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Involvement of Laminin Binding Integrins and Laminin-5 in Branching Morphogenesis of the Ureteric Bud during Kidney Development

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Branching morphogenesis of the ureteric bud (UB) [induced by the metanephric mesenchyme (MM)] is necessary for normal kidney development. The role of integrins in this complex developmental process is not well understood. However, the recent advent of *in vitro* model systems to study branching of UB cells and isolated UB tissue makes possible a more detailed analysis of the integrins involved. We detected integrin subunits α3, α6, β1, and β4 in both the UB and cells derived from the early UB. Blocking the function of each of these integrin subunits individually markedly inhibited branching morphogenesis in cell culture models. However, inhibiting individual integrin function with blocking antibodies in whole kidney and isolated UB culture only partially inhibited UB branching morphogenesis, suggesting that, in these more complex *in vitro* systems, multiple integrins are involved in the branching program. In whole organ and isolated bud culture, marked retardation of UB branching was observed only when both α3 and α6 integrin subunits were inhibited. The α6 integrin subunit can be expressed as both α6β1 and α6β4, and both of these β subunits are important for UB branching morphogenesis in both cell and organ culture. Furthermore, laminin-5, a common ligand for integrins α3β1 and α6β4, was detected in the developing UB and shown to be required for normal UB branching morphogenesis in whole embryonic kidney organ culture as well as isolated UB culture. Together, these data from UB cell culture, organ culture, and isolated UB culture models indicate that both integrin α3 and α6 subunits play a direct role in UB branching morphogenesis, as opposed to being modulators of the inductive effects of mesenchyme on UB development. Furthermore the data are consistent with a role for laminin-5, acting through its α3β1 and/or α6β4 integrin receptors, in UB branching during nephrogenesis. These data may help to partially explain the renal phenotype seen in integrin α3 and α3/α6 subunit-deficient animals.

Key Words: **branching morphogenesis; kidney development; integrins; rat.**

INTRODUCTION

The development of the collecting system of the kidney begins with the interaction of the Wolffian duct with the metanephric mesenchyme (MM). The first phase of this process involves outgrowth of epithelial cells of the ureteric bud (UB) found near the MM; these cells will go on to form the entire urinary collecting system, from the bladder trigone through the collecting tubules. The UB grows a short distance until it contacts and penetrates the MM, leading to a period characterized by many iterations of branching morphogenesis. At each round of dichotomous branching, the number of UB tips doubles, and each of these tips is then available for the induction of additional nephrons (Davies and Davey, 1999; Pohl *et al.*, 2000c; Sakurai and Nigam, 2000; Stuart and Nigam, 2000; Wallner *et al.*, 1997, 1998).

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Cell–extracellular matrix interactions are thought to be an essential component of the normal development of the collecting system. In cell culture models of renal epithelial cell tubulogenesis in 3-D, the matrix composition is crucial to the phenotype observed (Pohl *et al.*, 2000a; Sakurai *et al.*, 1997; Santos and Nigam, 1993). The growing UB is believed to extensively remodel matrix as it grows, and the matrix in turn transduces signals that influence the growth characteristics of the cells. The predominant receptors that mediate the interactions between cells and extracellular matrix are integrins. Therefore, integrins and their interactions with various extracellular matrix components are believed to be essential in growth and branching of the UB and consequently for normal kidney development.

A role for integrins in renal development, and more specifically UB branching morphogenesis, has been demonstrated in genetically mutated mice. For example, α3-deficient mice exhibit a phenotype in the renal collecting system with fewer than normal collecting ducts in the papilla, suggesting decreased branching morphogenesis during development (Kreidberg *et al.*, 1996). Surprisingly, a renal phenotype is not described in α6-deficient mice (Georges-Labouesse *et al.*, 1996). However, when α3- and α6-deficient animals are crossed, the mice do not develop ureters (derivatives of the UB) and the kidneys have a similar phenotype to that seen in the α3-deficient animals (De Arcangelis *et al.*, 1999). α8 integrin-deficient mice also develop a severe renal abnormality with defective branching and growth of the UB and recruitment of mesenchymal cells into epithelial structures (Muller *et al.*, 1997). This phenotype is thought to be due to an abnormality of $\alpha 8\beta 1$ induction on the mesenchymal cells by the UB.

Nevertheless, there is a paucity of *in vitro* data that demonstrates a role of integrins in renal development. Integrin $\alpha 6\beta 1$ was shown to be important in *in vitro* conversion of metanephric mesenchyme to epithelial tubules in whole-organ culture models (Falk *et al.*, 1996). In the same model system, αv integrins were demonstrated to play a role in normal metanephric morphogenesis of the kidney with respect to proliferation of nephrons as well as organization of the UB branches (Wada *et al.*, 1996). While genetic and whole organ culture experiments suggest important roles for integrins in kidney development as a whole, they do not address the important question of whether these integrins participate largely in MM or UB morphogenesis. To date, there are no *in vitro* studies directly addressing the role of integrins on UB branching morphogenesis. Furthermore, although there is a good model to study *in vitro* branching morphogenesis of UB cells in 3-D culture (Sakurai *et al.*, 1997), with the exception of hyaluronic acid and its receptor CD44 (Pohl *et al.*, 2000b), there are no data on the role of specific matrix molecules and their receptors in this process. Likewise, while a robust model for growth and morphogenesis of the isolated UB (in the presence of soluble factors and matrix) has recently been developed (Qiao *et al.*, 1999), the roles of specific integrins and matrix molecules in this process are not clear and remain to be elucidated.

We therefore analyzed the role of integrins in *in vitro* models of the developing UB. These models included a cell culture model of UB cells, whole-organ kidney culture, and isolated UB culture (a system free of direct interactions with the MM). Utilizing these systems, we demonstrate that, similar to the knockout mouse, inhibiting integrin $\alpha 3\beta 1$ function hinders branching morphogenesis of the UB. In contrast to the α6 knockout animal where there is no renal phenotype, we demonstrate a direct role for integrin α6 subunit in UB branching morphogenesis which is, at least in part, $\alpha 6\beta 4$-dependent. We also demonstrate an additive effect by simultaneous blocking both α6 and α3 integrin subunits, which results in a more severe phenotype of the developing UB than blocking either of these integrins alone. Finally, we demonstrate that laminin-5, a common ligand for $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, is expressed in the UB, and a well-characterized functional blocking antibody directed against laminin-5 inhibits normal UB development in both whole-kidney and the isolated UB culture.

MATERIALS AND METHODS

Materials

Growth factor-reduced Matrigel and rat type I collagen were obtained from Becton Dickinson (Franklin Lakes, NJ). Growth factors (FGF1 and GDNF) were from R&D Systems (Minneapolis, MN). Antibodies against integrin subunits α1 (Ha31/8), α6 (GoH3), β1 (Ha2/5′, HMβ1-1, and 9EG7), and β4 (346-11A) were obtained from Pharmingen (San Diego, CA). Antibodies against integrin subunit α3 (Ralph 3.2) were obtained from Santa Cruz Laboratories (Santa Cruz, CA). Anti-laminin-5 antibodies (2778 and CM6) were isolated and purified as previously described (Plopper *et al.*, 1996; Gianelli *et al.*, 1997). Fluorescein-conjugated Dolichus biflorus lectin was from Vector Laboratories (Burlingame, CA). Except for the Transwells (clear, polyester, 0.4-μM pore; Costar, Cambridge, MA), all plasticware was from Falcon (Lincoln Park, IL). Anti-rat IgG antibody-coated Sepharose beads were from ICN/Cappel (Aurora, OH). [^{35}S]-Methionine and "Amplify" fluorographic reagent were obtained from Pharmacia-Amersham (Picataway, NJ). All other chemicals and reagents, unless otherwise indicated, were from Sigma (St. Louis, MO).

Cell Culture and Generation of Conditioned Medium

Immortalized UB and MM (BSN) cells were cultured and maintained as previously described (Sakurai *et al.*, 1997). BSN-conditioned medium was generated and harvested as previously described (Sakurai *et al.*, 1997). Renal papillae cells derived from either wild-type mouse E18 embryonic kidneys (B7 cells) or integrin subunit α3 (−/−) mouse E18 embryonic kidneys (B12 cells), as well as B12 cells reconstituted with the human α3 integrin subunit (R10 cells), were cultured and maintained as previously described (Wang *et al.*, 1999).

Three-Dimensional Cell Culture

Tubulogenesis of UB-derived cells (UB, B7, B12, and R10) cells was assayed in three-dimensional (3D) extracellular matrix gels composed of a 1:1 mixture of collagen [0.1 mg/ml type I collagen in Dulbecco's minimal essential media containing 20 mM Hepes (pH 7.2)] and growth factor-reduced Matrigel (Sakurai *et al.*, 1997). For quantification, cells that formed branching structures (defined as more than 1 branch), were counted in five randomly picked high-power fields.

Culture of Isolated Embryonic Kidneys

Embryonic kidneys isolated from either day 13 rat (Sprague–Dawley) or day 10.5 mouse (Swiss–Webster) embryos (day 0 of gestation coincided with appearance of the vaginal plug) were applied to the top of Transwell filters placed within individual wells of a 12-well tissue culture dish. The isolated kidneys were cultured (37°C and 5% CO_2/100% humidity) in DMEM/F12 media supplemented with 10% FBS with or without the antibodies described. Following 3 days in culture, the kidneys were fixed in 4% paraformaldehyde and stained with fluorescein-conjugated Dolichus bifluros lectin.

Isolation and Culture of UB

UBs separated from the MM of E13 rat kidneys were suspended within an extracellular matrix gel (see above) applied to the top of a Transwell filter and cultured in BSN-CM supplemented with 10% FBS, 125 ng/ml GDNF and 500 ng/ml FGF1 for 7–14 days in the absence or presence of indicated antibodies as previously described (Qiao *et al.*, 1999).

Lectin Staining and Immunohistochemistry

Whole cultured kidneys or UB cells grown in three-dimensional extracellular matrix gels were washed three times in PBS and fixed in 4% paraformaldehyde for 1 h at room temperature and stained with a fluorescein-coupled lectin from Dolichus biflorus (Qiao *et al.*, 1999). Samples were then washed overnight in PBS–saponin, counterstained with propidium iodine for 10 min, washed again in PBS–saponin, and visualized by confocal microscopy. Frozen sections of embryonic kidneys, either mouse (E11) or rat (E13) were fixed in 4% paraformaldehyde and processed for immunohistochemistry as previously described (Pohl *et al.*, 2000b). Localization of integrin subunits α6 or β4, as well as laminin-5, was determined using biotin-labeled secondary antibody and immunoperoxidase staining (Pohl *et al.*, 2000b).

RT-PCR

Day E13 UBs were separated from MM and Poly(A) RNA was isolated by using the Micro Poly(A) Pure Kit (Ambion). PCR was performed on the generated cDNAs where the annealing temperatures for the primers were 66°C for the integrins and 70°C for laminin-5. PCR was performed by using Hotstart PCR Taq DNA polymerase (Qiagen) and 35 cycles were performed. The primer pairs were as follows: integrin β1, forward GGAGGAATGTAACACGACTGC, reverse CAGATGAACTGAAGGACCACC with an expected product size of 701 bases; integrin α3, forward GTCTGGAAACCTTGTCAACCC, reverse CAACCACAGCTCAATCTCAGC with an expected product size of 671 bases; integrin α4, forward AATGGATAAAGCAGAGGACG, reverse CTGGGTGTTTTGATAATGG with an expected product size 229 bases; integrin α6, forward CCCAAGGAGATTAGCAATGGC, reverse CAGTCTTTGAGGGAAACACCG with an expected product size of 452 bases; integrin β4, forward GAGCGCAACTTTGTGTTCAAGG, reverse GGGTCTTATACTGGGTGTAGG with an expected product size of 560 bases; laminin-5 γ2, chain forward GACGAGAATCCTGACATTGAG, reverse CTGGTCCATCTGAGTCTTCAC with an expected product size of 600 bases.

Flow Cytometry

UB cells were disassociated from tissue culture dishes and exposed to diluted monoclonal antibodies of the appropriate integrin, followed by appropriate secondary antibodies (fluorescein isothiocyanate-coupled rabbit anti-rat or hamster immunoglobulin). Flow cytometry was performed with a FACScan instrument (Becton Dickinson).

Immunoprecipitation

Confluent monolayers of UB cells were labeled overnight in methionine-free growth medium in the presence of [^{35}S]-methionine (50 μCi/ml). Embryonic kidneys (~100) were isolated from day 10.5 mouse (Swiss–Webster) embryos (day 0 of gestation coincided with appearance of the vaginal plug) and applied to the top of a Transwell filter placed within a well of a 12-well tissue culture dish. The isolated kidneys were labeled overnight in methionine-free growth medium in the presence of [^{35}S]-methionine (1 mCi/ml). At the end of the labeling period, cells and kidneys were rinsed three times with PBS and then lysed on ice for 20 min in lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.05% Tween 20, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml each leupeptin, pepstatin A, and antipain)]. Samples were then triturated by repeated passages (three) through a tuberculin syringe. Lysates were placed in clean microfuge tubes and cleared of cellular debris by centrifugation at 14,000*g* for 30 min. Supernatants were removed to fresh microfuge tubes and precleared by incubation with ~50 μl of anti-rat IgG-coated Sepharose beads for 1 h at 4°C. Following preclearing, samples were centrifuged (14,000*g* for 5 min) to pellet the beads and the supernatants were removed and each was divided equally between two clean microfuge tubes. Supernatants were then incubated overnight at 4°C in the absence or presence of anti-α6 antibody. The next morning, anti-rat IgG-coated Sepharose beads were added to each tube and the samples were incubated for 1 h at 4°C. The beads were collected by centrifugation, washed five times in lysis buffer, boiled in sample buffer without reducing agents, and subjected to nonreducing SDS-PAGE on 4–20% gradient gels. The gels were subsequently fixed, stained with Coomassie blue, and destained. The gels were then treated with "Amplify" fluorographic reagent according to manufacturer's instructions, dried, and exposed to film.

Quantification of UB Branching

Confocal microscopic images of UBs stained with fluorsceinconjugated Dolichus biflorus lectin were analyzed by using Image-Pro Plus (Media Cybernetics). The degree of branching morphogenesis was quantified by using the number of endpoints as a correlative measure of the number of branching events. Assays were performed at least in triplicate and error bars represent the standard errors of the mean. *P* values were calculated with the Student's *t* test.

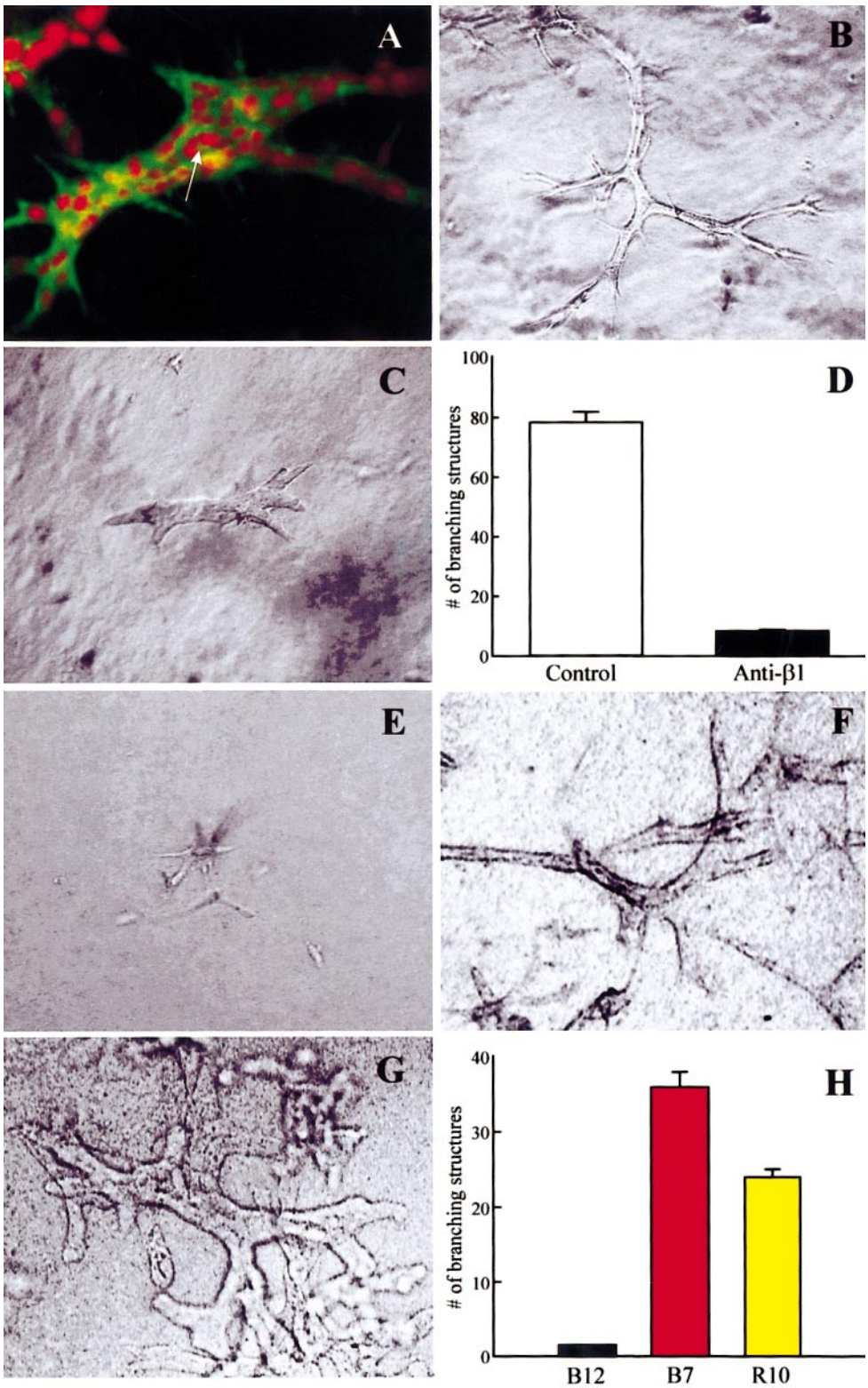


FIG. 1. Ureteric bud-cell morphogenesis is dependent on integrins $\beta 1$ and $\alpha 3$. (A) Fluorescent micrograph of cultured UB cells grown for 12 days in a 3-D extracellular matrix (ECM) gel composed of a 1:1 mixture of collagen (Type I) and Matrigel. The cells were stained with *D. biflorus* lectin (green) and propidium iodine (orange). The UB cells form multicellular-branched tubular structures with lumens (indicated

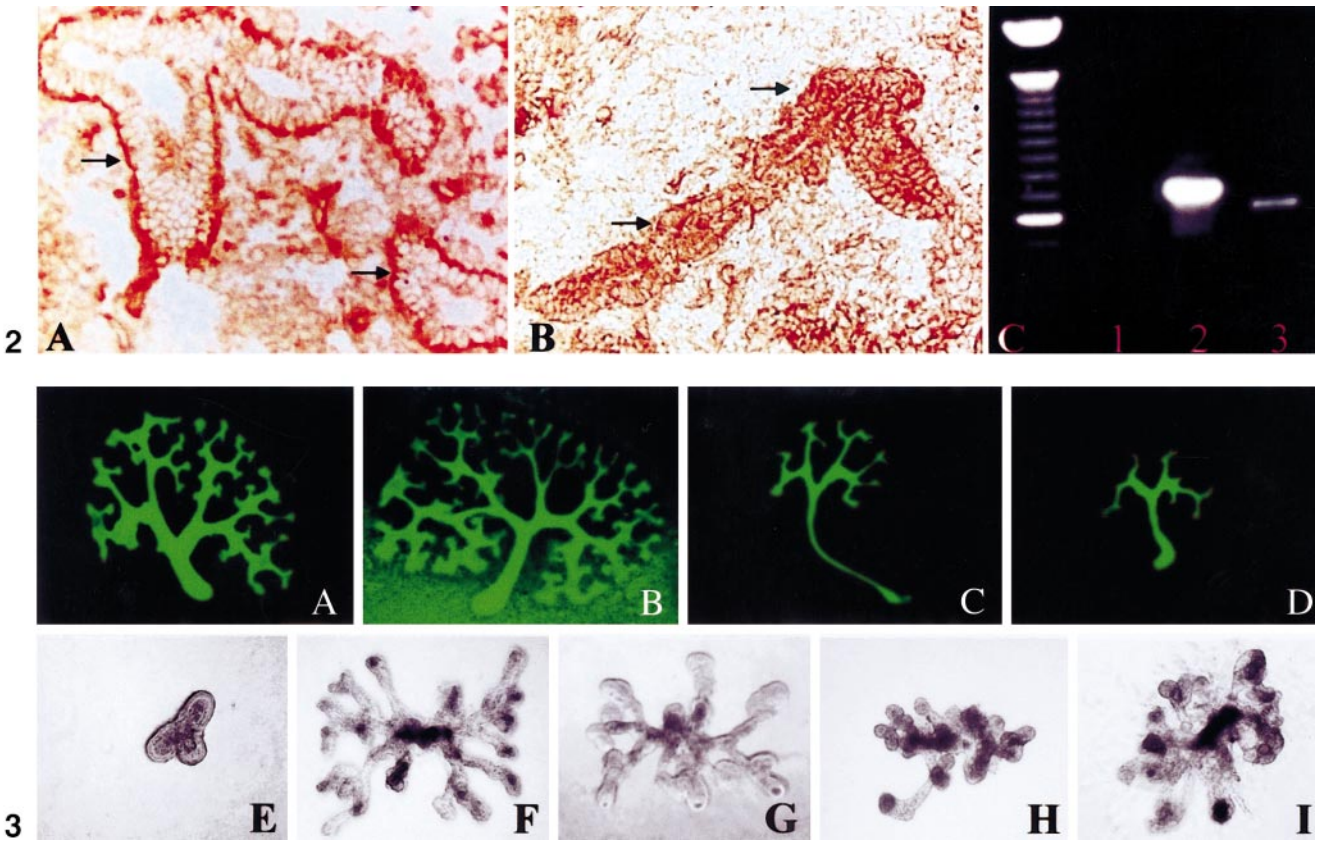


FIG. 2. Integrin α3 and β1 subunits are expressed in tubules derived from the ureteric bud. Immunohistochemical localization of integrin subunits α3 (A) and β1 (B) to the UB in frozen sections of embryonic day 13 rat kidneys utilizing antibodies Ralph 3.2 and HMβ1-1, respectively. These integrins are expressed by UB-derived structures (arrows). (C) RT-PCR analysis of the expression of integrin subunits α4 (lane 1), β1 (lane 2), and α3 (lane 3) in rat E13 ureteric bud.

FIG. 3. Integrin α3 and β1 subunits are necessary for normal ureteric bud development. (A–D) Fluorescent examination of the developing UB in whole rat kidneys stained with fluorescent-conjugated lectin from *D. biflorus*. Embryonic day 13 rat kidneys were grown for 3 days in the presence of either isotype IgG (A-control), anti-α1 antibody (Ha31/8-20 μg/ml) (B-negative control), anti-α3 antibody (Ralph 3.2-20 μg/ml) (C), or anti-β1 antibody (HA2/5-20 μg/ml) (D). (E–I) Phase contrast micrographs of UBs isolated from embryonic day 13 rat kidney and cultured in 3-D ECM gels for 14 days in the absence (F-control) or presence of either anti-α1 antibody (G), anti-α3 (H), or anti-β1 antibody (I). All of the buds grew from a small T-shaped structure (E).

RESULTS

Integrin α3β1 Is Required for Normal Branching Morphogenesis of UB

The renal collecting system in α3-deficient mice has a reduction in the number of medullary collecting ducts relative to normal mice. This abnormality may either be due to decreased branching morphogenesis of the UB or collecting ducts that are somehow lost after nephron formation (Kreidberg *et al.*, 1996). Utilizing cell and organ culture models, as well as the isolated UB culture system (in the absence of the MM) (Qiao *et al.*, 1999), we tested the

by the arrow). (B, C) Phase contrast photomicrographs of UB cells cultured for 12 days in 3-D ECM gels in the absence (B-control) or presence of a blocking anti-β1 integrin subunit blocking antibody (HA2/5) (C). UB cell tubule formation and branching morphogenesis is markedly inhibited (C). (D) Graph of inhibition by the anti-β1 integrin subunit blocking antibody (HA2/5) relative to control (quantified with respect to the number of branching structures per high power field). (E–G) Phase contrast photomicrographs of 3-D culture of renal papillae cells derived from E18 embryonic kidneys from either α3 integrin-deficient mice (B12) (E), wild-type mice (B7) (F), or B12 cells reconstituted with human α3 integrin subunit (R10) (G). B12 cells form markedly less branching multicellular structures (E) than B7 (F) or R10 cells (G). (H) Graph demonstrating the relative differences in branching multicellular structures formed by B12, B7, and R10 cells in 3-D ECM gels.

importance of integrin $\alpha3\beta1$ in branching morphogenesis of the UB.

When UB cells are grown in 3-D gels, they proceed through defined stages including the extension of cellular processes, formation of multicellular cords, and eventually, formation of branching tubular structures which bear a resemblance to the branching UB in the developing kidney (Figs. 1A and 1B) (Pohl *et al.*, 2000a,b; Sakurai *et al.*, 1997). This process is dependent on the functional integrity of $\beta1$ integrin subunits as UB branching morphogenesis is markedly inhibited by the addition of anti-$\beta1$ integrin functional blocking antibodies (Figs. 1C and 1D).

To test the importance of integrin $\alpha3\beta1$ in cells derived from the UB, we utilized well-characterized renal papillae cells (Wang *et al.*, 1999) derived from embryonic day 18 kidneys isolated from $\alpha3$ $(-/-)$-deficient mice (B12). As expected, B12 cells did not form tubules in culture (Figs. 1E and 1H). In contrast, renal papillae cells derived from wild-type animals (B7) as well as $\alpha3-/-$ cells (B12) that were stably transfected with the human $\alpha3$ integrin subunit (R10) undergo branching morphogenesis, similar to normal UB cells grown in 3-D gels (Figs. 1F and 1G). Although the morphology of the tubules formed by B7 and R10 cells were similar, there were somewhat fewer branching structures and the tubules were of larger diameter in the R10 cells (Figs. 1G and 1H). These differences in morphology between the wild-type and reconstituted cells may be explained by the fact that these cells express less $\beta1$ integrin-subunit than the wild-type cells (Wang *et al.*, 1999). Nevertheless, these results indicate that integrin $\alpha3\beta1$ is necessary for branching morphogenesis of embryonic renal collecting cells.

We next assessed the role of integrin $\alpha3\beta1$ in UB branching morphogenesis in the context of organogenesis. We first confirmed that $\alpha3\beta1$ integrins are expressed in the early developing kidney and more specifically in the developing UB. This was done utilizing immunohistochemistry to demonstrate $\alpha3$ and $\beta1$ integrin subunit expression in the developing collecting system of E13 rat kidneys (Figs. 2A and 2B) as well as by RT-PCR of cDNA derived from these structures (Fig. 2C).

Two model systems were utilized to determine whether integrin $\alpha3\beta1$ is important for normal UB morphogenesis, and hence, kidney development. In the first, rat embryonic kidneys were dissected on day 13, placed on filters, and allowed to grow over a period of 3 days. The kidneys were then stained with *D. biflorus* and viewed by confocal microscopy, allowing one to visualize only structures that are UB in origin (Laitinen *et al.*, 1987). As seen in the control, the metanephric kidney developed to form a well-defined structure associated with a multibranched UB (Fig. 3A). A similar result was observed when a blocking monoclonal antibody to integrin $\alpha1$ subunit was added to the medium (Fig. 3B). In contrast, when rat kidneys were grown in the presence of integrin $\alpha3$ subunit blocking antibodies, there was inhibition of branching morphogenesis (Fig. 3C). The metanephric kidney was much smaller (data not shown) and had far fewer branches of the UB. The results with the antibody to integrin $\alpha3$ subunits were similar to those observed when day 13 embryonic rat kidneys were exposed to $\beta1$ integrin subunit blocking antibodies (Fig. 3D). To control for both inter- and intra-experimental variation, we quantified the number of end points (ampullae) of the UBs in the developing kidney. This is a measure of UB branching where the number of end points correlates directly with the number of branching events. As seen in Table 1, the number of UB end points in both the $\alpha3$ ($P < 0.001$) and $\beta1$ ($P < 0.001$) antibody-treated kidneys is significantly different from control kidneys. These results indicate that $\alpha3\beta1$ integrins play a role in renal development.

TABLE 1

Functional Blocking of Integrin Subunits as well as Laminin-5 Inhibits Ureteric Bud Development in Cultured Whole Rat Kidney

Antibody treatment	Number of end points (ampullae) (% of control)
Control	100 ± 6.06
$\alpha3$	33.59 ± 4.45**
$\beta1$	30.54 ± 7.75*
$\alpha6$	40.92 ± 5.56**
$\alpha3/\alpha6$	20.07 ± 1.88**
Laminin-5	64.9 ± 7.99*

Note. Photomicrographs of cultured whole rat kidney stained with FITC-labeled *Dolichus biflorus* lectin were analyzed by using Image-Pro Plus (Media Cybernetics). The number of end points (ampullae) on each ureteric bud was determined. Mean ± SEM; $N \geq 4$; *, $P \leq 0.05$; **, $P \leq 0.001$.

One of the major drawbacks of whole-organ culture of kidneys is that it is difficult to differentiate the inductive effects of the mesenchyme from the development of the UB per se. Thus, it is possible that inhibiting integrin $\alpha3\beta1$ function may result in a UB phenotype due to alteration of the inductive effect of the mesenchyme. This question was addressed by utilizing a qualitative culture system in which isolated E13 rat UBs are grown in a 3-D matrix in the absence of mesenchyme (Qiao *et al.*, 1999). Under these conditions, the induced T-shaped UBs grew and branched to form multibranched structures (Figs. 3E and 3F). Addition of a functional blocking antibody to the $\alpha1$ integrin subunit did not affect UB branching (Fig. 3G); however, the addition of a functional blocking antibody to the $\alpha3$ integrin subunit disrupted both the branching and elongation of the UB (Fig. 3H). When $\beta1$ subunit function was inhibited, the UB branched somewhat less than control and the stalks did not grow (Fig. 3I). These results support the idea that integrin $\alpha3\beta1$ exerts its effect on the UB itself and suggest that the abnormalities seen in the $\alpha3\beta1$-deficient mouse are more likely to be due to decreased branching morphogenesis of the UB itself rather than collecting duct loss after nephron formation.

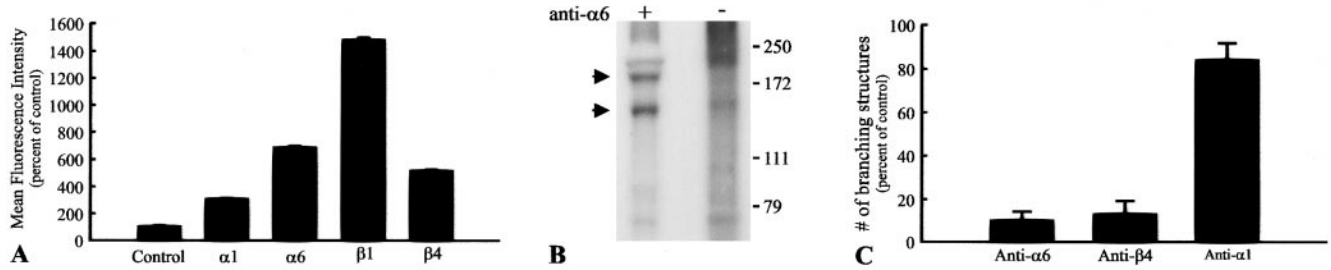


FIG. 4. Integrins $\alpha6\beta1$ and $\alpha6\beta4$ are necessary for UB cell branching morphogenesis. (A) Flow cytometry was performed on UB cells with monoclonal antibodies against integrin subunits $\alpha1$ (Ha31/8), $\alpha6$ (GoH3), $\beta1$ (9EG7), and $\beta4$ (346-11A). Expression of the various integrin subunits is expressed as mean fluorescence intensity (MFI) relative to control where only secondary antibody was used. (B) Autoradiograph of UB cells labeled with [^{35}S]-methionine and immunoprecipitated in the absence (−) or presence (+) of an anti-$\alpha6$ integrin antibody (GoH3). Bands of approximately 150 and 200 kDa, corresponding to the nonreduced molecular masses of $\alpha6$ and $\beta4$ integrin subunits, respectively, are apparent in samples exposed to anti-$\alpha6$ integrin antibody (+). (C) The effect of antibodies to integrin subunits $\alpha6$ (GoH3), $\beta4$ (346-11A), and $\alpha1$ (Ha31/8) on tubulogenesis in 3-D culture was quantified and demonstrated graphically as a percentage of control.

Integrin α6 Is Required for Normal Branching Morphogenesis of UB Cells as Well the UB

As seen above, neither the $\alpha3$ or $\beta1$ integrin subunit antibodies inhibited organ culture or UB culture as much as in the tubulogenesis model. This suggested that other integrin subunits might be playing a role in our models of UB branching morphogenesis. The $\alpha6$ integrin subunit was a likely candidate as integrin $\alpha6\beta1$ has been shown to play a role in renal development *in vitro* (Falk *et al.*, 1996). Furthermore, there is functional data that integrin $\alpha3/\alpha6$-deficient mice do not develop ureters (De Arcangelis *et al.*, 1999), which are derived from the UB. These data, together with the fact that $\alpha6\beta1$ and $\alpha6\beta4$ integrins are capable of interacting with basement membrane components found in the developing UB, prompted us to investigate the role of the $\alpha6$ integrin subunit in UB development.

To do this, we screened UB cells for integrin expression and confirmed that the integrin $\alpha6$-subunit is highly expressed (Fig. 4A). As $\alpha6$ preferentially heterodimerizes with $\beta4$ but will also heterodimerize with $\beta1$ (Hemler *et al.*, 1989), we determined which β integrin subunit $\alpha6$ predominantly heterodimerized with in UB cells. UB cells were metabolically labeled with [^{35}S]-methionine, lysed, immunoprecipitated with an anti-integrin $\alpha6$ antibody, and subjected to nonreducing SDS-PAGE. As demonstrated in Fig. 4B, bands of approximately 150 and 200 kDa, which correspond to the molecular masses of $\alpha6$ and $\beta4$ subunits, respectively, are present. There is no evidence of a 120-kDa band that would correspond with the $\beta1$ integrin subunit. These results suggest that $\alpha6$ is present predominantly in the form of integrin $\alpha6\beta4$ in the UB cell line. To test the functional significance of integrin $\alpha6\beta4$ in UB cell tubulogenesis, we grew UB cells in 3-D culture in the presence of blocking antibodies to either $\alpha6$ or $\beta4$ integrin subunits. As seen in Fig. 4C, both of these antibodies inhibited the formation of branching multicellular structures. In contrast, addition of an $\alpha1$ integrin subunit blocking antibody did not inhibit tubulogenesis despite the fact it was expressed, albeit at a lower level than $\alpha6$, $\beta1$, or $\beta4$ subunits (Fig. 4A). Thus, we conclude that both the $\alpha6$ and $\beta4$ integrin subunits are necessary for UB cell branching morphogenesis, and blocking either of these integrin subunits is sufficient to inhibit this process.

Prior to assessing the role of integrin subunit $\alpha6$ in UB branching morphogenesis, utilizing both whole-kidney and isolated UB culture, we reexamined its expression pattern in the developing kidney. There is controversy as to whether $\alpha6$ is expressed in the developing kidney as integrin $\alpha6\beta1$ alone or together with integrin $\alpha6\beta4$ (Falk *et al.*, 1996). Integrin $\alpha6\beta4$ is highly expressed in UB cells and laminin-5, a ligand for integrins $\alpha6\beta4$ and $\alpha3\beta1$ is highly expressed in both the adult (Mizushima *et al.*, 1998) and developing collecting system (Airenne *et al.*, 2000), suggesting that integrin $\alpha6\beta4$ may be present in the developing UB. We assessed the expression of integrin $\alpha6\beta4$ in the developing kidney, and more specifically, the UB by a number of techniques. First, we performed RT-PCR of cDNA extracted from E13 rat UB and demonstrated that both integrin $\alpha6$ and $\beta4$ subunits are present (Fig. 5A). Furthermore these transcripts are present throughout kidney development as shown by nonquantitative RT-PCR of cDNA extracted from whole-rat embryonic kidneys at days E13 through newborn fetus (Fig. 5B). We next assessed whether the $\alpha6$ integrin subunit primarily heterodimerized with the $\beta4$ subunit in the embryonic kidney. This was done by dissecting out mouse E13 kidneys, metabolically labeling them with [^{35}S]-methionine, and immunoprecipitating the lysate with an antibody to the $\alpha6$ integrin subunit. As seen in Fig. 5C, lane 1, we precipitated a prominent band with a molecular weight of 150 kDa and a less prominent band of approximately 200 kDa. These bands correspond to the expected molecular masses of $\alpha6$ and $\beta4$ integrin subunits under nonreducing conditions, respectively. Bands of similar molecular weight were immunoprecipitated from UB

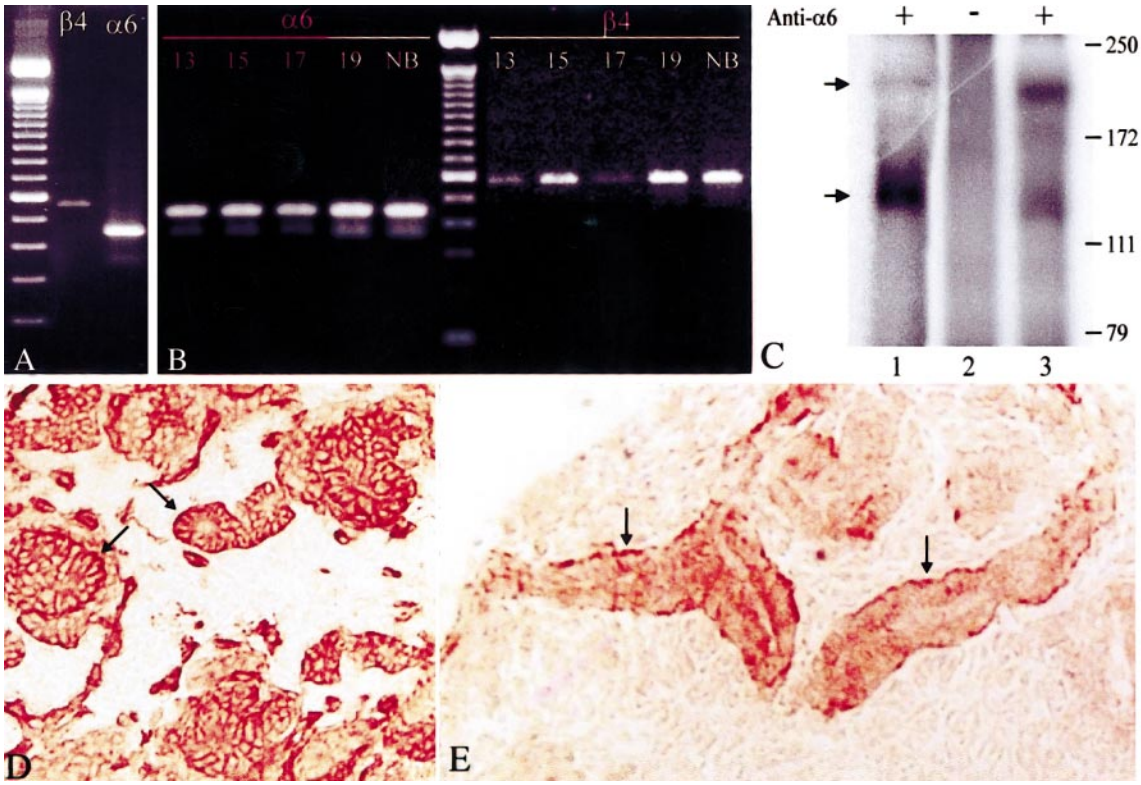


FIG. 5. Integrins $\alpha6\beta1$ and $\alpha6\beta4$ are expressed by the ureteric bud. (A, B) RT-PCR analysis of the expression of integrin subunits $\beta4$ and $\alpha6$ in rat E13 UBs (A) and in E13, E15, E17, E19, and newborn rat kidneys (B). (C) Autoradiograph of embryonic E13 mouse kidneys (1) and UB cells (2, 3) labeled with [^{35}S]-methionine and immunoprecipitated in the absence (−) or presence (+) of an anti-$\alpha6$ integrin antibody (GoH3). Bands of approximately 150 and 200 kDa corresponding to the nonreduced molecular masses of $\alpha6$ and $\beta4$ integrin subunits, respectively, are apparent in samples exposed to anti-$\alpha6$ integrin antibody (+). (D, E) Immunohistochemical localization of integrin subunits $\alpha6$ (D) and $\beta4$ (E) in cryostat sections of either embryonic day 13 rat kidneys (D) or embryonic day 14 mouse kidneys (E) utilizing antibodies GoH3 and 346-11A, respectively.

cells (Figs. 4B and 5C, lane 3) and, once again, there was no obvious band at 120 kDa, suggesting that $\beta1$ was not quantitatively coimmunoprecipitated with anti-integrin $\alpha6$ antibodies. This suggests that integrin $\alpha6$ subunit predominantly heteodimerizes with $\beta4$ in the embryonic kidney. Finally, to confirm that both $\alpha6$ and $\beta4$ integrin subunits are expressed in the UB, we undertook immunohistochemical studies of rodent embryonic kidneys. We demonstrate in Figs. 5C and 5D, respectively, that $\alpha6$ is present in UB-derived structures of E14 rat kidneys, and $\beta4$ is found on the UB-derived collecting tubules of E13 mouse kidney (no antibodies were available for rat $\beta4$). These results suggest that the integrin $\alpha6$ subunit is present in the UB predominantly as integrin $\alpha6\beta4$.

To assess the role of the integrin $\alpha6$ subunit in UB branching morphogenesis, we added $\alpha6$ integrin-blocking antibodies to both whole-kidney culture and isolated UB culture systems. As seen in Fig. 6B, addition of the $\alpha6$ antibody to whole-kidney culture resulted in decreased branching of the UB. This result is less severe than that seen in the presence of $\alpha3$ antibodies (Fig. 3B). When blocking antibodies to both $\alpha3$ and $\alpha6$ were added, there was marked inhibition of both growth and branching of the UB (Fig. 6C) and the inhibitory effect was much more severe than that seen with addition of antibodies to $\alpha3$, $\alpha6$, or $\beta1$ alone (compare Figs. 6C, 6B, 3C, and 3D). These effects were also evident when branchpoints were quantified (Table 1). The antibody to integrin subunit $\alpha6$ significantly inhibited the branch point number relative to controls ($P < 0.001$); however, the combined effect of the $\alpha3$ and $\alpha6$ antibody was greater than inhibition by any of the integrin subunit blocking antibodies alone (Table 1). In fact, this difference was statistically different when compared with either $\alpha3$ ($P < 0.02$) or $\alpha6$ ($P < 0.01$) antibodies alone; however, this was not the case when compared to the $\beta1$ antibody ($P < 0.2$). A similar result to whole-organ culture was seen in the isolated UB culture model. When the bud was exposed to integrin $\alpha6$ subunit antibody alone (Fig. 6E), it was smaller and did not elongate as much as the control buds (Fig. 6E). However, when the UB was exposed to both $\alpha3$

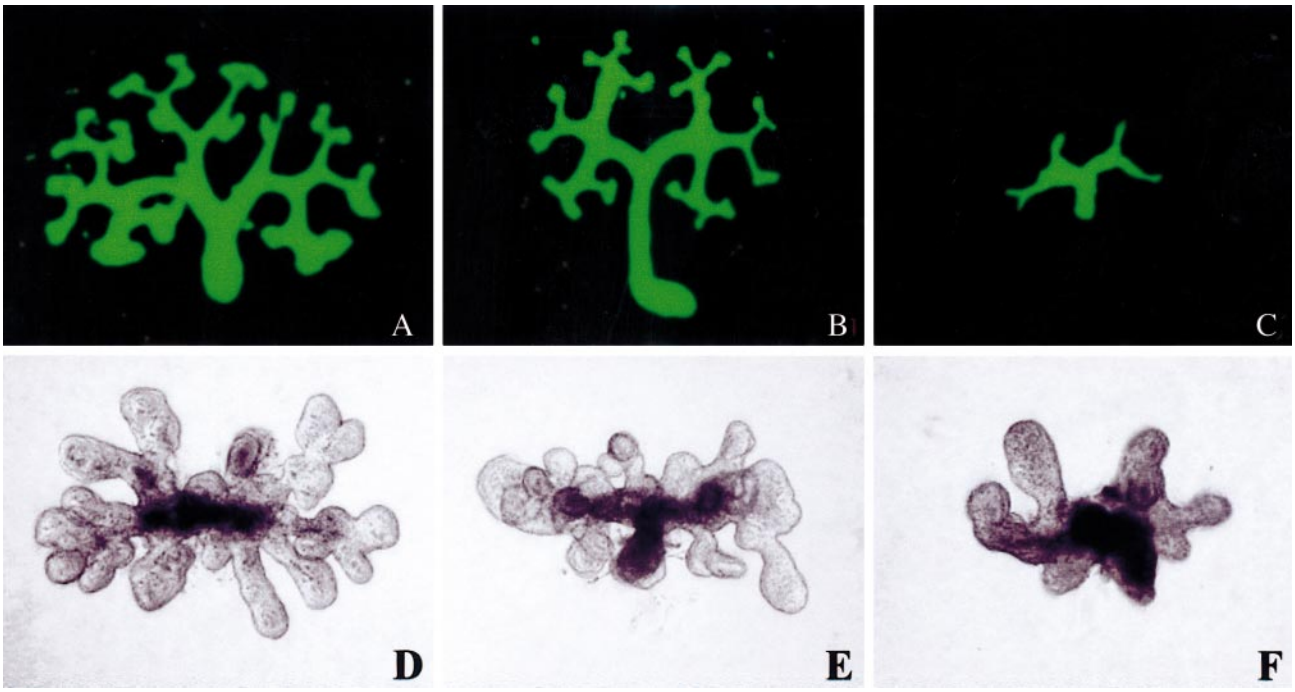


FIG. 6. Integrin α6 antibodies inhibit ureteric bud development and this is more profound when added to an α3 integrin antibody. (A–C) Photomicrographs of whole embryonic day 13 rat kidneys grown for 3 days in the absence (A-control) or presence of either anti-α6 antibody (GoH3-20 μg/ml) (B) or a combination of anti-α6 antibody (GoH3-20 μg/ml) and anti-α3 antibody (Ralph 3.2-20 μg/ml) (C). The developing UBs were visualized with fluorescent-conjugated *D. biflorus* lectin. (D–F) Phase contrast photomicrographs of isolated UBs grown for 7 days in the absence (D-control) or presence of either anti-α6 antibody (GoH3-20 μg/ml) (E) or a combination of anti-α6 antibody (GoH3-20 μg/ml) and anti-α3 antibody (Ralph 3.2-20 μg/ml) (F).

and α6 subunit antibodies (Fig. 6F), growth and branching were markedly inhibited. These results suggest that both integrin α3 and α6 subunits play a role in UB branching morphogenesis, and hence, renal development. Furthermore, their effects appear to be additive, which may explain the abnormalities of ureter development in α3/α6 integrin-deficient animals. These results also suggest that an integrin, other than either integrin α3β1 or α6β1, is playing a role in UB branching morphogenesis because the combined inhibitory effects of the α3 and α6 antibodies appear greater than the addition of β1 antibody alone. An obvious candidate integrin is α6β4.

We therefore assessed whether inhibiting the β4 integrin subunit with antibodies would result in abnormalities of UB branching morphogenesis. We were unable to locate blocking antibodies directed against rat β4 integrins and performed our organogenesis experiments in mice. Similar to rats, embryonic 11.5 day mice kidneys grow and branch when cultured on filters (Figs. 7A and 7B). However, when these kidneys were grown in the presence of the mouse anti-β4 blocking antibody, their development was inhibited, and they were smaller than controls with decreased branching morphogenesis of the developing UB (Fig. 7C). This result suggests that integrin α6β4 is not only present in the UB, but also plays a role in development of the UB and hence the kidney. Thus, the effects of α6 blocking antibodies is mediated, at least in part, by integrin α6β4 and not only α6β1 as previously reported (Falk *et al.,* 1996). Isolated UB cultures were not performed, as this technique is not yet possible in the mouse.

Laminin 5 Is Expressed in the Developing UB and Plays a Role in Kidney Development and UB Branching Morphogenesis

Laminin-5 is expressed on the basement membrane of both the Wolffian duct as well as the UB (day 11 in the mouse) (Airenne *et al.,* 2000). Furthermore, it is highly expressed in the collecting system of the adult kidney (Mizushima *et al.*, 1998). This fact, together with our results that the receptors for laminin-5, namely integrins α6β4 and α3β1, are present in the developing UB, suggested that laminin-5 might be important in the developing UB.

We verified expression of laminin-5 in developing rat kidneys by performing SDS-PAGE on E13 rat kidneys which we immunoblotted with a polyclonal antibody (2778) against the γ2 chain of laminin-5. As seen in Fig. 8A, there are at least three bands recognized by the antibody

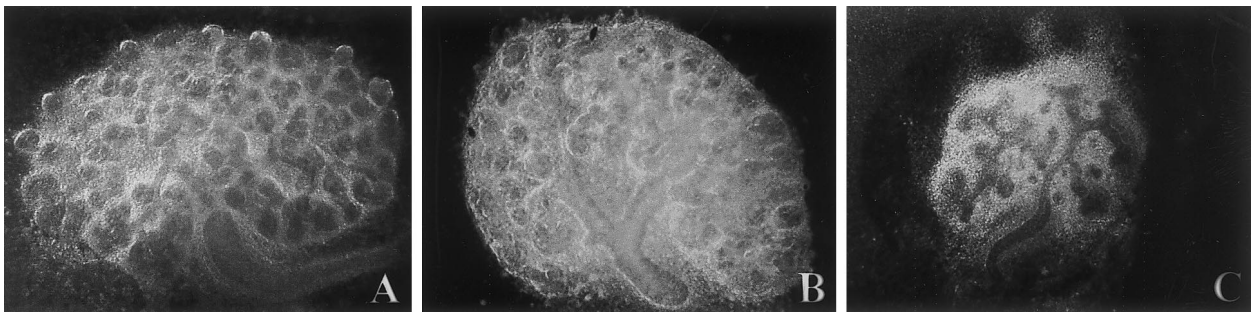


FIG. 7. Integrin $\beta 4$ antibodies inhibit ureteric bud development. Darkfield photomicrographs of day 11.5 mouse embryonic kidneys grown for 3 days in the absence (A-control) or presence of anti-$\alpha 1$ (B-negative control) or anti-$\beta 4$ antibody (346-11A, 20 μg/ml) (C).

and they correspond to the full-length as well as two cleavage products of the $\gamma 2$ chain of laminin-5 (Giannelli *et al.,* 1997). When immunohistochemistry was performed with the same antibody on E13 rat kidneys, laminin-5 was localized predominantly to the collecting tubules (Fig. 8B). RT-PCR of cDNA isolated from E13 rat UB confirmed that laminin-5 is expressed by the UB (Fig. 8C). Thus, we conclude that laminin-5, a common ligand for integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$, is expressed in the developing UB.

To assess whether laminin-5 played a role in renal development and more specifically in UB branching morphogenesis, we utilized the functional blocking antibody (CM6) directed against rat laminin-5 (Plopper *et al.,* 1996). This blocking antibody does not cross-react with mouse laminin-5, so we were unable to assess its effect in the UB cell branching morphogenesis assay. We were, however, able to test the effect of this antibody in both rat whole-organ as well as isolated UB culture. In whole-kidney culture (E13), the kidney was smaller than controls, with less UB branching morphogenesis when treated with anti-laminin-5 antibodies (Fig. 9B). The inhibitory effect of CM6 was significantly different from control kidneys ($P < 0.05$), but its effects were less than that seen with the blocking antibodies against integrin subunits $\alpha 3$, $\alpha 6$, or $\beta 1$ alone and certainly less severe than with a combination of $\alpha 3$ and $\alpha 6$ antibodies (Table 1). In the isolated UB system, CM6 also resulted in inhibition of UB growth and branching (Fig. 9D) similar to the effects seen by inhibiting $\alpha 3$, $\alpha 6$, or $\beta 1$ integrin subunits alone. Thus, laminin-5 appears play a role in UB branching morphogenesis.

DISCUSSION

Normal kidney development is dependent on branching morphogenesis of the UB, which induces differentiation of the metanephric mesenschymal mass (Pohl *et al.,* 2000c; Sakurai and Nigam, 2000; Stuart and Nigam, 2000). The mesenchyme in turn induces the iterative branching morphogenesis of the UB. Integrins are important in normal kidney development, as demonstrated in genetically mutated mice and whole-organ culture models (De Arcangelis *et al.,* 1999; Falk *et al.,* 1996; Georges-Labouesse *et al.,* 1996; Kreidberg *et al.,* 1996; Muller *et al.,* 1997; Wada *et al.,* 1996). However, little is known about the relative importance of integrins in UB branching morphogenesis, which is free of the inductive role of the mesenchyme. Here, we have used three model systems to investigate both the direct and indirect role of integrins in UB development. We demonstrated that integrin subunits $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$ are expressed by the UB as well as by cells derived from the early UB. Inhibiting the function of any of these integrin subunits individually inhibited branching morphogenesis of cells derived from the UB. However, in whole-kidney and isolated UB culture models, functional perturbations of integrin subunits $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$ alone only partially inhibited UB branching morphogenesis and combined inhibition of both $\alpha 3$ and $\alpha 6$ subunits were necessary to significantly inhibit UB branching morphogenesis. These data show that integrin subunits $\alpha 3$ and $\alpha 6$ are important for UB development per se and they are not simply modulators of the inductive effects of mesenchyme. Finally, we demonstrated that laminin-5, a common ligand for integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ is important for normal UB branching morphogenesis and kidney development.

The UB is composed of epithelial cells that will eventually form the entire urinary collecting system from the collecting ducts to the bladder trigone. All these structures are surrounded by a basement membrane, which consists of laminins, type IV collagens, nidogens (Miosge *et al.,* 2000), and proteoglycans (Colognato and Yurchenco, 2000). The literature on which integrins interact with the tubular basement membrane in the developing UB is unclear and somewhat controversial. In addition, there are no integrin expression data on UB cells. We demonstrate that integrin subunits $\alpha 3$ (data not shown), $\alpha 6$, $\beta 1$, and $\beta 4$ are expressed on UB cells. This integrin expression pattern is similar to that seen in the developing UB, where integrins $\alpha 3\beta 1$ (Kreidberg *et al.,* 1996), $\alpha 6\beta 1$ (Falk *et al.,* 1996), and $\alpha 6\beta 4$ are expressed by collecting duct tubules. To our knowledge, expression of integrin $\alpha 6\beta 4$ is a novel finding and is in contrast to other studies which suggest that only $\alpha 6\beta 1$ is

present in the developing kidney (Falk *et al.,* 1996). The expression of both integrin $\alpha6\beta1$ and $\alpha6\beta4$ is consistent with the fact that laminin-10/11 (Miner *et al.,* 1997; Sorokin *et al.,* 1997a,b) and laminin-5 (Miosge *et al.,* 2000) are expressed in the developing UB.

Studies of the role of matrix components in cell culture models of kidney tubule formation have been mostly limited to cells derived from adult canine kidney (MDCK cells) (Santos and Nigam, 1993). This cell culture model is probably not representative of the developing UB as MDCK cells are of adult origin and they form tubules in 3-D collagen I gels, an extracellular matrix which is not a major basement membrane component (Saelman *et al.,* 1995). In contrast to MDCK cells, UB cells grown in 3-D gels require basement membrane components in the form of Matrigel to form tubules (Pohl *et al.,* 2000a; Sakurai *et al.,* 1997). MDCK cell tubulogenesis is integrin $\alpha2\beta1$-dependent (Saelman *et al.,* 1995) while in embryonic collecting duct cells (UB and B12) it is an $\alpha6$ and $\alpha3$ integrin subunit-dependent process. Integrin $\alpha2\beta1$ is primarily a collagen receptor while the ligands for $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ interact with laminins that are found predominantly in basement membranes. The importance of integrin $\alpha6\beta4$ for tubulogenesis suggests that UB cells may be producing laminin-5 which is used for branching morphogenesis, similar to that described in breast cell branching morphogenesis on Matrigel (Stahl *et al.,* 1997) or else UB cells may be interacting with laminin10/11 (Kikkawa *et al.,* 2000).

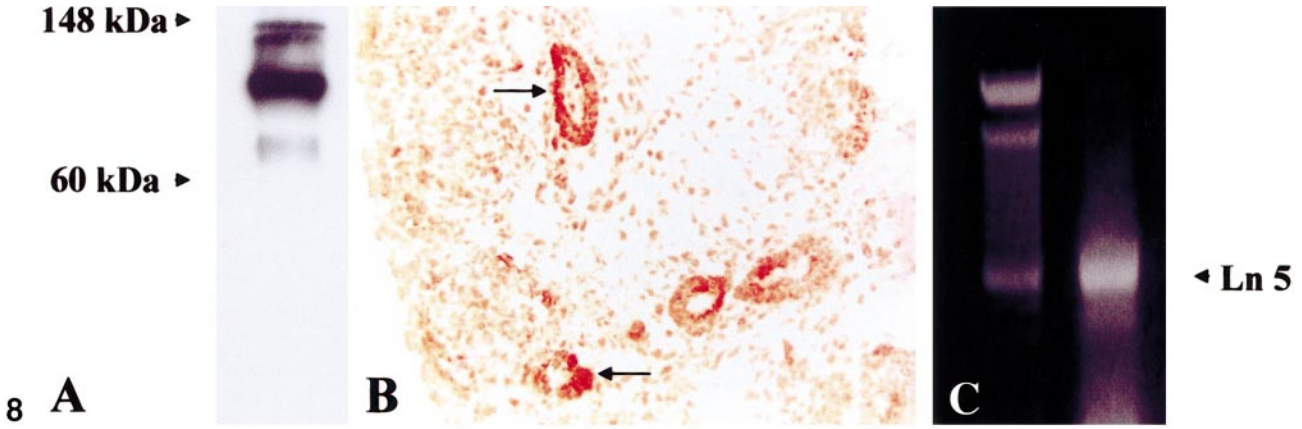
Our study not only provides evidence that integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ are expressed in the developing UB, but also that these integrins directly participate in UB morphogenesis. Furthermore, inhibition of isolated UB growth by antibodies to integrin $\alpha3$, $\alpha6$, and $\beta1$ subunits confirms that these integrins play a role in normal UB development independent of the inductive effects of the mesenchyme. In the isolated UB culture system, both integrin $\alpha3$ and $\alpha6$ subunit function must be blocked to induce a severe phenotype in the developing UB, suggesting functional redundancy of integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$. This result is not surprising, as multiple ligands for these integrins are present in the basement membranes of developing UBs.

Our organ culture results, where we observed a partial decrease in branching morphogenesis of the UB in the presence of $\alpha3$ blocking antibodies, complements data from integrin-deficient mice. The $\alpha3\beta1$-deficient mouse has about half the collecting ducts within the papillary region of the medulla as wild-type animals. The collecting ducts in the papillae are loosely packed with stromal cells, suggesting that the initial rounds of symmetrical branching of the UB were compromised (Kreidberg *et al.,* 1996). Furthermore, although there are decreased collecting ducts in the $\alpha3$-deficient animals, the number of glomeruli are normal. This abnormality could conceivably be due to either a loss of collecting ducts later on in kidney development after nephron induction or else increased induction of glomeruli along individual collecting ducts which are present in decreased number. Our studies, which indicate that there is decreased branching of the isolated UB in the presence of $\alpha3$ blocking antibodies, would support a direct role for integrin $\alpha3\beta1$ in branching morphogenesis as well as the idea that there is decreased branching morphogenesis of the UB rather than loss of collecting ducts after nephron formation.

Unlike the surprising result with the $\alpha6$ (Georges-Labouesse *et al.,* 1996)-deficient mice that do not have any renal phenotype, our experiments suggest that $\alpha6$ integrins do play a role in UB development. This discrepancy between our *in vitro* studies and the $\alpha6$-deficient mouse supports the notion of the redundancy of integrins required for epithelial cell–basement membrane interactions. This is even more evident when one compares our whole-organ and isolated UB culture results with the $\alpha3$ and $\alpha6$ antibodies to the kidney phenotype seen in the $\alpha3-/\alpha6-$ mice, which is no worse than that seen in the $\alpha3$-deficient animals. The $\alpha3/\alpha6$ animals do, however, fail to develop a ureter. This suggests that, although $\alpha6$-containing integrins play a role in *in vitro* UB branching morphogenesis, other basement membrane receptors (such as integrin $\alpha2\beta1$ or α-dystroglycan) can compensate for them in development of the upper collecting system in the intact animal. However, these basement membrane receptors are not sufficient for normal growth and development of the ureter where both $\alpha3$- and $\alpha6$-containing integrins are apparently necessary.

The discrepancy in phenotypes between our *in vitro* models of UB culture and that seen in the $\alpha6$- and $\beta4$-deficient animals may be also explained in a similar way to the role of $\alpha3$ and $\alpha6$ subunits in skin development. $\alpha6$ and $\beta4$ integrin-deficient mice have a "skin blistering phenotype," however the differentiation and stratification of the skin appears normal, implying that these integrins are important for maintaining aspects of the mechanical integrity of the skin at the dermo-epidermal junction (DiPersio *et al.,* 2000). Similarly, integrin subunits $\alpha6$ and $\beta4$ may only play a supporting role in the normal development of UB, but if the collecting system of the kidney were stressed a more severe phenotype may be seen.

We demonstrate that laminin-5 is not only expressed in the developing UB, but that this is of functional significance. This result is consistent with our data that functionally blocking integrins $\alpha3\beta1$ and $\alpha6\beta4$ (for which laminin-5 is a common ligand) results in abnormalities in UB development. The phenotype induced by the laminin-5 antibody more closely resembles that seen with either anti-$\alpha3$ or -$\alpha6$ antibodies alone rather than the severe phenotype observed with both blocking antibodies together. This implies that basement membrane components other than laminin-5 are important for UB development. Similar to the $\alpha6$- and $\beta4$-deficient mice, laminin-5-deficient mice have blistering skin abnormalities and do not have a significant renal phenotype (Ryan *et al.,* 1999). Although it is unclear to what extent the kidney has been examined in these mice, this reported absence of phenotype may speak to the issue of the so-called redundancy of laminin-5 in the developing UB. Obvious candidate basement membrane components that may compensate for the lack of laminin-5 are



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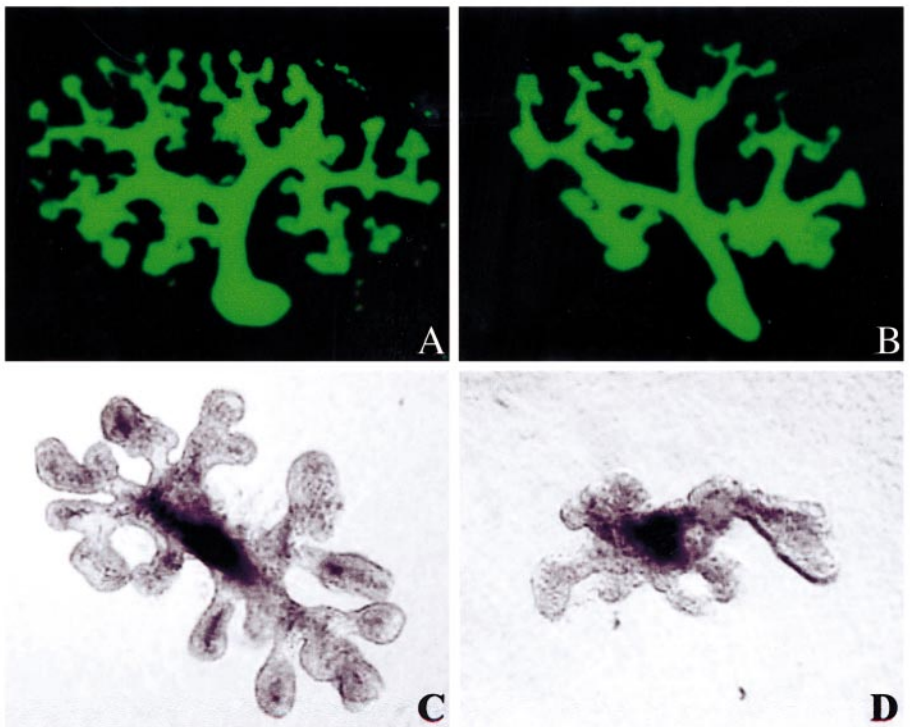

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FIG. 8. Laminin-5 is expressed by the developing kidney. (A) Western blot of embryonic day 13 rat kidneys probed for the presence of laminin-5. Kidneys were isolated, boiled in sample buffer, run in reduced conditions on a 4-20% gradient gel, and immunoblotted with a polyclonal antibody to the γ chain of laminin-5 (2778). The γ2 (140 kDa) and two cleavage products (Giannelli *et al.*, 1997), $\gamma2'$ (100 kDa) and γ2x (80 kDa), are demonstrated (A). (B) Immunohistochemical localization of laminin-5 in frozen sections of embryonic day 13 rat kidneys utilizing the same antibody as above. Laminin-5 is expressed primarily on the basolateral aspect of the developing ureteric tree (arrows). (C) RT-PCR analysis of the expression of laminin-5 in rat E13 UB.

FIG. 9. Functional blocking of laminin-5 inhibits ureteric bud development. (A, B) Fluorescent micrographs of the developing UB in whole kidneys stained with fluorescently-labeled lectin from *D. biflorus*. Whole kidneys from embryonic day 13 rats were cultured for 3 days in the absence (A-control) or presence (B) of a functional blocking antibody to laminin-5 (CM6-50 μg/ml). (C, D) Phase contrast photomicrographs of day 13 embryonic rat UBs grown in the absence (C-control) or presence of CM6 (50 μg/ml) (D).

laminin-10 and -11. These laminins have not only been shown to interact with integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$, but are necessary for normal glomerulogenesis and UB branching morphogenesis (Miner and Li, 2000).

In summary, we have demonstrated by *in vitro* models that integrins $\alpha3$ and $\alpha6$ as well as laminin-5 have a direct role in branching morphogenesis of the UB. Furthermore, the role of integrin $\alpha6$ subunit appears to be via both

integrin $\alpha 6\beta 1$ and $\alpha 6\beta 4$. The *in vitro* phenotypes induced by $\alpha 3$ and $\alpha 6$ antibodies are more severe than those seen in the genetically mutated deficient animals; however, it is possible that if these animals did not suffer perinatal lethality, worse abnormalities in the collecting system would be noted. Perhaps, by generating mice with UB-specific knockouts of integrins $\alpha 6$ and $\alpha 3$ and laminin-5, it may be possible to determine whether these components play a more significant role in the developing collecting system especially with reference to maintaining its mechanical integrity.

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