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Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis

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SUMMARY

A mutation was targeted to the murine *α3 integrin* gene. Homozygous mutant mice survived to birth, but died during the neonatal period. The mutation caused abnormal kidney and lung development. Mutant kidneys displayed decreased branching of the medullary collecting ducts, although the number of nephrons was not altered. Proximal tubules exhibited two distinct subsets of abnormalities, with the epithelial cells either containing excess lysosomes or becoming microcystic. In addition, glomerular development was markedly affected. In mutant kidneys, the extent of branching of glomerular capillary loops was decreased, with capillary lumina being wider than normal. The glomerular basement membrane was disorganized and glomerular podocytes were unable to form mature foot processes. Branching of the bronchi in lungs of mutant mice was also decreased and the large bronchi extended to the periphery. These results indicate a role for integrin receptors in basement membrane organization and branching morphogenesis.

Key words: alpha 3 beta 1 integrin, kidney, lung, basement membrane, branching morphogenesis, mouse

INTRODUCTION

Mesenchymal-epithelial interactions guide diverse morphogenetic processes during embryogenesis. Classical experiments in which mesenchyme and epithelium from different embryonic organs are recombined have demonstrated that both instructive and permissive information is passed from mesenchymal to epithelial cells through the secretion of diffusible growth factors and components of the extracellular matrix (ECM) (Wessels, 1970). Integrins are the major family of molecules that serve as cell surface receptors for components of the extracellular matrix (Hynes, 1992). Integrins are heterodimeric cell surface receptors, composed of a single α and β peptide subunit (Hynes, 1992). The extracellular domains of integrins interact with the ECM or other cell surface molecules, and some cytoplasmic domains have been shown to interact with the cytoskeleton (Chen et al., 1995; Otey et al., 1993). Within an integrin subfamily, a single β subunit is able to form heterodimers with several α integrins (Hynes, 1992). The extracellular domain of the α integrin confers the binding specificity for the heterodimer and the particular biological response to binding is determined by the α subunit cytoplasmic domain (Chan et al., 1992; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993). Upon integrin binding of components of the ECM, signals are transduced that control diverse cell behaviors such as cell adhesion and migration (Clark and Brugge, 1995). The cytoplasmic portion of the β chain is directly responsible for interacting with downstream signal transduction molecules such as $p59^{ILK}$ and Focal Adhesion Kinase (Hannigan et al., 1996; Schaller et al., 1995), and integrin-mediated binding of particular ligands such as collagen or fibronectin has been shown to trigger signal transduction cascades that activate MAP kinase (Schlaepfer et al., 1994). In cultured cells, binding of ECM components can result in the localization of certain integrins into focal contacts, in which actin stress filaments converge at points where cells are anchored to the substratum (DiPersio et al., 1995; Grenz et al., 1993). The cytoplasmic portion of the β chain has also been shown to physically interact with the cytoskeleton at these focal contacts by associating with the actin filament-binding molecules talin and α-actinin, supporting the premise that integrins form a structural link between the ECM and the cytoskeleton (Chen et al., 1995; Otey et al., 1993).

The *α3 integrin* gene is expressed during the development of many epithelial organs, including the kidney (Korhonen et al., 1991), lung (Mette et al., 1993) and skin (Hertle et al., 1991). A number of studies have defined multiple ligands for α3β1 integrin, including laminin, (Laminin-1 and -5), certain types of collagen, fibronectin and entactin (nidogen), (Carter et al., 1991; Dedhar et al., 1992; Delwel et al., 1994; Elices et al., 1991; Hemler et al., 1990; Weitzman et al., 1993). The usual basolateral distribution of α3β1 on epithelial cells and its binding specificities support its role as a receptor for the basement membrane. α3β1 has also been shown to participate

in homophilic (Sriramarao et al., 1993) and heterophilic interactions (Symington et al., 1993) with other integrins. Therefore, its expression along lateral membranes may involve a role for this integrin in cell-cell adhesion. Two alternatively spliced forms of α3 integrin have been described, which lead to two different cytoplasmic tails, referred to as α3a and α3b (Tamura et al., 1991). While the differential expression of these two forms amongst various tissues has not yet been described, an initial report shows α3a to be the predominant form expressed in lung (Tamura et al., 1991). As a major basement membrane receptor in both kidney and lung during embryogenesis, α3β1 is likely to be involved in mediating signals between the mesenchyme and epithelial cells in the kidney and lung.

This report focuses on abnormalities in kidney and lung development in α3 integrin-deficient mice. During normal development of the kidney, ductal growth and branching occurs as a consequence of reciprocal mesenchymal-epithelial interactions between the ureteric bud and the metanephric mesenchyme and, subsequently, its descendant stem cells (Saxen, 1987). These interactions also lead to the transformation of the metanephric mesenchyme into the renal epithelium, which further differentiates through the comma and S-shaped tubule stages into the distinct elements of the nephron. Coincident with the development of individual nephrons, the formation of collecting ducts during organogenesis of the kidney is the result of a defined series of branching events by derivatives of the original ureteric bud. α3β1 is expressed on ureteric buds and their derivative collecting ducts, glomerular endothelial cells and, most prominently, on glomerular visceral epithelial cells or podocytes (Adler, 1992; Ekblom et al., 1991; Patey et al., 1994; Rahilly and Fleming, 1992). The identification of α3β1 integrin on the ureteric bud suggests that this integrin is involved in mediating branching morphogenesis, to the extent that the ECM in contact with the ureteric bud is determined by the surrounding mesenchyme.

Lung development is the product of more typical mesenchymal-epithelial interactions, in that no inductive transformations occur. The original endodermally derived lung buds, proliferate and branch in response to mesenchymal influences. At later stages of lung development, the epithelium at the distal ends of the branches differentiates into the type I and type II pneumocytes that line the alveoli. In a classic group of experiments which demonstrated the importance of mesenchymal-epithelial interactions during organogenesis, lung bud epithelium was separated from its mesenchyme and recombined with mesenchyme from other developing organs. In several cases, the lung bud epithelium differentiated into an epithelium characteristic of the organ from which the mesenchyme was derived (Wessels, 1970).

MATERIALS AND METHODS

Construction of a targeting vector

Genomic clones of the murine *α3 integrin* gene were obtained from a library of partial *Mbo*I fragments from the D3 ES cell line (Doetschman et al., 1985), cloned into the *Bam*HI site of the λdash vector (Stratagene). A 9 kb subclone from one of four overlapping phage clones was cloned into the *Bam*HI site of pGEM-7 (Promega) and a detailed restriction map was obtained. A 0.5 kb *Bam*HI/*Xba*I fragment from the extreme 3′ end of this clone (5′-3′ orientation determined by direction of the cDNA) was isolated to be used as an external probe. The PGK-Neo-Poly(A) cassette was placed in a unique *Bcl*I site which was 5 kb downstream from the 5′ end of remaining 8.5 kb clone. Finally, the the genomic fragment containing the Neo gene was recloned into pKS(+) (Stratagene), along with the pMC-1 TK gene.

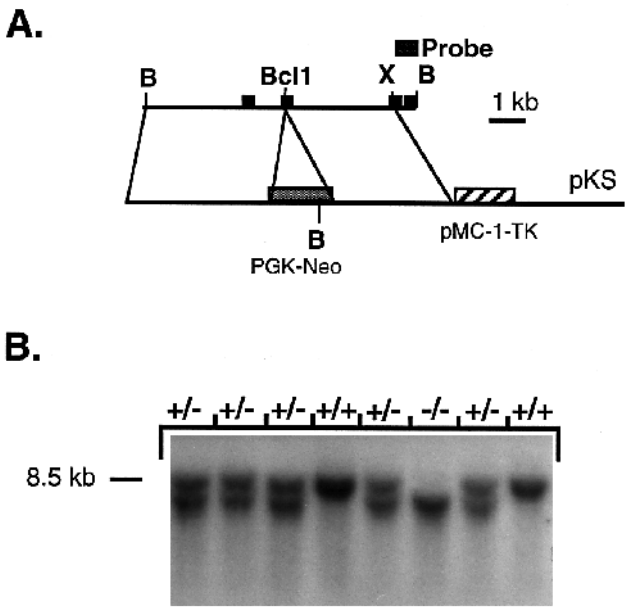

Fig. 1. Targeted mutagenesis of the α3 integrin gene. (A) The genomic clone of the α3 integrin gene is shown, with the position of the identified exons shown as black boxes. As discussed in the text, additional unmapped exons are present in this clone. The structure of the targeting vector is shown below, with the regions of homology specified. The external probe used for genotyping is shown above the chromosome map. Restriction sites: B, *Bam*HI; X, *Xba*I. (B) A Southern hybridization of a litter of newborn mice showing wild-type, heterozygote and homozygous mutant hybridization patterns.

ES cell culture, transfection and generation of chimeras

The J1 line of ES cells was grown and transfected as previously described (Li et al., 1992) ES cells were selected and screened as previously described (Kreidberg et al., 1993), using the DNA preparation of Laird et al. (1991). A *Bam*HI digest was used to detect homologous recombination using Southern blot hybridization on Hybond filter membrane (Amersham), using a ^{32}P-labelled probe as shown in Fig. 1. Chimeras were generated according to procedures described by Bradley (1987), with modification (Li et al., 1992).

Histological and immunohistochemical analysis

Newborn mouse tissues were fixed in 4% paraformaldehyde/phosphate-buffered saline, paraffin embedded and 4 μm-thick sections were cut. Serial sections were stained with Harris' hematoxylin and eosin, examined and photographed with an Axiophot microscope (Carl Zeiss). For lectin stains, newborn kidneys were fixed and embedded as above. 5 μm-thick sections were deparaffinzed, rehydrated and exposed to peroxidase-labeled Tetrogonolobus Purpureas (1:100) (Sigma: L1508) or Dolichos Biflorus (1:600) (Sigma:L4258) for 2 hours. Detection was with a liquid DAB substrate kit (Zymed, CA). For α3 integrin immunolocalization, E14 wild-type kidney and lung were fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 4-6 hours, dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. 5 μm paraffin sections were de-paraffinized, rehydrated and blocked with 1% BSA for 15 minutes. Sections were incubated with an anti-α3 integrin antibody (1:100) (DiPersio et al., 1995), for

2 hours at room temperature with detection by an avidin-biotin based alkaline phosphatase detection kit using Fast Red as the chromogen (BioGenex Labs, San Ramon, CA). Immunofluorescent analysis of β-1 laminin was done on frozen sections embedded in OCT (Miles), using an rat monoclonal anti-mouse laminin antibody from Life Technologies, Clone 5A2, with detection by a FITC-labelled anti-rat secondary antibody.

For sectioning of entire newborn lungs, lungs were inflated in situ via tracheal insufflation (at 23 cm H_2O) with 3% paraformaldehyde and 0.1% glutaraldehyde in PBS. After inflation for 30 minutes, the lungs were removed en bloc. The left lung was left intact and the right lung cut into three sagittal sections. The tissue was fixed for another 30 minutes in fresh fixative, washed in PBS (×3) and embedded in paraffin. At the time of embedding, the left lung was orientated so that the sections included the anterior surface, with the lateral airway branches exposed (Jones and Reid, 1978). Sections (5 μm) of the left lung, and of the apical, middle and basal regions of the right lung, were stained with hematoxylin and eosin.

Electron microscopy

Kidney samples for electron microscopy were fixed in 2% glutaraldehyde-1% paraformaldehyde, postfixed in osmium tetroxide and embedded in epoxy resin. 1 μm-thick sections were stained with toluidine blue for selection of appropriate areas. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips electron microscope.

RESULTS

Targeted mutation of the α3 integrin gene

We constructed a replacement type targeting vector using

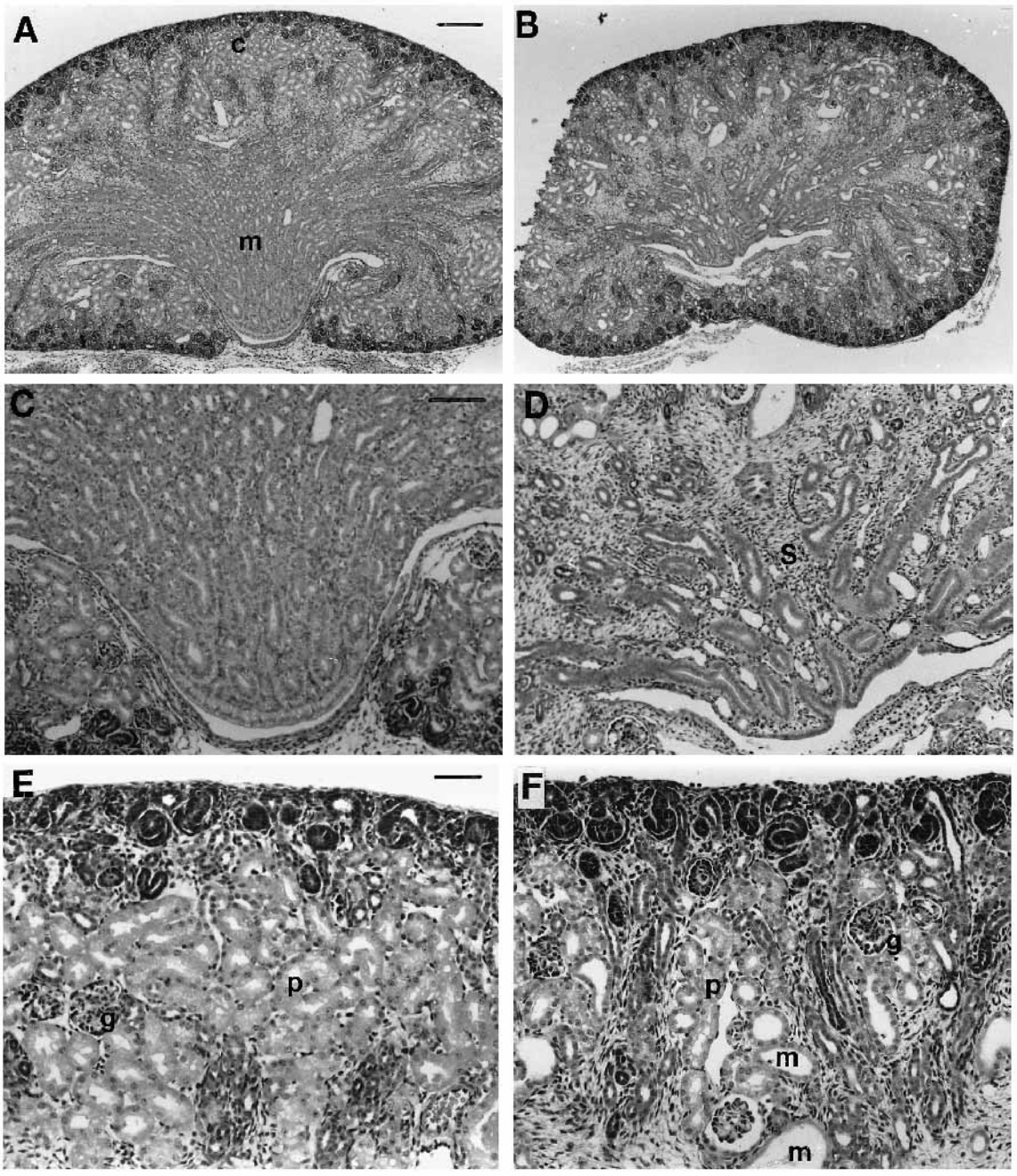

Fig. 2. Histological analysis of kidneys of wild-type and mutant newborn mice. (A,B) Saggital sections of wild-type (A) and mutant (B) kidneys. c, cortex; m, medulla The left side of the kidney in B was damaged during histological sectioning. (C,D) Papillae of wild-type (C) and mutant (D) kidneys. In the wild type, the papilla is densely packed with collecting ducts and capillaries. In the mutant a stromal component is present between collecting ducts. s, stroma. (E,F) Cortex of wild-type (E) and mutant (F) kidneys. The nephrogenic zone, where tubulogenesis is ongoing, is uppermost in each cortex. Below the nephrogenic zone, the cortex is mainly composed of proximal and distal convoluted tubules in the wild type. Fewer of these tubules are present in the mutant and many are microcystic. p, proximal tubules; g, glomeruli; m, microcysts. Bars (A,B) 0.2 mm; (C,D) 0.1 mm; (E,F) 50 μm.

restriction fragments from a genomic clone of the mouse *α3 integrin* gene obtained from a 129 strain mouse genomic library (Li et al., 1992). Sequencing of genomic subclones containing homologies to a human α3 integrin cDNA (Takada et al., 1991) revealed the presence of four exons, three of which were non-contiguous, implying the presence of additional exons within this clone. These exons are homologous to nucleotides 688-752, 1006-1159, 1471-1540 and 1541-1678 of the published mouse cDNA sequence (Takeuchi et al., 1995). The fragment containing the last two of these exons was used as an external probe to screen for recombinant clones (Fig. 1). A unique Bcl-1 restriction site was found at the site corresponding to cDNA nucleotide 1065 and the neomycin resistance gene was inserted into this site. This vector was used to target J1 ES cells (Li et al., 1992). Of 287 clones screened, 21 were homologous recombinants. All of these appeared to be single copy integrants of the targeting vector. When injected into embryos, one targeted ES cell clone generated a chimera that transmitted the mutation through the germ line. All of our results were obtained from mice outbred between C57Bl6/J and 129Sv/ter inbred strains.

Table 1. Genotypes of α3 integrin mutant mice and embryos

Age	+/+	+/–	–/–
E10.5-12.5	6	11	10
E13.5-18.5	23	30	24
Newborn	36 (2)	68 (4)	34 (16)
Adult	90	167	0

Genotypes of mice from heterozygous intercrosses. The numbers of newborns in parentheses represent the portion of the total which were dead or less vigorous at time of killing.

Heterozygous mice appeared normal and were intercrossed to obtain homozygous mutants. Homozygous mutant embryos were present at the expected 25% frequency throughout all stages of embryogenesis (Table 1). Neonatal homozygous mutants were able to breath and feed indistinguishably from their wild-type and heterozygous littermates for variable

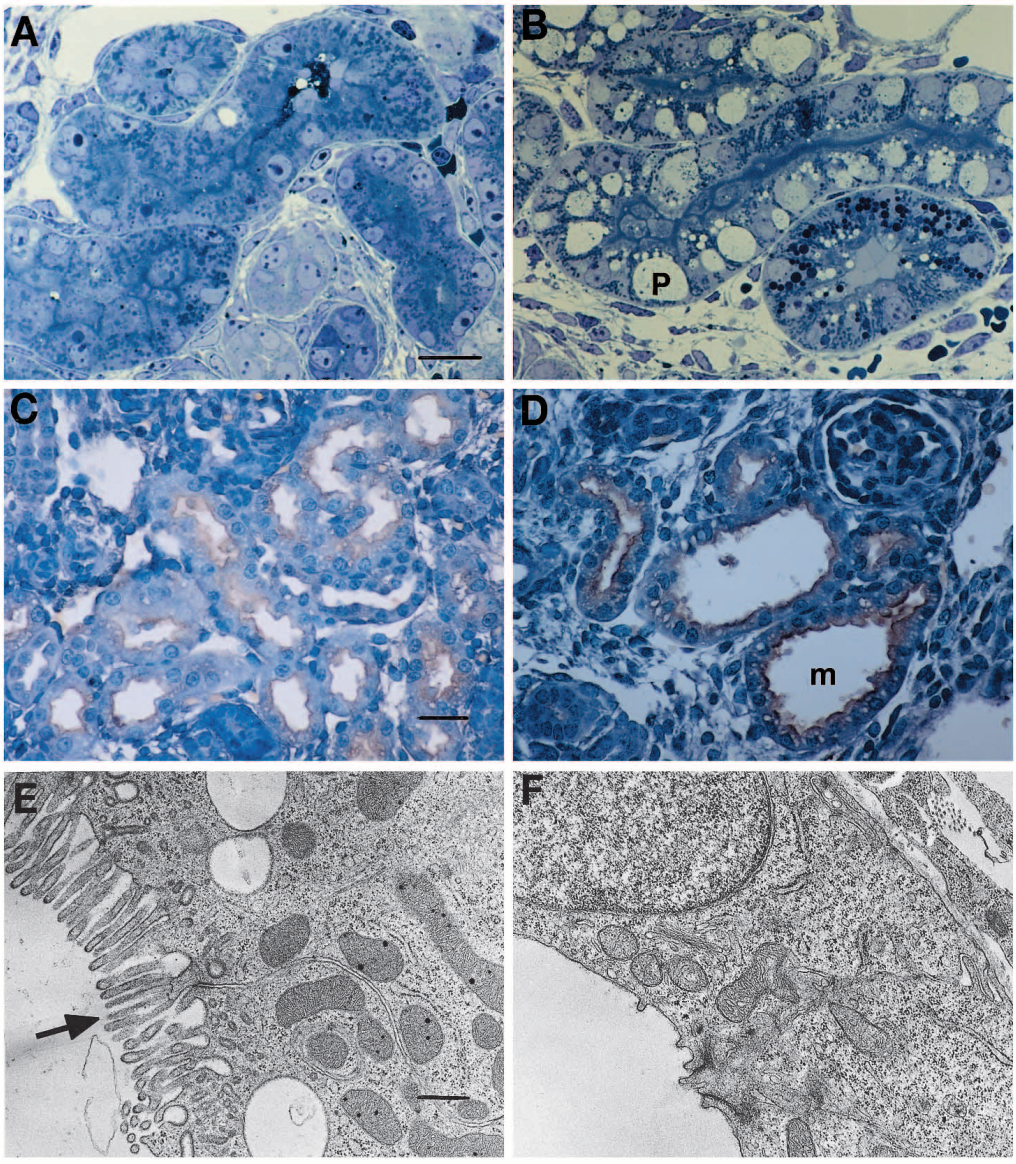

Fig. 3. Proximal tubules. (A,B) Proximal tubules in wild-type (A) and mutant (B) kidneys. Note the large vaculolar component in mutant epithelial cells. P, phagolysosomes. (C,D) Lectin Tetrogonolobus Purpureas staining of proximal tubules in wild type (C) and mutant (D), showing microcystic structures (m). (E,F) Electron microscopic analysis of microcysts. Only a few rudimentary microvilli are present. (E) Wild type; (F) mutant; arrow, microvilli. Bars (A,B) 5 μm; (C,D) 20 μm; (E,F) 0.5 μm.

periods of time. They then became progressively weaker and dehydrated, and all of them died during the first twenty four hours after birth (Table 1).

Abnormal nephrogenesis in mutant mice

Kidneys in mutant mice demonstrated marked abnormalities in normal renal architecture. The mutant kidneys were slightly smaller when compared with those of wild-type littermates (Fig. 2A,B). In the region of the renal medulla where the collecting ducts merge to form the renal papilla and ureter, serial sectioning through mutant and wild-type kidneys revealed an approximately two-fold reduction in the number of medullary collecting ducts (Fig. 2C,D), which were distinguished from distal tubules by staining with a specific lectin Dolichos Biflorus (data not shown). Instead of being tightly packed with collecting ducts, a stromal component was present in the papilla. This suggests that the initial rounds of symmetrical branching of the ureteric bud were compromised in the absence of α3β1 integrin. By immunofluorescent analysis, the ECM components surrounding collecting ducts, including laminin, type IV collagen, or the heparan-sulfate proteoglycan perlecan, were intact (data not shown). Electron microscopy also did not reveal any difference between the basement membrane of wild-type and mutant collecting ducts (data not shown).

The number of nephrons in the kidney theoretically should reflect the number of branches evolved from the ureteric bud. However, after the first few symmetrical branchings of the ureteric bud, which give rise to the inner medulla, a single main branch of the cortical collecting ducts usually induces several nephrons along its length in a process known as arcade formation. Arcades are the product of dichotomous branching events in the kidney cortex in which the main branch continues to elongate and a smaller terminal branch induces a nephron (Osathanondh and Potter, 1966a). Although the branching of collecting ducts in the medullary region appeared decreased in α3 integrin mutant newborn kidneys, the number of individual nephrons per kidney, as enumerated by counting glomeruli in serial sections through entire kidneys, appeared unchanged. We stained sections of kidney cortex with the collecting-duct-specific lectin Dolichos Biflorus in order to determine whether the retention of a normal nephron number in the mutant was the result of a compensatory increase in branching of collecting ducts after they had extended beyond the medulla into the cortex. However, similar to what is observed in the medulla, the number of cortical collecting ducts also appeared decreased in the mutant (data not shown). Two alternate explanations can be suggested to explain this apparent excess of nephrons over collecting ducts. Collecting ducts might be lost through an accelerated process of cell death, after having induced nephrons. Alternatively, during arcade formation in mutant kidneys, there may be increased induction of nephrons along individual collecting ducts. The latter possibility suggests a control mechanism that determines glomerular number during kidney formation and which is able to compensate for a dimunition in the number of collecting ducts.

Proximal and distal tubules in the kidney cortex are extensively convoluted and therefore histological sectioning through the cortex normally reveals multiple cross sections through the tubule associated with each individual nephron (shown in Fig.

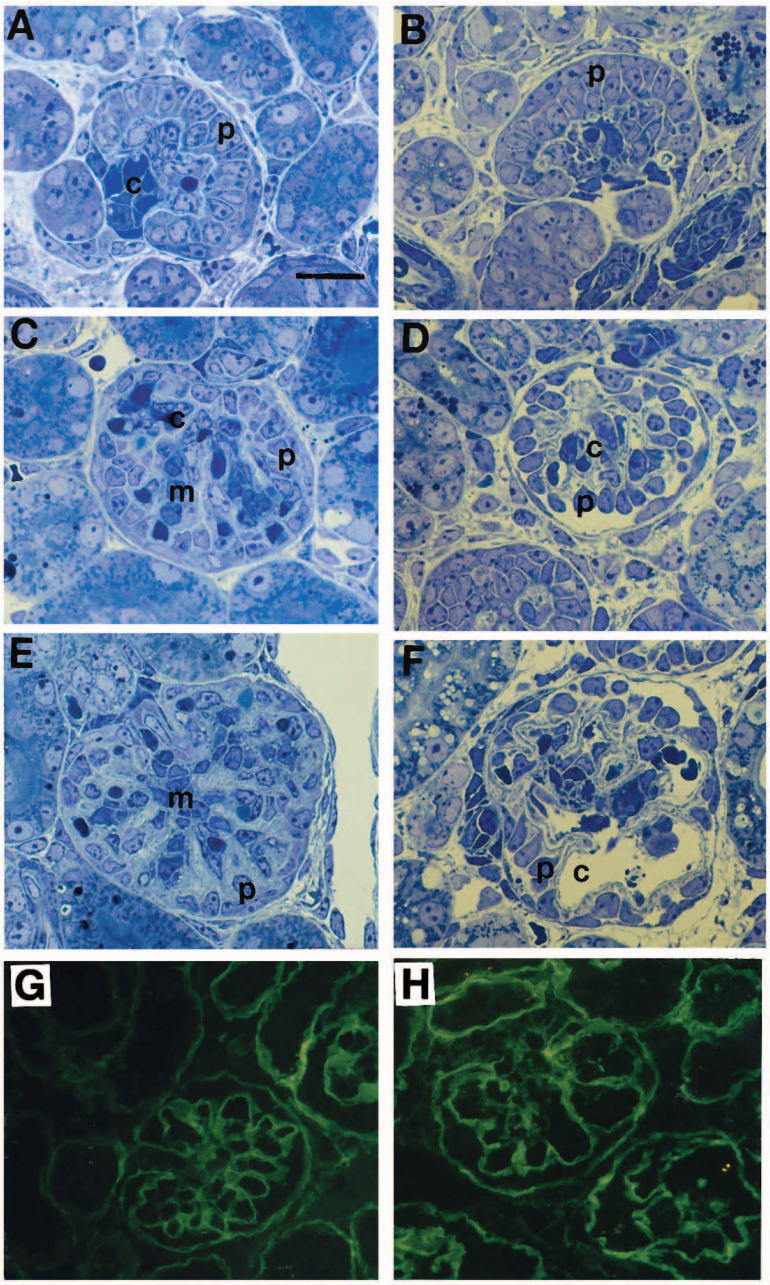

Fig. 4. Histological analysis of glomeruli from wild-type and mutant mice. (A,B) Early glomeruli from wild type (A) and mutant (B). (C,D) Immature glomeruli after formation of the first capillary loops (C, wild type; D mutant). Note the loss of lateral cell junctions in the mutant. (E,F) Mature wild-type (E) and mutant (F) glomeruli. (G,H) Immunfluorescent analysis of glomeruli, staining for β-1 laminin in wild-type (G) and mutant (H) glomerular basement membrane. In the mature mutant glomerulus (F, H), capillaries are wider and fewer. p, epithelial podocytes in all frames, at progressive stages of maturity; m, mesangial cells; c, capillary loops. Bar in A 8 μm; A-F are all same magnification.

2E). The similar number of nephrons between wild type and mutant contrasted with the reduced number of cross sections through proximal and distal convoluted tubules (Fig. 2E,F). The number of cross sections through tubules should directly relate to the extent of convolution, which, in turn, is related to the length of a tubule. This suggests that the length or extent of convolution of each proximal or distal tubule must be decreased in the mutant as compared to wild type. However, the formation of the tubular elements of the nephron appeared to have progressed through the normal stages and no morphological abnormalities were identified in the structures of the ureteric bud, comma and S-shaped tubules in developing mutant kidneys.

In addition to being decreased in length in mutant kidneys, proximal tubules appeared to have undergone two prominent changes from the wild-type appearance. One subset contained abundant cytoplasmic lysosomes and vacuoles (Fig. 3A,B). Otherwise, this subset of tubules retained their normal morphological appearance, including the elaboration of microvilli along their apical membranes (data not shown), which is a characteristic feature of proximal tubule epithelium. The other subset of proximal tubules exhibited microcystic changes, characterized by abnormally thin epithelial cells and widened lumina (Fig. 3C,D). These tubular epithelial cells had lost their normally abundant microvilli (Fig. 3E,F) and could only be positively identified as proximal tubules by staining their apical membrane with the lectin Tetrogonolobus Purpureas, which is specific for this type of tubule (Fig. 3C,D).

The abnormal renal architecture observed in mutant kidneys demonstrates a functional role for $\alpha3\beta1$ integrin during kidney development. $\alpha3\beta1$ appears to be required for the formation of the collecting system, and for the proper growth and maintenance of proximal tubules.

Growth and differentiation defects in glomeruli of mutant kidneys

The most striking abnormality in kidneys from mutant newborn mice was the failure of glomerular visceral epithelial cells, or podocytes, to undergo their normal program of differentiation. The glomerulus matures at the proximal end of the developing nephrogenic tubule, in a complex morphogenetic process that begins with the invasion of endothelial cells into the glomerular cleft, a space between the lower and middle limb of the late-S-shaped tubule (Potter, 1965). Lining the lower limb of the S-shaped tubule are the visceral epithelial cells that ultimately differentiate into the glomerular podocytes. The invading endothelial cells flatten and form the first capillary loop, which then becomes divided such that five or six (in human) main interconnecting branches are formed (Osathanondh and Potter, 1966b). As this capillary expansion proceeds, the vessels bulge into the podocyte layer and the podocytes become intimately associated with the capillary loops (Osathanondh and Potter, 1966b). The mesangial cells are also found in association with this vascular ingrowth. Finally, the afferent and efferent ends of the capillary system become constricted together to form the glomerular stalk, resulting in the appearance of the mature glomerulus (Potter, 1965). As the podocytes mature during this process, their basal surfaces develop into foot processes, forming a meshwork scaffold, which supports the capillary loops. $\alpha3\beta1$ is highly expressed along the basal surface of the podocyte and is the predominant integrin in this location.

In late S-shaped tubules, and very early glomeruli, the columnar epithelium that contains the presumptive podocytes was indistinguishable in mutant and wild-type kidneys (Fig. 4A,B). Mesangial cells also appeared present throughout all stages. However, as the capillary loops began to form, the podocytes of the mutant appeared to lose their lateral cellular attachments more readily than in the wild type (Fig. 4C,D). The loss of the lateral cell junctions in mutant glomerular epithelial cells supports the possibility that $\alpha3$ integrin may be involved in homophilic interactions, as has been suggested (Sriramarao et al., 1993). In more mature glomeruli, reduced numbers of capillary loops with widened lumina were observed in the mutant, suggesting that capillary branching is defective (Fig. 4E,F). Additionally, as glomeruli matured, the number of podocytes appeared decreased in mutant as compared with wild type, suggestive of a defect in cell division or increased cell death (Fig. 4E,F). Because it is difficult to visualize the number of capillary loops from sections prepared for light microscopy, we stained sections of wild-type and mutant kidney for laminin, which demonstrates the presence of the glomerular basement membrane between epithelial and endothelial cells. This demonstrated that capillary loops were indeed wider and fewer in the mutant kidneys (Fig. 4G,H).

In order to describe mutant glomeruli in more detail, we prepared sections of kidney for electron microscopic analysis. Electron microscopy showed that the cell bodies of podocytes in the mutant were unusually sequestered in the periphery of the glomerulus; these cells extended cytoplasmic projections inward that partially enveloped the widened capillaries (compare Fig. 5A,B). Electron microscopic examination of these mutant glomeruli also revealed that there was a dramatic absence of foot process formation by podocytes (Fig. 5A,B).

The glomerular basement membrane is disorganized in the absence of $\alpha3\beta1$ integrin

An additional observation in the mutant glomeruli indicates that integrins may play an important role in the organization of the ECM during organogenesis. We observed that the glomerular basement membrane(GBM) appeared disorganized and widened in the mutant (Fig. 5C,D). The GBM is believed to have a dual origin, arising as the product of a fusion of single basement membranes produced by podocytes and by the endothelial cells of the underlying capillary loops. In some electron microscopic views, this fusion had failed to occur (Fig. 5C,D). Higher magnification also revealed extreme disorganization and fragmentation of the GBM in many places along both the epithelial and endothelial borders (Fig. 5C,D). This observation suggests that, in addition to serving as a receptor for one or more components of the GBM, $\alpha3\beta1$ integrin may be importantly involved in initiating and maintaining the structural organization of the GBM.

Abnormal branching morphogenesis of the lungs in mutant mice

Mesenchymal-epithelial interactions are also important during the branching morphogenesis of embryonic lung buds that result in formation of the bronchi. In lungs of mutant newborn mice, a marked decrease in the number of branches arising from the major bronchi was observed, such that the wide

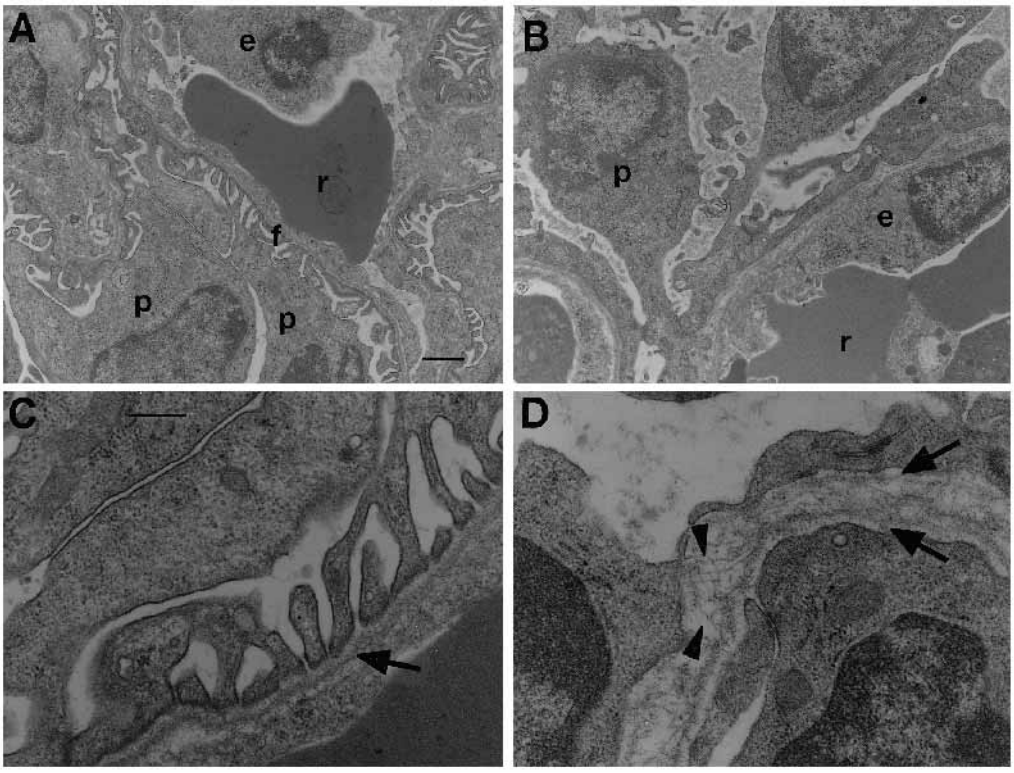

Fig. 5. Electron microscopic analysis of glomeruli (A,B) Podocytes (p) in wild type (A) have foot processes (f); r, red blood cell in capillary; e, endothelial cell. (B) In mutant, foot processes are absent from podocytes. (C,D) Basement membrane disorganization in mutant glomeruli; wild type (C) and mutant (D). The basement membranes are marked with arrows. In the wild type (C), the basement membrane has fused. In the mutant, fusion has not occurred and two basement membranes are present. Loose fragments of basement membrane in mutant (D) are marked with arrowheads. Bars, (A,B) 1.0 μm; (C,D) 0.3 μm.

mainstem bronchi could be followed to the periphery in a single histological section (Fig. 6A,B). The epithelial cells lining these terminal branches, or bronchiole-equivalents, in mutant lungs, were cuboidal, compared with the normally flattened epithelium of terminal bronchioles (compare Fig. 6C,D). This distal cuboidal epithelium in the mutant is most similiar to the cuboidal epithelium lining more proximal bronchi in late embryonic wild-type lung (Fig. 6E). In contrast, the differentiation of the alveoli appeared normal in mutant newborn lungs when compared with wild type. Therefore the decreased branching and immature bronchiolar epithelium suggests that this phenotype is due to a specific defect in bronchial development, rather than an overall immaturity of the lung in mutant newborns.

No abnormalities were found in the intestine or pancreas, two other organs where there is limited expression of α3β1 integrin. However, there are no sites within these organs where α3β1 is uniquely expressed to the exclusion of other integrins. In particular, acinar formation in the pancreas appeared normal, indicating that α3β1 is not required for this morphogenetic process, even though it is expressed in the ducts of this organ.

Expression of α3 integrin in embryonic kidney and lung

α3β1 integrin has previously been shown to be highly expressed in glomeruli of adult and fetal human kidney, and expressed at lower levels by ureteric bud derived structures, including the collecting ducts (Adler, 1992; Ekblom et al., 1991; Patey et al., 1994; Rahilly and Fleming, 1992). However, expression of α3β1 integrin on proximal tubules has not been observed. This raises the question of whether the proximal tubule pathology in mutant kidneys was a secondary effect due to abnormal glomerular development. In order to confirm that expression of α3 integrin in mouse embryonic and newborn kidney is similar to human, we stained kidneys with an antibody to the α3a cytoplasmic domain (DiPersio et al., 1995). Therefore, this analysis will not include expression of the 'b' form of α3. In agreement with these previous reports, we did not observe α3 integrin on proximal tubules, suggesting that the abnormalities observed in these tubules are indeed likely to be secondary to glomerular dysfunction. Also, in agreement with previously published reports, α3β1 was most highly expressed by podocytes in the glomeruli (Fig. 7A), and was observed on collecting ducts, and the branching derivatives of the ureteric bud in the nephrogenic zone of the newborn mouse kidney. This suggests that α3β1 is a receptor for the basement membrane of these cells. We also determined the expression on embryonic mouse lung. α3β1 was expressed basolaterally along the epithelial lobules of the lung buds (Fig. 7B), which is also consistent with a putative function as a basement membrane receptor. Since these expression patterns are consistent with previously published reports that used antisera that should recognize both α3a and α3b (Adler, 1992; Ekblom et al., 1991; Patey et al., 1994; Rahilly and Fleming, 1992), it is unlikely that there is significant expression of α3b in the kidney or lung by cells that do not also express α3a. α3a was not detected in mutant kidney (Fig. 7C) or lung (data not shown).

DISCUSSION

Mice deficient in α3 integrin die during the first day after birth, with severe abnormalities in the kidneys of homozygous mutant newborn mice. Glomerular podocytes lacked foot

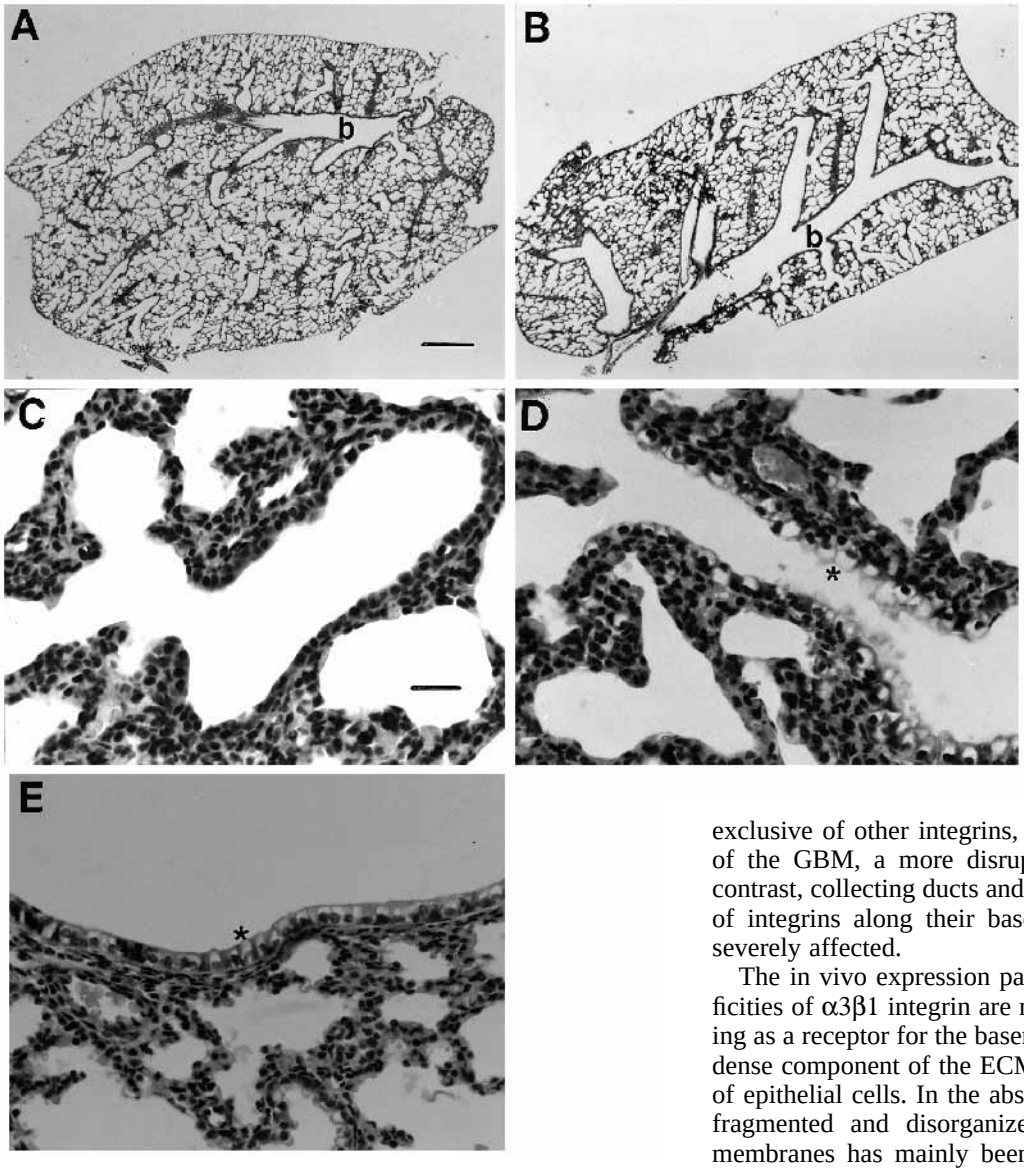

Fig. 6. Analysis of lungs from wild-type and mutant newborn mice and embryos. (A,B) Saggital sections through lungs of wild-type (A) and mutant (B) newborn mice. Major bronchi (b). (C,D) Bronchioles from wild-type (C) and mutant (D) newborn lungs. Cuboidal epithelial cells in mutant terminal bronchioles (*). (E) Cuboidal epithelial cells in wild-type E18 proximal airway (*). Bars (A,B) 0.4 mm; (C-E) 50 μm.

processes, and the glomerular basement membrane appeared fragmented and disorganized. Glomerular capillary loops were fewer in number and were wider than normal. Although mutant kidneys were smaller, the number of nephrons as enumerated by glomerular number appeared unchanged from wild type. Proximal tubules appeared damaged and became microcystic. Branching morphogenesis that gives rise to collecting ducts in the kidney and bronchi in the lung appeared decreased in both organs.

The severity of the defects in *α3 integrin* mutant mice can be related to the local expression of integrins other than α3β1. Other integrins expressed during kidney development include α2β1, which is expressed on glomerular endothelial cells, on epithelial cells of the distal tubules and collecting ducts (Korhonen et al., 1991). Addditionally, α6β1 is expressed on all tubular epithelial cells and transiently on podocytes (Adler, 1992; Ekblom et al., 1991; Patey et al., 1994; Rahilly and Fleming, 1992). During lung development, α2β1, α3β1 and α6β1 are all expressed on the bronchial epithelium (Bartolazzi et al., 1993; Mette et al., 1993). Where α3β1 is expressed exclusive of other integrins, such as along the epithelial side of the GBM, a more disruptive phenotype is observed. In contrast, collecting ducts and bronchi express a greater variety of integrins along their basement membranes, and are less severely affected.

The in vivo expression pattern and putative binding specificities of α3β1 integrin are most consistent with its functioning as a receptor for the basement membrane (basal lamina), a dense component of the ECM present along the basal surface of epithelial cells. In the absence of α3β1, the GBM appears fragmented and disorganized. The assembly of basement membranes has mainly been studied in vitro using purified components, which are able to selfassemble to varying extents, but little is known about how the different elements of this complex structure are joined together in vivo. Entactin, a putative ligand of α3β1 (Dedhar et al., 1992), has been shown to have the important role of serving as a cross-link between laminin-heterotrimers and type IV collagen multimers (Aumailley et al., 1993; Chung et al., 1993; Dong et al., 1995; Mayer et al., 1995). Transfection of cDNAs that encode α3 and β1 integrins into CHO cells confers the ability to assemble a matrix that contains entactin (Wu et al., 1995). This finding, in concert with our observations, raises the prospect that integrins are involved in either actively organizing the basement membrane or, at least, in maintaining the structural integrity of this portion of the ECM. This may occur simply by serving as nucleation points for assembly of the different components.

The cytoskeletal reorganization of podocytes that results in mature foot process formation appears highly dependent on the interaction of α3β1 integrin with the GBM. Loss of foot processes, which is observed in a variety of human glomerular diseases, and in experimental animal models of glomerular disease, is usually thought to be secondary to damage to the

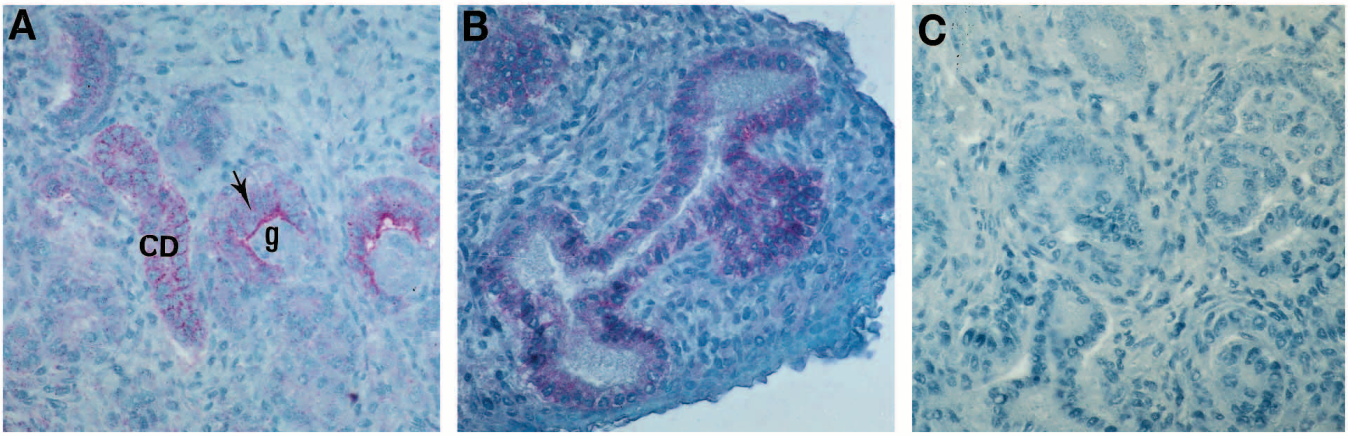

Fig. 7. Expression of α3a integrin in embryonic kidney and lung. Immunocytochemistry was used to demonstrate the presence of α3a integrin in developing organs of an E14 embryo. (A) Staining was present basolaterally in the presumptive podocytes of two early glomeruli (g) and also in an adjacent collecting duct (CD). (B) Basolateral staining is present in a lung bud. (C) No staining is present in the mutant kidney.

GBM. Whether the presence of an organized basement membrane and associated molecules is permissive or instructive for foot process formation and other aspects of epithelial differentiation will be determined by further study. However, to the extent that foot process formation is dependent on recognition of an organized basement membrane, α3β1 integrin is a candidate receptor to mediate this interaction. It is possible that the main role of α3β1 is to organize the GBM, and that other receptors recognize GBM organization and trigger foot process formation. This putative receptor is unlikely to be a known integrin, as α3β1 is the predominant integrin on the basal surface of the podocyte.

Abnormally widened capillary loops were observed in glomeruli from mutant kidneys. Since glomerular endothelial cells also express α3β1 integrin, this may be due either to a primary defect in the ability of these cells to develop the normal capillary structure of the glomerulus. Alternatively, the abnormal capillaries may be a consequence of the failure of the podocytes to provide an adequate scaffolding within which the divided capillaries would develop.

The formation of a branched epithelium during organogenesis is the consequence of mesenchymal-epithelial interactions that are in part mediated by components of the ECM adjacent to the epithelial cells. It has been suggested that mesenchymal cells exert their influence on branch formation by stabilizing the basement membrane in clefts and destabilizing or enzymatically degrading it where branches are to be initiated (Bernfield et al., 1984). If this model is correct, integrins are candidates for the relevant basement membrane receptors on epithelial cells that may transduce signals concerning the status of the basement membrane. Our observation of decreased branching of the medullary collecting ducts and bronchi suggests that signals transduced by α3β1 integrin may be involved in stimulating branch formation. Certainly other integrins are also likely to be involved in branch formation, perhaps even more importantly than α3β1, since the dimunition that we observe in the medulla is only two-fold. Integrins have already been suggested to be important in several cell lines that undergo branching in collagen gels. α2β1 integrin has been shown, using antisense RNA technology to be required for branching in vitro by MDCK cells or gland formation by mammary carcinoma cells (Keely et al., 1995; Saelman et al., 1995). In contrast, α3 integrin levels fall dramatically when a cell line derived from mammary gland epithelium undergoes branching (Berdichevsky et al., 1994). An antibody to α3β1 integrin that blocked adhesion to collagen also stimulated branching by these cells (Berdichevsky et al., 1994). While this result might a priori predict that loss of α3 integrin might lead to increased branching in mutant mice, our opposite results indicate the difficulty of extrapolating from tissue culture to the whole animal.

In α3-integrin-deficient mice, mature foot processes are unable to form. In humans, fusion of previously normal foot processes is commonly observed in a variety of pathological situations where there is damage to the GBM and the proteglycan matrix in which the foot processes are normally invested. Foot process fusion and GBM damage results in the loss of the normal glomerular filtration barrier, leading to heavy loss of protein in the urine, which is the central feature of a clinical state referred to as the nephrotic syndrome. Although we have been unable to obtain consistent measurements of protein concentrations in urine from newborn mice, we predict, based on appearance of the glomeruli, that mutant newborn mice suffer heavy proteinuria. α3β1 expression is retained along the GBM in less severe forms of the nephrotic syndrome (Baraldi et al., 1992; Shikata et al., 1995), In addition, we have examined a case of congenital nephrotic syndrome, a human disease in which normal foot process formation is thought not to occur. α3β1 integrin was present in the glomeruli of this individual (S. Goldstein, M. Donovan and J. Kreidberg, unpublished results). Together, these results suggest that expression of this integrin may be necessary, but not sufficient, for foot process formation. Unfortunately, the short life span of α3 mutant mice precludes long-term study of this potential model for kidney disease. We are currently breeding this mutation onto other strain backgrounds to determine whether we can extend the survival of mutant mice.

The targeted mutation of genes encoding several other integrin subunits has resulted in lethality during the embryonic period. Embryos deficient in β1 integrin are unable to undergo implantation (Fassler and Meyer, 1995; Stephens et al., 1995). Deficiency of α5β1 integrin, a fibronectin receptor, led to

embryonic demise at around E10 due to defects in mesodermal structures (Yang et al., 1993). Mutation of the gene encoding α4 integrin, which is both a fibronectin and VCAM receptor in association with β1 integrin, resulted in embryonic death due to placental and cardiac malformations that are probably due to the failure to bind VCAM rather than fibronectin (Yang et al., 1995). In contrast to these embryonic lethal phenotypes, a mutation in the α1 integrin gene did not result in any loss of viability or obvious phenotype (Gardner et al., 1996). Our results define a crucial role for α3β1 integrin in the development of the kidney and lung. While it is likely that the kidney and lung defects contributed to the neonatal lethality of α3 integrin mutant mice, we have not ruled out additional factors that remain to be determined. We are currently investigating other potential causes of neonatal mortality, such as defects in the nervous system.

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