Role of mesenchymal nidogen for epithelial morphogenesis in vitro

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SUMMARY

Recent biochemical studies suggested that the extracellular matrix protein nidogen is a binding molecule linking together basement membrane components. We studied its expression and role during development. By immunofluorescence and northern blotting, nidogen was found early during epithelial cell development of kidney and lung. Yet, in situ hybridization revealed that nidogen was not produced by epithelium but by the adjacent mesenchyme in both organs. Binding of mesenchymal nidogen to epithelial laminin may thus be a key event during epithelial development. This is supported by antibody perturbation experiments. Antibodies against the nidogen binding site on laminin B2 chain perturbed epithelial development in vitro in embryonic kidney and lung. Mesenchymal nidogen could be important for early stages of epithelial morphogenesis.

Key words: epithelium, laminin, nidogen

INTRODUCTION

Much has recently been learned about the expression and role of laminin chains during embryonic development (Adams and Watt, 1993; Ekblom, 1993), but less is known about another major basement membrane glycoprotein, nidogen, in these respects. Nidogen is a single $150×10^3$ M_r polypeptide chain, folded into two N-terminal globular domains, a C-terminal globular domain and connecting rod-like segments (Paulsson et al., 1986; Fox et al., 1991). Partial peptide sequences and the deduced complete RNA sequence from cDNA clones showed that nidogen is identical to entactin (Durkin et al., 1988; Mann et al., 1989). For simplicity, nidogen/entactin will here be referred to as nidogen.

The precise biological function of nidogen is not known. One of the EGF-type repeats of nidogen has an Arg-Gly-Asp (RGD) sequence which could be involved in cell binding (Durkin et al., 1988; Mann et al., 1989). Nidogen also binds to other basement membrane components such as type IV collagen, laminin and the proteoglycan perlecan (Aumailley et al., 1989; Fox et al., 1991; Battaglia et al., 1992). Nidogen binds to a single EGF-like repeat in domain III of laminin B2 chain (Gerl et al., 1991; Mayer et al., 1993b). Laminin chains studied so far do not contain collagen IV binding sites, and the adhesive bridge of nidogen may thus be critical for the supramolecular assembly of basement membranes (Timpl, 1989; Aumailley et al., 1993).

In extracts from a mouse tumour, nidogen forms a stable noncovalent complex with laminin chains (Paulsson et al., 1987). Nidogen and laminin can be extracted from mouse tissues in approximately equimolar amounts (Dziadek and Timpl, 1985), in agreement with findings that one laminin complex binds one nidogen molecule (Paulsson et al., 1987). Immunohistology has suggested that nidogen could be a ubiquitous component of basement membranes, and uniformly codistributed with laminin (Wu et al., 1983; Avner et al., 1983; Dziadek and Timpl, 1985; Desjardins and Bendayan, 1989). However, there are many laminin isoforms and some laminin chains are only transiently expressed during embryogenesis. Laminin A chain expression is largely confined to epithelial cells, whereas B1 and B2 chain expression is seen also in many other cell types (Kleinman et al., 1987; Edgar et al., 1988; Klein et al., 1988; 1990; Engvall et al., 1990). .

Detailed studies comparing nidogen expression with the expression of A or B chains of laminin are only available for a few tissues. In the developing eye, nidogen expression differed from the expression of both the laminin A and the B1 and B2 chains (Dong and Chung, 1991). In F9 embryonal carcinoma cells induced in vitro to differentiate, nidogen expression increased later and to a much lower degree than levels for laminin B chains (Carlin et al., 1982; Cooper et al., 1983). A similar dissociation of nidogen and laminin B chain expression was found during preimplantation mouse embryonic development. Laminin B chains are expressed already in 2-4 cell stage, whereas nidogen seems to appear with laminin A chain at the 16 cell stage morula (Cooper and MacQueen, 1983; Dziadek and Timpl, 1985).

Although nidogen is found in epithelial basement

membranes, it might be produced also by mesenchymal cells (Hogan et al., 1982). We therefore studied the expression and role of nidogen during embryonic development in embryonic kidney and lung, two tissues where mesenchymal cells are required for the branching of the epithelium. In the kidney but not in the lung, some of the mesenchymal cells convert into new epithelial tubule cells as a result of the epithelial-mesenchymal interactions (Grobstein, 1956). Our data suggest that nidogen is produced by the mesenchyme, but is required for epithelial development.

MATERIALS AND METHODS

Tissues and organ culture

NMRI×129 hybrid mouse embryos were used. Nidogen expression during de novo formation of a basement membrane was studied in metanephrogenic mesenchyme induced to differentiate into epithelium. Kidney mesenchyme from 11 day-old embryos was isolated, and cocultured in a transfilter culture with embryonic spinal cord as an inducer in I-MEM medium (Gibco) supplemented with transferrin and 10% fetal calf serum (Grobstein, 1956; Weller et al., 1991).

Antibody perturbation experiments were performed with kidneys from 12-day old and lungs from 11- and 12-day old mice. Organs were cultured in defined medium (Klein et al., 1988) supplemented with 10% FCS (kidney) or 50 µg/ml transferrin (lung) for 2 or 3 days. The number of tubules in kidneys and end buds in lung were counted independently by three persons from photographs taken by stereomicroscopy. Non-immune IgG, PBS, or antibodies against laminin fragment P1 were used in control experiments (Mayer et al., 1993b). Direct immunofluoresence showed that antibodies penetrated into the tissue. Rudiments were examined by stereomicroscopy and histology (Klein et al., 1988). Formation of epithelial basement membranes was studied in frozen sections stained with monoclonal antibody 200 detecting the E3 domain of laminin A chain (Sorokin et al., 1992). DNA content of 5 antibody-treated embryonic day 12 kidneys and 5 control kidneys was measured as described by Weller et al. (1991).

Antibodies

Nidogen was detected with an affinity-purified rabbit antibody against mouse nidogen (Paulsson et al., 1986). Immunofluoresence results in the kidney were confirmed with rat monoclonal antibody against nidogen (Dziadek et al., 1988). The specificity of the antiserum was verified by immunoblotting. In extracts from a mouse tumour the affinity-purified antiserum against nidogen reacted with 150×10^3 M_r nidogen but not with laminin A or B chains. The smaller bands recognized by the anti-nidogen antiserum (Fig. 1, lane A) are the expected proteolytic degradation products of nidogen (Dziadek et al., 1985, 1988). This is supported by the northern blotting for nidogen mRNA. In poly(A)$^+$ RNA a single full length 6 kb signal could be seen (Fig. 1, lane B). Laminin chains were studied with rabbit laminin antiserum reacting with A and B chains of laminin (Klein et al., 1988) and with rat monoclonal antibodies 200 or 201 reacting with the 400×10^3 M_r Ae chain of laminin (Sorokin et al., 1992). FITC- and TRITC-labelled second antibodies were from Jackson Laboratories.

Rabbit antisera against laminin fragment P1 and the recombinant fragment B2III3-5, which block nidogen binding to laminin were those described by Gerl et al. (1991); Mayer et al. (1993b). Antibody purification and characterization by radioimmunoassays followed standard protocols (Timpl, 1982). Antibodies against B2III3-5 were purified on a fragment P1 immunoadsorbent. Successive immunoadsorptions on a B2III3-5 column followed by a fragment P1 column was used to separate the antibodies from the anti-P1 antiserum into two fractions that recognize different epitopes.
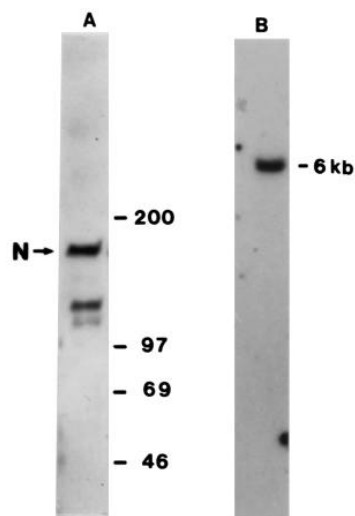

Fig. 1. Reactivity of the antiserum against nidogen. (A) Immunobotting was performed on extracts from the mouse Engelbreth-Holm-Swarm tumour. A reaction is seen with the 160×10^3 M_r nidogen (N) polypeptide and its proteolytic fragments. The apparent molecular mass is indicated to the right ($\times10^{-3}$). (B) Northern blotting hybridization. Nidogen cDNA was probed to 2 µg poly (A)$^+$ RNA from day-13 embryonic lung. Only a full length 6 kilobase (kb) message was detected.

Immunofluorescence

Frozen sections (5 µm) were fixed with −20˚C methanol and washed in 1% BSA in PBS. Antiserum against nidogen was diluted 1:100, antiserum against laminin, 1:1000, and purified monoclonal antibody 200 or 201 against laminin A chain was used at 10 µg/ml. TRITC or FITC-labelled second antisera against rabbit IgG were diluted 1:200. Rat monoclonal antibodies were detected with biotin-conjugated anti-rat antiserum (1:200) and FITC-conjugated streptavidin diluted 1:500 (Amersham).

Immunoblotting

Tissues were sonicated in Tris-buffered saline, pH 7.4 containing 10 mM EDTA buffer and protease inhibitors (Paulsson et al., 1987). Homogenates were kept on ice for 1 hour, centrifuged, and the protein contents of the supernatants were determined. Samples were boiled for 5 minutes in Laemmli buffer containing dithiotreitol, proteins were then separated on a 5-15% gradient SDS polyacrylamide gel and transferred to nitrocellulose filters. As blocking solution 3% bovine serum albumin and 0.05% Tween-20 was used. Antisera against nidogen or laminin were used at 1:100 and 1:1000, respectively. Bound antibodies were detected as described by Klein et al. (1990).

Northern blotting

Tissues were frozen in liquid nitrogen, and total RNA was isolated as described (Chirgwin et al., 1979). Poly(A)$^+$ RNA was isolated by two passages through an oligo(dT) cellulose column. For northern hybridization, 10 µg total RNA or 2 µg poly(A)$^+$ RNA was used for each lane. RNA was denatured with glyoxal, separated by electrophoresis on 1.1% agarose gels (Sambrook et al., 1989), transferred onto Hybond N membranes (Amersham-Buchler, Braunschweig, FRG), and baked for 1-2 hours at 80˚C at 0 torr. Filters were preincubated for 2 h at 42˚C in 50% formamide (Fluka), 2.5 × Denhardt's solution, 2× SSPE, 0.2% SDS and 50-100 µg/ml E. coli tRNA. Hybridization was performed with the same solution for 16-20 hours at 42˚C with the following mouse cDNA probes: a 1.5 kb EcoRI insert (clone 6c, Mann et al., 1989) corresponding to nidogen mRNA, a 1.6 kb SphI-HindIII fragment of cDNA clone corresponding to mouse laminin B1 mRNA (Oberbäumer, 1986); a 0.6 kb EcoRI-SalI fragment of a cDNA clone pPE9 corresponding to laminin B2 mRNA (Barlow et al., 1984); a 1.5 kb EcoRI fragment (clone PA01, Sasaki et al., 1988) corresponding to the middle coding region of laminin A-chain mRNA; a 1.2 kb PstI fragment of mouse β-actin cDNA clone pAL41 (Minty et al., 1983). The amount of RNA on filters was also evaluated by staining the filters with 0.04% methylene blue. cDNA inserts were labeled with [^{32}P]dCTP (Amersham) to a specific activity of about 2×10^8 cts per minute/µg DNA by nick translation (Bethesda Reseach

Laboratories) or oligolabeling (Pharmacia, Uppsala). After hybridization, filters were washed with a final stringency of $0.1 \times$ SSC, 0.2% SDS at 52-56°C, and were exposed to Kodak XAR films at −70°C in the presence of intensifying screens.

In situ hybridization

In situ hybridization was performed according to Ernfors et al. (1990) with minor modifications. Briefly, sections were fixed in 4% paraformaldehyde, rinsed in PBS, dehydrated in a graded ethanol series including a 5 minute wash in chloroform. For hybridization we used a synthetic 45-mer oligonucleotide probe 5′-TCG CCC CCA GTG ATG CTA AAC CCA TTC TTG AAC CCA TCC TGC TCC-3′ complementary to nucleotides 1505-1549 of mouse nidogen (Durkin et al., 1988). The probe was labelled at the 3′ end with α-^{35}S-dATP (Amersham) using terminal deoxyribonucleotidyl transferase (Scandinavian Diagnostic Services) to a specific activity of 1×10^9 cts per minute/µg. Hybridization was performed in 50% formamide, $4 \times$ SSC, $1 \times$ Denhardt's solution, 10% dextran sulfate, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl, 0.02 M Na_2HPO_4 (pH 7.0), 0.05 M dithiothreitol, using 10^7 cts per minute/ml for each probe. Sections were hybridized at 42°C for 15-18 h, washed extensively at 56°C in $1 \times$ SSC. Control sections hybridized with the same amount of labelled probe plus unlabelled probe in excess did not show any specific binding. Slides were dipped in Kodak NTB-2 photo emulsion (diluted 1:1 in water), exposed for 3 weeks at 4°C, developed, fixed and counterstained with cresyl violet.

RESULTS

Nidogen during in vitro conversion of metanephric mesenchyme to epithelium

Undifferentiated kidney mesenchymes from 11-day embryonic mice were isolated and induced to differentiate. During the in vitro culture, mesenchymal cells differentiate into polarized epithelial tubules surrounded by a basement membrane (Grobstein, 1956). Epithelial cell polarization begins at 36 hours of in vitro culture. Nidogen was expressed in a diffuse punctate pattern within the mesenchyme in the uninduced mesenchyme, and a slightly stronger punctate expression pattern was seen in mesenchyme induced for 24 hours (Fig. 2A). Expression was particularly strong in the interphase between loose mesenchyme and areas that will become condensates (Fig. 2B). At 48 hours of culture, nidogen was enriched in the newly formed epithelial basement membranes, although the surrounding mesenchyme also still showed some staining (Fig. 2C). The staining pattern with anti-nidogen antiserum was thus similar to that obtained with antiserum detecting both laminin A and B chains (Klein et al., 1988).

Northern blotting showed that nidogen mRNA expression was low in uninduced mesenchyme. A dramatic increase occurred on day 1 of in vitro culture, and it then remained at the same level on day 2 of culture (Fig. 3). Nidogen mRNA expression during in vitro development of kidney tubules thus increased early and coordinately with laminin B1 and B2 chain mRNA expression but not with laminin A chain mRNA expression (Ekblom et al., 1990).

Northern blotting for nidogen, laminin A, B1, and B2 mRNA during in vivo development of kidney and lung

An apparent coexpression of nidogen with the B1 and B2 chains was also observed by northern blotting of different
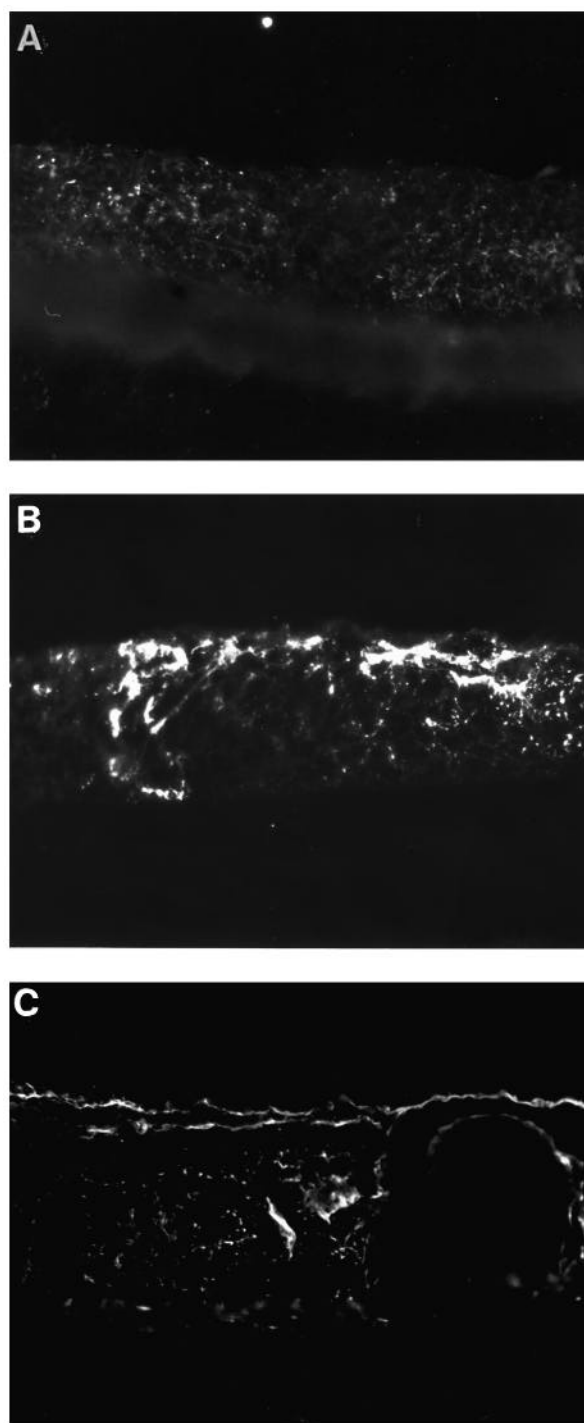

Fig. 2. Nidogen polypeptide expression during in vitro conversion of metanephric mesenchyme to epithelium. Immunofluorescence staining with anti-nidogen antiserum of kidney mesenchyme isolated from 11-day old mouse embryos showing that nidogen is expressed in the uninduced mesenchyme (A) as small punctate deposits. A similar somewhat stronger expression pattern is seen in the in vitro induced, but not yet differentiated mesenchyme at 24 hours of in vitro culture (B). At 48 hours of in vitro culture, nidogen is also deposited in the basement membrane of the newly formed epithelial kidney tubules (C). Bar, 30 µm.
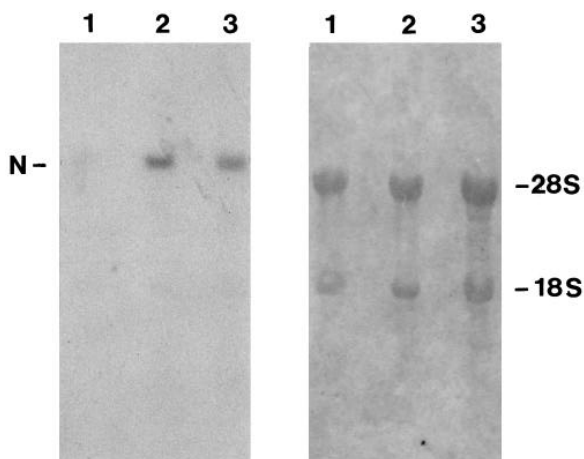
Fig. 3. Increased expression of nidogen mRNA during in vitro conversion of the metanephrogenic mesenchyme to epithelium. Northern hybridization with a nidogen cDNA probe to total RNA isolated from uninduced kidney mesenchyme of 11-day embryonic mice (lane 1) and mesenchyme cultured in vitro for 24 (2) and 48 hours (3). An embryonic spinal cord was used as an inducer. The 6 kb signal for nidogen (N) is already expressed at 24 hours of induction (2) and remains at the same level at 48 hours of in vitro culture (3). Methylene blue staining of the filter (right panel) shows the relative amounts of RNA present in each lane.

Fig. 4. Northern hybridization with nidogen, laminin B1, B2 and A-chain cDNA probes to total RNA from embryonic and newborn kidney and lung. 10 µg total RNA from 14-day embryonic kidney (lanes 1,5), 13-day embryonic lung (2,6) and newborn kidney (3,7) and newborn lung (4,8) was loaded. One filter was hybridized with nidogen and laminin B2 cDNA (lanes 1-4), and another filter with cDNA to laminin A and B1 chain (lanes 5-8). The filters were stained with methylene blue to show the relative amounts of RNA present in each lane (lowest panel). The position of 18S and 28S mRNA are indicated. N, nidogen; B1, laminin B1 chain mRNA; B2, laminin B2 chain mRNA; A, laminin A chain mRNA.

stages of kidneys and lungs developing in vivo. Laminin B1, B2 and nidogen mRNAs were expressed in both stages studied (embryonic day 13-14 and newborn), and the ratio between the nidogen and laminin B1 and B2 signal intensities was similar in all samples (Fig. 4). In contrast, laminin A chain expression was high in the early developmental stage and lower in the newborn stage in both tissues, but particularly in the lung (Fig. 4).

Localization of nidogen in the developing kidney in vivo

During in vivo development of kidney epithelia, the epithelial ureter bud locally induces mesenchymal cells to convert into a new epithelium. These new epithelial areas are surrounded by a loose mesenchyme, which in vivo also contains many blood vessels. The first sign of conversion of mesenchyme to epithelium is condensation of the mesenchyme around the tip of the ureter. At this stage, nidogen was expressed in the basement membranes of ureter epithelium and endothelial cells (Fig. 5A). In addition, punctate expression was seen in the interphase between the condensate and uninduced mesenchyme. Very little nidogen expression was noted between the cells within the condensate whereas expression was seen in the loose mesenchyme outside the condensate (Fig 5A). In contrast, laminin A chain was seen only in the basement membrane of the epithelial ureter buds, and no expression was seen in endothelial basement membranes or in mesenchyme (Fig. 5B). At later stages, when new tubular and glomerular basement membranes had formed, nidogen was expressed in these basement membranes, and in basement membranes of blood vessels (Fig. 6 A,B). In addition some expression was seen in mesenchyme surrounding the epithelium. Expression in mesenchyme was uneven, with strong expression around develop-
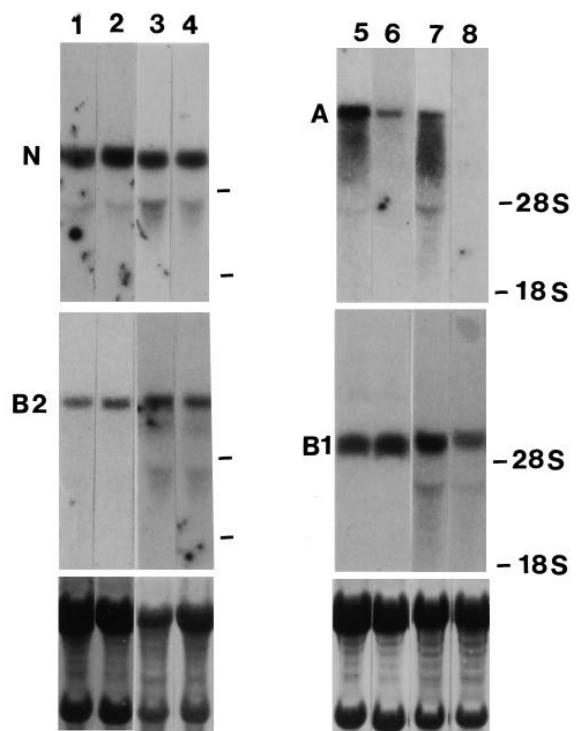
ing tubules (Fig. 6A) and very little if any expression in the stroma close to the stalk of the ureter (Fig. 6B).

In situ hybridization showed nidogen mRNA uniformly in all parts of the mesenchyme, both in mesenchyme close to the stalk of the ureter (Fig. 6C) and close to developing tubules (Fig. 6D). No nidogen mRNA expression was noted in the epithelium in any part of the embryonic kidney (Fig. 6E,F). Because of the uniform mesenchymal distribution of the grains, only some of the expression in the mesenchymal area can be due to endothelial cells.

Localization of nidogen in the embryonic lung in vivo

In sections from embryonic lung, the anti-nidogen antiserum stained both the bronchial and the blood vessel basement membranes (Fig. 7A), whereas monoclonal antibody against A chain stained only the basement membranes of bronchial epithelial cells (Fig. 7B). Lower magnifications revealed that the staining pattern with anti-nidogen antiserum was the same in all parts of the developing lung (Fig. 7C). In mesenchyme between the endothelial and epithelial cells little staining for nidogen was observed. Yet, in situ hybridization showed nidogen mRNA expression uniformly and exclusively in the mesenchymal compartment (Fig. 7D) in all parts of the devel-
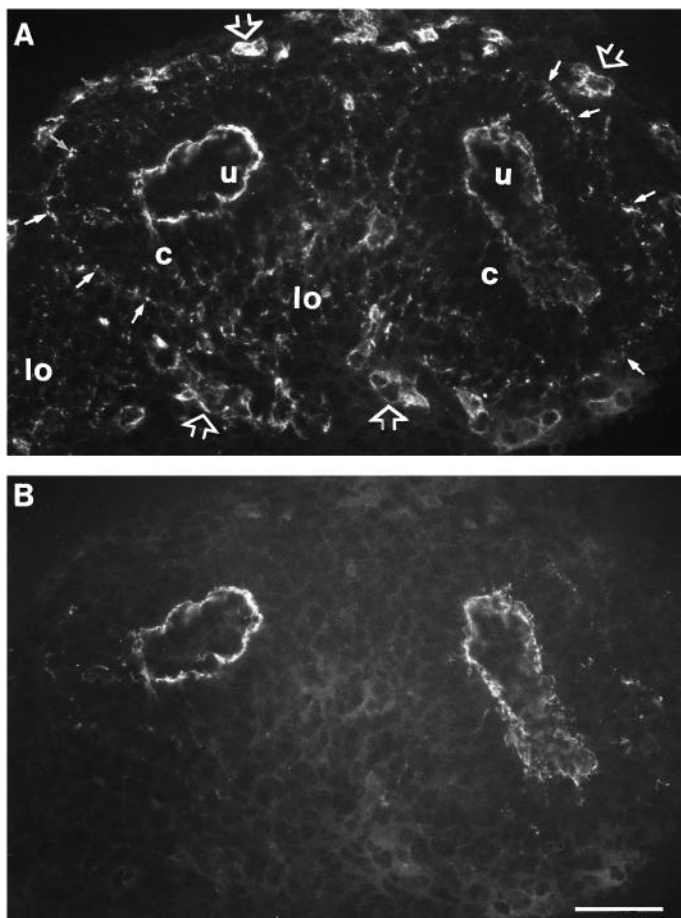
Fig. 5. Expression of nidogen (A) and laminin A chain (B) in a 12-day embryonic kidney, where the first condensates have formed around two branches of the ureter epithelium. Double immunofluoresence was performed with rabbit antiserum against nidogen (A) and mAb 201 against rat anti-mouse laminin A chain (B). The basement membrane of the two ureter buds (u) express nidogen and laminin A chain. Nidogen is also seen (small closed arrows) in the interphase between the condensing mesenchyme (c) and the loose mesenchyme (lo), in the loose mesenchyme itself, and in basement membranes of capillaries (open arrows). Bar, 50 µm.

Table 1. Binding specificites of affinity-purified antibodies used in inhibition of laminin-binding to nidogen

Antibody against (code No)	Antigen-binding capacity (ng/µg antibody) for	
	laminin P1	B2III3-5
B2III3-5 (N-283)	40	16
laminin P1 (N-285)*	10	5
laminin P1 (N-286)†	46	<0.1

*Affinity-purified on B2III3-5.
†Affinity-purified on laminin fragment P1 after depletion on B2III3-5.

Table II. Development of tubules in antibody treated kidney explants

Antibody	Number of tubules (% of controls)	Number of explants
–	100	10
N-283 (50 µg/ml)	90	10
N-283 (100 µg/ml)	40-45	10
N-283 (120 µg/ml)	30	18
N-285 (100 µg/ml)	55-65	10
N-286 (100 µg/ml)	100	10

The variations in the given percentage values indicate observer variation.

oping lung, as seen clearly in lower magnifications (Figs 7E, 6F).

Perturbation of development with antibodies blocking laminin-nidogen interaction

Three different antibodies were used to test whether the binding of mesenchymal nidogen to laminin is important for morphogenesis of the kidney and lung. Antisera were raised against the $18×10^3$ M_r recombinant laminin fragment B2III3-5 and the larger fragment P1 ($200×10^3$ M_r) which both share the laminin-binding site for nidogen. These antisera were previously shown to inhibit laminin-nidogen interaction in vitro by binding close to a single EGF-like repeat responsible for nidogen binding (Mayer et al., 1993b). For use in organ culture studies, antibodies were purified by affinity chromatography and characterized by radioimmunoassays (Table 1). From the antiserum produced against fragment P1 two different antibody populations were obtained. The first one was obtained by

binding to B2III3-5 (antibody No. N-285) and the second one was obtained by a subsequent affinity purification of the non-bound antibodies on a fragment P1 immunoadsorbent (antibody No. N-286). Both the antibody raised against recombinant B2III3-5 (No. N-283) and N-285 showed distinct binding to fragment P1 and B2III3-5 but differed in antigen binding capacity. They inhibited laminin-nidogen interaction in ligand assays, with antibody N-283 being about 10 times more active than antibody N-285. The third antibody (N-286) showed distinct binding for fragment P1 but not for B2III3-5 (Table 1) and was used for control purposes.

At concentrations above 100 µg/ml antibody N-283 drastically altered morphogenesis of 12-day embryonic kidneys in organ culture (Table 2). Kidneys grown for 3 days in the presence of N-283 had fewer branches of the ureter and less new epithelium formed around the tips of the ureter (Fig. 8A). However, it appears that N-283 did not primarily affect growth as during culture of control 12-day kidneys DNA content increased on the first day of culture from 0.4 µg to approximately 1.5 µg and the same increase occurred in the presence of 100 µg/ml N-283. Subsequently, kidneys treated with N-283 (100 µg/ml) did not increase their size as much as control kidneys.

N-283 had only a limited effect on kidney development at 50 µg/ml (Table 2). Kidneys grown in the presence of 100 µg/ml of purified non-immune IgG were normal (Fig. 8B). Kidneys grown in the presence of antibody N-285 (100 ug/ml) were slightly altered (Fig. 8C), whereas kidneys grown in the presence of antibody N-286 (100 µg/ml) developed normally (8D, Table 2). Thus the effect of the antibodies in kidney organ culture correlated with the binding capacity of the antibodies to B2III3-5 (Table 1).

Kidneys treated for 3 days with 100 µg/ml antibody N-283 showed some branching of the ureter epithelium. However, only very few new tubules had formed (Fig. 8E) and in the area
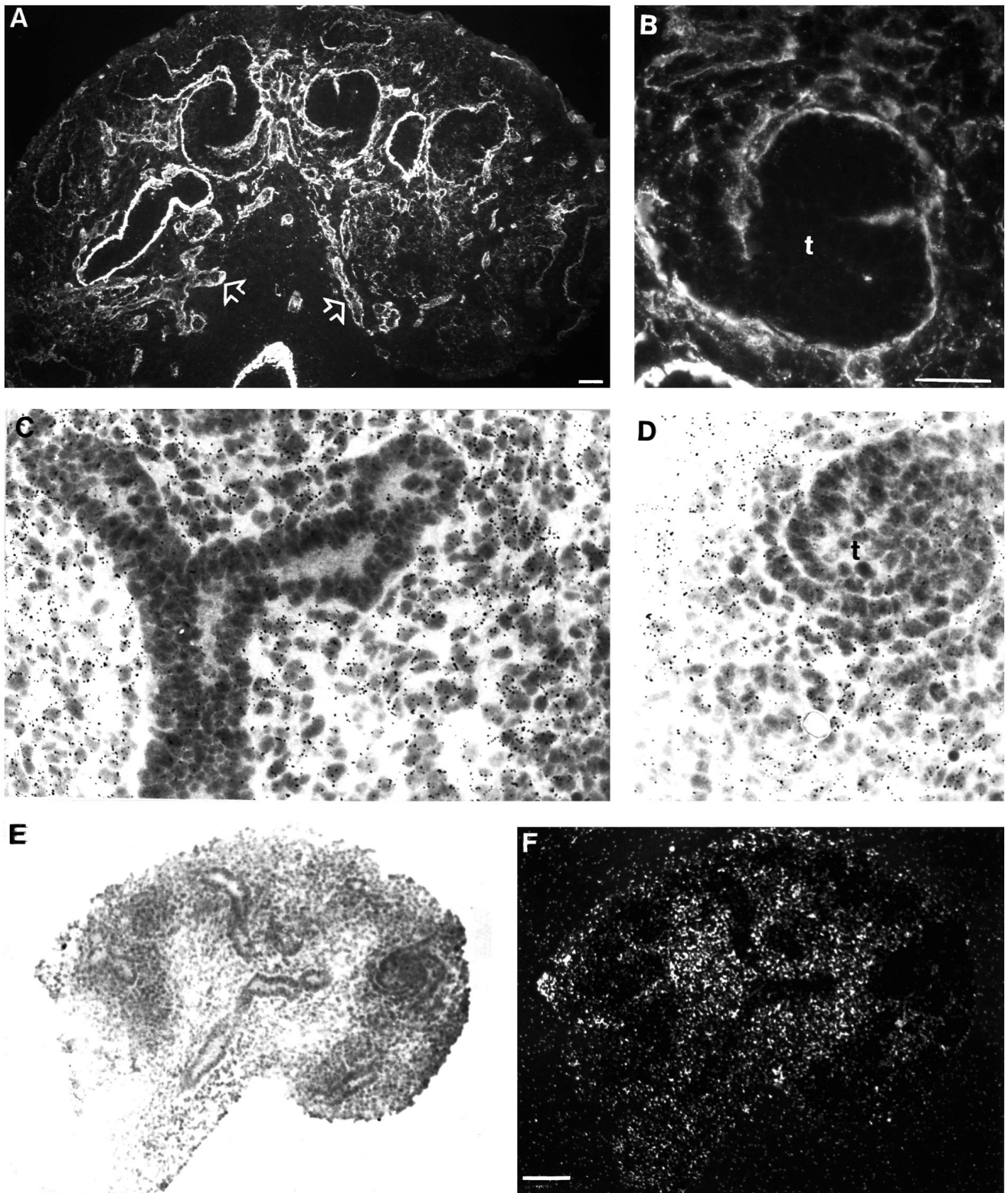
Fig. 6. Comparison of nidogen polypeptide and mRNA expression in a 14-day old embryonic kidney. Immunofluoresence with anti-nidogen antiserum revealed expression in the basement membranes of blood vessels (open arrows) and tubules, glomeruli and of collecting ducts (A). Uneven expression was noted also in loose mesenchyme. Larger magnifications of a developing epithelial S-shaped tubule (t) showed expression in developing basement membranes, and in mesenchyme surrounding the epithelium (B). Bright-field micrographs of in situ hybridization with the ^{35}S-labelled 45-mer oligonucleotide showed grains both in the mesenchyme close to the collecting ducts (C) and close to developing S-shaped tubules (D). Lower magnification of the nidogen in situ hybridization in bright-field (E) and dark-field (F) illumination revealed many grains evenly distributed in all parts of the mesenchyme but no grains above background in epithelial structures. Bar in A and B, 10 μm. Bars for C-F, 60 μm.
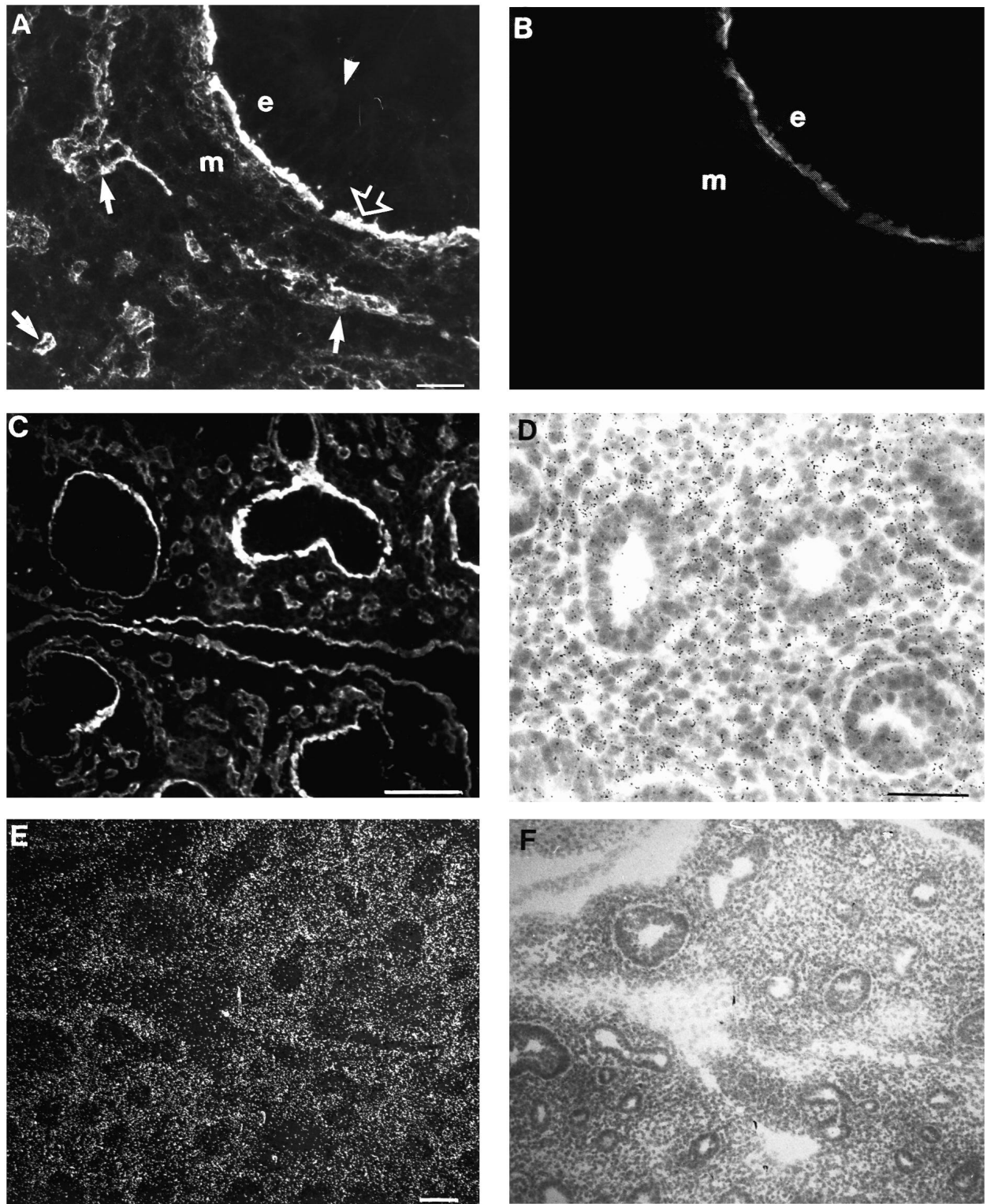
Fig. 7. Nidogen polypeptide and mRNA expression in the developing lung of 14-day old embryos. Double immunostaining for nidogen (A) and laminin A chain (B) showed reactivity for nidogen in basement membranes of a large trachea with its epithelium (e) and in basement membranes of blood vessels (arrows) but no reactivity in mesenchyme. Laminin A chain was not seen in blood vessel basement membranes. Comparison of immunostaining for nidogen in the developing lung (C) and a bright-field micrograph of a section hybridized with the antisense oligonucleotide to detect nidogen mRNA by in situ hybridization (D) revealed nidogen immunoreactivity only in basement membranes of epithelium and endothelium, but expression of nidogen mRNA in the mesenchyme. Low magnification dark-field (E) and bright-field (F) micrographs of the in situ hybridizations showed that all parts of the lung mesenchyme express nidogen mRNA and no signals above background were detected in the epithelial structures. Bars, 4 µm (A-B); 80 µm (C-D); 80 µm (E-F). Closed arrows, endothelial cell basement membranes; arrowhead, apical border of epithelium; open arrow, basal border of epithelium.
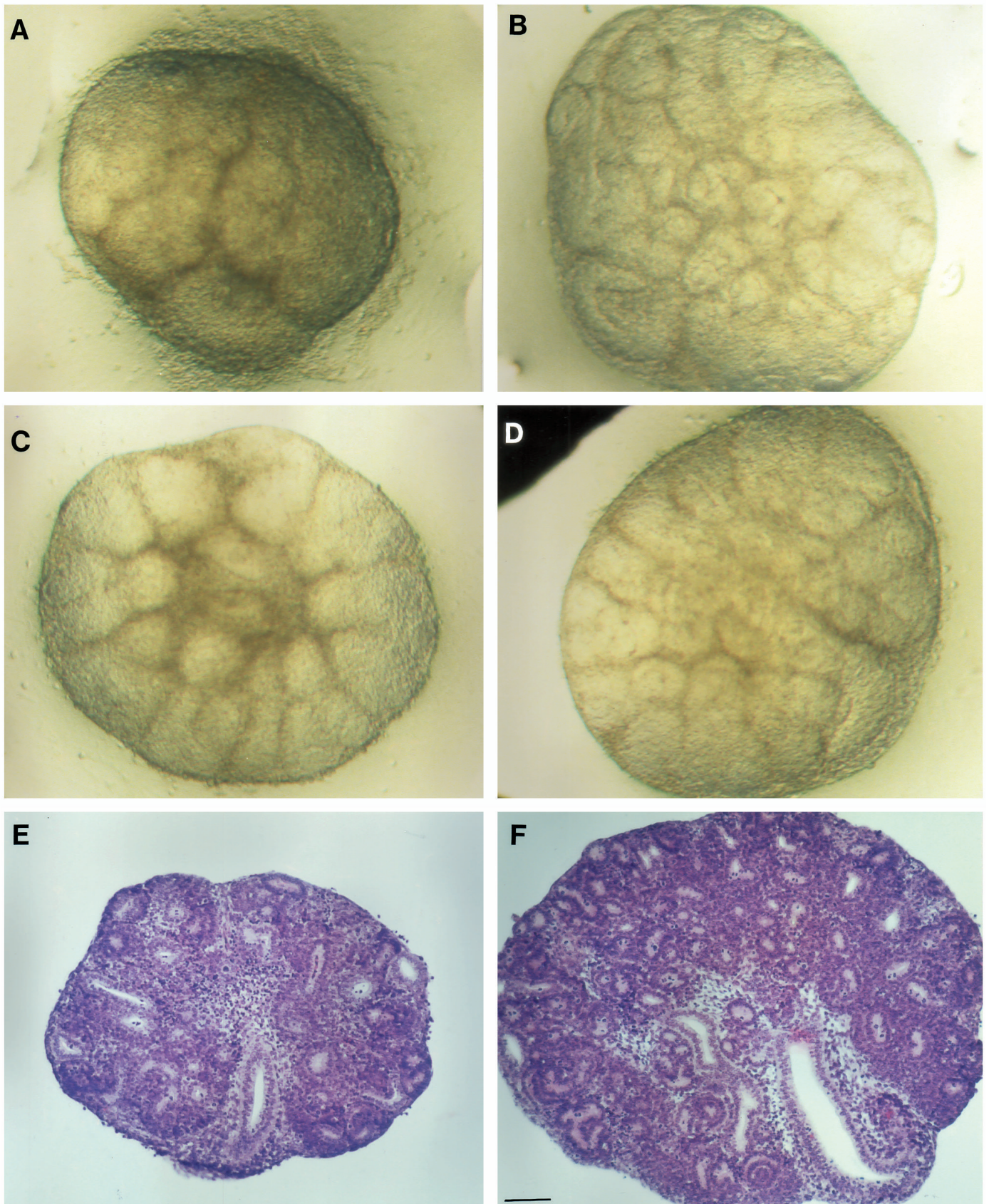
Fig. 8. Inhibition of kidney tubule development in vitro by antibodies against domain III3-5 of laminin B2 chain. Kidneys from 12-day old mice were cultured in the presence of 100 µg/ml antibody N-283 (A,E), non-immune IgG (B,F), N-285 (C) or N-286 (D) and examined after 2 days by stereomicroscopy (A-D) and after 3 days by light microscopy of cross-sections (E,F). Bar, 100 µm.
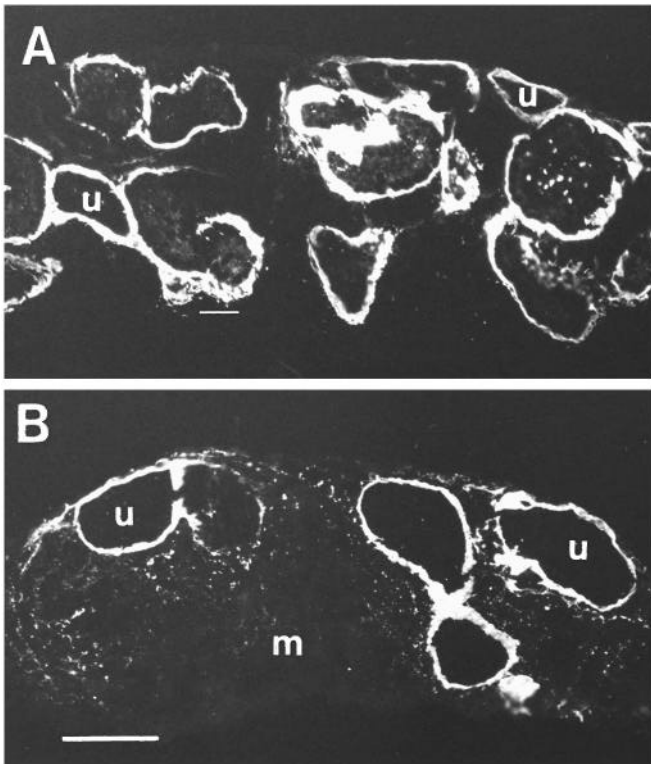
Fig. 9. Perturbation of tubular and glomerular basement membrane assembly in organ culture by antibodies against domain B2III3-5 of laminin B2. Organ culture was performed for 2 days, and basement membrane assembly was then monitored by immunofluorescence staining of frozen sections with monoclonal antibody 200 detecting laminin A chain (Sorokin et al., 1992). Laminin A chain was detected in the ureter (u) basement membrane both in kidneys treated with non-immune IgG (A) and in kidneys treated with N-283 (B). In kidneys grown in the presence of non-immune IgG, tubular and glomerular basement membrane also formed (A), but in kidneys grown in the presence of 50 µg/ml of antibody N-283 formation of new basement membranes in mesenchyme (m) was severly inhibited (B). Bar, 20 µm.

where the first tubules should have formed, some necrotic cells were seen instead. In more cortical parts, an abundant mesenchyme was seen but very few tubules (Fig. 8E). Kidneys grown in the presence of non-immune IgG showed numerous tubules, well developed ureter epithelium and mesenchyme around the ureter (Fig. 8F). In a first attempt to study molecular differences between treated and untreated explants, we analyzed the expression of an epithelial marker, laminin A chain on day 2 of in vitro development. In controls, both the ureter epithelium and the new tubular epithelium expressed laminin A chain in their basement membranes (Fig. 9A), but in explants treated with 100 ug/ml antibody N-283, basement membrane staining was seen only in the ureter epithelium. In mesenchyme, punctate expression of laminin A chain was noted but only in a few areas, and no new basement membranes had formed (Fig. 9B).

Antibody N-283 also inhibited branching of 11 day lung rudiments. Fewer end buds formed and the lungs were smaller (Figs 10, 11). In contrast, 11 day lung rudiments cultured with antibody N-286 did not differ from control cultures (Figs 10, 11). The effect of antibody N-283 appeared to be stage-

dependent in lung and the effect was already marginal in E12 lung rudiments. The stage-dependent effect shows that the N-283 solution was not toxic to cells.

DISCUSSION

Mesenchyme is known to be important for epithelial morphogenesis, and it has been suggested that some extracellular matrix proteins of mesenchyme such as tenascin (Chiquet-Ehrismann et al., 1986) and epimorphin (Hirai et al., 1992) are involved in the interactions. Although there is some evidence that tenascin could be involved in lung epithelial morphogenesis (Young et al., 1994), other recent findings suggest that neither tenascin nor epimorphin are crucial for epithelial morphogenesis in general (Hirai et al., 1993; Erickson, 1993). Other mesenchymal factors may be required and we now suggest that nidogen could be one such factor required for epithelial morphogenesis.

Several lines of evidence suggest that binding of mesenchymal nidogen to the laminin B2 chain is crucial for initiation of epithelial basement membrane assembly and for organ development. First, in situ hybridization showed that nidogen mRNA was predominantly expressed in the mesenchymal tissue rather than the epithelial compartment. Similar in situ hybridization results were recently reported by Thomas and Dziadek (1993). Second, nidogen polypeptide was found in basement membranes very early during the development of epithelial and endothelial cells in the kidney and lung. Third, antibodies against the nidogen binding site on laminin B2 chain perturbed basement membrane assembly and development in organ culture. Antibodies to adjacent EGF-like repeats on the P1 fragment of laminin did not perturb these events.

The early presence of nidogen at sites of initiation of basement membrane assembly in the developing kidney was shown by immunofluorescence. The in vitro culture system for the development of kidney tubule cells (Grobstein, 1956) allowed a detailed comparison of the expression patterns of laminin chains and nidogen during the formation of a new basement membrane. In this system, the conversion of mesenchymal cells to polarized epithelium is accompanied by the formation of a new basement membrane. Northern hybridization showed a dramatic increase in the expression of nidogen mRNA already on day 1 of in vitro culture, and its expression thus seems to be coregulated with the laminin B1 and B2 chain mRNA rather than with laminin A chain mRNA (Klein et al., 1988; Ekblom et al., 1990; Sorokin et al., 1990).

Some nidogen was already present as diffuse punctate deposits in the undifferentiated, uninduced metanephrogenic mesenchyme together with laminin B chains. On day two of in vitro culture of the mesenchyme, nidogen polypeptide expression increased and subsequently nidogen became enriched in the newly formed tubular basement membrane. At the stage of formation of new tubular basement membranes, several layers of mesenchymal cells that do not themselves become tubular cells expressed nidogen. The evaluation of the staining pattern in areas close to forming tubules during in vivo development was somewhat ambiguous because of expression of nidogen in blood vessel walls. It nevertheless seemed clear that nidogen was present in mesenchyme close to tubules, whereas very little if any nidogen polypeptide could be

Fig. 10. Inhibition of lung branching in vitro by antibody against the nidogen binding site of laminin. Lung rudiments from 11-day embryos were cultured in the presence of 30 µg/ml antibody N-283 (A), 30 µg/ml antibody N-286 (B) and PBS (C) and examined, after 2 days, under stereomicroscopy. Bar 200 µm.
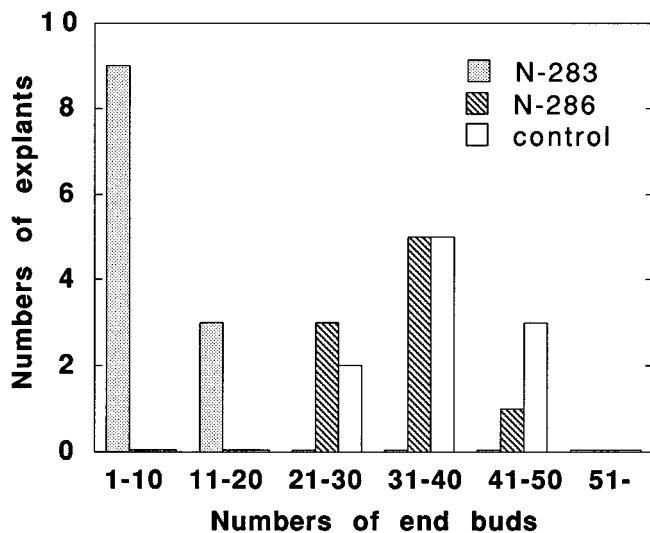

Fig. 11. A semi-quantitative analysis of lung branching in antibody-treated explants. 11-day lung rudiments were cultured in the presence of antibody N-283 or N-286 for 2 days. The number of end buds in each explant was counted.

detected in the mesenchymal area close to the stalk of the ureteric tree.

Based on the immunofluoresence the cellular origin of nidogen could not be judged with certainty, but it seemed likely that the epithelial and endothelial cells were the main producers, while some mesenchymal cells may also contribute. However, in situ hybridization revealed a striking expression of nidogen mRNA exclusively in the mesenchymal-endothelial compartments. Although the nidogen polypeptide expression seemed to be focal, mRNA for nidogen was found evenly in all parts of the mesenchyme, also close to the stalk of the ureter tree. An abundant and even mRNA expression pattern in the mesenchyme was also found in the developing lung, where none of the mesenchymal cells convert into epithelium. Using another probe to study nidogen mRNA, similar expression patterns were recently reported (Thomas and Dziadek, 1993).

Previous immunofluorescence studies of some other tissues

have suggested that nidogen can be expressed outside basement membranes. Nidogen was found in the dermis of neonatal rat skin (Hogan et al., 1982) and in virgin rat mammary gland (Warburton et al., 1984). Likewise, mammary fibroblasts in culture have been reported to express nidogen, whereas mammary epithelial and myoepithelial cells, which synthesize other basement membrane proteins, laminin and collagen IV, do not synthesize nidogen (Warburton et al., 1984).

Taken together, current data suggest that the nidogen-laminin complex in epithelial basement membranes forms extracellularly as a result of an epithelial-mesenchymal cooperation. The importance of similar cooperations in basement membrane assembly has been suggested for the developing gut (Simon-Assmann et al., 1988). Our previous studies showed that both laminin A chain and the integrin $\alpha6$ subunit of the laminin receptor are produced by epithelial cells (Ekblom et al., 1990; Sorokin et al., 1990), but as shown here nidogen is derived from the mesenchyme. It appears that B chains of laminin could be produced by both cell types. It is noteworthy that no nidogen polypeptide was detected in the mesenchyme surrounding the stalk of the ureter and yet these mesenchymal areas expressed nidogen mRNA. One possibility is that nidogen is rapidly degraded in areas lacking all constituents necessary for basement membrane assembly. Tissue-bound nidogen is extremely sensitive to proteases (Dziadek et al., 1985; 1988). Soluble nidogen is highly vulnerable to various tissue proteases with major cleavage sites between the two N-terminal globules and within the C-terminal laminin binding domain G3 (Mayer et al., 1993a). Binding of nidogen to laminin structures prevented cleavage in G3 and a similar stabilization may occur in situ in basement membranes.

If extracellular formation of complexes between epithelial laminin A-B1-B2 and mesenchymal nidogen at the epithelial-mesenchymal interphase is important for basement membrane assembly, it should be possible to perturb development in organ culture with antibodies that block binding of nidogen to laminin. It has not been possible to produce blocking antibodies directed against nidogen (Mann et al., 1989; Mayer et al., 1993a,b), and therefore we prepared an antibody against the nidogen binding site of laminin B2. Current knowledge

strongly suggests that this antibody (N-283) specifically blocks laminin-nidogen binding and does not interfere with other assembly events such as laminin trimer formation, which occurs intracellularly and is initiated by the carboxy-terminal ends. Here we demonstrate that antibody N-283 against the nidogen binding site on the B2 chain of laminin (Mayer et al., 1993b) altered both kidney and lung morphogenesis in organ culture. An unexpected finding was that the antibodies were active only in explants from very early stages in the lung. The reason for this is unclear at the moment. The functional studies suggest that the early binding of nidogen to laminin B2 chain is important during initial stages of epithelial-mesenchymal interactions. Nidogen-laminin binding might also be important for formation of new basement membranes during conversion of mesenchyme to epithelium in the kidney. The subsequent event, formation of kidney basement membranes containing laminin A chain was severely perturbed when nidogen-laminin B2 chain binding was blocked. It is likely that the blocking antibodies in both tissues primarily acted at the epithelial-mesenchymal interphase, but we cannot exclude that the antibodies acted indirectly by affecting the development of mesenchymal or endothelial cells. It remains to be seen to what extent N-283 treatment secondarily induces apoptosis, which has been shown to occur when epithelial cell-matrix interactions are disrupted (Frisch and Francis, 1994).

The expression studies also suggest a role for nidogen during blood vessel formation. Blood vessels are found in large amounts within embryonic mesenchyme, and endothelial cells produce basement membranes at early stages of angiogenesis (Ekblom, 1981). Our current data show that all morphologically identified capillaries express both nidogen and the B (B1 or B2) chains of laminin at the earliest developmental stage detectable. In contrast, they seem to express low amounts or lack the A chain of laminin. This was already noted in previous studies using polyclonal antibodies (Klein et al., 1990), and could now be confirmed with monoclonal antibodies against the mouse 400×10^3 M_r A chain (Sorokin et al., 1992). Laminin A chain is apparently not important for embryonic angiogenesis in the tissues analyzed. It is thus notable that in vitro studies have implicated laminin A chain in angiogenesis (Grant et al., 1989) and that it has been detected in many basement membranes of endothelial cells of adult tissues (Sanes et al., 1990; Wewer et al., 1992). This could indicate differences between embryonic and adult tissues or differences between subsets of blood vessels. A likely possibility is that only some endothelial cell types express the 400×10^3 M_r A chain typical for many embryonic epithelial cells, while other endothelial cells may express laminin with another type of A chain. This was in fact shown for endothelial cells from aorta and pulmonary arteries which produce two distinct A chains both with similar molecular mass of about 400×10^3 (Tokida et al., 1990).

Our findings showing low amounts of the first described laminin A chain both in mesenchyme and in capillary basement membranes are in apparent conflict with a recent study on lung development. By in situ hybridization and immunoprecipitation Schuger et al. (1992) provided evidence for an equal expression of this A chain in embryonic mouse lung mesenchyme and epithelium. However, in their in situ hybridization it could not be judged whether the intensity of the signal differed from background intensity. Moreover, the identity of

their large 400×10^3 M_r polypeptide chains seen in immunoprecipitation of the lung mesenchyme was not established and it could be a novel laminin chain. We thus consider it likely that embryonic mesenchyme and capillaries in the lung and kidney and many other organs express very litte laminin A chain as compared with the expression of epithelial cells. Based on in situ hybridization for the A chain mRNA, similar conclusions were recently made by Thomas and Dziadek (1993).

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