Differential expression and function of cadherin-6 during renal epithelium development

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SUMMARY

The cadherin gene family encodes calcium-dependent adhesion molecules that promote homophilic interactions among cells. During embryogenesis, differential expression of cadherins can drive morphogenesis by stimulating cell aggregation, defining boundaries between groups of cells and promoting cell migration. In this report, the expression patterns of cadherins were examined by immunocytochemistry and in situ hybridization in the embryonic kidney, during the time when mesenchymal cells are phenotypically converted to epithelium and the pattern of the developing nephrons is established. At the time of mesenchymal induction, cadherin-11 is expressed in the mesenchyme but not in the ureteric bud epithelium, which expresses E-cadherin. The newly formed epithelium of the renal vesicle expresses E-cadherin near the ureteric bud tips and cadherin-6 more distally, suggesting that this primitive epithelium is already patterned with respect to progenitor cell types. In the s-shaped body, the cadherin expression patterns reflect the developmental fate of each region. The proximal tubule progenitors express cadherin-6, the distal tubule cells express E-cadherin, whereas the glomeruli express P-cadherin. Ultimately, cadherin-6 is down-regulated whereas E-cadherin expression remains in most, if not all, of the tubular epithelium. Antibodies generated against the extracellular domain of cadherin-6 inhibit aggregation of induced mesenchyme and the formation of mesenchyme-derived epithelium but do not disrupt ureteric bud branching in vitro. These data suggest that cadherin-6 function is required for the early aggregation of induced mesenchymal cells and their subsequent conversion to epithelium.

Key words: Cadherin, Kidney development, Cell adhesion, Proximal tubule, Mouse

INTRODUCTION

The development of the renal epithelium from the metanephric mesenchyme and the ureteric bud requires inductive interactions, cellular proliferation and extensive tissue remodeling (Saxen, 1987; Ekblom, 1989). Much of the renal epithelium is derived from the metanephric mesenchyme, a group of morphologically distinct cells at the posterior end of the intermediate mesoderm. Upon induction by the ureteric bud epithelium, the metanephric mesenchymal cells aggregate and become polarized to generate a primitive, proliferating epithelial vesicle that ultimately generates the glomerular, proximal tubular and distal tubular epithelium. The analysis of mouse mutants and the use of in vitro organ culture methods have delineated many genes that are essential for these early inductive events and for the subsequent differentiation of the mesenchyme (Lechner and Dressler, 1997). Transcription factors that are essential during patterning and differentiation of the renal epithelium include Pax-2 (Torres et al., 1995), WT-

1 (Kreidberg et al., 1993), N-myc (Stanton et al., 1992) and Lim-1 (Shawlot and Behringer, 1995). In addition, the secreted signaling molecules Wnt-4 (Stark et al., 1995), BMP-7 (Dudley et al., 1995; Luo et al., 1995), GDNF (Pichel et al., 1996; Moore et al., 1996; Vega et al., 1996) and its receptor (Schuchardt et al., 1993) are also required for correct renal epithelial differentiation. How these developmental regulators exert their morphogenetic effects remains unclear. Ultimately, conversion of the metanephric mesenchyme must also be mediated, at least in part, by structural proteins that specify polarity, differential cell adhesion and cell migration.

Among the best characterized molecules that mediate cell-cell adhesion, the cadherins are calcium dependent, homophilic cell adhesion molecules that are implicated in pattern formation during development and signal transduction (Takechi 1995; Gumbiner 1996). The cadherins generally contain five extracellular repeats, termed CAD domains, a transmembrane domain and a cytoplasmic tail that binds to the catenin family of cytoskeletal anchoring and signal transducing

proteins (Ozawa et al., 1989, 1990). These CAD domains are responsible for the specificity of homophilic interactions between cells expressing the same cadherin. The prototype family member, E-cadherin, selectively interacts through the first extracellular CAD domain (Nose et al., 1990), the structure of which has recently been solved (Overduin et al., 1995). The ability of cells that express diverse cadherins to sort out and aggregate with cells expressing only like cadherins provides a molecular mechanism for tissue patterning, boundary formation and cell migration.

In this report, we have examined the expression of cadherins during the differentiation of the renal epithelium and the remodeling of the nephron in the mouse. In the process, we have also identified the mouse homologue of rat K-cadherin, or human cadherin-6, and generated antibodies specific for the cadherin-6 protein. Four cadherins were examined in a temporal sequence beginning with cadherin-11 in the uninduced mesenchyme, cadherin-6 in the mesenchymal aggregates, renal vesicle and proximal tubule progenitors, P-cadherin in the glomerular tuft and podocyte progenitors, and E-cadherin in the ureteric bud, collecting ducts and mature tubules. Differential expression of cadherin-6 and E-cadherin in the proximal and distal halves of the renal vesicle demonstrates, for the first time, that this primitive renal epithelium is already patterned with respect to its future derivatives. Furthermore, antibodies against the extracellular domain of cadherin-6 can interfere with the aggregation of induced mesenchymal cells and block the formation of mesenchyme-derived epithelium in kidney organ cultures.

MATERIALS AND METHODS

Screening of embryonic kidney cDNA libraries

A mouse embryonic day 17 kidney cDNA library was screened using degenerate PCR primers for cadherin cytoplasmic sequences as described by Suzuki et al. (1991) but with the addition of cloning nucleotides compatible with the CLONEAMP vector (Gibco BRL). The PCR conditions were: 5 cycles at 94°C, 80 seconds; 45°C, 2 minutes; 72°C, 2 minutes ramp; 72°C, 1 minutes; followed by 25 cycles at 94°C, 80 seconds; 55°C, 80 seconds; 72°C, 90 seconds. The PCR template DNA was isolated from a random primed, E17 mouse kidney cDNA library. A 50 μl PCR reaction contained 400 nmol of each primer and 2.5 units Taq polymerase (Boehringer Mannheim), 0.5 mM dNTPs, and 1× buffer. The PCR products were ligated into the CLONEAMP vector and sequenced using the di-deoxy method (Sequenase, USB). The cadherin-6 PCR product was used to screen a mouse E17 kidney cDNA library. Several overlapping clones were identified that were greater than 97% identical to the rat K-cadherin sequence in the coding domain. In an independent screen, mouse cadherin-6 was isolated from an E9.5 cDNA library (gift of B. Hogan) under low-stringency conditions (Coffmann et al., 1990) using the entire coding region of *Xenopus* F-cadherin (Espeseth et al., 1995). The entire 789 amino acid cadherin-6 coding region was encompassed in a single clone. Sequence comparison revealed that both kidney-derived and whole-embryo-derived cDNAs encoded the same protein.

In situ hybridization

In situ hybridization with cadherin-11 was done essentially as described by Mackem and Mahon (1991) except ^{33}P-UTP was substituted for ^{35}S-UTP in the labeling reactions. The antisense cadherin-11 probes were derived from a cDNA clone that contained the entire coding region, as previously defined (Kimura et al., 1995;

Hoffman and Balling, 1995). The cadherin-11 probes used were a *Sty*I-*Eco*RV fragment (nucleotides 1960-2535) and an *Eco*RV-*Xba*I fragment (2535-2890) that gave identical results upon hybridization to E11.5 and E15.5 sections. The antisense Pax-2 probe was a *Bam*HI-*Eco*RI fragment described previously (Dressler et al., 1990). Labeling reactions contained 1 μg of linearized plasmid DNA and 100 μCi of ^{33}P-UTP (2000 Ci/mM, Amersham) and generally produced 3.5-$4×10^7$ dpm of probe. Probes were diluted to 50,000 dpm/μl for hybridization and the slides were exposed for 28 days after emulsion coating.

Generation of cadherin-6 antibodies and Fab fragment purification

To produce a polyhistidine-tagged fusion protein in *E. coli*, a 1023 bp *Bam*HI fragment, corresponding to amino acids 68-410, of the cadherin-6 cDNA 12A was inserted into the *Bam*HI site of pRSET-B (In Vitrogen Inc.). Protein expression was induced with IPTG in the BL21 strain of *E. coli* and the protein was purified under denaturing conditions by nickel affinity chromatography. Purified protein was dialyzed stepwise into PBS. The cadherin-6 fusion protein was injected into rabbits (Babco Antibody Co.) and the animals were killed after the third boost. The antiserum was first purified by protein-A sepharose chromatography and then affinity purified using the cadherin-6 fusion protein fixed to an amino-link agarose column (Pierce). Fab fragments were prepared from affinity-purified α-cadherin-6 antibodies (cad6-Fab) or preimmune serum IgG (PI-Fab) by papain digestion (10 μg papain/mg Ab) in 100 mM NaOAc, pH 5.5, 50 mM cysteine, 1 mM EDTA for 2-4 hours at 37°C. Iodoacetamide was added to the reaction to a final concentration of 75 mM to stop the digestion. After 30 minutes at room temperature, the digested antibodies were dialyzed in 4 changes of 1 liter of PBS, for at least 8 hours per liter.

Cell aggregation

Mouse L cells (ATCC CCL-1) were transfected with a MMuLV retroviral vector (Rubenstein et al., 1984) containing the complete cadherin-6 coding region and transformants selected with 800 μg/ml G418 in DMEM/10% fetal calf serum. Individual colonies were picked, expanded and tested for cadherin-6 expression by western blotting. Two lines were identified as cadherin-6 positive (LK14 and LK17) and utilized for short-term aggregation assays as described by Nakagawa and Takeichi (1995). Monolayers of LK14 cells were treated with 0.01% trypsin and 1 mM CaCl$_2$ in Ca^{2+} and Mg^{2+} free 10 mM Hepes-buffered saline solution (HCMF) for 15 minutes at 37°C on a gyrotary shaker. The cells were pelleted and, after discarding the supernatant, 100 μl of 0.5% (w/v) soybean trypsin inhibitor in HCMF was added to the cell pellet. The cells were washed three times with ice-cold HCMF. Cells were then resuspended at 2-$5×10^5$ cells/ml in HCMF using Pasteur pipette to get single-cell suspension. Aggregation assay was performed in 24-well plate precoated with 1% BSA in HCMF. 500 μl of cell suspension in HCMF, with 1 mM CaCl$_2$, with 1 mM CaCl$_2$ and 17 μg/ml PI-Fab, or with 1 mM CaCl$_2$ and 17 μg/ml cad6-Fab was added to each well. The plate was rotated on a gyroshaker at about 80 revs/minute at 37°C. After 2 hours of incubation, cells in each well were photographed.

Immunostaining

FVB/N inbred mice were mated and the day of the vaginal plug was designated E0. Embryos were dissected free of extraembryonic tissue and frozen on dry ice. Commercial reagents utilized were: anti-Laminin Ab (Sigma #L-9393); anti-E-cadherin Ab (Sigma #U-3254); anti-WT1 Ab (Santa Cruz #sc-192); anti-Pan Cytokeratin Ab (Sigma #C-9687); anti-P-cadherin Ab (Zymed Labs. #13-2000); FITC-conjugated Lotus Tetragonobulus (LTA, Sigma #L-9254). Immunostaining was performed as described by Harlow and Lane (1988). Cryostat sections of fresh frozen embryos were cut at 8 μm, collected on gelatinized slides and air dried for 30-60 minutes. Sections

were fixed in 4% paraformaldehyde/PBS for 10 minutes, then washed twice in PBS/0.05% Tween-20 for 5 minutes. Antibody dilutions were 1:400 for E-cadherin Ab, 2 μg/ml for α-cadherin-6 Ab, 1:100 for WT1 Ab, 1:20 for P-cadherin Ab and 10 μg/ml of α-Pax-2 IgG in 2% goat serum in PBS. Fluorescein-conjugated Lotus Tetragonolobus was diluted 1:20. Slides were incubated at room temperature with 20 μl of antibody in a humid chamber and then washed twice in PBS/0.1% Tween-20 (PBST). The second antibodies were diluted 1:400 for the Texas Red-conjugated anti-rabbit (Molecular Probes) and 1:20 for the FITC-conjugated anti-rat (Sigma) in 2% goat serum in PBS. After a 30 minutes incubation, slides were washed twice in PBST and covered with gelvatol. Control sections were incubated with a preimmune rabbit IgG fraction and both second antibodies. No specific staining could be detected with the preimmune IgG from rabbits immunized with the cadherin-6 antigen (data not shown).

For whole-mount antibody staining of organ cultures, kidney rudiments were fixed in 100% methanol for 10 minutes and washed twice in PBST for 20 minutes. Tissues were incubated for 4-5 hours with primary antibodies at 4°C and washed overnight in PBST at 4°C. Incubation with secondary antibodies was 4-5 hours at 4°C and multiple washes were performed with a large volume PBST overnight at 4°C. Whole-mount tissues were placed under coverslips in gelvatol for microscopy.

Organ culture

Kidneys were dissected from E11.5 littermates in Ham's F12 media and cultured on Transwell filter inserts (Costar) with 3.0 μm pore sizes. Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin was used for all experiments. For the antibody blocking experiments, the kidney rudiments were incubated for 1 hour in DMEM with 30 μg/ml of PI-Fab or cad6-Fab before placing on the filters and then cultured for an additional 48 hours in the presence of antibodies.

RESULTS

Cadherin genes in the embryonic kidney

In order to more precisely define the different cadherins that are expressed in the developing kidney, we screened an E17 kidney cDNA library using degenerate PCR primers designed to amplify the conserved cadherin cytoplasmic domain. The PCR products were cloned into a plasmid vector and 21 independent clones were identified. Sequence analysis revealed that three clones were identical to mouse uvomorulin, or E-cadherin (Nagafuchi et al., 1987; Ringwald et al., 1987), three clones corresponded to P-cadherin (Nose et al., 1987), six clones were identical to rat K-cadherin (Xiang et al., 1994) or mouse cadherin-6 (Inoue et al., 1997), and nine clones were identical to cadherin-11 (Kimura et al., 1995; Hoffman and Balling, 1995). The mouse cadherin-6 PCR fragment was used to isolate the entire coding region from an E17 embryonic kidney cDNA library. Concurrently, a mouse E9.5 cDNA library was screened with a *Xenopus* F-cadherin probe under low-stringency conditions. A single 3.3 kb clone was identified (c12A) that contained the entire cadherin-6 open reading frame. The sequence was determined to be identical with that reported by Inoue et al. (1997).

Expression of cadherins in the developing kidney

In order to determine how the cadherins might function in the differentiation of the renal epithelium, the temporal and spatial expression patterns of the different cadherins were analyzed by in situ hybridization or immunocytochemistry in the developing kidney. Cadherin-11 is expressed primarily in mesenchymal cells throughout the embryo and has been described in detail (Kimura et al., 1995; Hoffman and Balling, 1995). However, in the induced mesenchyme of the E11.5-day kidney, cadherin-11 is expressed more strongly and overlaps with the Pax-2-positive mesenchymal cells at this stage (Fig. 1A-D). Cadherin-11 is not expressed in any epithelial structures, including the ureteric bud (Fig. 1C) and the developing renal tubules (Fig. 1E) that are derived from the cadherin-11-expressing mesenchymal cells. Cadherin-11 continues to be expressed in the interstitial mesenchymal cells of the kidney at later stages (Fig. 1E). Although high levels are initially detected in the induced mesenchymal cells, cadherin-11 is down-regulated as these induced cells become polarized and form the undifferentiated epithelium of the renal vesicle.
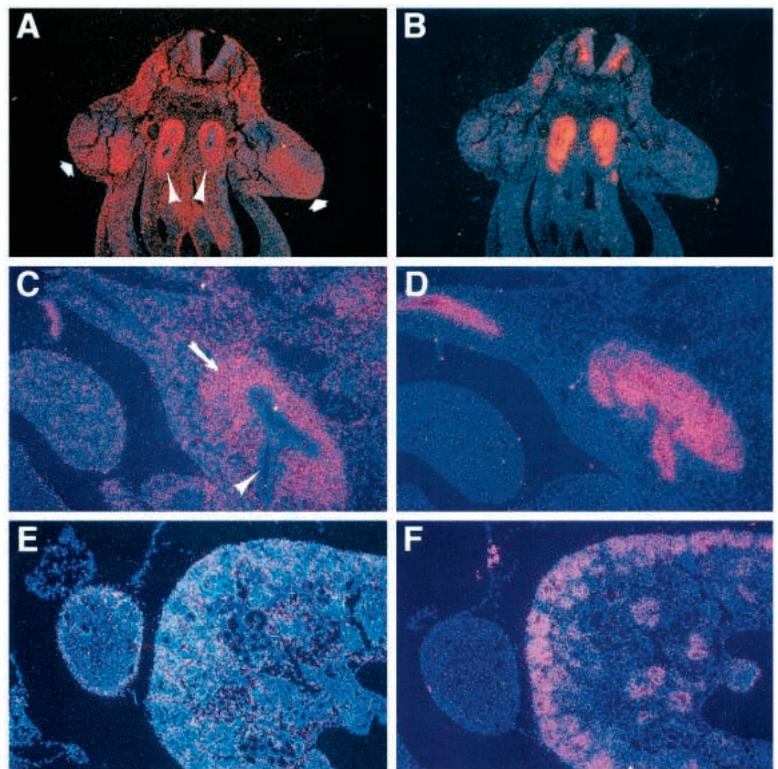

Fig. 1. Expression of cadherin-11 and Pax-2 mRNAs in the developing kidney. In situ hybridization using probes specific for cadherin-11 (A,C,E) and Pax-2 (B,D,F) are shown in serial sections through the kidney at various embryonic stages. (A,B) Transverse section through an E11.5 embryo showing cadherin-11 in the metanephros (arrowhead), limb buds (arrow) and other mesenchymal cell types. Pax-2 is restricted to the metanephros and the neural tube. (C,D) Sagittal sections through an E11.5 kidney. Note, cadherin-11 is only in the mesenchymal cells (arrow) and not in the ureteric bud epithelium (arrowhead), whereas Pax-2 is expressed in both cell types. (E,F) Sagittal sections through an E14.5 kidney showing persistent cadherin-11 expression in the interstitial mesenchymal cells but not in the mesenchymal aggregates along the periphery, where Pax-2 expression is still high. Micrographs were taken with a Nikon Microphot-FXA and 35 mm film with a total magnifications of 12× (A,B), 64× (C,D), 32× (E,F).
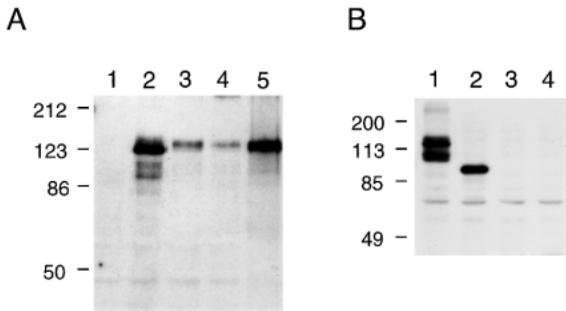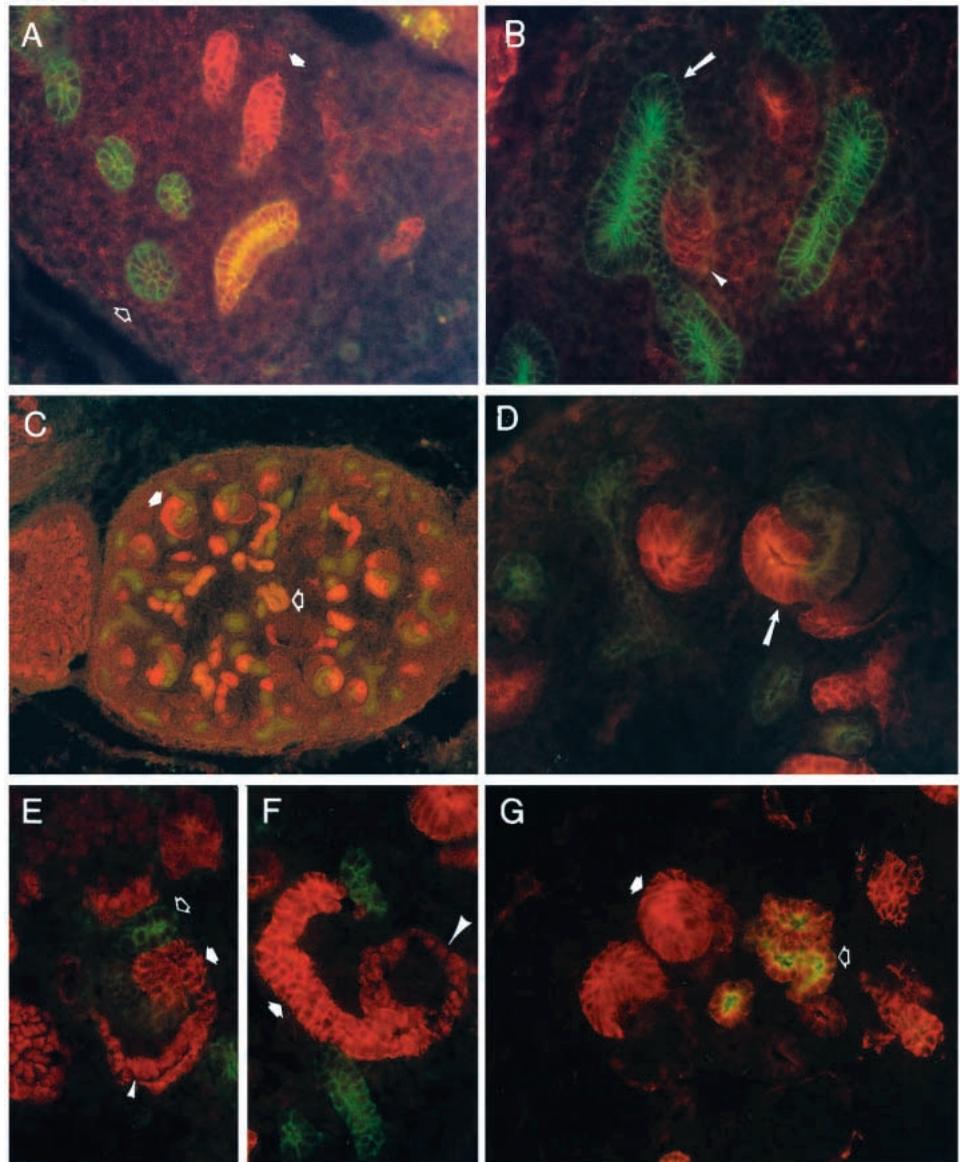
Fig. 2. Specificity of α-cadherin-6 antibodies. Western blots probed with affinity-purified α-cadherin-6 antibodies. (A) Lane 1, control vector transfected LTK- cells; lane 2, CMV-cadherin-6 transfected LTK- cells; lane 3, renal cell carcinoma line UCK111; lane 4, renal cell carcinoma line UCK117; lane 5, E17.5 kidney protein lysate. (B) NIH 3T3 transiently transfected cells. Lane 1, CMV-cadherin-6; lane 2, CMV-ΔC-cadherin6; lane 3, CMV-cadherin-11; lane 4, CMV control vector.

In order to analyze the protein product of the cadherin-6 gene, antibodies were generated against a bacterial protein that contained amino acids 68 to 410 fused to a polyhistidine tag. The polyclonal rabbit antibodies were purified first over a protein-A agarose column and subsequently by affinity chromatography to the fusion protein. Specificity of the purified α-cadherin-6 antibodies is demonstrated by western blotting (Fig. 2). The α-cadherin-6 recognizes a single protein in extracts of embryonic kidney that migrates at approximately 120×10^3 M_r. A similar species is detected in human renal carcinoma cell extracts, consistent with the reported expression of cadherin-6 in renal cell carcinoma (Xiang et al., 1994). A similar size protein is detected in LTK– cells transfected with a CMV-cadherin-6 expression plasmid, but not in vector only transfected cells. Since the predicted molecular mass of the mature peptide would be approximately 81×10^3 M_r, the increase in apparent molecular mass is probably due to post-translational modifications such as N-linked glycosylation.

Because cadherin-11 is the most closely related to cadherin-

Fig. 3. Immunocytochemistry of E-cadherin and cadherin-6 in the embryonic kidney. (A) E12.5 mesonephros showing E-cadherin (green) along the dorsal side (open arrow) where the nephric duct is located and cadherin-6 (red) at the ventral end (solid arrow) of the tubules. Both E and cadherin-6 are expressed in a transition zone (yellow) located between the nephric duct and the mesonephric glomeruli. (B) E12.5-day metanephros showing E-cadherin (green) in the ureteric bud (arrow) and cadherin-6 (red) in the renal vesicle (arrowhead). Note E-cadherin expression in renal vesicle cells directly abutting the ureteric bud termini. (C) E15.5 metanephros showing E-cadherin (green) and cadherin-6 (red). Cadherin-6 is still prominent in the s-shaped bodies (solid arrow) but more mature tubules express both K and E-cadherin (open arrow). (D) E15.5 comma-shaped body (arrow) expressing cadherin-6 (red) at the proximal end and E-cadherin (green) at the distal end, adjacent to the ureteric bud. (E) E15.5 S-shaped body expressing E-cadherin (green) at the distal end, cadherin-6 (red, cell surface) more proximally, and WT1 (red, nuclear) in the podocyte progenitors of the glomerular cleft. (F) E15.5 Glomerulus expressing WT1 (red, nuclear) and proximal tubule expressing cadherin-6 (red, cell surface). Note the absence of cadherin-6 in the glomerular epithelium. (G) E15.5 metanephros expressing cadherin-6 (red) in the comma- and s-shaped bodies and the developing proximal tubules which also stain with the LTA (green). Total magnification is 25× (C) and 100× (A,B,D-G).

6, being 61% identical across the extracellular domain used for immunization, additional specificity controls were done to exclude potential cross reactivity of α-cadherin-6 to cadherin-11 (Fig. 2B). The α-cadherin-6 antibodies recognized the major $120×10^3\,M_r$ form and several smaller bands in cadherin-6 transiently transfected 3T3 cells . Given the high levels of expression observed upon transient transfection, it is likely that the smaller bands represent unmodified cadherin-6. Cells transfected with a vector expressing a truncated form of cadherin-6 (ΔC), minus the cytoplasmic domain, exhibited a single band at approximately $90×10^3\,M_r$. Cells transfected with a cadherin-11 expression vector did not show any proteins detectable with α-cadherin-6 above control vector transfected cells. Cross reactivity to E-cadherin is unlikely given only 36% homology in the extracellular domain between E-cadherin and cadherin-6.

Antibodies against cadherin-6, E-cadherin and Pax-2 were used to stain embryonic kidneys at various stages of development. The pattern of *Pax-2* expression has been described in detail (Dressler et al., 1990; Dressler and Douglass, 1992; Ryan et al., 1995). At E12.5, the nephric duct extends from anterior to posterior within the more dorsal intermediate mesoderm and expresses E-cadherin (Fig. 3A). Along the more anterior nephric duct, the mesonephric tubules are still present as a linear array in the sagittal plane. These mesonephric tubules are transient embryonic structures that consist of a glomerular capsule, near the dorsal aorta, and a convoluted tubule emptying into the nephric duct. These tubules are patterned along the dorsal-ventral axis with respect to cadherin expression (Fig. 3A). The mesonephric tubules express cadherin-6 in the most ventral region, near the glomerular capsule. As the tubules extend dorsally towards the nephric duct, there is a region of overlap where both cadherin-6 and E-cadherin are expressed. Finally, as the tubules reach the nephric duct, only E-cadherin remains.

At the posterior end of the E12.5 nephric duct, the ureteric bud has grown out and induced the metanephric mesenchyme to generate the definitive kidney or metanephros. *Pax-2* expression is prevalent in both ureteric bud epithelium, induced metanephric mesenchyme and the epithelial derivatives of the mesenchyme (Dressler et al., 1990; Dressler and Douglass, 1992; Ryan et al., 1995). E-cadherin is prevalent in the ureteric bud and its branches. The expression of cadherin-6

can first be detected in the mesenchymal aggregates that will form the epithelium of the renal vesicle at E12.5 (Fig. 3B). Initially, cadherin-6 is detected on the apical side of the newly formed epithelium and subsequently demarcates the entire cell surface. Cadherin-6 expression is highest in those cells furthest from the ureteric bud termini, whereas some cells adjacent to the ureteric bud termini express E-cadherin. The expression of cadherins in the E15.5 kidney is complex and reflects the ongoing morphological changes in the developing renal epithelium. Cadherin-6 is still prevalent in the renal vesicles at the periphery of the kidney, with E-cadherin throughout the ureteric bud epithelium and the major collecting ducts derived from the ureter (Fig. 3C). In the comma-shaped bodies (Fig. 3D), cadherin-6 is strong in the future proximal end of the body, furthest from the ureteric bud, while E-cadherin is expressed in the more distal part, nearest to the ureter. To minimize confusion, we will use proximal and distal with respect to the future position of the glomerulus, as is the case with the mature nephron. Only a small region of overlap can be detected between cadherin-6 and E-cadherin at this time. As the glomerular cleft is formed at the proximal end of the comma-shaped body to generate the s-shaped body (Fig. 3E,F), cadherin-6 is down-regulated in the epithelium of the glomerular cleft, the prospective podocytes. The podocyte progenitors can be stained for the nuclear antigen WT1 (Fig. 3E,F) and clearly show little cadherin-6 on the cell surface. These WT1-expressing podocyte progenitor cells have also down-regulated the Pax-2 gene (Ryan et al., 1995). Cadherin-
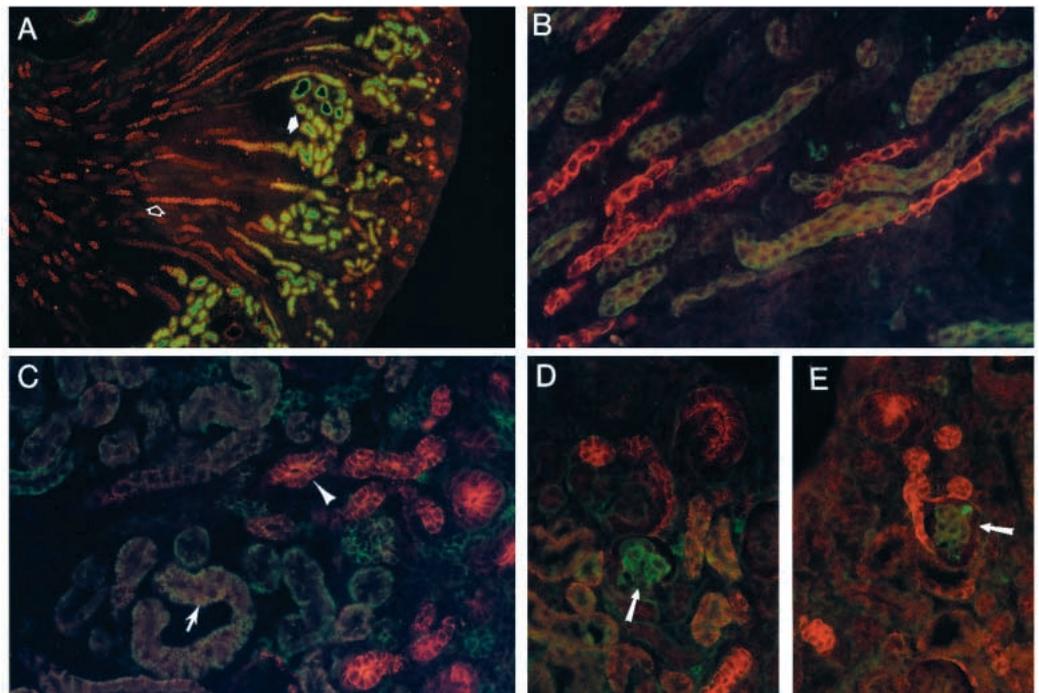

Fig. 4. Immunostaining of cadherins in the newborn kidney. (A) Cadherin-6 (red) persists in the nephrogenic zone along the periphery and in the medullary zone (open arrow). Mature proximal tubules stain with LTA (green) and have down-regulated cadherin-6 (solid arrow). (B) Newborn renal medulla showing E-cadherin (green) in the collecting ducts and cadherin-6 (red) in the ascending and descending limbs of Henle's loop. (C) Newborn renal cortex exhibits less cadherin-6 (red) in maturing proximal tubules with punctate E-cadherin (green) now detectable in most epithelial cells. (D,E) Newly formed glomeruli express P-cadherin (green) in cells of Bowmans capsule and cadherin-6 in the proximal tubules exiting the glomerulus. Total magnification is 25× (A) and 100× (B-E).

6 is still strong in the prospective proximal tubule cells of the s-shaped body with E-cadherin marking the prospective distal tubule cells nearest the developing collecting ducts. As in the developing mesonephros, there is a transition zone within the s-shaped body where E-cadherin and cadherin-6 are co-expressed.

The cadherin-6-expressing cells are primarily destined to become proximal tubule epithelium as indicated by the expression of the lectin-binding sites for Lotus Tetragonolobus (LTA, Laitinen et al., 1987). In the E15.5 kidney, fluorescein-conjugated LTA stained only cadherin-6-expressing cells. These double-labeled cells were generally more mature than cadherin-6-expressing cells located along the peripheral, nephrogenic zone, which did not express the LTA-binding site (Fig. 4A). In the newborn kidney, cadherin-6 expression is strong in two particular regions, in the nephrogenic zone where the mesenchymal-to-epithelial transition is still ongoing and in the medullary zone where proximal tubules are extending radially inward to form the descending and ascending loops of Henle (Fig. 4 B). The more mature proximal tubules are easily marked with LTA and have down-regulated cadherin-6 expression. E-cadherin is now detected in those cells that had expressed cadherin-6 at earlier times. E-cadherin is also prevalent in the collecting ducts of the medullary zone (Fig. 4 C) but does not overlap with cadherin-6 in this region. In the glomerular epithelium, the expression of P-cadherin can now be detected in the podocyte epithelium. The expression of P-cadherin is similar to previously reported observations (Tassin et al., 1994).

Antibodies against cadherin-6 inhibit aggregation and formation of tubules

Given the expression of cadherin-6 in the mesenchymal aggregates that form at the tip of the ureteric bud, we hypothesized that cadherin-6 may mediate the aggregation and polarization of these mesenchymal cells to generate proximal tubule epithelium and perhaps other parts of the nephron. In order to test this hypothesis, Fab fragments were made by papain digestion of the affinity-purified anti-cadherin-6 polyclonal antibodies (cad6-Fab) and from the pre-immune IgG fraction (PI-Fab). The antibodies were tested for their ability to inhibit cadherin-6-mediated aggregation using transfected L cells. LK14 cells stably expressing cadherin-6 were generated by retroviral-mediated gene transfer and utilized in short-term aggregation assays (Fig. 5). Calcium-dependent cellular aggregates could be observed with LK14 cells whereas the parental L cell line showed no evidence of aggregation (data not shown). Aggregation of LK14 cells could be inhibited by the addition of cad6-Fab but not with the control PI-Fab fraction (Fig. 5). Thus, cadherin-6-dependent homophilic adhesion could be abrogated with the polyclonal antibodies against the extracellular domain.

In order to study the effects of cadherin-6 inhibition during renal development, the antibodies were added to the medium of E11 kidney rudiments and the kidneys cultured for 48 and 96 hours. The degree of development of cultured kidneys was examined by immunohistochemistry using antibodies against E-

cadherin, cytokeratins, cadherin-6, laminin, Pax-2 and WT1 (Fig. 6). Three separate experiments utilizing at least 6 E11 kidney rudiments for each culture condition were performed. In all cultures examined, there was no significant difference between kidneys cultured in DMEM alone and DMEM supplemented with 30 µg/ml of PI-Fab. Ureteric bud branching occurred repeatedly as detected by both cytokeratin and E-cadherin staining (Fig. 6A,C). Mesenchyme-derived epithelium was easily distinguishable as Pax-2-positive, comma- or s-shaped tubules adjacent to the ureteric bud branches (Fig. 6A). The mesenchyme-derived epithelium did not stain with the cytokeratin antibodies, but did have a laminin-containing basement membrane (Fig. 6E) and expressed either E-cadherin or cadherin-6 (Fig. 6C). Similar to the situation in vivo, mesenchyme-derived tubules expressed E-cadherin near the ureteric bud and cadherin-6 further from the bud. Strikingly, cadherin-6 was first visible on the mesenchymal cells at the extreme tip of the bud, on the prospective apical side of the cells undergoing polarization (Fig. 6C arrowhead).

In contrast, kidney rudiments cultured in the presence of 30 µg/ml cad6-Fab exhibited significant differences in the formation of mesenchyme-derived epithelium. The cad6-Fab had no effect on ureteric bud branching. However, the ability of mesenchymal cells to condense near the ureteric bud tips and generate epithelium was severely compromised. Activation of Pax-2 is an early marker of mesenchymal induction and appeared undisturbed by any antibody treatment (Fig. 6B). However, in the cad6-Fab-treated kidneys, Pax-2-expressing
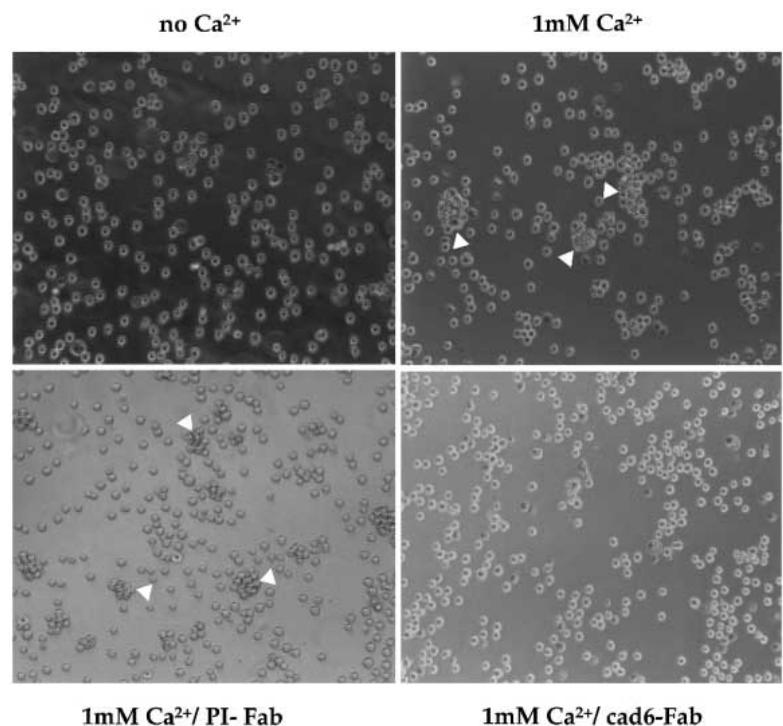

Fig. 5. Inhibition of cadherin-6-dependent aggregation by cad6-Fab. LK14 cells expressing cadherin-6 were trypsinized and allowed to reaggregate in the presence or absence of calcium. Note the formation of cellular aggregates (arrowheads). Cad6-Fab but not control PI-Fab antibodies inhibit the calcium-dependent aggregation.

cells at the ureteric bud tips were very loosely associated and many Pax-2-positive cells were localized throughout the rudiment. In contrast, controls showed tightly associated Pax-2-positive cells separated by Pax-2-negative, presumably interstitial, mesenchyme (Fig. 6A). The formation of comma- and s-shaped bodies was severely inhibited in cad6-Fab-treated cultures as clearly indicated by the lack of polarized epithelium with a laminin-staining basement membrane (Fig. 6F). Almost all of the epithelium in the cad6-Fab-treated cultures was derived from the ureteric bud as indicated by cytokeratin (Fig. 6F) and E-cadherin staining (Fig. 6D). The whole cadherin-6 antibodies detected very little cadherin-6 reactivity in the cad6-Fab-treated samples (Fig. 6D). Notably, those cells that were cadherin-6 positive were localized in the interior of the organ culture and did appear to be epithelial. After 96 hours, the presence of glomerular structures were detected using an antibody against WT1, which is highly expressed in the podocyte precursor cells of the s-shaped body and in mature glomeruli (Ryan et al., 1995). Kidney rudiments cultured with cad6-Fab exhibited few, if any normal glomerular structures (Fig. 6H), whereas controls showed round clusters of WT1-positive cells (Fig. 6G) indicative of glomerular podocyte development.

DISCUSSION

The development of the nephron from the two primary progenitor cell types, the ureteric bud epithelium and the metanephric mesenchyme, requires a phenotypic conversion of mesenchymal cells to an epithelial phenotype and a complex series of morphogenetic events that pattern the epithelium along the proximal-distal axis. The ability to concentrate urine, sense and regulate osmolarity, and continuously feed back information to the glomerular apparatus requires a precise three-dimensional architecture in the nephron that is intimately linked to proper function. How this architecture is specified during development must be regulated not only by specific transcription factors but also by proteins that provide the motor forces underlying cell movement and tissue remodeling.

The expression pattern of cellular adhesion molecules can demarcate the developing renal epithelium into functionally and morphologically distinct regions as the mesenchyme aggregates, forms the renal vesicle and ultimately

generates a mature nephron. The data presented in this report are summarized schematically in Fig. 7. The *cadherin-11* gene is expressed in a large variety of mesenchymal cell types
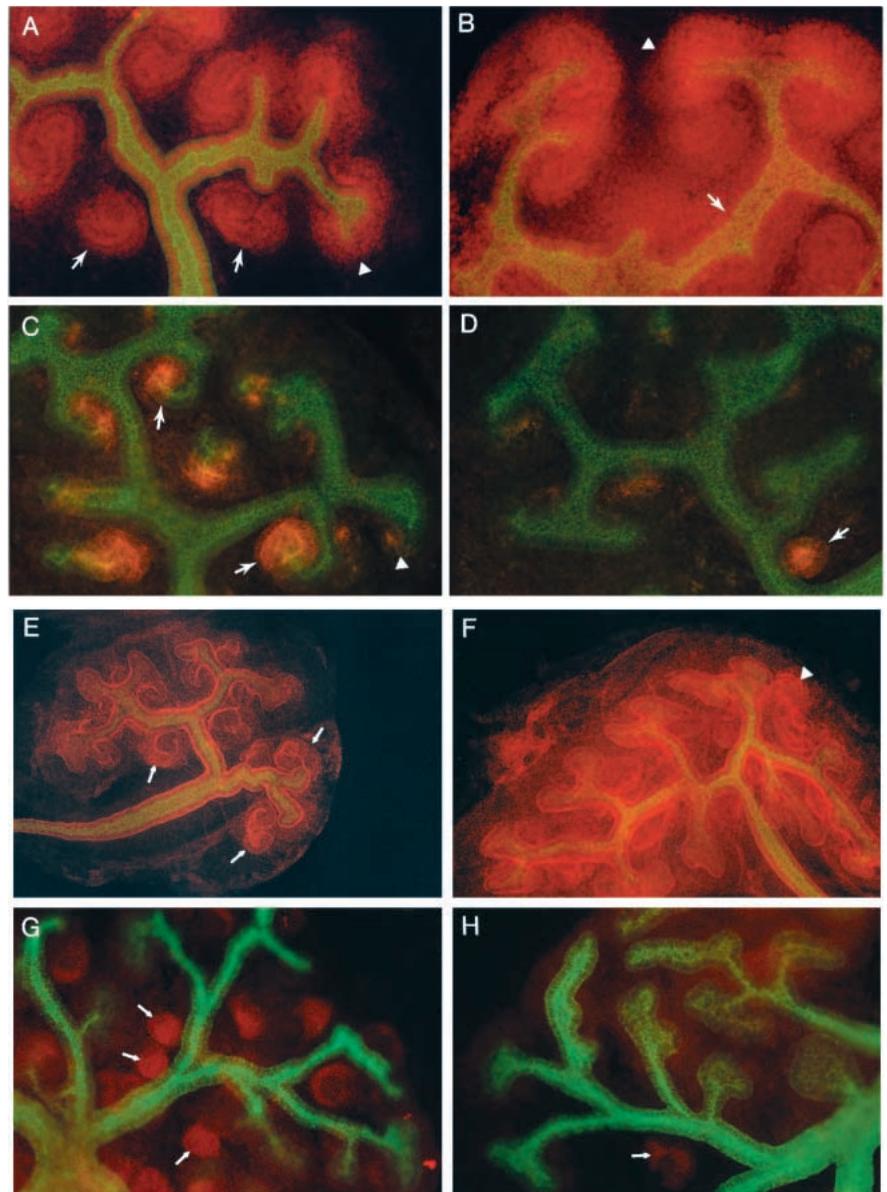

Fig. 6. Inhibition of mesenchyme-derived tubulogenesis by α-cadherin-6 antibodies. E11.5 metanephric rudiments cultured for 48 hours with PI-Fab (A,C,E) or cad6-Fab (B,D,F) and stained whole mount with specific antibodies. (A,B) Staining with Pax-2 (red) and cytokeratin (green) shows loosely associated induced mesenchyme (B, arrowhead) at the tips of ureteric bud in cad6-Fab cultures. Controls exhibit tight association (A, arrowhead) and comma shaped bodies (A, arrows). Also note Pax-2-positive cells in B (arrow) that line the more mature ureteric bud epithelium, this is never observed in controls. (C,D) Staining for cadherin-6 (red) and E-cadherin (green) reveals well-developed comma- and s-shaped bodies in controls (C, arrows) and little cadherin-6 protein in cad6-Fab-treated cultures; the occasional positive structure (D, arrow) appears epithelial. (E,F) Staining for laminin (red) and cytokeratin (green) reveals well-developed basement membranes around comma- and s-shaped bodies in controls (E, arrows). In cad-6-Fab-treated cultures, basement membrane staining is clear in ureteric bud epithelium, positive for cytokeratin, but diffuse staining in cells adjacent to the ureteric bud tips. Only occasional, poorly developed structures show characteristic basement membrane formation in mesenchyme-derived epithelium (F, arrowhead). (G,H) Staining for WT1 (red) and cytokeratin (green) after 4 days shows well-developed glomerular structures (G, arrows) in controls and few, poorly developed glomeruli in cad6-Fab-treated cultures (H, arrow).
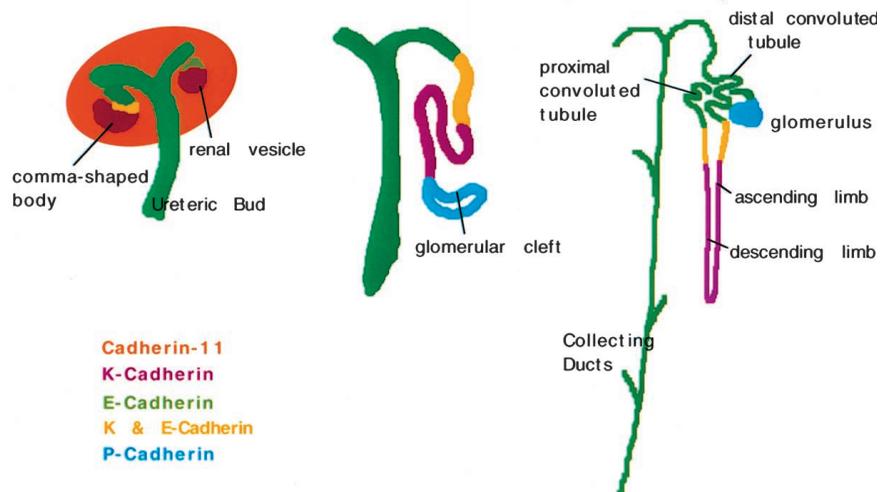
Fig. 7. Summary of cadherin expression during nephron development. Induced mesenchymal cells repress cadherin-11 and activate cadherin-6, mesenchymal cells closest to the ureteric bud activate E-cadherin. The s-shaped body is patterned with respect to cadherin expression such that glomerular precursors express P-cadherin, proximal tubule precursors express cadherin-6 and more distal tubule precursors express E-cadherin. In newborns, cadherin-6 becomes down-regulated in maturing proximal tubules but can still be seen in Henle's loop.

throughout embryogenesis, with strikingly high levels in the metanephric mesenchyme, relative to surrounding mesoderm. The cells of the uninduced metanephric mesenchyme can be distinguished morphologically from surrounding tissue because they are more tightly associated. Whether this is due to increased levels of cadherin-11 is unclear. After induction occurs at E11, cadherin-11 expression levels do not appear to be higher in the cells directly adjacent to the ureteric bud tips where the aggregates first form and levels are quickly down-regulated as evidence of epithelial transformation is apparent. Yet, cadherin-11 could still function in the earliest adhesion response to induction, perhaps by the controlled activation of other factors that interact with the intracellular domain such as the catenin family of proteins (Ozawa et al., 1989, 1990) thus providing a rapid response to the inductive signals.

Cadherin-6 is first detected in the mesenchymal aggregates and in the renal vesicle before a polarized epithelium, complete with basement membrane, is generated. Particularly in organ culture, where the timing of events may be slower, cadherin-6 is first detected as a patchy strip around the extreme ureter bud tips, suggesting that its expression is first localized to one side of the mesenchymal cell and precedes the formation of the polarized epithelium. At E12.5, the renal vesicle already appears patterned with respect to cadherin expression. The cells adjacent to the ureteric bud expresses primarily E-cadherin whereas the more distal cells, relative to the ureteric bud, express cadherin-6. Thus, the epithelium of the renal vesicle is not a homogenous population of primitive epithelial cells. The proximity to the ureteric bud and its inductive signals may determine the type of cadherin expressed in the vesicle and the ultimate fate of the epithelium. Alternatively, the expression of E-cadherin may in fact reflect the origin of the cells in that part of the renal vesicle, as recent evidence points to a population of cells that can delaminate from the ureteric bud and contribute to the tubular epithelium that was previously thought to derive only from the mesenchyme (Qiao et al., 1995). At the s-shaped body stage, differential cadherin expression clearly demarcates the precursor cells of distal tubules as E-cadherin positive, the proximal tubules that express cadherin-6, and the glomerular epithelium that begins to express P-cadherin (Tassin et al., 1994). There is a small region of overlap between the cadherin-6 and E-cadherin

expression domains that is more prominent on the apical side of the epithelium. This overlap does not appear to be due to cell mixing, rather it appears that individual cells can express both E-cadherin and cadherin-6 simultaneously. Expression of cadherin-6 marks the differentiating and proliferating proximal tubule cells in the nephrogenic zone. In the newborn kidney, cadherin-6 expression is also high in the medullary zone as the ascending and descending limbs of Henle's loop grow towards the renal papilla. These ascending and descending limbs are probably the last epithelial structures formed in the developing nephron.

The in vitro antibody inhibition experiments clearly point to a function for cadherin-6 in the aggregation and/or polarization of induced mesenchyme. Induced Pax-2-positive metanephric mesenchyme appears loosely organized with little evidence of tubule formation when cadherin-6 blocking antibodies are added to kidney organ cultures. Although cadherin-6 is not expressed in the WT1-positive cells of the glomeruli, glomerular development is inhibited with the blocking antibodies most probably because the requisite steps leading to s-shaped body formation cannot occur. Cadherin-6 expression appears down-regulated in the presence of blocking Fab fragments and suggests that expression levels may be potentiated by aggregation. Alternatively, the Fab fragments that cover the extracellular domain of cadherin-6 may react poorly with the secondary antibody made against the whole rabbit IgG molecule.

Several lines of evidence support our interpretation that the cadherin-6 Fab fragments function by inhibition of cadherin-mediated cell-cell adhesion. First, the antigen used spans amino acids 68-410 of the cadherin-6 propeptide and includes most of the extracellular domain. The proposed N-terminal signal sequence and precursor peptide spans amino acids 1-53 (Xiang et al., 1994); the first CAD domain encompasses amino acid residues 54-159 and is followed by four additional CAD repeats. This amino terminal CAD domain is essential for homophilic interactions of class I cadherins (Nose et al., 1990). Although it is not clear whether the type II cadherins interact with similar affinities and sequence requirements, conserved elements within this amino terminal CAD domain can be identified. Our antigen includes 87% of the first CAD domain, including the conserved βD, βE and calcium-binding domain

(Overduin et al., 1996), the entire 2nd and 3rd CAD repeat and approximately half of the 4th CAD repeat. More importantly, the cadherin-6 antibodies are able to inhibit calcium-dependent aggregation of cadherin-6-expressing L cells. Mouse L cells do not express any endogenous cadherins, show little cell-cell contact in monolayers, and have been used extensively to assay cadherin function (Urushihara et al., 1979). Initial attempts to generate cadherin-6 transformants using plasmid expression vectors were unsuccessful, perhaps due to high levels of expression. Using retroviruses that integrate only a single copy of the mouse *cadherin-6* gene, we were able to generate several expressing lines and demonstrate cadherin-6-induced aggregation similar to what has been reported for chicken cadherin-6B (Nakagawa and Takeichi, 1995). Similarly, in cultured kidney rudiments exposed to cad6-Fab, induced metanephric mesenchymal cells are more spread out and found along the entire ureteric bud epithelium, whereas control mesenchymal cells are generally tightly associated and never found directly adjacent to mature ureteric bud epithelium.

The activation of cadherin-6 in the mesenchyme-derived renal vesicle precedes the establishment of epithelial polarity. Strikingly, expression of E-cadherin is sufficient in culture to redistribute the localization of cell surface proteins and reorganize the membrane cytoskeleton (McNeil et al., 1990). Whether cadherin-6 is a similar determinant of polarization in the renal vesicle epithelium or merely one protein among many that are necessary to form adhesive junctions remains to be determined.

In summary, the ability of cells to undergo cadherin switching may be potent driving force for morphogenesis. The data presented in this report demonstrate that cadherins can demarcate the newly formed mesenchyme-derived epithelial cells from the ureteric bud epithelium and surrounding interstitial mesenchyme. Furthermore, as this new epithelium proliferates into the s-shaped body, it becomes compartmentalized into glomerular, proximal tubular and distal tubular epithelium. Differential expression of cadherin-6, E-cadherin and P-cadherin may provide repellent forces that aid in cleft formation as well as pattern the developing nephron with respect to cell types along the proximal-distal axis.

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Note added in proof

A recent report by Rosenberg et al. (*Dev. Biol.* **187**, p. 55, 1997) demonstrates that the expression of R-cadherin is very similar to cadherin-6 in the developing kidney. R-cadherin is a type-I cadherin and does not react with our cadherin-6 antibodies. Thus, a role for R-cadherin in mesenchymal cell aggregation must also be considered.