., 2000. High and low affinity heparin-binding sites in the G domain of the mouse laminin α4 chain. J. Biol. Chem. 275, 29458–29465.

Yurchenco, P.D., Cheng, Y.S., 1993. Self-assembly and calcium-binding sites in laminin. A three-arm interaction model. J. Biol. Chem. 268, 17286–17299.