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# Dystroglycan binding to laminin α1LG4 module influences epithelial morphogenesis of salivary gland and lung *in vitro*

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**Abstract** Dystroglycan is a receptor for the basement membrane components laminin-1, -2, perlecan, and agrin. Genetic studies have revealed a role for dystroglycan in basement membrane formation of the early embryo. Dystroglycan binding to the E3 fragment of laminin-1 is involved in kidney epithelial cell development, as revealed by antibody perturbation experiments. E3 is the most distal part of the carboxyterminus of laminin α1 chain, and is composed of two laminin globular (LG) domains (LG4 and LG5). Dystroglycan-E3 interactions are mediated solely by discrete domains within LG4. Here we examined the role of this interaction for the development of mouse embryonic salivary gland and lung. Dystroglycan mRNA was expressed in epithelium of developing salivary gland and lung. Immunofluorescence demonstrated dystroglycan on the basal side of epithelial cells in these tissues. Antibodies against dystroglycan that block binding of α-dystroglycan to laminin-1 perturbed epithelial branching morphogenesis in salivary gland and lung organ cultures. Inhibition of branching morphogenesis was also seen in cultures treated with polyclonal anti-E3 antibodies. One monoclonal antibody (mAb 200) against LG4 blocked interactions between α-dystroglycan and recombinant laminin α1LG4–5, and also inhibited salivary gland and lung branching morphogenesis. Three other mAbs, also specific for the α1 carboxyterminus and known not to block branching morphogenesis, failed to block binding of α-dystroglycan to recombinant laminin α1LG4–5. These findings clarify why mAbs against the carboxyterminus of laminin α1 differ in their capacity to block epithelial morphogenesis and suggest that dystroglycan binding to α1LG4 is important for epithelial morphogenesis of several organs.

**Key words** laminin · dystroglycan · organ culture · lung morphogenesis · salivary gland morphogenesis

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## Introduction

Epithelial branching morphogenesis stimulated by the adjacent mesenchyme is crucial for development of many organs. Growth factors and basement membrane components are key regulators of epithelial-mesenchymal interactions (Ashkenas et al., 1996; Cunha et al., 2000), but the precise mode of action of many individual ligand-receptor interactions is not well known. Laminins, trimeric proteins composed of α, β, and γ chains, are major biologically active components of basement membranes. Laminins are cross-shaped molecules with three short and one long arm. The amino-terminal short arms of all three chains mediate laminin polymerization (Colognato and Yurchenco, 2000). A distinct feature of the C-terminus of the α-chains is the tandem of five laminin globular (LG) modules forming the distal end of the long arm (Timpl et al., 2000). Of the 5 known α chains, α1 is expressed in embryonic epithelia but is absent from most other embryonic basement membranes

(Ekblom et al., 1998), suggesting that its main function is to promote epithelial morphogenesis. The α1LG1–3 modules contained in the proteolytic E8 fragment participate in binding to several integrins (Colognato and Yurchenco, 2000), whereas fragment E3 (composed of α1LG4–5) binds with high affinity to another cell surface receptor, dystroglycan (Gee et al., 1993; Brancaccio et al., 1995).

Studies with the embryonic kidney led to the hypothesis that α1E3 binding to dystroglycan could be involved in onset of epithelial morphogenesis (Klein et al., 1988; Durbeej et al., 1995). Genetic studies in mice suggest that dystroglycan function is required for the organization and assembly of extracellular matrix components into a basement membrane structure (Williamson et al., 1997; Henry and Campbell, 1998). These data indicate that dystroglycan plays a critical role in tissue morphogenesis. However, dystroglycan-deficient embryos fail to progress beyond the early egg cylinder stage, thereby restricting functional analysis to the period before gastrulation (Williamson et al., 1997).

Laminin-1 and its α1 chain have been suggested to play a role for development of many epithelia, but data on dystroglycan are available only for a few tissues (Durbeej et al., 1995; Jiang et al., 2001). For the salivary gland and mammary epithelium, it was shown that antibodies against the E3 fragment block epithelial development (Streuli et al., 1995; Kadoya et al., 1995), but the receptors involved were not identified. Work on lung development has so far focused on other domains of laminin-1 than E3 (Dunsmore and Rannels, 1996). Schuger et al. (1991; 1995) showed that monoclonal antibody (mAb) AL-5 against globular regions of β1 or γ1 chain and mAb AL-1 against α1 chain domains within the central cross region perturbed epithelial branching morphogenesis in the lung, whereas mAbs against other central parts (AL-2, AL-3) or the carboxyterminal end of α1 chain (AL-4) failed to do so. AL-4 was subsequently found to cause subtle morphological abnormalities of developing peribronchial smooth muscle cells, but its inability to affect epithelial development was confirmed (Schuger et al., 1997). Further observations with the AL-antibodies and recombinant proteins suggest that globular regions of β1 chain are necessary for laminin polymerization, which in turn promotes epithelial cell polarization (Schuger et al., 1991; 1995; 1997; 1998). Similar antibody perturbation experiments suggest that the nidogen binding site of laminin γ1 chain could be required for lung branching morphogenesis (Ekblom et al., 1994). The studies with β1 and γ1 chains suggest that polymerization of laminins into networks is required for lung branching morphogenesis but do not discriminate between the roles of laminin isoforms, as these chains are shared by several laminins (laminin-1, -2, -10) present in developing lung (Klein et al., 1990; Miner et al., 1997; Sorokin et al., 1997; Flores-Delgado et al., 1998; Mollard and Dziadek, 1998).

In order to act on the developing lung epithelial cells, laminin polymers should bind cell surface receptors. For laminin-1, two domains have so far been identified as binding sites for embryonic lung epithelial cells: α1 chain domains in the central cross-section (Schuger et al., 1995) for which no receptors are known so far, and integrin binding α1LG1–3 modules of the E8 fragment (Matter and Laurie, 1994; Canigga et al., 1996). In mice carrying null mutations of both the integrin α3 and α6 genes, early lung development was clearly retarded, whereas many of the early stages of epithelial development were scored as nearly normal (DeArcangelis et al., 1999). This emphasized the role of α3 and α6 integrins for lung morphogenesis and suggested that other receptors such as dystroglycan and integrin α2β1 are sufficient for kidney morphogenesis to occur (DeArcangelis et al., 1999). Yet, the results do not rule out the possibility that dystroglycan could participate in epithelial morphogenesis of lung. We therefore studied the role of dystroglycan binding to E3 (α1LG4–5) for both lung and salivary gland development *in vitro*.

A major dystroglycan binding site was recently mapped to some domains within the α1LG4 module (Andac et al., 1999; Talts et al., 1999). Nine mAbs against the α1LG4–5 domains have previously been characterized by Sorokin et al. (1992), but only one of these (mAb 200) clearly retarded kidney tubulogenesis *in vitro*. It has remained unclear why only mAb 200 was function-blocking in these assays and why other mAbs also reacting with the carboxyterminus of laminin α1 have failed to perturb epithelial branching morphogenesis (Schuger et al., 1995) or affect lung epithelial cells (Chen et al., 1997). To understand how laminin-1 acts through discrete receptors, it should be clarified whether these mAbs differ in their capacity to block discrete receptor-laminin α1 interactions. For this reason, we determined whether the epithelial branching-blocking mAb 200 and three non-blocking mAbs (188, 199, AL-4) differ in their capacity to perturb binding of purified dystroglycan to recombinant α1LG4–5. We demonstrate that mAb 200 completely blocks binding of α1LG4–5 to dystroglycan in solid-phase assays, whereas 188, 199, and AL-4 showed no such activity. We also show that dystroglycan is expressed during early stages of developing lung and salivary gland. Moreover, mAb 200 and anti-dystroglycan mAb IIH6, which blocks laminin-1 dystroglycan interactions, retard salivary gland and lung branching epithelial morphogenesis *in vitro*.

## Methods

### Tissues and organ cultures

NMRI hybrid mouse embryos were used. Eleven-day-old embryonic lungs and thirteen-day-old embryonic salivary glands were isolated for whole organ cultures and cultured in I-MEM, supplemented with heat-inactivated 10% fetal calf serum and 1% L-gluta-

mine, in the presence or absence of antibodies as described (Ekblom et al., 1994; Kadoya et al., 1995). Salivary gland cultures were also carried out in serum-free conditions, supplemented with 50 μg/ml transferrin and 1% L-glutamine (Kadoya et al., 1995). After two days in culture, explants were photographed with an Olympus SZH stereomicroscope. Photographs were used to count the number of end buds and to evaluate the morphology of the explants. An unpaired t-test for independent means was used to statistically evaluate the differences in numbers of branches (95% confidence level).

### Sources of antibodies and proteins

Monoclonal antibodies (mAbs) IIH6 and XIXC2 (both IgM isotypes) have previously been described (Ervasti and Campbell, 1991; 1993) and used in various *in vitro* studies (Durbeej et al., 1995; Brown et al., 1999; Colognato et al., 1999; García-Castro et al., 2000). mAbs 188, 199 and 200 against mouse laminin E3 fragment and MTn15 against mouse tenascin-C (all rat IgG isotypes) have been described previously and used in organ cultures (Sorokin et al., 1992; Talts et al., 1997). mAb AL-4 (rat IgG) against the carboxyterminal end of laminin α1 chain (Skubitz et al., 1988; Schuger et al., 1995) was from a commercial source (Chemicon International Inc., Temecula, CA, USA). Purified rat IgG was purchased from Sigma Immuno-chemicals. Polyclonal rabbit anti-E3 antibodies were raised against pure E3 fragment, and IgG fractions were purified by affinity chromatography on a laminin column (Streuli et al., 1995). Purified recombinant laminin α1LG4–5 was produced as described (Talts et al., 1999). Chicken kidney α-dystroglycan was a kind gift from Dr. A. Brancaccio (Rome, Italy).

### Northern blot analysis

Tissues were frozen in liquid nitrogen, and total RNA was isolated. 10 μg total RNA was denatured with glyoxal and electrophoresed on a 1% agarose gel. After transfer to Zeta-Probe GT membrane (Bio-Rad), RNA was fixed by UV cross-linking with a Stratalinker (Stratagene) under conditions recommended by the manufacturer. Membranes were pre-hybridized for 30 minutes at 65°C in solution containing 0.25 M $Na_2HPO_4$ and 7% sodium dodecyl sulphate (SDS). Hybridization was performed in the same solution for 18–24 h at 65°C with the following cDNA probes: a 1.5 kb EcoRI fragment of the cDNA clone R43-B, corresponding to rabbit dystroglycan (Ibraghimov-Beskrovnaya et al., 1992) and a 1.1 kb human G3PDH cDNA probe (Clontech) as control. cDNA inserts were labeled with $^{32}P$ to a specific activity about $2 \times 10^8$cpm/μg DNA by oligolabelling kit (Pharmacia). After hybridization, filters were washed $2 \times 1$ h in 20 mM $Na_2HPO_4$, 5% SDS at 65°C and $2 \times 1$ h in 20 mM $Na_2HPO_4$, 1% SDS at the same temperature and exposed to Hyperfilm MP film at −70°C in the presence of intensifying screens. Quantification of data was performed using a Lumi Imager F1 and the software Lumi Analyst 3.x (Boehringer/Roche).

### *In situ* hybridization

To detect dystroglycan mRNA by *in situ* hybridization, we used a synthetic 45-mer oligonucleotide probe 5′-CAC TCG AAA TGA GCG CCC GAC AAC AGC CGT ACC GTC TGG AAT GCC-3′, complementary to nucleotides 1523–1568 of mouse dystroglycan mRNA (Brancaccio et al., 1995) as described (Durbeej et al., 1995). Control sections hybridized with the same amount of labeled probe plus unlabelled probe in excess did not show any specific binding.

### Immunofluorescence

Tissues were frozen in Tissue Tek. Unfixed cryostat sections (7 μm) were washed in PBS and blocked in 3% BSA in PBS for 20 minutes. Since IIH6 did not react well in immunofluorescence, sections were incubated with affinity-purified sheep polyclonal antibodies specific for fusion protein B (anti FP-B) which recognize both α- and β-dystroglycan (Ibraghimov-Beskrovnaya et al., 1992) and affinity-purified sheep polyclonal antibodies specific for β-dystroglycan, sheep 43-DAG (Ohlendieck and Campbell, 1991). Anti FP-B and sheep 43-DAG were diluted 1:5. Bound antibodies were visualized using donkey anti-sheep IgG conjugated with FITC. Slides were examined under a Zeiss Axioplan microscope equipped with epi-fluorescence optics. A similar staining was obtained with the two antibodies but only figures for anti FP-B staining are shown.

### Immunoblot analysis

Glycoprotein preparations from salivary glands and lungs were obtained according to Durbeej et al., (2000). Glycoproteins were resolved on 3–12% gradient gels and transferred to nitrocellulose membranes. Immunoblot staining was performed as previously described (Ohlendieck and Campbell, 1991) except that the blots were developed using enhanced chemiluminescence (SuperSignal, Pierce Chemical Co.).

### Solid-phase inhibition assay

Solid-phase assays were carried out with α-dystroglycan (5 μg/ml) coated onto the plastic surface of microtiter wells at 4°C. All further incubations were at room temperature. Wells were blocked for 2 h with 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$ (wash buffer) with 1% bovine serum albumin (BSA; Sigma). Laminin α1LG4–5 (20 μg/ml) was mixed with serial dilutions of antibodies (mAbs 188, 199, 200, and AL-4) and incubated for 2 h. Wells were incubated with mAb pre-incubated laminin α1LG4–5 for 1 h. After washing, bound ligand was detected with a specific rabbit antisera, diluted to give an absorbance of 1.5–2.0 at 490 nm in regular ELISA. After a further wash, the bound antibodies were detected by addition of horseradish-peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc) followed by addition of 1 mg/ml 5-amino-2-hydroxybenzoic acid (Sigma), 0.001% $H_2O_2$.

## Results

### Northern blot analysis of dystroglycan mRNA in developing lung

The dystroglycan complex is composed of the extracellular peripheral membrane protein α-dystroglycan, which is non-covalently attached to the membrane spanning β-dystroglycan. The two subunits are encoded by a single messenger RNA expressed in several adult tissues (Ibraghimov-Beskrovnaya et al., 1992). To demonstrate the presence of dystroglycan during lung and salivary gland development, we first performed Northern blot analyses. Expression was seen at all stages, but the signal peaked at birth. In two-week-old lungs, dystroglycan mRNA expression had declined to levels seen during embryogenesis (Fig. 1). The 5.8 kb transcript was also expressed in salivary glands from 13-d-old embryos and from newborn fetuses (not shown).

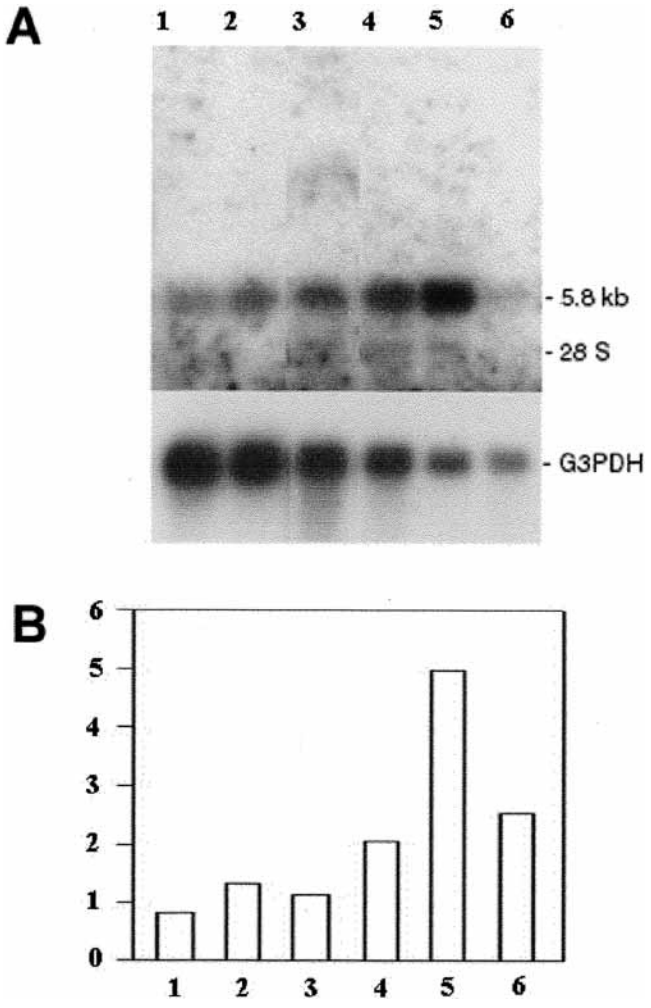



**Fig. 1** Dystroglycan mRNA expression in developing lung. (**A**) Northern blot analysis of dystroglycan mRNA expression during mouse lung development *in vivo*. Each lane was loaded with 10 μg of total RNA, blotted and hybridized to a cDNA probe recognizing a 5.8 kb dystroglycan transcript. Total RNA was from lungs of 12-d-old (*lane 1*), 13-d-old (*lane 2*), 16-d-old (*lane 3*), 18-d-old (*lane 4*) embryonic mice, newborn mice (*lane 5*) and 2-week-old mice (*lane 6*). The same filter was also hybridized with a G3PDH cDNA probe. (**B**) Quantification of signal intensities normalized to the signal intensities of the control hybridization.

## Localization of dystroglycan mRNA and polypeptides in developing salivary gland and lung

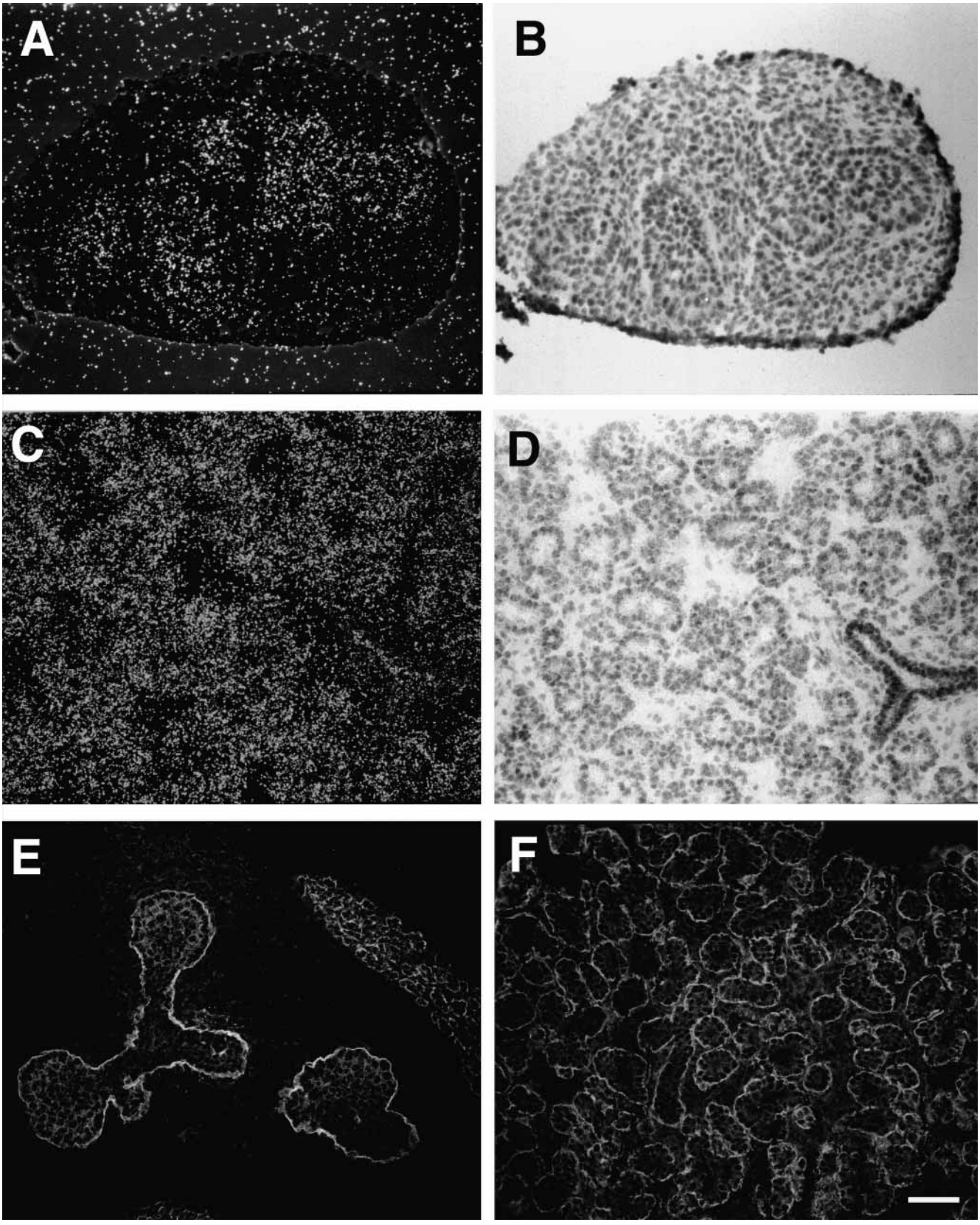
To examine the distribution of dystroglycan mRNA in salivary glands, we carried out *in situ* hybridization experiments using an oligonucleotide probe against mouse dystroglycan mRNA. Salivary glands from 13-d-old embryos display only rudimentary epithelial branches, and dystroglycan mRNA was detected exclusively in the epithelium (Fig. 2A, B). In 17-d-old embryonic salivary glands, the epithelium is highly branched and several terminal lobules and ducts have formed. Strong expression of dystroglycan mRNA was seen in all epithelial structures and very little, if any, in mesenchymal cells (Fig. 2C, D). Indirect immunofluorescence showed that α/β-dystroglycan was expressed in epithelial cells in 13 d-old embryonic salivary gland and enriched on the basal side of the epithelial clusters and the epithelial stalk (Fig. 2E). All epithelial basement membrane regions were positive for α/β-dystroglycan staining in 17-d-old embryonic salivary glands (Fig. 2F).

In 13-d-old embryonic lung, dystroglycan mRNA was mainly confined to epithelial cells (Fig. 3A, B), as shown (Durbeej et al., 1995). Expression of dystroglycan mRNA in epithelial cells persisted in 18-d-old embryonic lung (Fig. 3C, D). Indirect immunofluorescence revealed that in 12- and 14-d-old embryonic lungs, α/β-dystroglycan was localized to the basal cell surface of the bronchial epithelium both around the tips and the distal part of the buds. Immunoreactivity was slightly weaker around the tips of buds than the distal part (Fig. 3E), which might be related to the thinning of immunoreactivity of several basement membrane components at the tips (Mollard and Dziadek, 1998). Strong expression of α/β-dystroglycan was also noted in smooth muscle (Fig. 3F). The molecular mass of α-dystroglycan can vary due to differences in glycosylation. For example, α-dystroglycan from adult skeletal muscle and kidney is 156 kDa, whereas that of brain is 120 kDa (Ervasti and Campbell, 1993; Durbeej and Campbell, 1999). To ascertain the molecular mass of α-dystroglycan in developing salivary gland and lung, we performed immunoblot analysis on glycoprotein preparations from newborn and adult tissues. Staining with monoclonal antibody IIH6 (against α-dystroglycan) revealed the presence of a typically broad α-dystroglycan band of 150–160 kDa in salivary gland and lung from newborn mice and also in the corresponding adult tissues (Fig. 4).

## Monoclonal antibody 200 blocks dystroglycan binding to recombinant α1LG4–5

Branching-blocking antibody 200 and three non-blocking antibodies (188, 199, AL-4) were tested for their ability to inhibit α-dystroglycan-laminin α1LG4–5 interactions. MAb 200 inhibited the binding of recombinant laminin α1LG4–5 to α-dystroglycan effectively (>50% inhibition was obtained at 5 μg/ml), whereas no inhibition could be seen with mAbs 188, 199, or AL-4 (Fig. 5). A schematic drawing showing the laminin α1 globular do-

**Fig. 2** Detection of dystroglycan mRNA and polypeptides in the embryonic salivary gland. Sections of 13-d-old (**A** and **B**) and 17-d-old (**C** and **D**) embryonic mouse salivary glands were analyzed by *in situ* hybridization with an oligonucleotide probe against dystroglycan. Dystroglycan mRNA is exclusively expressed in the epithelium of developing salivary gland. Sections of 13-d-old (**E**) and 17-d-old embryonic (**F**) salivary glands were analyzed by indirect immunofluorescence with antibody FP-B against α- and β-dystroglycan. α/β-Dystroglycan is enriched on the basal side of epithelial cells in close contact with basement membranes. *Bar*, 85 μm.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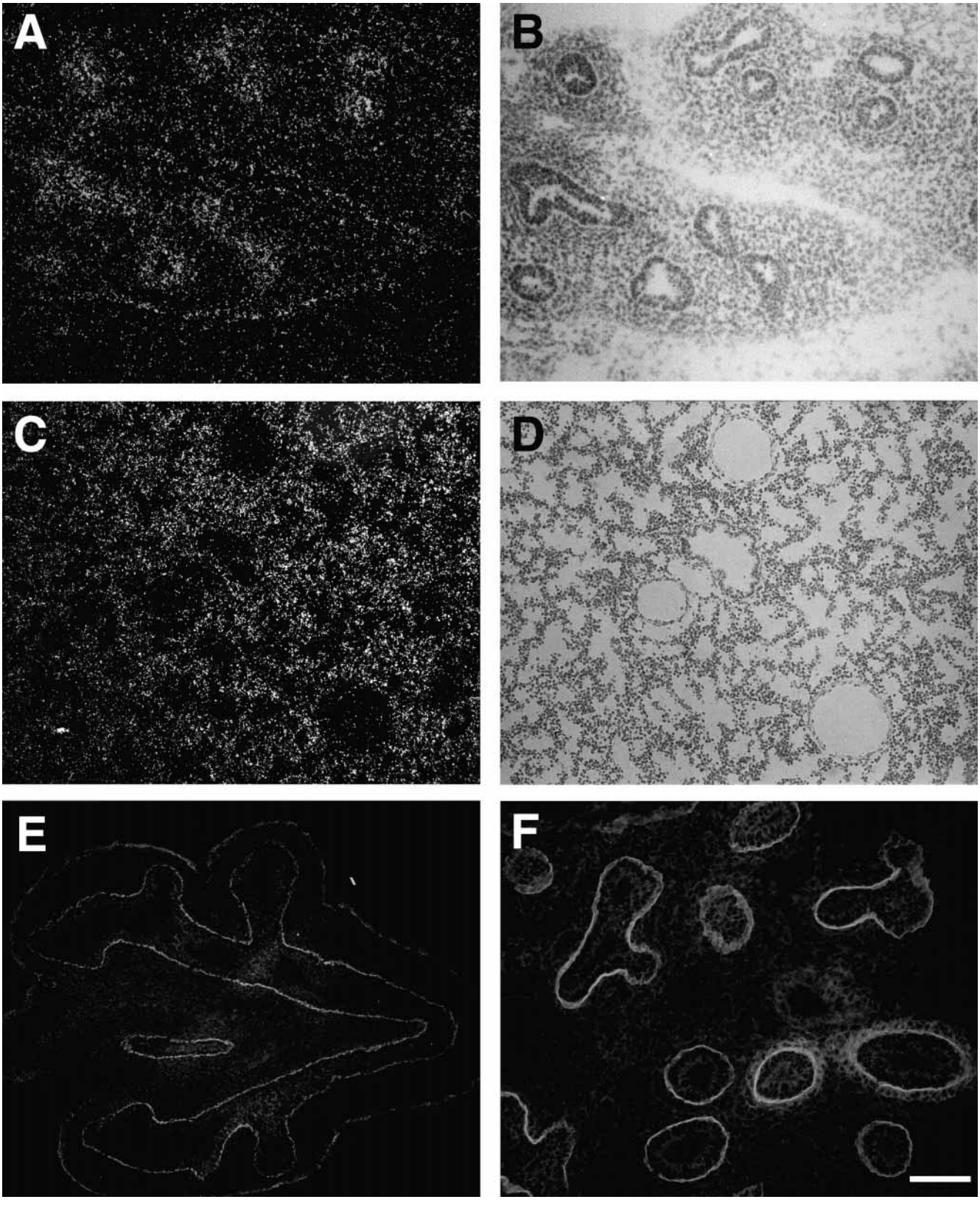
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main with LG module borders, and the areas to which the mAbs have been mapped is included for reference (Fig. 6).

## Salivary gland development is perturbed by antibodies blocking the laminin-dystroglycan interaction

The monoclonal antibody IIH6 that blocks α-dystroglycan binding to laminin-1 has previously been shown to perturb kidney development (Durbeej et al., 1995), to induce a dystrophic phenotype of primary muscle cultures (Brown et al., 1999), and to perturb laminin polymerization on muscle cell surfaces (Colognato et al., 1999). The same antibody recognized α-dystroglycan from mouse salivary gland and lung (Fig. 4). Thus, we used IIH6 for subsequent antibody blockade experiments. First, we carried out perturbation experiments in medium supplemented with 10% fetal calf serum. At 100 μg/ml, mAb IIH6 caused abnormal branching of the epithelium in salivary gland organ cultures. The epithelium did branch, but large abnormal terminal lobules were formed compared to salivary glands cultured without antibodies (data not shown). The effect was enhanced by raising the concentration of IIH6 to 200 μg/ml, and at this concentration, the number of epithelial end buds was also significantly reduced compared to control explants (Figs. 7A and 8A). The control cultures included explants grown without antibodies (Figs. 7C and 8A) and explants grown in the presence of IgM antibodies against dystrophin (mAb XIXC2) (Figs. 7B and 8A), IgG antibodies against tenascin-C (mAb MTn15) (Fig. 8A), and non immune rat IgG antibodies (Fig. 8A). At 200 μg/ml, the control antibodies did not alter salivary gland development in any detectable way (Fig. 8A). Similar results were obtained when antibody perturbation experiments were carried out in serum-free conditions. IIH6 at 200 μg/ml severely reduced the number of end buds, while XIXC2 had no such effect (Fig. 8B).

## Lung development is perturbed by antibodies blocking the laminin-dystroglycan interaction

We next tested whether mAb IIH6 also could perturb epithelial branching morphogenesis in organ cultures of embryonic lung. The end buds in IIH6-treated (200 μg/ml) explants appeared enlarged and displayed no clear lumen (Fig. 9A). Also, the number of end-buds was sig-

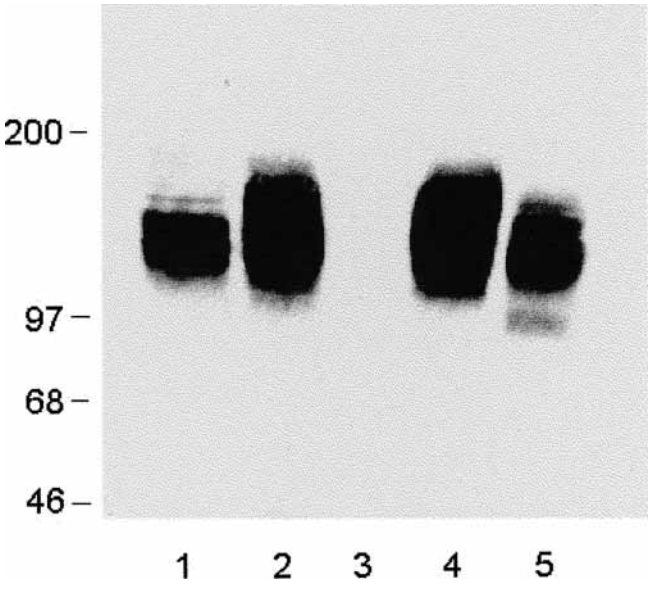



**Fig. 4** Immunoblot analysis of α-dystroglycan expression in newborn and adult salivary gland and lung. Glycoprotein preparations (20 μg) from salivary gland (*1*) and lung (*2*) of newborn mice and from salivary gland (*4*) and lung (*5*) of adult mice were analyzed by SDS-PAGE and immunoblotting using antibody IIH6 against α-dystroglycan. *Lane 3* is empty. Molecular masses are indicated in kDa.

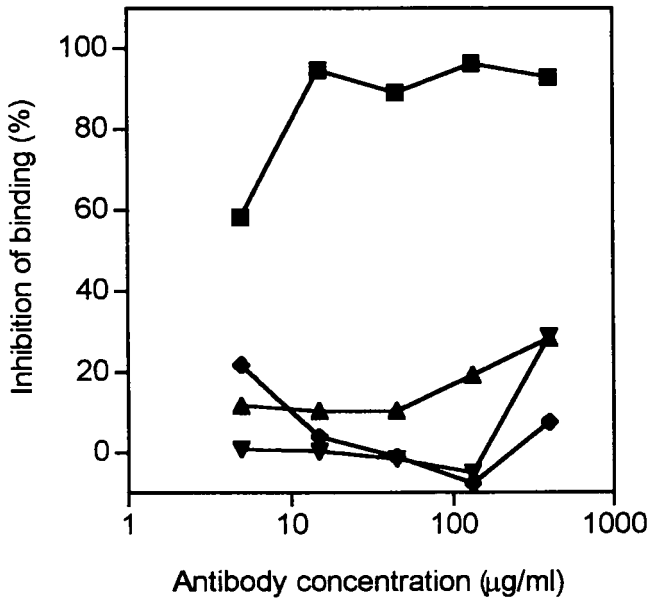



**Fig. 5** Inhibition of laminin α1LG4–5 binding to α-dystroglycan by mAb 200. A fixed amount of laminin α1LG4–5 (20 μg/ml) was mixed with increasing concentrations of mAb 200 (■), mAb 188 (▲), mAb 199 (▼), and AL-4 (◆) prior to incubation with immobilized α-dystroglycan. Detection of binding was with a polyclonal antiserum against laminin E3.

**Fig. 3** Detection of dystroglycan mRNA and polypeptides in the embryonic lung. Sections of 13-d-old (**A** and **B**) and 18-d-old (**C** and **D**) embryonic mouse lungs were analyzed by *in situ* hybridization with an oligonucleotide probe against dystroglycan. Dystroglycan mRNA is exclusively expressed in the epithelium of developing lung. Sections of 11-d-old (**E**) and 14-d-old embryonic (**F**) lungs were analyzed by indirect immunofluorescence with antibody FP-B against α- and β-dystroglycan. α/β-dystroglycan is enriched on the basal side of epithelial cells in close contact with basement membranes. Note that staining also can be detected in smooth muscle. *Bar*, 100 μm.

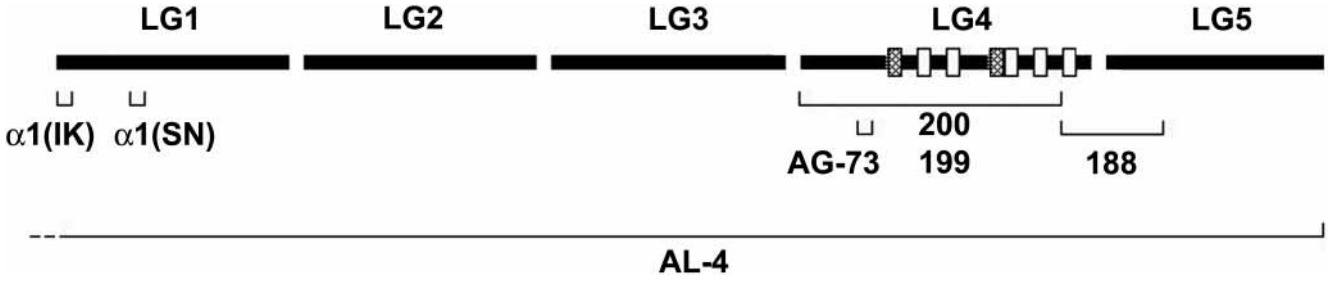



**Fig. 6** Epitopes of monoclonal antibodies specific for the carboxy-terminus of laminin α1 chain. Laminin globular (LG) module borders are denoted by *interruptions in the black line*. The E3 fragment consists of LG4 and LG5. Basic residues known to be important for α-dystroglycan binding are *boxed in white*. Residues coordinating the calcium ion important for α-dystroglycan binding are denoted by *hashed boxes* (Andac et al., 1999; Talts et al., 1999; Timpl et al., 2000). Shown below are the areas to which the mAb epitopes have been mapped so far. Antibodies α1(SN) and α1(IK) were raised against the indicated peptides (Matter and Laurie, 1994; Chen et al., 1997; 1998). Epitopes of mAb 200 and 188 were mapped by binding assays using laminin fragments (Sorokin et al., 1992; Andac et al., 1999). AL-4 binding to intact laminin-1 was mapped by electron microscopy after rotary shadowing (Skubitz et al., 1988). The long line for AL-4 indicates that its epitope within the carboxyterminus has not yet been precisely mapped. The location of residues representing peptide AG-73 (Kadoya et al., 1998) is also indicated.

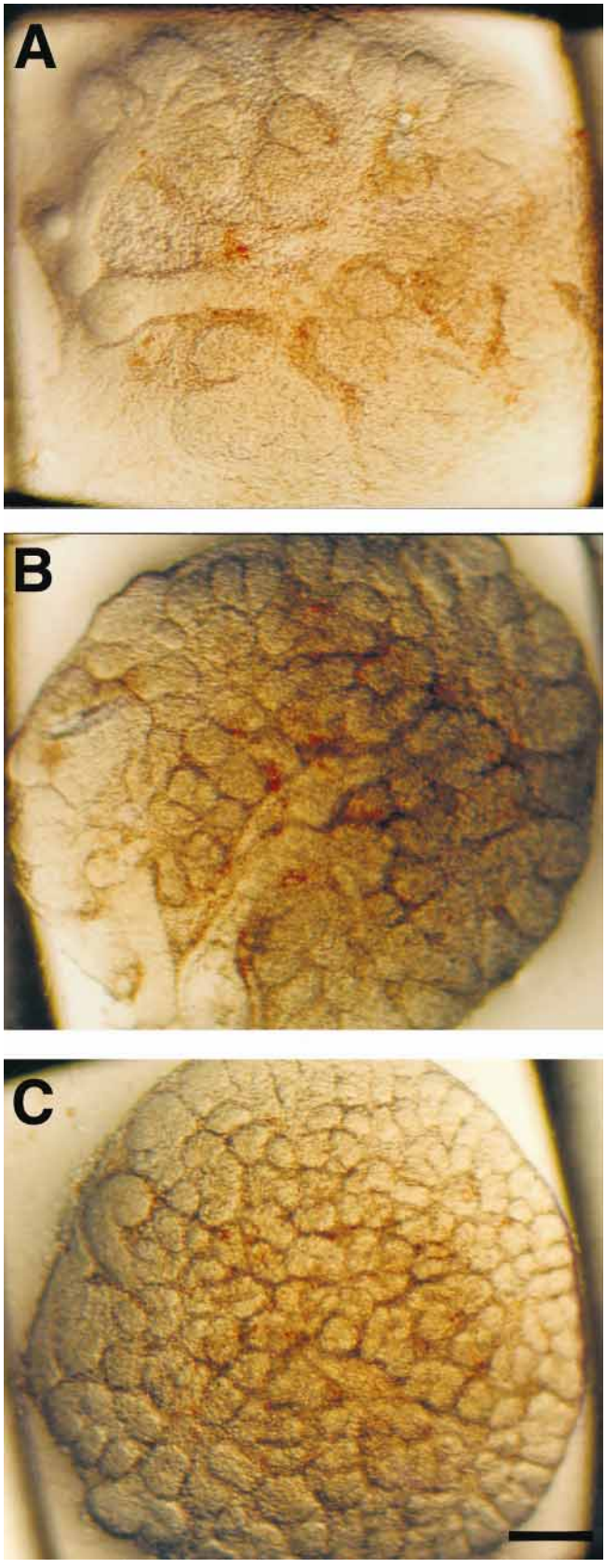
nificantly reduced (Figs. 9A and 10). No effects were observed in cultures incubated with 200 μg/ml of monoclonal antibody XIXC2 (Figs. 9B and 10), 200 μg/ml of monoclonal antibody MTn15, or without antibodies (Figs. 9C and 10). The anti-E3 polyclonal antibodies have previously been used to block casein expression in mouse mammary epithelial cells (Streuli et al., 1995). MAb 200 has previously been shown to perturb kidney development (Sorokin et al., 1992) and branching morphogenesis of salivary gland (Kadoya et al., 1995). Anti E3 polyclonal antibodies and mAb 200 were tested here in organ cultures of 11-d-old embryonic lungs. At 100 μg/ml of polyclonal anti E3 antibodies, lung branching morphogenesis was seriously retarded (Figs. 9D and 10). Fewer end buds formed and the lungs were smaller. The polyclonal anti E3 antibodies also disturbed kidney and salivary gland development (data not shown). MAb 200 also retarded lung development at 100 μg/ml, albeit not as much as the polyclonal E3 antibodies (Figs. 9E and 10). In contrast, rat IgG at the same concentration had no effect on lung development (Figs. 9F and 10).

## Discussion

Laminins are large extracellular components, but several sites active on cells may reside in discrete smaller domains. The roles of such domains can be revealed in biological assays with approaches that specifically target these sites. Here we show that antibodies that block dystroglycan binding to the α1LG4 domain perturb epithelial development of salivary gland and lung *in vitro*. Our data suggest that this cell-matrix interaction is involved in early stages of epithelial development.

Out of 12 tested monoclonal antibodies directed against the carboxyterminal end of laminin α1 chain (Schuger et al., 1991; Sorokin et al., 1992; Matter and Laurie, 1994), only antibody 200 has so far been shown to clearly act as a blocking antibody for epithelial cells. A distinct but partial inhibition was seen with mAb 200 in kidney and salivary organ cultures (Sorokin et al., 1992; Kadoya et al., 1995), whereas AL-4 (Schuger et al., 1991; 1995), α1(SN), or α1(IK) (Matter and Laurie, 1994; Chen et al., 1997; 1998) did not affect epithelial cells of the lung. The apparent discrepancy could be due to cell-type specific requirements for receptors but is more likely due to different binding epitopes of the antibodies. This issue was clarified by several observations in the current study. Antibody 200 was also shown to partially block lung epithelial morphogenesis *in vitro*. This antibody reacts with α1LG4 at dystroglycan-binding sites or domains close to it (Sorokin et al., 1992; Andac et al., 1999), but it has remained unclear whether it can block defined protein-protein interactions. Here we show that this antibody completely blocks binding of recombinant α1LG4–5 to dystroglycan in solid-phase assays, whereas three other α1 LG antibodies (mAbs 188, 199, AL-4) showed no such activity. The three other antibodies tested were selected as suitable controls since they do not block epithelial branching morphogenesis and also react with α1LG4 (mAb 199), α1LG4–5 (mAb 188), or not yet determined α1LG modules (mAb AL-4) and therefore potentially with the dystroglycan-binding site. The antibodies α1(SN) and α1(IK), which are unable to block lung epithelial attachment to laminin-1 (Matter and Laurie, 1994) or differentiation functions of lacrimal epithelial cells, bind to α1LG1 domains (Chen et al., 1997; 1998) far from the dystroglycan-bind-

**Fig. 7** Branching morphogenesis in the presence of antibodies against α-dystroglycan and dystrophin. Salivary glands from 13-d-old embryonic mice were cultured (**A**) in the presence of mAb IIH6 against α-dystroglycan (200 μg/ml), or (**B**) in the presence of antibody XIXC2 against dystrophin (200 μg/ml), or (**C**) without added antibodies. Cultures were examined after 2 days by stereomicroscopy. *Bar*, 70 μm.



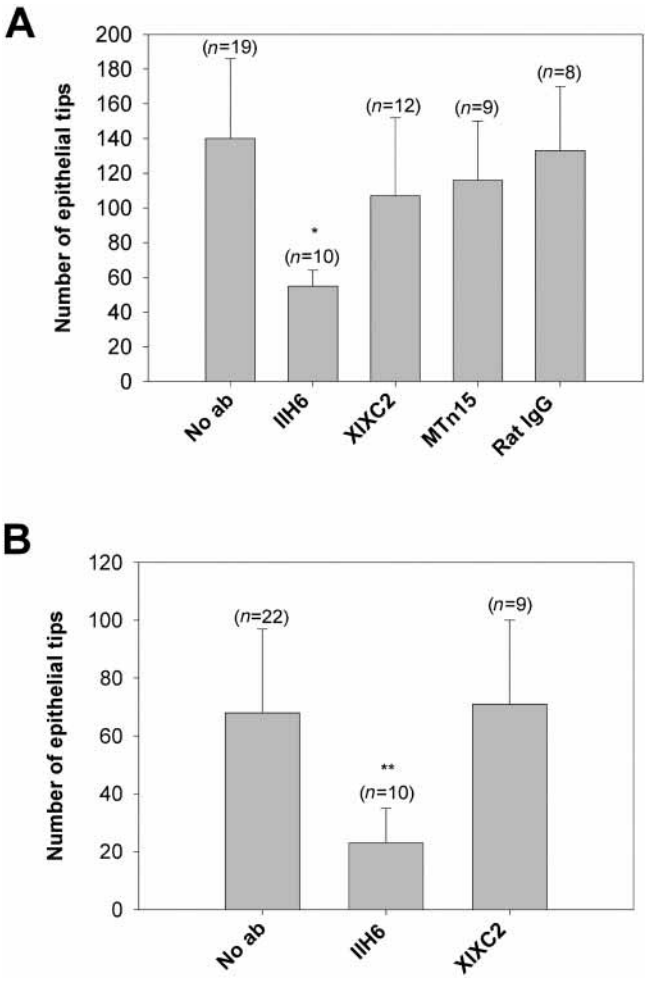
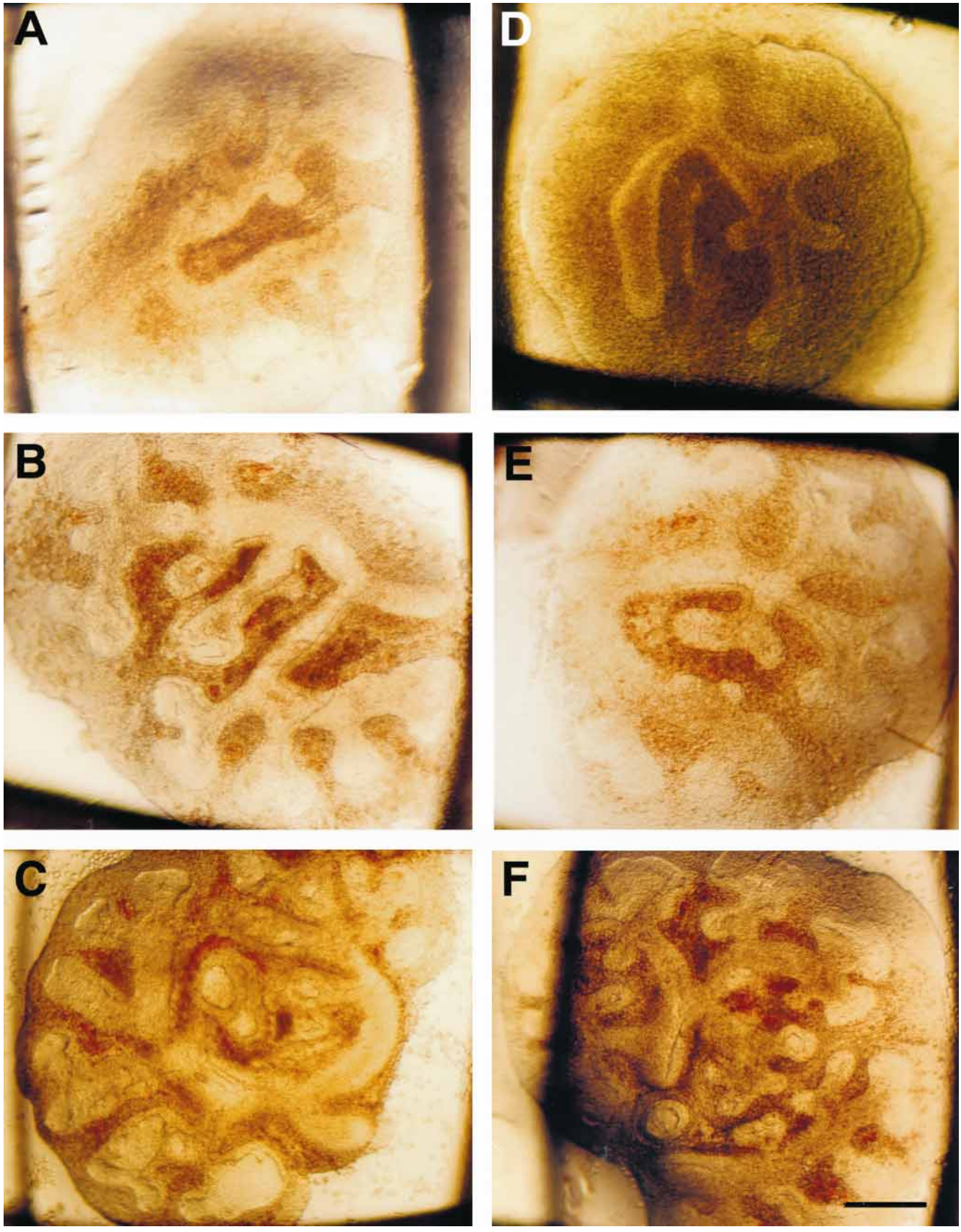




**Fig. 8** Number of end lobules in organ cultures of embryonic submandibular glands. (**A**) Glands from 13-d-old embryonic mice were cultured for two days in 10% fetal calf serum in the absence or presence of antibodies at 200 μg/ml. End buds were counted from photographs taken by stereomicroscopy on day 2 of culture, and results were evaluated statistically using an unpaired t-test for independent means. *Significantly different from controls ($P<0.0019$). (**B**) Glands from 13-d-old embryonic mice were cultured for two days in defined medium in the absence or presence of antibodies. End buds were counted from photographs taken by stereomicroscopy on day 2 of culture and results were evaluated statistically using an unpaired t-test for independent means. **Significantly different from controls ($P<0.0002$).

ing site. A synthetic peptide, AG-73, an inhibitor of submandibular gland epithelial morphogenesis (Kadoya et al., 1998), is composed of residues *2719–2730* in α1LG4 (Nomizu et al., 1995). These residues are not implicated in dystroglycan binding (Andac et al., 1999; Timpl et al., 2000), but it cannot be excluded that AG-73 interferes with dystroglycan-laminin-1 interactions.

Based on available perturbation studies in several organ culture systems using antibodies against different α1LG domains, we suggest that α1LG4-dystroglycan interactions are of importance for branching epithelial morphogenesis in several organs. This view is supported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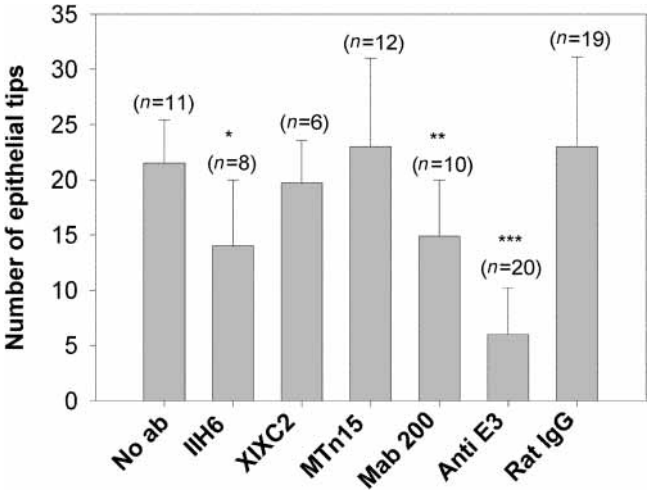


**Fig. 10** Number of end buds in organ cultures of embryonic lungs. Lungs from 11-d-old embryonic mice were cultured for two days in 10% fetal calf serum in the absence (No ab) or presence of antibodies at 200 μg/ml (IIH6, XIXC2, MTn15) or 100 μg/ml (Mab 200, Anti E3, Rat IgG). End buds were counted from photographs taken by stereomicroscopy on day 2 of culture and results were evaluated statistically using an unpaired t-test for independent means. *Significantly different from controls ($P<0.055$). **Significantly different from controls ($P<0.013$). ***Significantly different from controls ($P<0.0001$).

by the dystroglycan expression data and antibody perturbation studies using an anti-dystroglycan antibody. Dystroglycan mRNA was expressed by epithelium throughout salivary gland and lung development, and α/β-dystroglycan was localized to the basement membrane regions of the epithelial stalk and the epithelial clusters. Moreover, monoclonal antibody IIH6, known to perturb laminin-1 dystroglycan interactions and cell binding to laminin-1 (Ervasti and Campbell, 1993) but not cell binding to laminin-10/11 (Ferletta and Ekblom, 1999), inhibited epithelial branching morphogenesis in organ cultures of salivary glands and lungs. Salivary glands treated with the dystroglycan blocking antibody displayed large abnormal terminal lobules, compared to control explants. Similarly, in salivary glands treated with mAb 200 against α1LG4, the epithelial tips became enlarged (Kadoya et al., 1995). Also in lungs, similar clear alterations in the morphology of epithelial sheets were noted in explants treated with IIH6.

It is still unclear how the interaction between one LG module and dystroglycan affects epithelial morphogenesis at the molecular level, but the identification of mAb 200 as an inhibitor of this interaction allows a more precise interpretation of the current organ culture results and some previous findings (Kadoya et al., 1995; Jiang et al., 2001). Mab 200 causes a distinct disruption of basement membranes in developing salivary gland, not seen with antibodies against integrin α6 (Kadoya et al., 1995). Our findings thus support the hypothesis that the formation of the laminin-1 dystroglycan complex could be an early step in the formation of epithelial basement membrane assembly (Henry and Campbell, 1998; 1999) and defines the α1LG4-dystroglycan interaction as one potential nucleation event in such processes. Our findings may also explain why mAb AL-4 is unable to block lung epithelial development (Schuger et al., 1991; 1995) and only slightly alters peribronchial cell shape and smooth muscle differentiation (Schuger et al., 1997); it does not interfere with a major dystroglycan-laminin-1 interaction.

As shown for the developing kidney (Klein et al., 1988; Sorokin et al., 1992), polyclonal anti-E3 antibodies inhibited branching morphogenesis in developing lung more efficiently than mAb 200 against the single LG4 module. Polyclonal antibodies against E3 have also been shown to reduce mammary gland epithelial gene expression stimulated by laminin-1 (Streuli et al., 1995). The interacting regions on both α1LG4 and α-dystroglycan are fairly large (Andac et al., 1999; Hohenester et al., 1999), and polyclonal antibodies might be more efficient as inhibitors of dystroglycan binding to α1LG4. Since mAb 200, at least in the solid-phase assays, completely inhibited α1LG4–5 binding to dystroglycan, a more likely explanation is that E3 or adjacent domains harbor additional sites important for branching of epithelia. The polyclonal antibodies might perturb E3 interactions with cell surface proteoglycans such as syndecan-1 (Salmivirta et al., 1994; Muschler et al., 1999) or several extracellular matrix components known to bind E3 (Timpl et al., 2000). Furthermore, it has been reported that polyclonal anti E3 antibodies can also inhibit cell binding to the E8 fragment in attachment assays (Aumailley et al., 1990), perhaps by steric hindrance of integrin-mediated interactions. In contrast, no inhibition of cell binding to E8 was observed with mAb 200 (Sorokin et al., 1992).

Our findings are in line with notions that non-E3 laminin-1 receptors also are required for epithelial branching morphogenesis. Genetic studies indicate that laminin-binding members of the integrin β1 family are involved both in basement membrane assembly or epithelial branching morphogenesis (Brakebusch et al., 1997). Disorganized basement membranes were seen in teratomas derived from β1 integrin null ES cells, but here the mechanisms of the disorganization are complex, as lack of β1 integrin decreased laminin-1 expression (Sasaki et al., 1998; Aumailley et al., 2000). Genetic studies of discrete integrin α chains provide clear evidence for a role of integrins in epithelial branching mor-

**Fig. 9** Perturbation of branching epithelial morphogenesis in embryonic lung organ cultures. Lungs from 11-d-old embryonic mice were cultured in the presence of 200 μg/ml of antibody IIH6 (**A**), in the presence of 200 μg/ml of antibody XIXC2 (**B**), without added antibodies (**C**), in the presence of 100 μg/ml of anti E3 antibodies (**D**), in the presence of 100 μg/ml mAb 200 (**E**), or in the presence of 100 μg/ml of rat IgG (**F**). Cultures were examined after 2 days by stereomicroscopy. *Bar*, 80 μm.

phogenesis. For example, bronchial branching is slightly decreased in lungs of integrin α3 −/− mice (Kreidberg et al., 1996), and defects are more severe in a compound mutant lacking both the α6 and α3 integrin subunits. Interestingly, lung defects in the compound mutant are not clearly related to a disorganization of the basement membrane (DeArcangelis et al., 1999), even though both α3 and α6 integrins are major receptors for several laminins (Delwel et al., 1994; Gu et al., 1999; Talts and Timpl, 1999; Kikkawa et al., 2000). One possibility is that other integrins or dystroglycan in some sites are more crucial for basement membrane assembly, whereas integrins α3 and α6 are more involved in other processes such as signaling. In other sites where the receptor-ligand repertoire is different, α3 or α6 integrins may also more directly participate in basement membrane assembly. One such site is skin, where some disorganization of basement membranes was seen in mice lacking either the α3 or α6 integrin subunit (Georges-Labouesse et al., 1996; DiPersio et al., 1997). Obviously, the dissection of the distinct (Muschler et al., 1999) and overlapping functions of all the laminins and their receptors in different tissues is a formidable task and is still in its infancy. The here-defined reagents that specifically block the interaction of only one LG domain to dystroglycan should be useful tools to study the role of laminin-1 in locations where it is mainly found, broadly, during the early stages of epithelial development (Klein et al., 1990; Virtanen et al., 2000) and in a more limited set of adult epithelia (Falk et al., 1999; Virtanen et al., 2000).

During onset of salivary gland development, laminin α1 chain is expressed only very transiently (Kadoya et al., 1995) and is also gradually lost from developing lung epithelium (Sorokin et al., 1997; Pierce et al., 2000). Dystroglycan is expressed throughout development and remains present in adult epithelia (Durbeej et al., 1998; 1999). α-dystroglycan binds several basement membrane components (Henry and Campbell, 1999; Talts et al., 1999), which could be important for epithelial development at later developmental stages. In adult lung, dystroglycan may participate in epithelial repair after injury (White et al., 2001). The mRNA of laminin α5 chain is abundantly expressed in epithelial cells throughout salivary gland development (Durbeej et al., 1996). Also, several potential dystroglycan binding laminins are present during kidney (Ekblom et al., 1998) and lung development either in developing myofibroblasts (Schuger et al., 1997; Flores-Delgado et al., 1998) or in epithelium (Galliano et al., 1995; Durbeej et al., 1996; Miner et al., 1997; Sorokin et al., 1997). Structural predictions suggest that LG domains of the α3, α4, and α5 chains should not bind dystroglycan (Hohenester et al., 1999; Tisi et al., 2000), but recombinant laminin α5LG1–5 produced in bacteria has some α-dystroglycan binding activity (Shimizu et al., 1999). It will therefore be interesting to determine whether α-dystroglycan also binds native laminin α5 chain complexed in laminin-10/11.

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