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Importance of nidogen binding to laminin γ1 for branching epithelial morphogenesis of the submandibular gland

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SUMMARY

Epithelial-mesenchymal interactions are major driving forces for the development of most solid organs. The importance of these interactions was first shown for the embryonic submandibular gland more than 40 years ago. We here present evidence that interactions between two basement membrane components, nidogen (entactin) and laminin γ1 chain, could be important for epithelial-mesenchymal interactions in this gland. Nidogen mRNA was detected by in situ hybridization in the mesenchyme, and yet the protein was detected in epithelial and endothelial basement membranes. The role of nidogen-laminin interactions for epithelial morphogenesis was studied by applying antibodies to submandibular gland organ cultures. Antibodies reacting strongly with the nidogen-binding site of laminin γ1 chain drastically perturbed branching epithelial morphogenesis. Electron microscopy of the epithelial-mesenchymal interface showed that blocking antibodies disrupted the formation of the basement membrane. Epidermal growth factor was shown to increase the expression of nidogen in mesenchyme, and could counteract the effect of the blocking antibodies. We suggest that nidogen could be an important mesenchymal factor for submandibular gland development.

Key words: entactin, laminin, nidogen, epithelial-mesenchymal interaction, basement membrane, mouse, submandibular gland, epidermal growth factor

INTRODUCTION

In most solid tissues, the development of the epithelium requires the presence of mesenchymal cells. The biological importance of epithelial-mesenchymal interactions was recognized a long time ago (Grobstein, 1953) but the molecular basis of these interactions has remained poorly understood until recently. Multiple molecular processes operating simultaneously are apparently needed for proper epithelial branching. There is now good evidence that growth factors and their receptors play crucial roles in many of these interactions (Birchmeier and Birchmeier, 1993; Minoo and King, 1994; Thesleff et al., 1995; Hogan, 1996). In many cases, the growth factor requirement seems to be fairly tissue specific, as exemplified by the interactions of glial-derived neurotrophic factor and its tyrosine kinase receptor c-ret, which are crucial for the first epithelial-mesenchymal interactions of embryonic kidney and for innervation of gut (Schuhardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). Tissue-specific transcription factors for epithelial-mesenchymal interactions have also been described (Hatini et al., 1996).

Extracellular matrix components provide another important control system for epithelial-mesenchymal interactions. Basement membranes are located between mesenchyme and epithelium and form early during embryogenesis. A variety of organ culture approaches have suggested that the integrity of the basement membrane is important for epithelial morphogenesis (Bernfield et al., 1972, 1984; Adams and Watt, 1993; Gullberg and Ekblom, 1995). Many recent studies (Schuger et al., 1995; Streuli et al., 1995; DeArcangelis et al., 1996) support the proposal that laminin-1 is involved in epithelial morphogenesis (Klein et al., 1988; Ekblom, 1996). Laminin-1 is a large heterotrimeric glycoprotein of basement membranes. Because numerous laminin chains have been discovered, a new laminin nomenclature has recently been adopted. The first-described laminin, previously called EHS-laminin with the chains A, B1 and B2, was renamed to laminin-1 with the α1, β1 and γ1 chains (Burgeson et al., 1994). In the current study, we have focused on one aspect of epithelial development, the possible role of interactions between laminin γ1 chain and nidogen for branching morphogenesis.

Nidogen is one of the mesenchymal extracellular matrix glycoproteins suggested to be involved in epithelial-mesenchymal interactions. Other such proteins include epimorphin and tenascin-C. Antibodies against epimorphin and tenascin-C have been shown to perturb lung epithelial morphogenesis (Hirai et al., 1992; Young et al., 1994). Antibodies that block nidogen-laminin binding have been shown to perturb development of kidney and lung epithelium (Ekblom et al., 1994). By immunofluorescence tenascin-C can be seen focally in mesenchyme

close to embryonic epithelium (Chiquet-Ehrismann et al., 1986; Ekblom and Aufderheide, 1989), whereas epimorphin is found widely distributed in many embryonic mesenchymal compartments (Hirai et al., 1992). Nidogen, also called entactin (Durkin et al., 1988; Mann et al., 1989), is a basement membrane protein that binds with high affinity to laminin γ1 chain (Mayer et al., 1993a). The few crucial residues important for nidogen binding on a single epidermal growth factor-like module of the γ1 chain (III4) have been identified (Timpl and Brown, 1996) and the structure of the loops where these reside has been clarified in detail (Baumgartner et al., 1996; Stetefeld et al., 1996). These and many other studies suggest that nidogen forms a crucial link between basement membrane components (Timpl and Brown, 1996). It is thus intriguing that nidogen nevertheless seems to be produced by mesenchyme rather than by epithelium during embryogenesis. Mesenchymal nidogen expression is usually not evident by immunofluorescence, but becomes apparent by in situ hybridization of the mRNA (Thomas and Dziadek, 1993, 1994; Ekblom et al., 1994). Mesenchymal cells were also identified as the principal sources of nidogen by analyses of cultured primary cell lines from neonatal lung (Senior et al., 1996). Nidogen is easily proteolytically cleaved unless it is bound to laminin γ1 chain (Mayer et al., 1993b, 1995), which might explain the weak signal seen in mesenchyme by immunofluorescence.

The roles of extracellular matrix proteins such as nidogen, tenascin-C and epimorphin for morphogenesis are still not well known. Mice lacking tenascin-C develop normally (Saga et al., 1992; Forsberg et al., 1996), raising the possibility that tenascin-C is not vital for epithelial-mesenchymal interactions. We recently failed to block epithelial development in the embryonic submandibular gland with antibodies against epimorphin (Kadoya et al., 1995), although they affect lung epithelial morphogenesis (Hirai et al., 1992). More subtle effects of the antibodies could have gone unnoticed in our assays, but another possibility is that epimorphin has tissue-specific functions (Kadoya et al., 1995). Nidogen and tenascin-C may likewise have tissue-specific roles. Alternatively, some of these molecules may represent the 'mesenchyme common' factors postulated by Grobstein (1967). It was thus of interest to study whether nidogen binding to laminin γ1 chain is important in embryonic submandibular gland, a much-studied model system for branching epithelial morphogenesis.

As in many other embryonic tissues (Dziadek, 1995), nidogen mRNA was found to be produced by the mesenchyme and endothelium in the submandibular gland, and yet the protein was detected clearly only in basement membranes of epithelium and endothelium. The localization studies were compatible with the view that nidogen is a mesenchymal product required for epithelial morphogenesis in this gland. Antibody preparations with different affinities to the nidogen binding site of laminin γ1 chain (Ekblom et al., 1994) were therefore tested in organ cultures of embryonic salivary gland. It was found that antibodies which block laminin-nidogen binding drastically retard branching epithelial morphogenesis.

MATERIALS AND METHODS

Tissues, cells and organ culture

A fibroblast cell line PA6M (Kodama et al., 1982) was grown in 1% fetal calf serum in Dulbecco's modified minimum essential medium (DMEM; Gibco) with or without the addition of epidermal growth factor (EGF). Organ cultures were performed with submandibular gland with or without sublingual gland from day-13 mouse embryos (NMRI). Organs were placed on Nuclepore filters (pore size 0.05 μm) and cultured (Grobstein, 1953) in DMEM or improved minimum essential medium (IMEM-ZO) supplemented with 50 μg/ml transferrin (Collaborative Research) (Kadoya et al., 1995). In some experiments bovine serum albumin (Sigma) was supplemented at 1 mg/ml. For perturbation experiments, antibodies were added at 10-100 μg/ml to the medium at onset of culture. Appropriate antibody penetration into the cultured organs was confirmed by direct immunofluorescence. In some experiments epidermal growth factor (EGF; Boehringer-Mannheim) and transforming growth factor β1 (TGFβ1; either courtesy of Dr Miyazono, Ludwig Institute for Cancer Research, Uppsala, Sweden, or purchased from Boehringer-Mannheim) were added. After 2 or 3 days in culture, organs were photographed with an Olympus SZH stereomicroscope. The photographs were used to count the number of end buds and to evaluate the morphology of the individual epithelial branches. Duncan's test was used to evaluate the differences in number of branches statistically (Kirk, 1968). Explants were also collected for immunohistochemistry, electron microscopy or isolation of RNA.

Antibodies and peptides

Affinity-purified polyclonal antibodies N-283, N-285, and N-286 were prepared as described (Mayer et al., 1993a; Ekblom et al., 1994). Briefly, antibody N-283 was raised against recombinant fragment of the third to fifth EGF-like repeats (LE modules) in domain III (γ1 III3-5) of laminin γ1 chain. Antibody N-285 was raised against laminin-1 fragment P1 and first affinity-purified on γ1 III3-5. Subsequently antibody N-286 was obtained by purification of the unbound antibody fraction on fragment P1 (Ekblom et al., 1994). Rat monoclonal antibody 200 against E3 fragment of laminin-1 (Sorokin et al., 1992) was used in the organ cultures, as described previously (Kadoya et al., 1995). Rabbit polyclonal antiserum (Paulsson et al., 1986) and rat monoclonal antibody BE1/4 (Dziadek et al., 1988) were used to study nidogen expression. Control rabbit IgG was from Sigma (St Louis, MO).

Peptides that block nidogen-binding to laminin included the recombinant 27 kDa γ1 III3-5 fragment (formerly B2III3-5, Mayer et al., 1993a), and a synthetic 3 kDa peptide comprising loop a,c of the binding module on γ1 III4 (Pöschl et al., 1994).

Immunohistochemistry and transmission electron microscopy

Tissues were frozen in Tissue Tek (Miles, Naperville, IL). Frozen sections (7 μm) were fixed with methanol at –20°C for 5 minutes or with acetone for 2 minutes, treated with 10% goat serum in PBS for 20 minutes, and incubated with the primary antibodies. Monoclonal antibody BE 1/4 against mouse nidogen (Dziadek et al., 1988) was used at 1-20 μg/ml. Bound antibodies were visualized using goat anti-rat IgG conjugated with FITC (Sigma) or Cy3™ (Jackson) and goat anti-rabbit conjugated with TRITC (Jackson). Slides were examined under a Zeiss Axiophot microscope equipped with epifluorescence optics. For electron microscopy, cultures were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) and processed (Kadoya and Yamashina, 1991). Ultrathin sections were stained with both lead and uranyl acetate and examined under a Philips CM-10 electron microscope.

In situ hybridization

Nidogen mRNA was detected with a previously described 45-mer oligonucleotide probe: 5′-TCG CCC CCA GTG ATG CTA AAC CCA TTC TTG AAC CCA TCC TGC TCC-3′ (Ekblom et al., 1994), complementary to nucleotides 1505-1549 of mouse nidogen (Durkin et al., 1988). The oligonucleotide was labelled at the 3′ end with [α-

^{35}S]dATP (Amersham Sweden AB, Solna). In situ hybridization was performed as described (Ekblom et al., 1994) except that sections after hybridization were washed four times at 60°C in 1× SSC and were exposed for 2-3 weeks at +4°C.

Gel electrophoresis and immunoblotting

Tissues were sonicated in solubilizing buffer (10 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM PMSF, 0.1% Trasylol and 0.1% N-ethylmaleimide) on ice. After incubating for 1 hour on ice, the sonicated tissues were centrifuged at 9,000 ***g*** for 5 minutes. For polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), supernatants were resolved in SDS sample buffer with 100 mM DTT and separated using 5-15% gradient polyacrylamide gels (Bio-Rad). After electrophoresis, immunoblotting was carried out with anti-nidogen antiserum (Paulsson et al., 1986) diluted 1:100. Protein concentration was determined with the Bio-Rad Protein Assay Kit using bovine gamma globulin as a standard.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was purified from submandibular gland rudiments of day-13 embryos according to Chomczynski and Sacchi (1987). RT-PCR was carried out with GeneAmp RNA PCR Kit and thermocycler (Perkin Elmer, Norwalk, CO) as described (Kadoya et al., 1995). The RT reaction was carried out with 1 μg RNA using random hexamer as primers. PCR cycles were: 1 minute denaturation at 94°C, 2 minutes annealing at 55°C, and 2 minutes extension at 72°C. After 35 cycles, PCR products were analyzed on a 1.2% agarose gel. Control PCR reactions were carried out without reverse transcription. Primers used for laminin β1 chain were: sense primer 5′-ACCACTGTCGCCCTTGCCCTTGT-3′ (pos. 2912-2934) and antisense primer 5′-CACAGGCTCGGCATTCCACATCA-3′ (pos. 3552-3574) (Sasaki et al., 1987); for laminin γ1 chain: sense primer 5′-GAACAGCAGACCGCCGACCAA-3′ (pos. 4231-4251) and antisense primer 5′-AGCCATCCCCGCCATCATCAT-3′ (pos. 4696-4716) (Sasaki and Yamada, 1987); and for nidogen: sense primer 5′-TCCAGGGGCAGGCGTTCAATC-3′ (pos. 2438-2458) and antisense primer 5′-TCCAGGGGCAGGCTTCAATC-3′ (pos. 2864-2884) (Durkin et al., 1988). The specificity of PCR products was confirmed by restriction enzyme analysis.

Northern blotting

Total RNA was isolated (Chirgwin et al., 1979) from cells and cultured glands, fractionated by agarose gel electrophoresis, transferred to Zeta-Probe GT membranes (Bio-Rad) and hybridized to ^{32}P-oligolabelled cDNA clones in a 0.25 M sodium-phosphate solution, pH 7.2, containing 7% SDS at 65°C. Filters were subsequently washed several times in 0.02 M sodium phosphate solutions containing 5% and 1% SDS before autoradiography. cDNA clones used were: a 1.2 kb fibulin-2 probe MK 10 detecting mouse fibulin-2 (Pan et al., 1993), a 1.6 kb clone 6c detecting mouse nidogen (Mann et al., 1989), and a 1.1 kb human G3PDH clone (Clontech Laboratories, Palo Alto, CA). Quantitation of signal intensities was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values were normalized to G3PDH signal intensities of the same lanes.

RESULTS

Expression of nidogen and laminin chains in developing submandibular gland

By immunoblotting with anti-nidogen antibodies, the expected 150 kDa band was detected throughout pre- and post-natal developmental stages and in adult submandibular glands. The intensity of the band increased slightly by advancing development. In glands from 6-week-old mice we also detected a 100 kDa band, presumably a proteolytic cleavage product (Fig. 1). Previous immunoblotting experiments with antiserum ISH-1 have shown an abundant expression of either laminin β1 or γ1 chain in the embryonic day 13 (E13) submandibular gland (Kadoya et al., 1995). Since the ISH-1 antiserum does not distinguish between the different chains of laminin-1 (Klein et al., 1988) and nidogen binds only to laminin γ1 chain (Gerl et al., 1981), we used reverse transcriptase (RT)-PCR to verify the presence of the different chains. RT-PCR analysis of RNA from E13 submandibular glands yielded the expected bands representing mRNAs for laminin β1, and γ1 chains and nidogen (Fig. 2). The specificity of the bands was further demonstrated by the presence of proper restriction enzyme cutting sites (data not shown). The presence of some laminin α1 chain at this stage has been demonstrated previously (Kadoya et al., 1995).

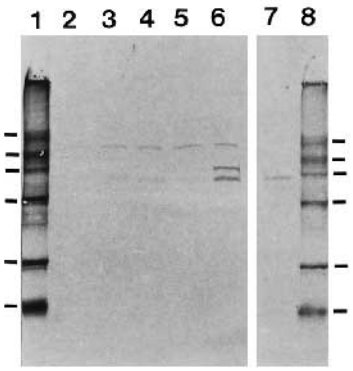

Fig. 1. Immunoblotting for nidogen in mouse submandibular gland. 40 μg of EDTA extract of submandibular gland from 14-day embryos (lane 2), 17-day embryos (lane 3) and newborn (lane 4), 1-week-old (lane 5) and 6-week-old mice (lanes 6 and 7) were separated on a 5% to 15% gradient gel by SDS-PAGE in the presence of a reducing agent. Immunoblotting was performed with anti-nidogen antiserum and second antibody (lanes 2-6) or with second antibody alone (lane 7). Locations of the molecular mass markers corresponding to 220, 132, 94.5, 79.5, 68 and 55 ($\times 10^{-3}$ M_r, top to bottom) are shown in lanes 1 and 8.

Detection of nidogen mRNA and protein by in situ hybridization and immunofluorescence

In frozen sections of E14 glands a strong signal was seen in the mesenchyme by in situ hybridization with the oligonucleotide specific for nidogen, whereas no signal over back-

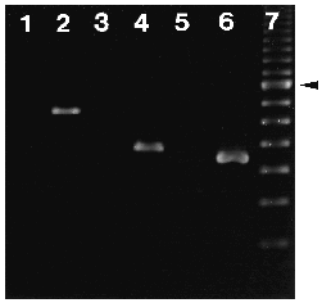

Fig. 2. Detection of laminin β1 and γ1 chains and nidogen mRNAs in embryonic submandibular gland by RT-PCR. PCR amplification reactions on samples from submandibular gland of 13-day-old mouse embryos were carried out with primers specific for laminin β1 chain (lanes 1,2), laminin γ1 chain (lanes 3,4) and nidogen (lanes 5,6) either without reverse transcriptase (lanes 1,3,5) or with reverse transcriptase (lanes 2,4,6). The expected fragments for laminin β1 chain (663 bp), laminin γ1 chain (486 bp) and nidogen (447 bp) were obtained only when reverse transcriptase was present. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide. Lane 7 shows molecular markers that differ by 100 bp and the 800 bp band is marked (arrow).

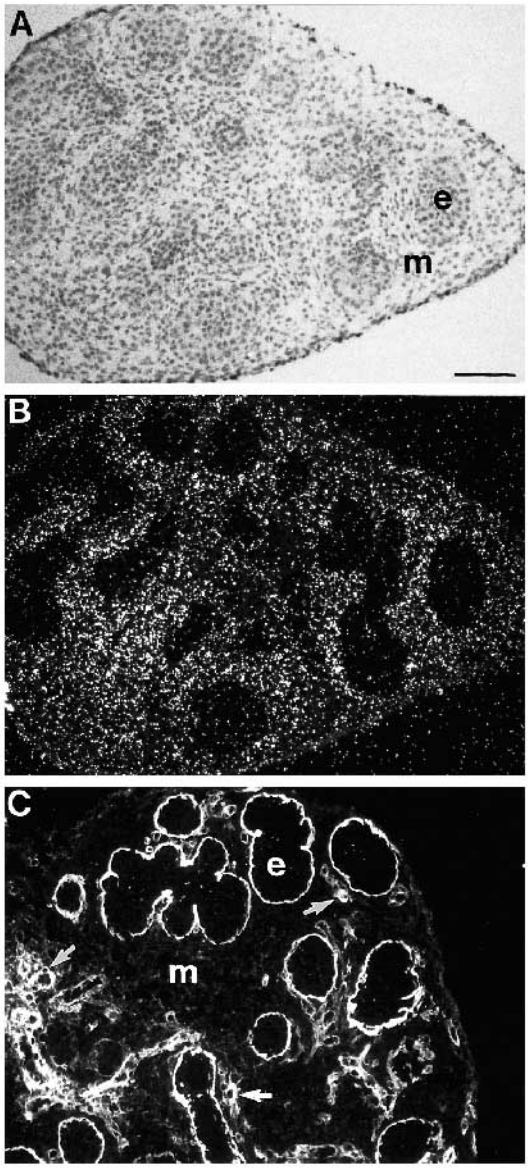

Fig. 3. Localization of nidogen mRNA and protein in embryonic submandibular gland. Frozen sections of submandibular gland of 14-day-old mouse embryos containing approximately an equal amount of epithelium (e) and mesenchyme (m) were probed with an antisense oligonucleotide specific for nidogen and micrographs were taken under bright-field (A) and dark-field (B) illumination. A similar section was stained with monoclonal antibody BE1/4 against nidogen. Bound antibodies were detected by indirect immunofluorescence (C). Some blood vessels in C are indicated with arrows. e, epithelium; m, mesenchyme. Bar, 100 μm.

ground was seen in the epithelium. The signal was equally strong in all parts of the mesenchyme (Fig. 3A,B). Some blood vessels are found within the mesenchyme but the signal was not stronger in blood vessels than in the mesenchyme. By immunohistochemistry only weak if any expression of nidogen was noted in the mesenchyme of E14 glands, and instead strong expression of nidogen was detected in basement membranes of the epithelia, and the blood vessels (Fig. 3C).

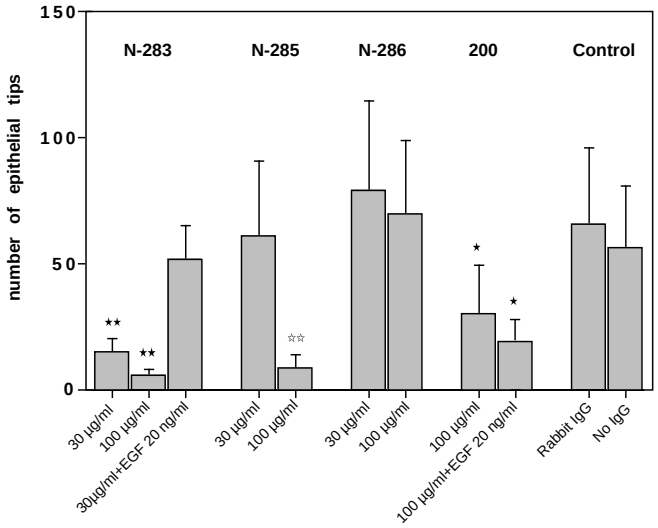

Fig. 4. Number of end lobules in organ cultures of embryonic submandibular glands treated with antibodies against different domains of laminin-1. Glands from 13-day-old embryonic mice were cultured for 2 days in defined medium in the absence or presence of antibodies against laminin fragments, which either block (N-283, N-285) or do not block (N-286) laminin-nidogen interaction, or against laminin fragment E3 (200). Some of the assays contained in addition epidermal growth factor (EGF). End buds were counted from photographs taken by stereomicroscopy on day 2 of culture and results were evaluated statistically using Duncan's test. Each bar represents the mean ± s.e.m. of at least 7 explants and the total number of explants analyzed was 109. *Significantly different from controls but not from each other (Duncan's test; $P<0.01$). **Significantly different from controls, 30 μg/ml of N-283 supplemented with EGF (20 ng/ml) and each other (Duncan's test; $P<0.001$). ☆☆Significantly different from controls and 30 μg/ml N-285 (Duncan's test; $P<0.001$).

Perturbation of epithelial morphogenesis with antibodies against different domains of laminin-1

The number of end buds was counted in explants cultured for 2 days in the presence or absence of antibodies (Fig. 4) or synthetic laminin peptides. In addition, the morphology of the end buds was evaluated (Fig. 5). In control cultures grown in the presence of purified rabbit IgG or without IgG, the number of end buds was around 60 (Figs 4, 5A). Antibody preparation N-283 has a high affinity for the nidogen-binding site, the affinity of antibody preparation N-285 is approximately threefold lower, whereas N-286 binds poorly if at all to the nidogen binding site but binds well to laminin fragment P1 (Ekblom et al., 1994). In explants treated with 30 μg/ml of antibody N-283 the number of end buds was reduced by 75% (Figs 4, 5D), whereas no such effect was seen with this concentration of N-285 (Figs 4, 5B) or N-286 (Fig. 4). At 100 μg/ml, N-285 also severely reduced the number of end buds (Figs 4, 5C), but at this concentration of N-286 could still not reduce the number of end buds (Figs 4, 5F). The minimum effective dose of the antibody preparations required for reduction of number of end buds was thus inversely proportional to the capacity to bind to recombinant fragment γ1III3-5 (Ekblom et al., 1994). Similar results were obtained when antibody perturbation experiments were carried out in medium supplemented with 10% fetal calf serum, but higher concen-

trations of the perturbing antibodies had to be used (data not shown).

Epidermal growth factor (EGF) has been shown to promote epithelial branching of mouse submandibular rudiments (Nogawa and Takahashi, 1991) and growth of embryonic mesenchyme in tooth and kidney (Partanen et al., 1985; Weller et al., 1991). We therefore tested if exogenous EGF could affect development in explants treated with antibodies against different domains of laminin-1. Addition of EGF alone at 20 ng/ml did not affect the number of epithelial branches during submandibular gland morphogenesis. EGF (20 ng/ml), but not TGFβ1, to a large extent rescued the explants from the inhibition of epithelial branching caused by N-283 (Figs 4, 5F). The same concentration of EGF failed to rescue the inhibition of epithelial branching caused by monoclonal antibody 200 (Kadoya et al., 1995), which reacts with fragment E3 of laminin α1 chain (Figs 4, 5H).

A recombinant laminin peptide γ1 III3-5 was previously shown to bind nidogen with the same affinity as laminin-1 (Mayer et al., 1993a). This peptide was tested in organ culture over a wide concentration range (0.5-50 μM). At least five explants were anlayzed for each concentration. No statistically significant differences in the number of epithelial tips were seen between controls and treated explants at any tested concentration (Duncan's test). A small synthetic peptide loop a,c from the binding region (Pöschl et al., 1994) was also tested (50 μM and 100 μM, at least eight explants each), but according to a statistical analysis (Duncan's test) the number of epithelial tips did not differ significantly from control cultures.

Morphology of end lobules

End lobules were small and rounded in control cultures (Fig. 5A). In the explants treated with concentrations of N-283 or N-285 antibodies, which blocked branching morphogenesis, the poorly branched epithelial structures were thin and rod-like and completely lacked rounded end lobules (Fig. 5B,D). The end lobules of explants treated with N-283 together with EGF were indistinguishable (Fig. 5E) from control lobules. The epithelium in the explants treated with N-286 (100 μg/ml) branched equally as well as control explants, but some morphological abnormalities were noted; an apparent fusion between neighbouring lobules could be noted in many of the explants (Fig. 5F). In explants treated with antibody 200 against laminin α1 chain fragment E3, the epithelial tips became enlarged both in the absence (Fig. 5G) and presence of EGF (Fig. 5H), in clear contrast to the morphology of the tips in either control cultures or in explants treated with antibodies N-283 or N-286. The formed end lobules in explants treated with recombinant fragment γ1 III3-5 were larger than those seen in explants treated with antibody N-283 (Fig. 5I).

Integrity of the basement membrane in organ cultures of submandibular gland treated with antibody N-283

By transmission electron microscopy the epithelial basal surface had a smooth contour and appeared normal in control cultures (Fig. 6A), but an irregular contour of the basal surface of the epithelium was evident already at low magnification in explants treated with N-283 (Fig. 6B). Larger magnifications of the basal surface of epithelial cells at the tip of the epithelial branches revealed a normal basement membrane in control cultures (Fig. 6C) but a complete absence of the basement membrane in explants treated with N-283 (Fig. 6D).

Nidogen expression in cultured explants and its modulation by epidermal growth factor

In explants cultured in the presence of control IgG, nidogen was detected by immunofluorescence throughout the basement membrane of the epithelium (Fig. 7A), but in explants cultured in the presence of antibody N-283 at 30 μg/ml, nidogen was seen only in a few locations in the interface between mesenchyme and epithelium (Fig. 7B). In sharp contrast to these expression patterns, a large number of immunoreactive spots was seen throughout the mesenchyme and in the epithelial basement membrane in explants treated with a combination of N-283 and EGF (Fig. 7C). We therefore tested whether EGF can increase the expression of nidogen in a fibroblast cell line

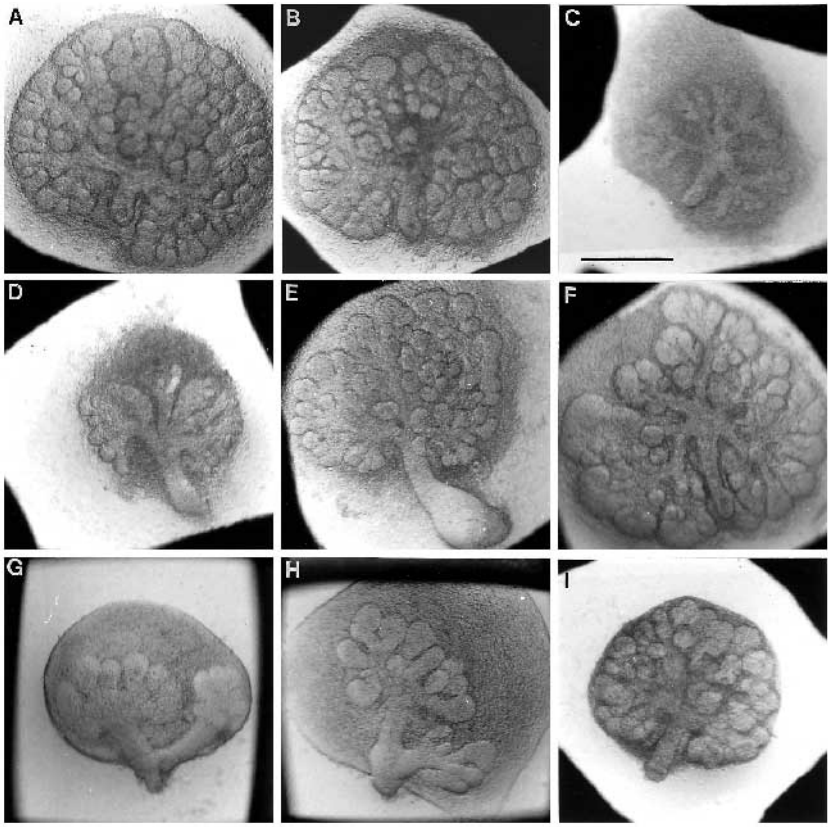

Fig. 5. Branching epithelial morphogenesis in embryonic submandibular glands, cultured in the presence or absence of various laminin peptides and antibodies against fragments of laminin-1. All reagents were added at onset of culture and photographs were taken by stereomicroscopy on day 2 of culture. (A) Control culture with rabbit IgG (100 μg/ml). (B) Antibody N-285 at 30 μg/ml. (C) Antibody N-285 at 100 μg/ml. (D) Antibody N-283 at 30 μg/ml. (E) Antibody N-283 at 30 μg/ml together with EGF at 20 ng/ml. (F) Antibody N-286 at 100 μg/ml. (G) Antibody 200 at 100 μg/ml. (H) Antibody 200 at 100 μg/ml together with EGF at 20 ng/ml. (I) Recombinant laminin fragment γ1 III3-5. Bar, 0.5 mm.

and in cultured submandibular gland. Expression was monitored by northern blotting (Fig. 8). As judged by Phosphorimage quantitation of signal intensities, EGF at 1 ng/ml already increased the expression of nidogen mRNA more than twofold in fibroblasts, and no further increase was seen at 10 ng/ml. In contrast, the same concentrations of EGF did not alter the expression of the mRNA for another mesenchymal extracellular matrix protein, fibulin-2 (Fig. 8A). In submandibular glands cultured for 2 days, EGF was tested at the concentrations used to perturb the effect of antibody N-283. At 20 ng/ml EGF increased the expression of nidogen mRNA approximately 2.3-fold and at 40 ng/ml the increase was 2.5-fold (Fig. 8B).

DISCUSSION

The importance of mesenchyme for epithelial branching morphogenesis was first demonstrated for the submandibular gland (Borghese, 1950; Grobstein, 1953). Subsequently, it became evident that epithelial-mesenchymal interactions are major driving forces for development of many organs (Grobstein, 1967; Saxén and Kohonen, 1969). Although several aspects of epithelial morphogenesis and basement membrane formation have been clarified for the submandibular gland (Bernfield et al., 1984; Kadoya and Yamashina, 1989, 1991; Takahashi and Nogawa, 1991), few if any mesenchymal molecules required for epithelial morphogenesis have been defined in this classical model system. Our current studies raise the possibility that nidogen could be such a factor.

Nidogen mRNA was detected exclusively by the mesenchymal compartments of the embryonic submandibular gland. The protein was nevertheless found by immunocytochemistry in basement membranes of the branching epithelium but not in mesenchyme. The weak expression in the mesenchyme, despite synthesis by mesenchyme, could be explained by the extreme sensitivity of nidogen to proteases, and the presence of nidogen in epithelial basement membrane by the decreased susceptibility to proteolysis when bound to laminin γ1 chain (Mayer et al., 1993b, 1995). The antibody perturbation experiments suggest that nidogen-laminin binding is involved in branching epithelial morphogenesis of submandibular gland. The three antibody preparations used showed a differential affinity for the nidogen-binding region on laminin γ1 chain and to the larger fragment P1 (Ekblom et al., 1994). We emphasize that antibodies N-286 and N-285 were

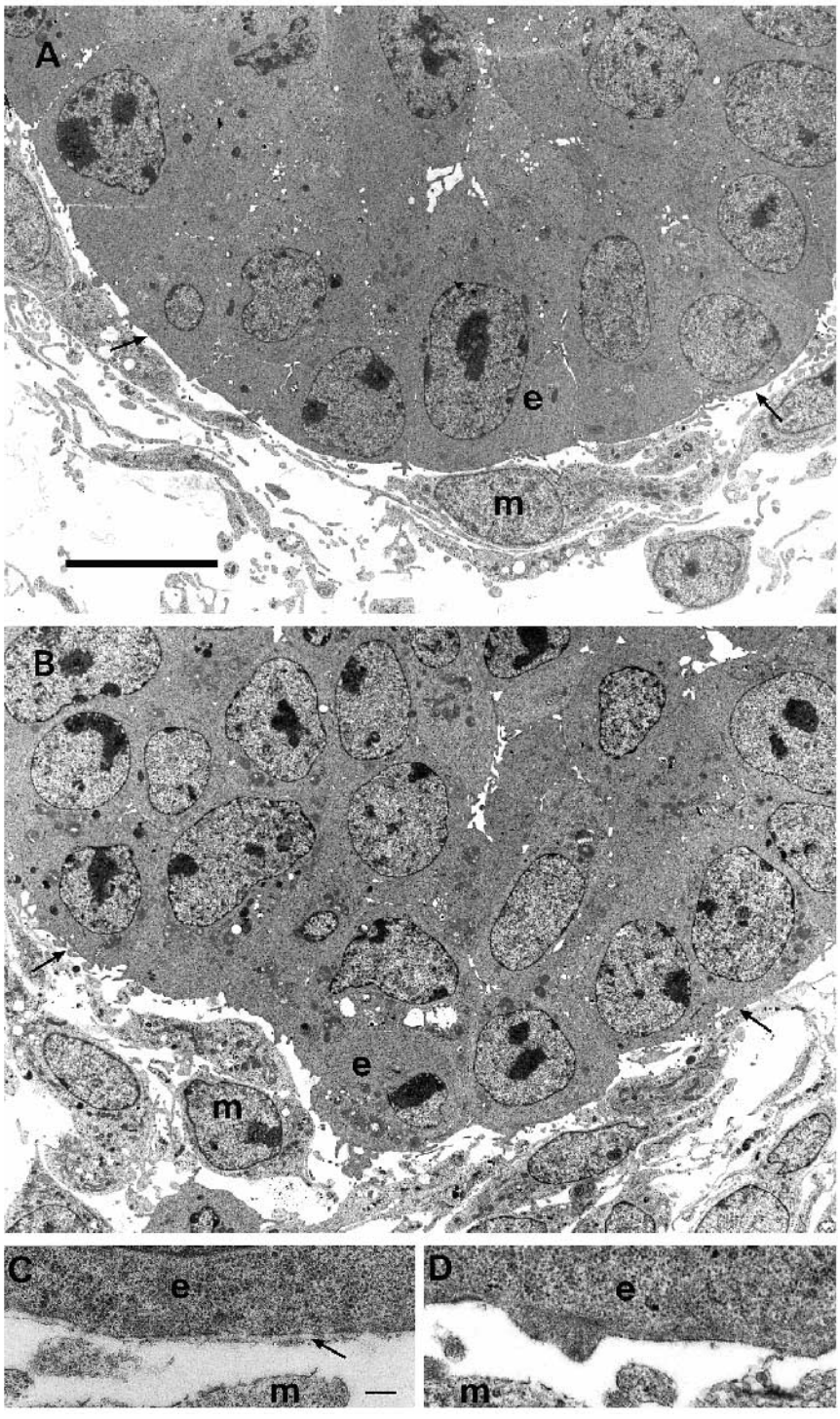

Fig. 6. Integrity of the basal surface of epithelial cells in organ culture. Electron micrographs were taken of the tip of the branching epithelium of embryonic day 13 submandibular glands that had been cultured for 2 days either in the presence of a control IgG (A,C), or antibody N-283 at 30 μg/ml (B,D). The micrographs reveal a flat basal surface in control cultures (A) and larger magnifications reveal a well-developed basement membrane (C). In explants treated with antibody N-283, the basal surface is highly irregular (B) and larger magnifications reveal an absence of a basement membrane along the basal surface (D). e, epithelium; m, mesenchyme; arrows denote the epithelial-mesenchymal interface. Bar, 10 μm (A), 0.2 μm (C).

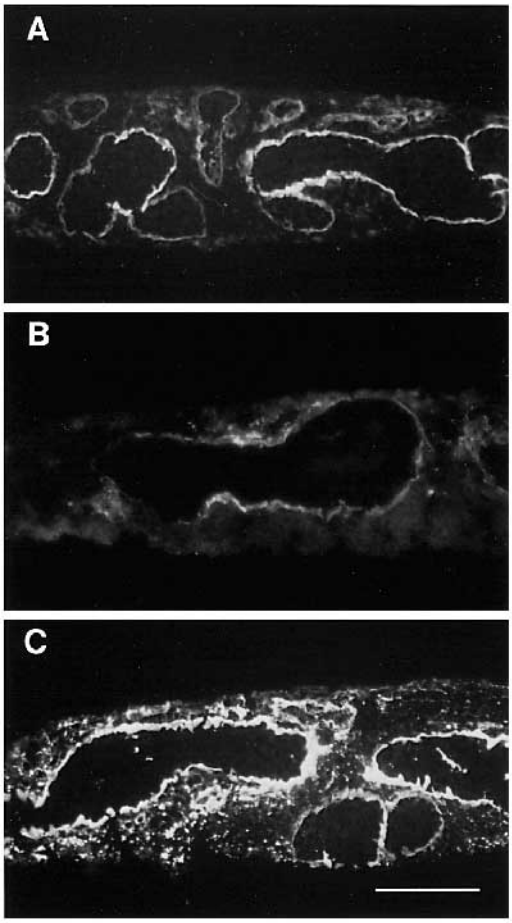

Fig. 7. Expression of nidogen in organ cultures of embryonic submandibular gland. Frozen sections of explants cultured for 2 days in vitro in the presence of control IgG (A), antibody N-283 at 30 μg/ml (B) or antibody N-283 at 30 μg/ml together with EGF at 20 ng/ml (C), were stained with monoclonal antibody BE1/4 against nidogen. Bound antibodies were detected with fluorescein-conjugated second antibody. Bars, 100 μm.

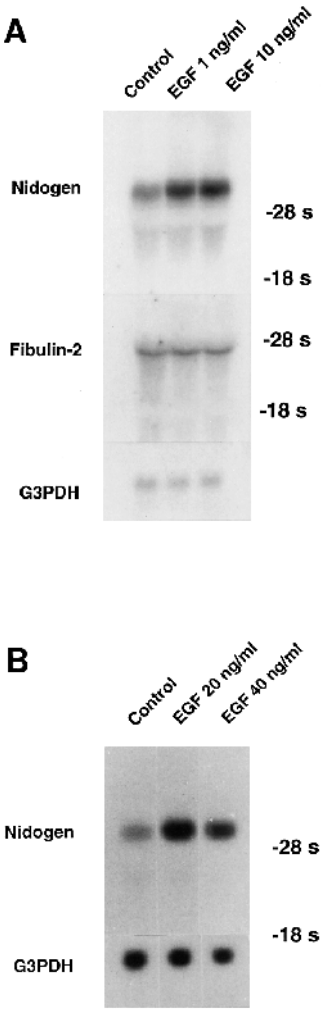

Fig. 8. Stimulation of nidogen mRNA expression by epidermal growth factor (EGF). Total cellular RNA was loaded onto each lane, run on a 1% agarose gel, transferred to a nylon filter and hybridized to 32P-labelled cDNA clones for mouse nidogen, fibulin-2 or human G3PDH. The positions of 18S and 28S rRNA are indicated. (A) Northern blot analysis of total RNA (15 μg/lane) from PA6M cells grown for 2 days in 1% fetal bovine serum only (control), or in 1% fetal bovine serum supplemented either with 1 ng/ml EGF or 10 ng/ml of EGF. (B) Northern blots of total RNA (10 μg/lane) from submandibular glands of 13-day-old mouse embryos cultured for 2 days in the presence or absence of EGF (20 ng/ml and 40 ng/ml).

derived from the same antiserum by affinity purification. The antibody preparations were therefore excellent controls of each other. The antibody preparation N-283, with the strongest affinity for the nidogen binding region, dramatically perturbed branching epithelial morphogenesis even at a low concentration, whereas the same concentration of N-285 was without such an effect. The affinity of antibody N-285 to the nidogen-binding site is approximately three-fold lower than N-283 (Ekblom et al., 1994). It was therefore significant that N-285 was a good inhibitor of branching epithelial morphogenesis when used at a three-fold higher concentration than N-283. Moreover, N-286, which showed the strongest affinity for laminin-1 fragment P1 but only weakly if at all bound to the nidogen-binding site, did not seem to reduce the number of epithelial branches, even at a higher concentration. Taken together, the antibody perturbation and the expression studies provide strong evidence that nidogen binding to laminin γ1 chain is critical for branching epithelial morphogenesis.

Binding studies with isolated proteins or their fragments provided the first clear evidence that nidogen could act as a crucial link between laminins and other basement membrane components (Fox et al., 1991; Gerl et al., 1991; Mayer et al., 1993a; Timpl, 1996). It therefore became important to establish that binding of mesenchymal nidogen to laminin γ1 chain is required for basement membrane biogenesis in intact tissues. Our organ culture studies provided such evidence. The antibody preparation N-283, which blocked epithelial morphogenesis, was shown by electron microscopy to disrupt the basement membrane.

It is notable that a monoclonal antibody against E3 fragment of laminin α1 chain also seemed to act by disrupting basement membrane assembly in the same model system (Kadoya et al., 1995). However, epithelial morphology of the explants treated with the different antibodies was distinct. In the explants treated with antisera against the nidogen-binding site, the epithelium remained thin and in the branches that formed we could not detect any signs of formation of larger end lobules. This was in contrast to the explants treated with antibodies against E3 fragment, where the tips of the few formed epithelial branches became abnormally enlarged, in an apparent attempt of cells to grow despite the inability to form branches. We therefore suggest that the nidogen-binding site of laminin γ1 chain and E3 fragment of laminin α1 chain influence epithelial morphogenesis in distinct fashions. As has been suggested for other tissues, E3 fragment might act by binding to cell surface dystroglycan (Durbeej et al., 1995).

Another antibody, N-286, which binds to laminin-1 fragment P1 but not to the nidogen binding site, could not reduce the number of epithelial branches. However, a clear abnormality, fusion between the epithelial end lobules, could be noted in the explants treated with antibody N-286. It was not unexpected that antibody N-286 had some effect. In addition to nidogen binding, domain P1 contains sites that may promote cell growth (Panayotou et al., 1989). A cryptic cell-binding RGD sequence has also been demonstrated in mouse laminin α1 chain on P1 fragment (Aumailley et al., 1990). Finally, Schuger et al. (1991) have shown that certain monoclonal antibodies against P1 can perturb branching epithelial morphogenesis of lung. We conclude that the N-283 binding sites, which harbour the nidogen-binding site, could be of major importance for early stages of epithelial morphogenesis, and that other domains in the P1 fragment may be involved in fine-tuning of the branching process at later developmental stages.

In some experiments initially designed to study the role of growth factors for morphogenesis, some explants had been treated both with epidermal growth factor (EGF) and antibody N-283. Unexpectedly we noted that EGF drastically reduced the effect of antibody N-283. This could be confirmed in a larger series of experiments. In contrast, EGF could not reduce the inhibition of branching morphogenesis caused by antibody 200 against laminin α1 chain. Since growth factors may act by stimulating extracellular matrix synthesis, we investigated whether EGF can stimulate nidogen synthesis in the submandibular gland. Northern blotting showed that EGF increased nidogen mRNA levels more than twofold, both in the gland and in a fibroblast cell line. In explants treated by EGF and the antibody N-283, strong expression of nidogen protein could be seen in the mesenchyme, in sharp contrast to the weak expression in control explants or explants treated only with antibody. Taken together, these results suggest that EGF could have counteracted the effect of the function-blocking antibodies by increasing the synthesis of nidogen. These experiments serve as controls demonstrating that effects of N-283 are unlikely to be the result of toxic effects, but they do not necessarily imply a physiological role for EGF in branching morphogenesis. It remains to be seen whether EGF also increases expression of laminin chains or modulates the levels of tissue proteases or their inhibitors. Lower levels of active proteases known to cleave nidogen (Mayer et al., 1993b, 1995) could reduce the depletion of mesenchymal nidogen. Such potential effects of EGF remain to be analyzed in the future.

Epithelial-mesenchymal interactions are major driving forces for morphogenesis of most solid tissues. Our previous (Ekblom et al., 1994) and current antibody perturbation studies provide important new insights about the molecular basis of these interactions. Whereas antibodies against epimorphin apparently block epithelial development of only some organs (Hirai et al., 1992) and have no apparent effect on morphogenesis of 13-day-old embryonic submandibular gland in 2-day assays (Kadoya et al., 1995), antibodies against the nidogen binding site of laminin γ1 chain have so far blocked branching epithelial morphogenesis in all three tissues studied. Epithelial-mesenchymal interactions require both tissue-specific and common mesenchymal factors (Grobstein, 1953, 1967). Our data suggest that nidogen is an important mesenchymal factor for at least three epithelial-mesenchymal interactions. Other extracellular matrix components, and different types of growth factors (Birchmeier and Birchmeier, 1993; Hogan, 1996) could be involved in tissue- or stage-specific epithelial-mesenchymal interactions.

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