The Cell Adhesion Molecule L1 Is Developmentally Regulated in the Renal Epithelium and Is Involved in Kidney Branching Morphogenesis

Hanna Debiec,* Erik Ilsø Christensen,‡ and Pierre Marie Ronco*

*Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 489, Hôpital Tenon, 75020 Paris, France; and
‡Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-800 Aarhus C, Denmark

Abstract. We immunopurified a surface antigen specific for the collecting duct (CD) epithelium. Microsequencing of three polypeptides identified the antigen as the neuronal cell adhesion molecule L1, a member of the immunoglobulin superfamily. The kidney isoform showed a deletion of exon 3. L1 was expressed in the mesonephric duct and the metanephros throughout CD development. In the adult CD examined by electron microscopy, L1 was not expressed on intercalated cells but was restricted to CD principal cells and to the papilla tall cells. By contrast, L1 appeared late in the distal portion of the elongating nephron in the mesenchymally derived epithelium and decreased during postnatal development. Immunoblot analysis showed that expression, proteolytic cleavage, and the glycosylation pattern of L1 protein were regulated during renal development. L1 was not detected in epithelia of other organs developing by branching morphogenesis. Addition of anti-L1 antibody to kidney or lung organotypic cultures induced dysmorphogenesis of the ureteric bud epithelium but not of the lung. These results suggest a functional role for L1 in CD development in vitro. We further postulate that L1 may be involved in the guidance of developing distal tubule and in generation and maintenance of specialized cell phenotypes in CD.

Key words: collecting duct • epithelium • cell adhesion molecules • morphogenesis • organ culture

THE formation of an organism requires coordination of cell behaviors and thus is highly dependent on communication among cells mediated by cell surface receptor proteins and their ligands on the adjacent cells or presented in the extracellular matrix (Clark and Brugge, 1995; Cunningham, 1995; Gumbiner, 1996; Miller and Moon, 1996). Renal development involves reciprocal inductive interactions between an epithelial structure, the ureter bud (a caudal outgrowth of the mesonephric duct also called the Wolffian duct), and a surrounding mesenchyme, the metanephric blastema. Signals from the tips of ureteric bud epithelium induce the metanephric mesenchyme to undergo a sequence of events leading to its transformation into an epithelial structure that gives rise to the glomerular and tubular epithelia of the mature kidney. On the other hand, the transdifferentiated mesenchyme induces branching morphogenesis of the ureter bud, leading to the development of the collecting duct (CD)[1] system (Davies, 1996; Vainio and Müller, 1997). As development proceeds, the CD epithelium itself turns from an embryonic inductor into an excretory epithelium composed of two intermingled functionally and morphologically different cell types, the principal (P) cells and the intercalated (IC) cells (Evan et al., 1991; Tisher and Madsen, 1996). In the last few years, studies on early stages of renal embryogenesis have revealed a complex cascade of inducing and signaling events implicating transcription factors, growth factors and their receptors, extracellular matrix constituents, and extracellular matrix degrading enzymes (Lechner and Dressler, 1997; Lelongt et al., 1997; Wallner et al., 1997). By contrast, still very little is known about the molecular and cellular events that control later stages of renal development. These postinductive stages include segmental organization and functional maturation of individual nephron segments, branching and growing of the CD, and generation of its cellular heterogeneity.

Address correspondence to Hanna Debiec, INSERM U 489, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France. Tel.: 33 1 56 01 65 12. Fax: 33 1 56 01 62 17. E-mail: biblio.inserm@tnn.ap-hop-paris.fr

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; CCD, cortical collecting duct; CD, collecting duct; CNT, connecting tubule; IMCD, inner medullary collecting duct; IC, intercalated; IgSF, immunoglobulin superfamily; OMCD, outer medullary collecting duct; P, principal.

To identify novel morphoregulatory molecules possibly involved in kidney development, we generated a murine monoclonal antibody (mAb) against a cell surface antigen of the CD cell line RC.SVtsA58 (Prié et al., 1991). This source of antigen was chosen because the cell line has a principal cell phenotype, but with some characteristics of intercalated cells (Prié et al., 1994), suggesting that it could express developmentally regulated antigens. In this work, we show that the mAb-recognized antigen is a splicing isoform of the neural cell adhesion molecule L1.

L1 is a member of the immunoglobulin superfamily (IgSF). It is composed of six Ig-like domains followed by five fibronectin type III-like repeats, a transmembrane domain, and a cytoplasmic domain that confers cytoskeletal association via linker proteins (Davis and Bennett, 1994; Brümmendorf and Rathjen, 1996; Dahlin et al., 1997). This molecule is engaged in complex extracellular interactions, with multiple binding partners on cell surface and in the extracellular matrix, triggering distinct signal transduction pathways (Kamiguchi and Lemmon, 1997; Walsh and Doherty, 1997). L1 is primarily expressed in the embryonic and adult nervous system, where it is involved in diverse neuronal processes (Brümmendorf and Rathjen, 1996; Hortsch, 1996). Consistent with a role in development, mutations in the human *L1* gene are associated with a variable phenotype including mental retardation and anomalous development of the nervous system, referred to as "CRASH" syndrome (Fransen et al., 1997; Brümmendorf et al., 1998). Similarly, mutations in the *Drosophila* L1 homologue, neuroglian, result in abnormal guidance of motorneurons (Bieber et al., 1989; Hall and Bieber, 1997). Phenotypes of L1-deficient mice (Cohen et al., 1997; Dahme et al., 1997) confirm that L1 is crucial for embryonic brain histogenesis, in particular for the development of axon tracts and pyramidal decussation. Outside the nervous system, the effects of *L1* gene mutation or invalidation have not been analyzed; however, L1 appears to be important for interactions between leukocytes and between epithelial crypt cells of the small intestine (Brümmendorf and Rathjen, 1995; Kadmon and Altevogt, 1997).

To understand the role of L1 in kidney morphogenesis, we first studied its ontogeny by a combined immunohistochemical and immunoblotting approach. We showed that among organs developing by branching morphogenesis, the kidney was the only one that expressed L1. L1 was detected since the mesonephric stage and was developmentally regulated both in its segment distribution and molecular processing. In vitro using the organotypic culture model, we found that addition of anti-L1 antibody perturbed the development of the ureter bud epithelium, suggesting a functional role for L1 in CD development. Based on the properties of L1 and its distribution pattern, we further suggest a possible role of L1 in the guidance of developing distal tubule and in the generation and maintenance of the terminal phenotype in the CD.

Materials and Methods

Tissue Isolation, Fractionation, and Protein Extraction for Immunoblotting and Protein Purification

Membranes were prepared from embryonic, postnatal, or adult New Zealand White rabbits, from human tissues, and from various CD epithelial cell lines generated in our laboratory, including: RC.SVtsA58 (Prié et al., 1991), RC.SV3 (Vandewalle et al., 1989), and HCD (Prié et al., 1995). Tissues or cells were homogenized in hypotonic buffer (50 mM Tris, pH 7.4, 1 mM EDTA) in the presence of protease inhibitors (10 μg/ml leupeptin, aprotinin, and pepstatin), followed by low-speed centrifugation at 1,000 *g* to remove nuclei and cell debris. The supernatant was further centrifuged at 100,000 *g* for 1 h. The pellet was extracted for 30 min at 4°C with lysis buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2 mM EDTA, and protease inhibitors) containing 1% (wt/vol) NP-40. Insoluble material was pelleted by centrifugation at 50,000 *g* for 1 h, and the supernatant was used for further analysis. Protein concentration was determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

In some experiments, SDS denatured membrane proteins were deglycosylated with PNGase F (Boehringer Mannheim GmbH, Mannheim, Germany) using buffers and conditions as recommended by the manufacturer.

For macrodissection of cortex, outer, and inner medulla, E29 kidneys were excised, and the three zones were isolated with dissection scissors. For microdissection of nephron segments, the adult rabbit kidney was perfused with cold saline until blood free, followed by cold DME (Sigma Chemical Co., St. Louis, MO) containing 1.5 mg/ml collagenase. The kidney was removed, cut into coronal slices, and incubated at 37°C for 30 min in the DME-collagenase solution. Slices were rinsed with ice-cold PBS, placed into DME medium containing 1.0% FBS, and maintained at 4°C during microdissection. Glomeruli, proximal convoluted tubule, medullary thick ascending limb, cortical thick ascending limb, cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD) segments were dissected under microscope.

Purification of Antigen by Immunoaffinity Chromatography

mAb 272, produced and tested as described previously (Prié et al., 1994), was purified from mouse ascites by chromatography on HiTrap protein G (Pharmacia Biotech AB, Uppsala, Sweden). Purified mAb was bound to CNBr-activated Sepharose 4 B (Pharmacia Biotech AB) by overnight incubation and then washed with the buffers as recommended by the manufacturer.

Aliquots of solubilized membranes prepared from kidney, brain, or cells were loaded onto the affinity column at a flow rate of 3 ml/h. The column was extensively washed with three buffers successively: (*a*) 20 mM Tris, pH 7.4, 1% NP-40; (*b*) 20 mM Tris, pH 8.0, 0.5% NP-40, and 0.5 M NaCl; and (*c*) 50 mM Tris, pH 9, 0.5 M NaCl. Finally the antigen was eluted with 50 mM triethanolamine, pH 11.5, containing 150 mM NaCl and 1% octyl glucoside, and quickly neutralized with HCl. Fractions were analyzed using SDS-PAGE, and peak fractions were concentrated in a Centricon-30 (Amicon, Inc., Beverly, MA).

Gel Electrophoresis, Immunoblotting, and Protein Sequence Analysis

The membrane fractions were diluted in SDS sample buffer, boiled for 3 min, and separated in 6% SDS–polyacrylamide gels according to Laemmli (1970). For Western blotting, proteins were transferred from gels to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 10% nonfat dry milk in PBS–0.1% Tween and 0.1 mM levamisole for 2 h at 37°C and then probed with purified mAb 272 (1.0 μg/ml) followed by alkaline phosphatase–conjugated secondary antibody (Promega, Heidelberg, Germany) diluted 1: 5,000. Primary antibodies were omitted, or isotype-matched murine IgG was used as the primary antibody for control blots. The immunoreaction was visualized by enzymatic reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate complex.

For protein sequencing, the immunopurified samples were deglycosylated with PNGase F (Boehringer Mannheim GmbH) and separated on 8% SDS gels. After transfer to PVDF membrane (Trans-Blot Transfer Medium; Bio-Rad Laboratories) protein spots were excised from Coomassie brilliant blue–stained membranes. NH_2-terminal sequencing was performed by automated Edman degradation using a protein sequenator (model 473A; Applied Biosystems, Inc., Foster City, CA).

Immunolocalization of L1

In rabbit embryonic kidney, L1 was localized by immunoperoxidase stain-

ing of 4-μm frozen sections. Tissue was fixed in 2% PFA for 2 h and then immersed in 30% sucrose for 12 h. Samples were frozen in Tissue-Tek over liquid nitrogen, and sections were cut at −20°C in a cryostat. Sections were preincubated in PBS containing 0.05 M glycine and 5% FCS with 2% nonfat dry milk and then incubated overnight at 4°C with mAb 272 or a control isotype-matched murine IgG (5 μg/ml). After extensive washing, sections were treated with horseradish peroxidase–conjugated anti–mouse secondary antibody (Amersham, Buckinghamshire, UK) for 4 h at room temperature. Peroxidase was visualized with 3-amino-9-ethyl carbazole (Sigma Chemical Co.).

Immunolocalization of L1 in the adult kidney was studied using techniques on frozen ultramicrotome sections. Tissues were prepared and fixed as previously described (Christensen et al., 1995). Ultrathin sections of 0.8 μm and 70–90 nm were obtained at −80°C for light microscopy and −100°C for electron microscopy, respectively, using a cryoultramicrotome (model Ultracut S; Reichert-Jung, Vienna, Austria), and collected as described previously (Christensen et al., 1992). For immunolabeling, sections were preincubated in PBS containing 0.05 M glycine and 0.1% nonfat dry milk and subsequently incubated with mAb 272 or a control isotype-matched murine IgG (5–10 μg/ml) overnight at 4°C. For light microscopy, sections were further incubated with peroxidase-conjugated secondary antibody (Daco A/S, Glostrups, Denmark). Peroxidase was visualized with diaminobenzidine. For electron microscopy, bound immunoglobulins were visualized with 10-nm gold-labeled goat anti–mouse Ig (Bio Cell, Cardiff, UK). The sections were embedded in methylcellulose and examined with an electron microscope (model CM 100; Phillips Electron Optics, Mahwah, NJ). Immunolocalization of L1 by electron microscopy in E29 kidney was done by using the same method as for the adult tissues.

Organ Culture Experiments

Rabbit embryos were surgically extracted at 11.5 d of gestation (E11.5) from pregnant New Zealand rabbits. Embryonic kidneys and lungs were isolated by microdissection and were cultured on sterile 1-μm Nucleopore filters (Costar Corp., Cambridge, MA) in 24-well plates in the presence of serum-free, chemically defined medium, which was changed every 24 h. The medium was composed of an equal volume of improved minimum essential medium (Richter et al., 1972) and Ham F12 (GIBCO BRL, Eragny, France) supplemented with 30 nM sodium selenite, 50 μg/ml transferrin, and 4 mM glutamine (Sigma Chemical Co). When indicated, 10, 50, 100, or 150 μg/ml of mAb 272, purified from ascites fluid by HiTrap protein G, or 150 μg/ml isotype-matched murine IgG, prepared in an identical manner, were added. Organ cultures were examined after 24 or 96 h. For each experiment, the effects of mAb 272 at a given concentration and of control antibody were tested on kidneys or lungs sampled from the same embryo to avoid variations due to asynchronic maturation within the same littermate. Morphological alterations were analyzed in kidneys cultured for 96 h, fixed in 2% PFA, dehydrated, embedded in paraffin, and sliced into tissue sections before staining with hematoxylin and eosin. Antibody penetration into tissue was tested by direct whole mount immunofluorescence. Kidneys grown in medium supplemented with anti-L1 antibody were fixed in 2% PFA, blocked in PBS containing 0.05 M glycine and 5% FCS with 2% nonfat dry milk, and further incubated with FITC-conjugated anti–mouse antibody (Biosys, Compiègne, France). They were then mounted in the mounting medium (Daco Co., Carpinteria, CA) and viewed under a microscope (model Axioplan 2; Carl Zeiss, Inc., Thornwood, NY). All experiments were repeated at least eight times.

Results

Antigen Detection, Purification, and Its Identification as L1 Cell Adhesion Molecule

To identify the target antigen of mAb 272 selected for its specificity for the rabbit CD (Prié et al., 1994), membrane-rich fractions were prepared from three CD cell lines established in our laboratory and analyzed by Western blotting. Under nonreducing conditions, mAb 272 revealed immunoreactive bands in all three cell lines. In addition, distribution of the antigen in adult rabbit and human tissues was analyzed by Western blot of membrane fractions. Substantial amounts of antigen were found in only two tissues, that is the kidney and the brain. Although the reactivity pattern looked very different before deglycosylation, *N*-glycanase treatment revealed the same two protein bands: a minor band at 150 kD and a strong one at 100 kD in all tested samples including the CD cell lines and tissues. Summarized results are presented in Fig. 1, *A* and *B*.

To characterize the kidney and brain rabbit antigen, the NP-40–solubilized membranes were submitted to immu-

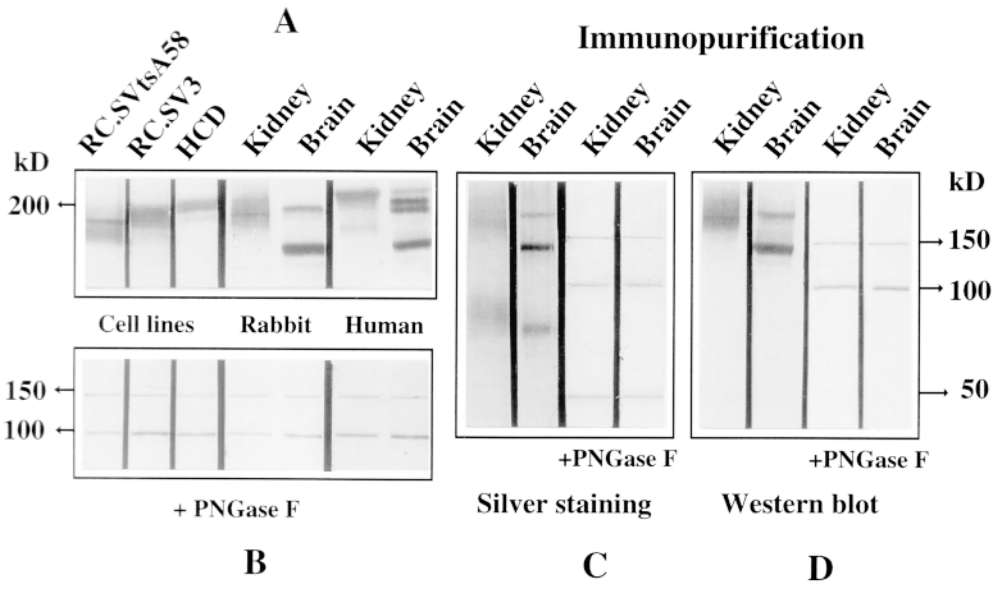

Figure 1. Immunodetection and purification of the antigen identified by mAb 272. (*A* and *B*) Western blot analysis of proteins solubilized from membrane fractions prepared from CD cell lines or kidney and brain isolated from rabbit and human species. Equal amounts of protein (50 μg) were loaded in all lanes, subjected to SDS-PAGE, electrotransferred, and probed with mAb 272 (*A*). *N*-glycosidically linked carbohydrates were removed from proteins by digestion with PNGase F before Western blot analysis. Note the same pattern of reactivity of all membrane preparations (*B*). (*C* and *D*) The antigen purified by affinity chromatography from rabbit kidney and brain was resolved by SDS-PAGE and visualized by silver staining (*C*) or immunostained with mAb 272 after transfer on PVDF membrane (*D*) before and after deglycosylation with PNGase F. Note that the 50-kD band does not react on immunoblot (*D*).

Table I. NH_2-terminal Sequences of Immunoaffinity Chromatography–purified Rabbit Proteins: Homologies with Known Proteins

150-kD	1																								
Kidney	?	Q	I	P	E																				
Brain	I	Q	I	P	E																				
100-kD																									
Kidney	I	Q	I	P	E	E	–	–	–	–	–	L	L	E	P	P	V	I	T	E	Q	S	P		
Cell line	I	Q	I	P	E	E	–	–	–	–	–	L	L	E	P	P	V	I	T	E					
Brain	I	Q	I	P	E	E	Y	E	G	Y	H	V	L	E	P	P	V								
Mouse*	I	Q	I	P	D	E	Y	K	G	H	H	V	L	E	P	P	V	I	T	E	Q	S	P		
Rat*	I	Q	I	P	D	E	Y	K	G	H	H	V	L	E	P	P	V	I	T	E	Q	S	P		
Human*	I	Q	I	P	E	E	Y	E	G	H	H	V	M	E	P	P	V	I	T	E	Q	S	P		
50-kD	845																								
Kidney	H	V	H	K	G	H	V	V	V	P	A	D	T	T	S	A	I	L							
Cell line	H	V	H	K	G	H	V	V	V	P	A	D	T	T	S										
Brain	H	V	H	?	G	H	V	V	?	P	A	D	T	T	S	A	I	L							
Mouse*	H	I	H	K	S	H	I	V	V	P	A	N	T	T	S	A	I	L							
Rat*	H	V	H	K	S	H	M	V	V	P	A	N	T	T	S	A	I	L							
Human*	H	I	H	K	D	H	V	V	V	P	A	N	T	T	S	V	I	L							

*Sequences of the neuronal form of mouse, rat, and human L1 have been taken from Moos et al. (1988), Miura et al. (1991), and Hlavin and Lemmon (1991), respectively. Identical residues are squared.

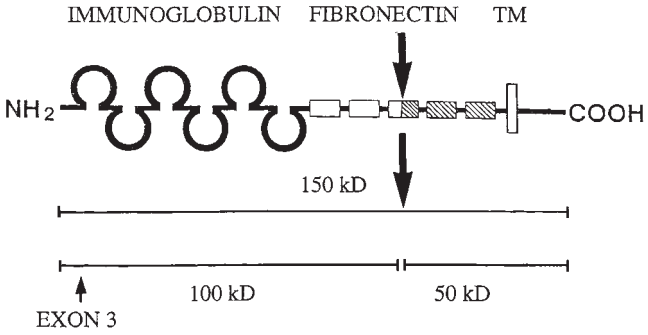

Figure 2. Model of the domain structure of L1. Ig domains are shown as loops and fibronectin III repeats are shown as boxes. The transmembrane region is indicated by a vertical bar. The arrow indicates the phylogenetically conserved cleavage site at amino acid 845. The linear presentation explains the origin of the immunopurified components and shows the place of exon 3.

noaffinity chromatography using mAb 272. The unreduced eluate was analyzed by SDS-PAGE and silver staining (Fig. 1 *C*) and by Western blotting (Fig. 1 *D*) before and after PNGase F treatment. In addition to the expected bands previously detected by Western blotting in kidney and brain membrane extracts (Fig. 1, *A* and *B*), an additional protein component was copurified both in kidney and brain. After PNGase F treatment, the immunopurified antigen resolved in three bands at 150, 100, and 50 kD in the kidney as well in the brain (Fig. 1 *C*), but the 50-kD band was not reactive with the antibody (Fig. 1 *D*), which suggests that this protein component is strongly associated with the first two by noncovalent bonds.

To identify the nature of the antigen and of the tightly associated protein eluted from the immunoaffinity column, the eluate was resolved by SDS-PAGE and electrotransferred to PVDF membrane, and the NH_2-terminal sequence of each of the three bands was determined. We analyzed the antigen purified from rabbit kidney and brain because of its peculiar abundance in these two tissues. We also sequenced the antigen isolated from the rabbit CD cell line RC.SV3 because CD cells represent a minor cell population in the whole kidney, which is composed of many other different cell types. All NH_2-terminal sequences are shown in Table I. The search for homologous sequences in the Genbank protein sequence database revealed close homology (85 to 90% identity) of the NH_2-terminal sequences of the rabbit brain 150-, 100-, and 50-kD components with corresponding protein sequences of the mouse (Moos et al., 1988), rat (Miura et al., 1991), and human (Hlavin and Lemmon, 1991) neuronal cell adhesion molecule L1. The degree of homology between rabbit and the other species was quite similar, to the degree of interspecies homology previously reported. We therefore concluded that the kidney and brain antigen was the rabbit orthologue of the murine and human L1.

The basic structural plan of L1 molecule is presented in Fig. 2. The NH_2-terminal sequence of the 150- and 100-kD components corresponds to that of the mature protein predicted by the cDNA sequence, whereas the NH_2-terminal end of the 50-kD component starts at amino acid 845 in the middle of the third fibronectin type III repeat. As the other members of its group, L1 is synthesized as a 150-kD precursor polypeptide, subsequently cleaved at a conserved sequence in the third fibronectin type III repeat. The resulting 100- and 50-kD polypeptides remain tightly associated in cell membranes as heterodimers. This explains why although the antibody was not reactive with the 50-kD component, this polypeptide was purified with its 100-kD partner, which expresses the reactive epitope.

Sequencing the 100-kD component of the rabbit kidney L1 revealed an unexpected finding. Five amino acids were missing in both the kidney and the RC.SV3 cell line antigens compared with the sequence identified in the brain peptide (Table I). In addition, the first amino acid after this deletion was substituted, with a leucine in the kidney replacing a valine in the brain antigen (Table I). Comparison with the genomic structure of the mouse and human genes (Coutelle et al., 1998) shows that the missing sequence and the substitution of the next amino acid correspond exactly to the splicing event of the mini-exon 3, which was called exon 2 or B until an additional untranslated exon was identified at the 5′ end of the gene (Kallunki et al., 1997). Thus, the kidney epithelium expresses an alternative splicing isoform of the brain L1 characterized by the skipping of exon 3 sequence.

Immunohistochemical Localization of L1 in the Developing and Adult Rabbit Kidney

To explore the possibility that L1 may participate in the development of the vertebrate urinary excretory system, we examined its spatial and temporal distribution by immunohistochemistry on tissue cryosections. Within the excretory system, L1 starts to be weakly expressed in the mesonephros, in the Wolffian duct (E10) (Fig. 3 *B*). Immunostaining predominated at the cranial end of the duct and was absent at the caudal end, from which the ureteric bud outgrows (not shown). In contrast, the mesonephric

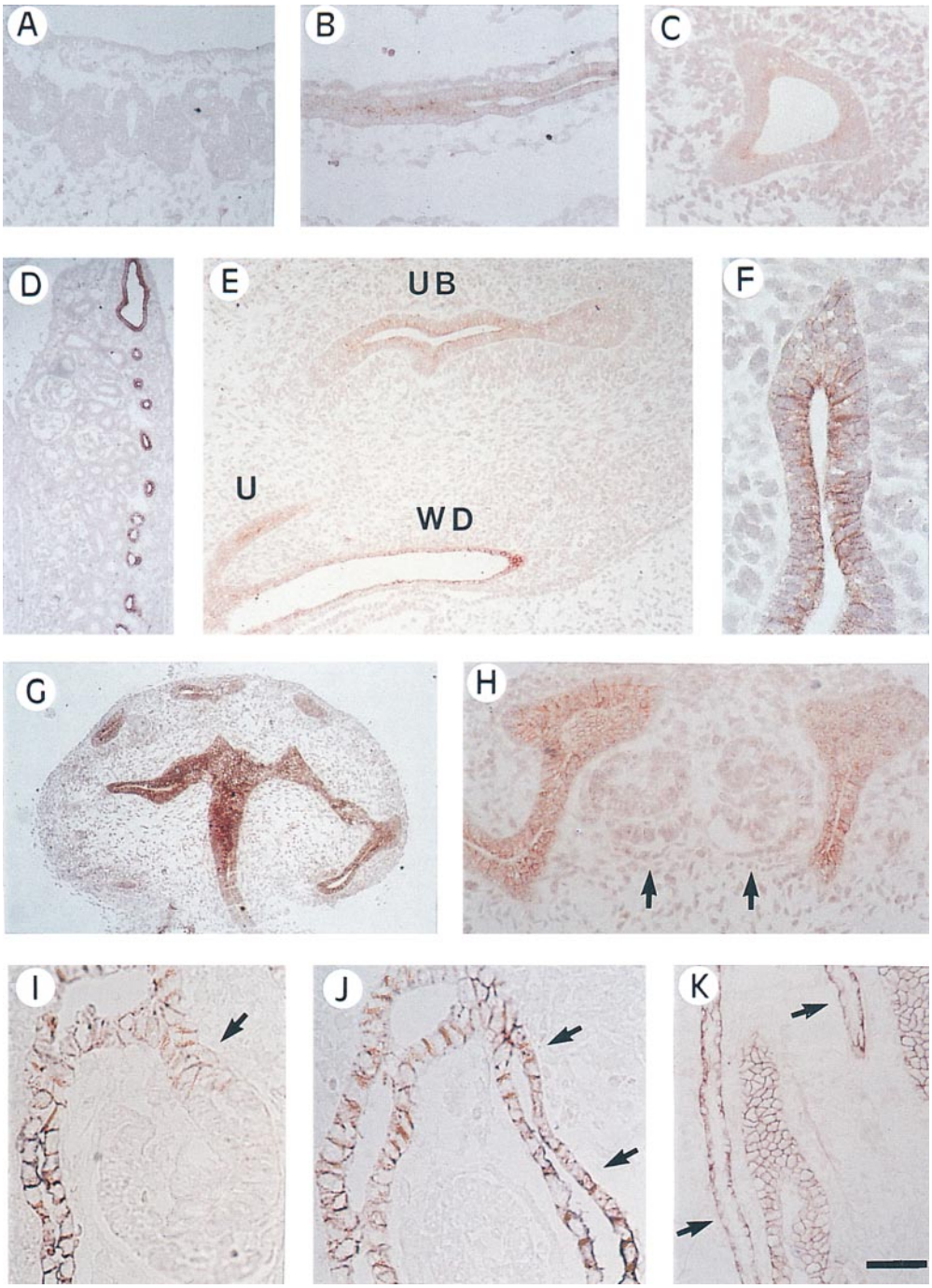

Figure 3. Immunohistochemical localization of L1 protein during development of rabbit kidney. Cryosections were incubated with mAb 272 and visualized by indirect peroxidase staining. (*A* and *B*) L1 is expressed at low level in the Wolffian duct (*B*) but not in mesonephric tubules (*A*) as shown in a longitudinal section (*B*) and a transverse section (*A*) through the E10 mesonephros. (*C*) Longitudinal section through the E11.5 metanephros. L1 starts to be expressed in the ureteric bud epithelium (which starts to branch) but is not detected in induced mesenchyme. (*D*) E12.5 mesonephros. Note strong expression in the Wolffian duct visible on a transverse section. (*E* and *F*) E12.5 metanephros. L1 is expressed in the T-shaped ureteric bud (*UB*), ureter (*U*), and Wolffian duct (*WD*) but still not in the mesenchyme (*E*). Higher magnification showing predominant apical and lateral subcellular localization of L1 in a ureter bud branch (*F*). (*G* and *H*) E15. Note strong labeling of ureter bud branches (*G*) and lack of staining of S-shaped bodies in the nephrogenic zone (*arrows*, *H*). (*I–K*) E27. In addition to its persistent expression in the CD system, L1 is now detected in mesenchyme derivatives where it is first expressed in the distal segment of S-shaped bodies (*arrows*, *I*) hooking up with the terminal ampulla. In more mature nephrons, L1 is detected in the connecting segment (*arrows*) and the collecting duct (*J*) and is also visible in the medullary region in elongating tubules that form the loops of Henle (*arrows*, *K*). *E–K* are longitudinal sections. Bar: (*A–C*) 50 μm; (*D* and *E*) 100 μm; (*F*) 40 μm; (*G*) 160 μm; (*H–K*) 50 μm.

tubules were unstained (Fig. 3 *A*). Subsequently L1 expression increased, and at E12.5, the mesonephric duct (Wolffian duct) was heavily stained (Fig. 3 *D*).

The formation of definitive kidney (metanephros) is initiated by interaction of the ureteric bud with the metanephric blastema that occurs at E11.0 in the rabbit (Saxen, 1987). At E11.0, the rising ureteric bud grows caudally into an adjacent region of the metanephric mesenchyme. At E11.5, after ureteric bud–mesenchyme interaction, a weak staining became visible in the ureteric bud epithelium, mainly at the apical aspect of the cells (Fig. 3 *C*). The antigen was not detected in the induced condensing mesenchyme (Fig. 3 *C*). At E12.5, L1 was clearly expressed in the T-shaped ureteric bud that has just divided within the metanephric mesenchyme, as well as in the developing ureter (Fig. 3 *E*). Staining predominated on the apico–lateral cell membranes, whereas the basal side remained negative (Fig. 3 *F*). Later on, the ureter bud branches repeatedly

and the condensing mesenchyme surrounding the tips of the bud branches starts its epithelial differentiation. At E15 (Fig. 3, *G* and *H*), L1 expression was still restricted to the epithelium of the ureter bud derivatives (Fig. 3 *G*). No staining was observed in mesenchyme-derived structures, including condensates, comma-shaped bodies, and S-shaped bodies (Fig. 3 *H*). At E27 (Fig. 3, *I–K*), the S-shaped bodies elongate and join with the terminal ampulla of the CD (Fig. 3 *I*). L1 immunoreactivity then appeared at the lateral membranes of the cells, which form the most distal part of the newly formed primitive nephron (Fig. 3 *I*). In more mature nephrons, in which the distal tubule has further elongated (Fig. 3 *J*), L1 was still detected in connecting tubule segments in the cortex (Fig. 3 *J*) as well as in the elongating tubules that will form the loops of Henle (Fig. 3 *K*). L1 expression in these tubule segments was, however, transient and declined after birth. L1 immunoreactivity was not observed at any time in proximal tubules and glomeruli. It was not detected on the ureter smooth muscle cells, vascular endothelium, or stroma cells.

In the adult kidney, we had previously reported that the antigen recognized by mAb 272 was a marker of P cells in the cortical segment of the CD (Prié et al., 1994). Our new ontogenic data, and particularly L1 expression in the embryonic distal tubule, led us to reappraise L1 distribution in the adult kidney. In the distal tubule, L1 did persist albeit in small amounts, on the basal plasma membrane of connecting cells, but it was not detected on IC cells (Fig. 4 *A*). In the CD, there was no reaction on IC cells but strong reactivity was seen on the basolateral cell membrane of the principal cells in cortex, as well as in outer and inner medulla (Fig. 4, *A–C*). Serial adjacent sections showed absence of L1 staining from CD cells that expressed H^+-ATPase, a marker for IC cells (not shown). In the deep segment of the IMCD where IC cells and ciliated P cells are replaced by progressively taller cells with less basolateral plasma membrane infoldings, L1 protein then became restricted to the lateral membrane of the tall cells (Fig. 4 *D*). The same subcellular distribution was seen in papilla-forming cells.

L1 was not detected in endoderm-derived structures, including urethra and urinary bladder (not shown). It was also not detected during ontogeny of other organs, including the lung, intestine, salivary gland, and pancreas, which develop as the kidney by branching morphogenesis of an epithelial structure (not shown).

Immunoelectron Microscopic Localization of L1 in the Embryonic and Adult Collecting Duct Epithelium

To gain insight into the distribution of L1 in specific cellular domains in the embryonic and terminally differentiated CD epithelium, an immunoelectron microscopy study was carried out. In the developing CD epithelium at E27, L1 was localized in both lateral membranes of adjacent epithelial cells (Fig. 5 *A*). At that time, the embryonic CD showed no expression of H^+-ATPase and band 3, two markers for IC cells (not shown). In the adult kidney, the terminally differentiated CD epithelium is composed of P cells and of IC cells (Evan et al., 1991; Tisher and Madsen, 1996). Cross-sections of CD in cortex and outer and inner medulla showed gold particles exclusively along the basal infoldings (Fig. 5 *D*) and lateral membranes of P cells,

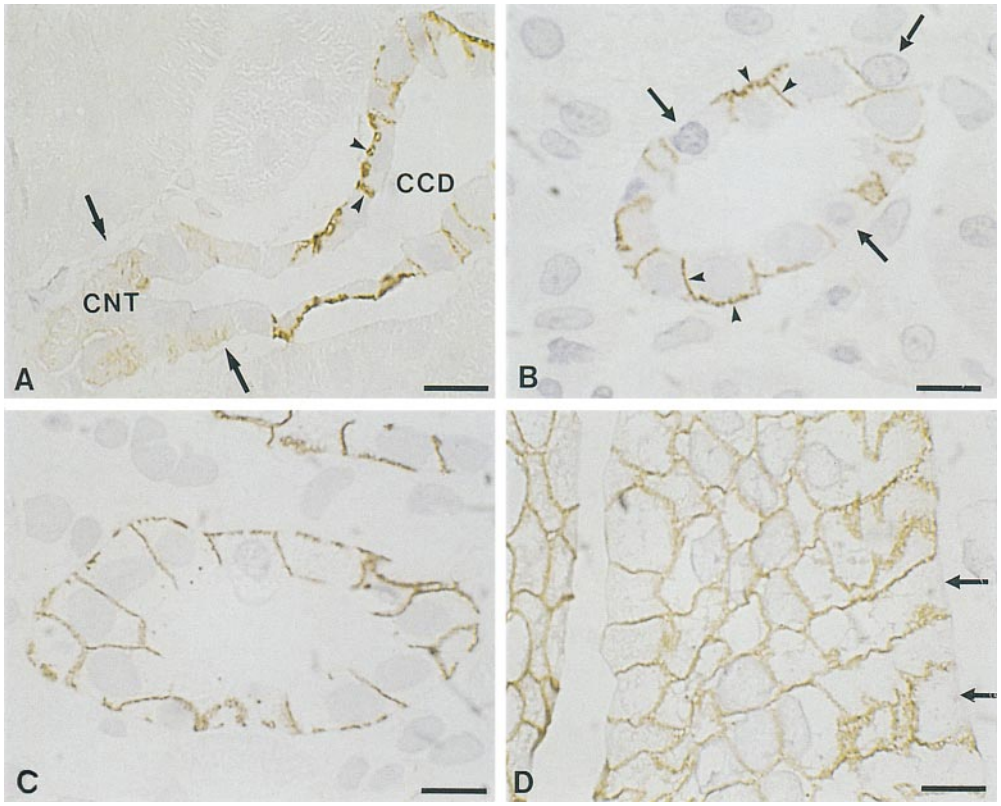

Figure 4. Immunolocalization of L1 protein within different adult renal segments. Semithin cryosections were incubated with mAb 272 and visualized by indirect peroxidase staining. (*A*) Transition between the connecting tubule (*CNT*) and the initial collecting duct (*CCD*). Note very weak immunostaining of the CNT confined to basal infoldings of connecting cells (*arrows*). By contrast in the initial CCD, L1 is strongly expressed at the basolateral side (*arrowheads*). (*B*) Cortical CD showing strong lateral and basal staining (*arrowheads*) of principal cells. Arrows point to unstained intercalated cells. (*C*) Inner medullary CD showing staining of lateral and basal membrane of principal cells. (*D*) Papillary duct. Basal (*arrows*) and apical membranes are unstained in contrast to the intensely stained lateral membranes. Bars, 10 μm.

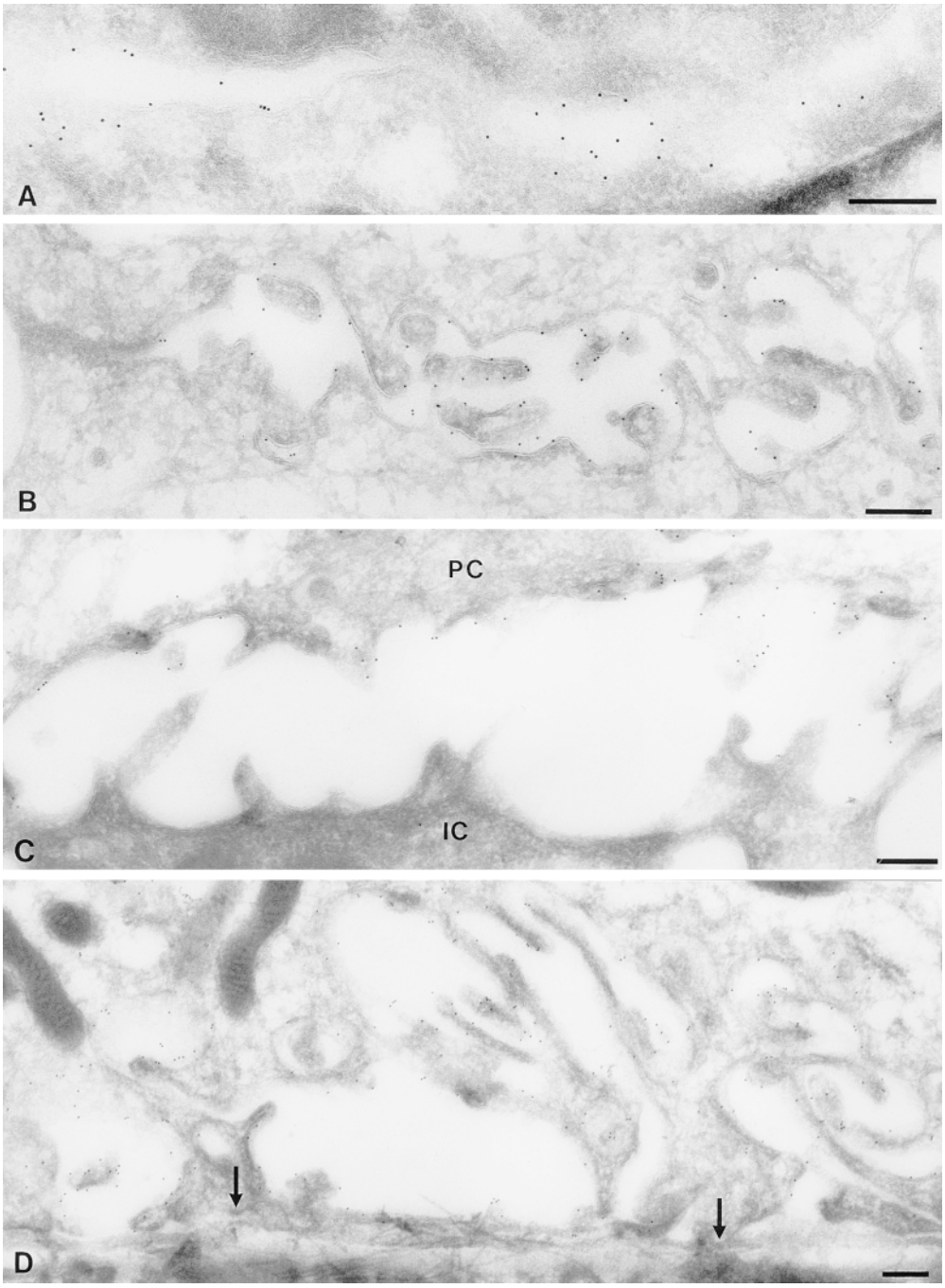

Figure 5. Immunogold electron micrograph showing ultrastructural localization of L1 in the embryonic and fully differentiated adult CD epithelium. (*A*) Embryonic kidney at E27, outer medulla. The gold particles are seen along the lateral membranes of two epithelial cells. (*B*) Adult kidney, inner medulla. The gold particles are localized along the lateral membranes of two adjacent principal cells. (*C* and *D*) Adult kidney cortex. (*C*) Lateral cell domain. The gold particles are visible on the membrane of principal cell (*PC*), but not of intercalated cells (*IC*). (*D*) Basal domain. The gold particles are localized on deep basal membrane infoldings but are absent at contact points with basement membrane (*arrows*). Bars, 0.25 μm.

whereas the facing lateral domain of IC cells (Fig. 5 *C*) as well as their basal membrane were negative. The basal side of P cells in contact with the basement membrane (Fig. 5 *D*) consistently failed to express L1, excluding the contribution of L1 to cell interactions with basement membranes. Furthermore, the basement membrane itself, extracellular matrix components, and the luminal surface were always L1 negative. When two P cells were in contact, uniform gold labeling of both adjacent lateral membranes was seen (Fig. 5 *B*). In the IMCD and the papilla, immunogold labeling was restricted to lateral membranes of the tall epithelial cells (not shown). These data provide evidence that in the adult CD epithelium, L1 is down-regulated only on the IC cells. This observation suggests the involvement of L1 in the establishment and maintenance of P cell phenotype.

Molecular Forms of L1 Expressed during Renal Ontogeny

To investigate the qualitative expression of L1 during renal development, we used Western blotting to analyze membrane fractions prepared from the whole mesonephros, metanephros, and postnatal kidney at various time points and from microdissected adult renal segments. The antigen was first detected at E10 in mesonephros (Fig. 6

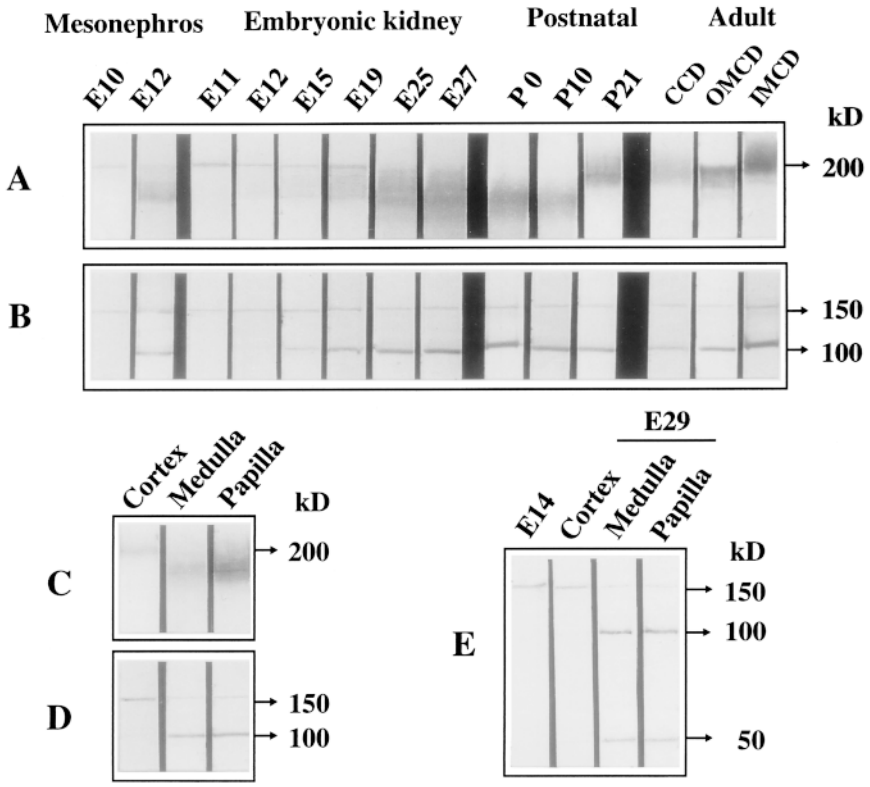

Figure 6. Immunochemical analysis of L1 ontogeny in rabbit kidney. Kidneys were isolated at indicated times from embryonic, postnatal and adult rabbits. (*A–D*) Western blot analysis. Proteins were solubilized from membrane fractions prepared from whole kidneys, adult microdissected segments (*A* and *B*), or from E29 dissected renal zones (*C* and *D*). Equal amounts of protein (50 μg) were loaded in all lanes, subjected to SDS-PAGE, electrotransferred, and probed with mAb 272. Proteins were analyzed before (*A* and *C*) and after (*B* and *D*) PNGase F treatment. (*E*) SDS-PAGE analysis of L1 purified by immunoaffinity using mAb 272 from E14 kidney, and from superficial cortex, medulla, and papilla of E29 kidney. Samples were treated with PNGase F before electrophoresis. Gels were silver stained.

A). The amount of antigen increased with time in parallel to substantial changes of its electrophoretic pattern (Fig. 6 *A*). In the adult kidney, L1 was predominantly detected in membrane fractions prepared from the CD segments isolated from CCD, OMCD, and IMCD, confirming the results of immunohistochemical localization (Fig. 6 *A*), whereas immunoreaction was not visible on the immunoblots when glomeruli, proximal tubule, or thick ascending limb were analyzed (not shown). After removal of N-linked glycans, the antigen resolved in 150- and 100-kD components in all tested samples (Fig. 6 *B*), indicating that the changing electrophoretic pattern of L1 during development was mainly due to increasing carbohydrate content. PNGase F treatment also showed a changing ratio of the 150- and 100-kD components. Expression of the 150-kD component was relatively higher in early stages of renal development (Fig. 6 *B*), which means that L1 was first synthesized mainly as a precursor molecule. In later stages of development and in the adult kidney, the 100-kD component predominated (Fig. 6 *B*), suggesting that L1 was then expressed as a heterodimer composed of the two associated 100- and 50-kD chains originating from the 150-kD precursor.

To support the assumption of a maturation process of L1 during development, L1 expression was examined by Western blotting in the zonal sections of E29 kidney (Fig. 6, *C* and *D*) and by immunoaffinity chromatography of E14 and E29 samples (Fig. 6 *E*). At E29, the cortical area consists predominantly of the growing CD tips, undifferentiated mesenchyme, and its early differentiation stages. This zone essentially showed the 150-kD component (Fig. 6 *D*). In deeper parts of the kidney that contain late and terminal stages of epithelial differentiation, the 100-kD component dominated. By comparison in the adult kidney, where the nephrogenic zone has disappeared from the upper cortex, L1 was expressed as a heterodimer with a prevailing 100-kD component in the CD segments throughout kidney zones (Fig. 6 *B*). Since our antibody did not recognize the 50-kD polypeptide, further characterization of the embryonic kidney antigen was performed by immunoaffinity chromatography followed by SDS-PAGE after antigen deglycosylation (Fig. 6 *E*). At E14 and in the superficial cortex of E29 kidney, L1 was predominantly expressed as the 150-kD precursor, whereas in the rest of E29 kidney, L1 was mainly composed of the 100- and 50-kD polypeptides as in the adult kidney.

Effect of Anti-L1 Antibody on Kidney Morphogenesis in Organ Culture

To provide further insight into the role of L1 in early kidney development, antibody perturbation experiments were performed in vitro using the organotypic culture model. Kidneys were explanted from E11.5 embryos and cultured for 24–96 h. We first verified that the organ culture model initially established for mouse kidney could be adapted to the rabbit. In E11.5 rabbit kidney, the ureter bud has penetrated the mesenchyme but has not divided yet (Fig. 7 *A*). T-shaped branching of the ureter bud occurred within the first 24 h of culture (Fig. 7 *B*). Later on, growth and maturation of rabbit kidney proceeded similarly to in vivo (compare Figs. 3 *H* and 8 *A*). After 96 h in

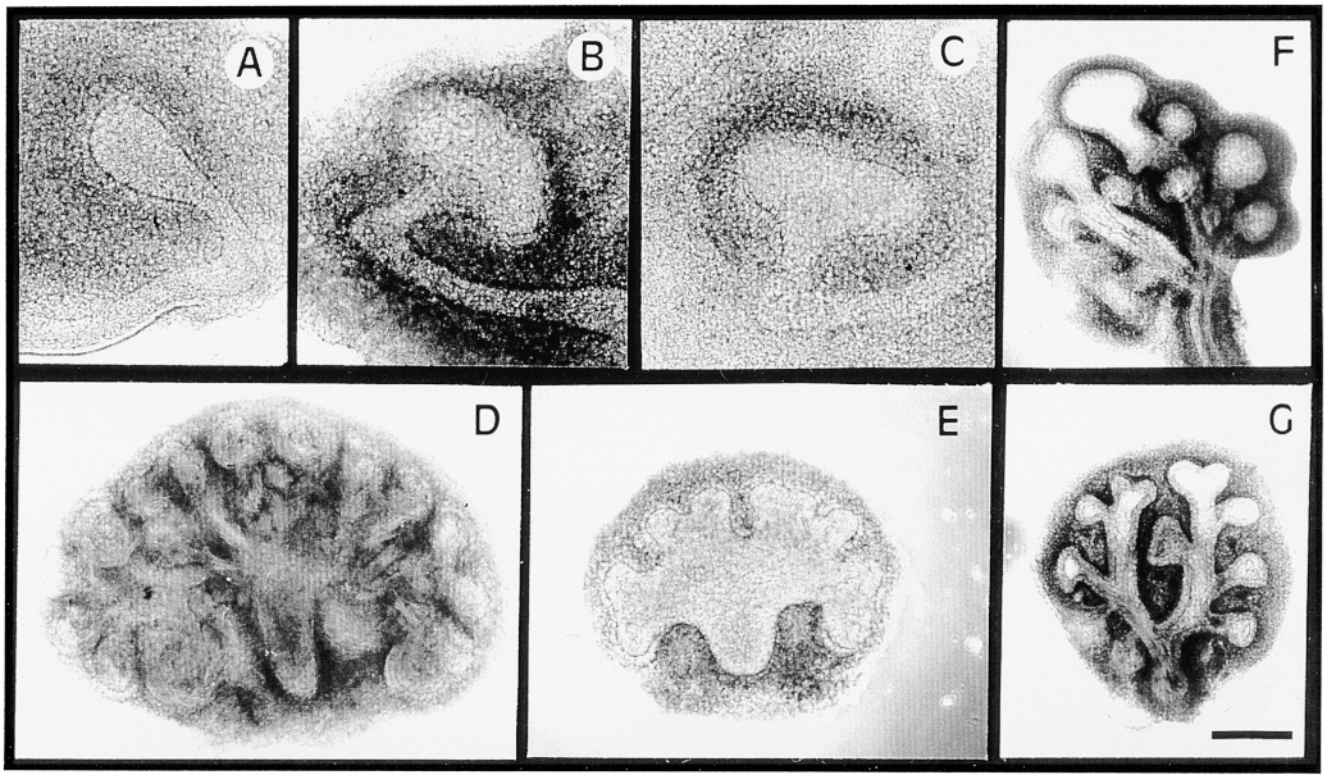

Figure 7. Effect of anti-L1 antibody on kidney and lung branching morphogenesis. Kidney (*A–E*) and lung rudiments (*F* and *G*) were isolated from E11.5 rabbit embryos. Kidney explants sampled at E11.5 (*A*) were grown on Nucleopore filters for 24 h (*B* and *C*) or 96 h (*D* and *E*) in the presence of 150 μg/ml of anti-L1 antibody (*C* and *E*) or control mouse IgG (*B* and *D*). Note that anti-L1 antibody did not affect T-shaped branching (*C* versus *B*) but dramatically impaired further branching morphogenesis of the ureter bud (*E* versus *D*). In contrast, lung morphogenesis was not affected by anti-L1 antibody (*G*) after 96 h of culture in the same conditions (control, *F*). Bar: (*A–C*) 75 μm; (*D–G*) 160 μm.

culture, the ureter bud has divided many times (Fig. 7 *D*), and mesenchymal condensates as well as comma-shaped bodies were detected around the branches (Fig. 8 *A*).

E11.5 kidneys were then cultured in the presence of monoclonal anti-L1 antibody (mAb 272) or mouse monoclonal IgG as a control. Antibody penetration in tissue was demonstrated by direct immunofluorescence of kidneys with FITC-linked anti–mouse IgG to localize anti-L1 mAb (not shown). Treatment with anti-L1 antibody did not alter T-shaped branching of the ureter bud (Fig. 7 *C*) in the first 24 h of culture. By contrast, after 96 h (Fig. 7 *E*), it induced dramatic alterations of branching morphogenesis and a marked reduction of kidney size (Fig. 7 *E* and Fig. 8, *B* and *C*). The ureteric bud and its primary and secondary divisions were notably shorter and dilated, while the tertiary divisions were rudimentary. The tips of the ureteric bud branches, the site of nephron formation, were blunted. Nevertheless, the ureteric bud was still able to induce nephrogenesis since condensates and early epithelial differentiation stages, including renal vesicles and comma-shaped bodies, had developed in the periphery of the organ (Fig. 8, *B* and *C*). Impairment of ureter bud morphogenesis by anti-L1 antibody was concentration dependent (Fig. 9). The effects of anti-L1 antibody became visible at 50 μg/ml and were maximum at 150 μg /ml. No alteration was observed in the kidney grown in basal medium or in the presence of mouse IgG in the same concentration range.

Lung rudiments sampled from the same E 11.5 embryos were grown in parallel with kidneys under the same experimental conditions. Contrary to kidney explants, anti-L1 antibody did not induce significant alterations of lung branching morphogenesis after 96 h of culture (Fig. 7, *F* and *G*). These results indicate that the antibody has no effect on nonrenal epithelial structures developing by branching morphogenesis but that it specifically impairs kidney development.

Discussion

In this work, we have identified the antigen defined by mAb 272 in the CD epithelium as an isoform of the neuronal cell adhesion molecule L1, a member of IgSF. We analyzed its renal expression during ontogeny by a combined biochemical and immunohistochemical approach. In addition, we first demonstrate that L1 is implicated in branching morphogenesis of the ureter bud in organ culture.

Identification of the CD antigen as L1 is based on the following data: (*a*) The rabbit immunopurified polypeptides have 85–90% amino acid identity with the corresponding regions of human, mouse, and rat L1 protein. (*b*)

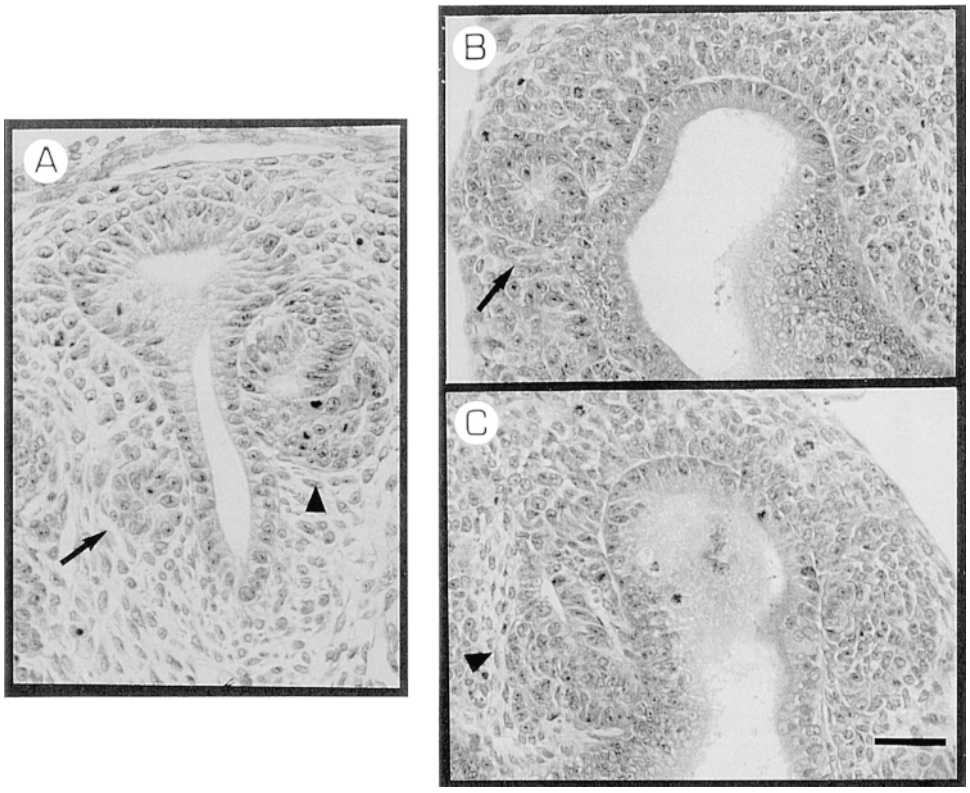

Figure 8. Photomicrographs of paraffin sections from E11.5 kidneys grown for 96 h in control IgG (*A*) and in the presence of anti-L1 mAb (*B* and *C*). Control IgG and anti-L1 mAb were added to culture media at a concentration of 150 μg/ml. Sections were stained with haematoxylin and eosin. In control conditions (*A*), mesenchymal condensation has occurred around the tips of ureter bud branches. Early stages of epithelial differentiation, including vesicle (*arrow*) and comma-shaped bodies (*arrowhead*) are visible. Note marked cystic dilation of ureter bud branches in anti-L1 antibody–treated cultures (*B*). However, early signs of epithelial differentiation, including palisadic linear organization of mesenchymal cells around the tips as well as vesicles (*arrow*, *B*) and comma-shaped bodies (*arrowhead*, *C*), are still visible. Bar, 40 μm.

The 100- and 50-kD polypeptides originate from one 150-kD precursor molecule and are noncovalently associated in the cell membrane, a common feature of all members of the L1 subfamily. (*c*) The missing sequence in the NH_2 terminus of the rabbit kidney antigen corresponds exactly to the mini-exon detected in the human and mouse *L1* genes (Coutelle et al., 1998), which suggests that at least at the 5′ end of the coding sequence, the rabbit *L1* gene has the same organization as those genes. The skipping of exon 3 that we detected in the kidney has already been described in B lymphocytes (Ebeling et al., 1996), but it has never been observed in cells of neuronal origin (Miura et al., 1991). The region encoded by exon 3 precedes the Ig domains endowed with strong homophilic binding activity (Zhao and Siu, 1995). A recently found mutation in a patient with X-linked hydrocephalus leads to exon 3 skipping (Jouet and Kenwrick, 1995). Thus, the region encoded by exon 3 appears to be crucial to the function of the neuronal L1 protein but not to that of the renal and the lymphocytic proteins. L1 protein is encoded by a single gene composed of 29 exons, with only two splicing products as yet identified. The other reported splicing concerns exon 28, a 12-bp exon located in the 3′ region of the gene, which is spliced out in L1 mRNA in the peripheral nervous system (Miura et al., 1991) and leukocytes (Ebeling et al., 1996). By using reverse transcription PCR surrounding exon 28, we found that this exon was also spliced out in the rabbit epithelial cell line RC.SV3 (data not shown). Whether splicing of small exons 3 and 28 can occur independently in renal cells and whether it is developmentally regulated is not yet known. A formal proof that these particular combinations of exons may exist as RNA transcripts would require additional work beyond the scope of this paper.

For some time, kidney expression of L1 has been controversial. Searches for L1 protein (Mujoo et al., 1986) or mRNA (Moscoso and Sanes, 1995) in renal tissue were unsuccessful. On the other hand, it was reported that anti-L1 antibody, used as a marker for neurons in the metanephros, also stained ureteric bud epithelial structures (Sainio et al., 1994, 1997). However neither the precise localization of L1 in the kidney nor its role in nephrogenesis had been defined. We show that the protein is expressed in a temporally dynamic and spatially restricted pattern within the developing and adult kidney epithelium, but with contrasting kinetics in ureter bud and mesenchyme derivatives. L1 expression can be considered as constitutive in epithelial structures that originate from the mesonephric duct (Wolffian duct)—that is, the collecting duct system and the urogenital tract epithelia (Kujat et al., 1995)—since it appears early and persists in all cells except for intercalated cells in which L1 is downregulated in the terminally differentiated CD. By contrast in the metanephric mesenchyme derivatives, L1 appears late in the distal portion of elongating newly formed tubules and disappears from the distal tubule after its patterning has been completed. Only connecting cells show a persistent but weak basal expression.

In parallel to its spatio–temporal regulation during ontogeny, L1 undergoes substantial posttranslational modifications, including proteolytic cleavage and glycosylation. These modifications may play an important role in renal organogenesis. The degree of proteolytic cleavage increases during renal development parallel with epithelial

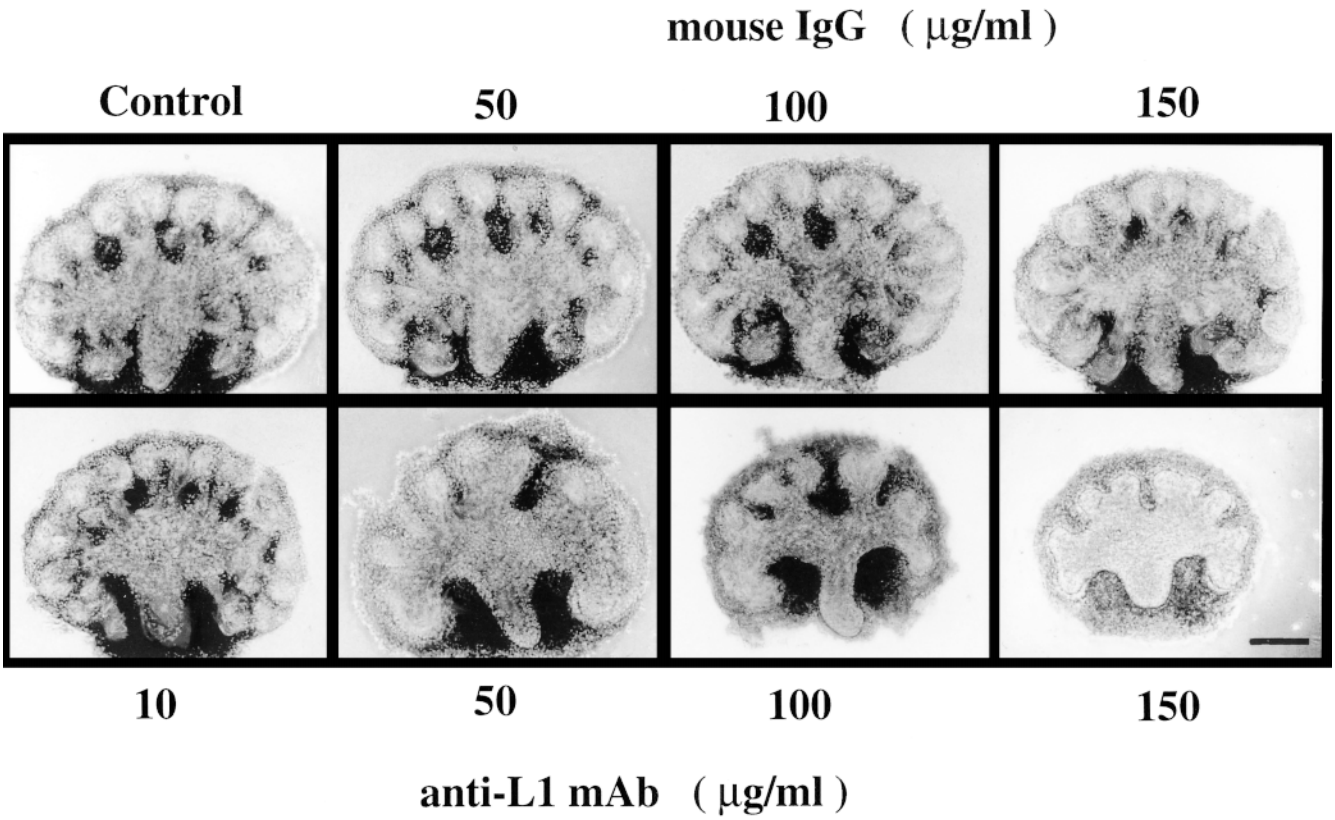

Figure 9. Concentration-dependent effect of anti-L1 mAb on branching morphogenesis of kidney. Kidney explants were cultured for 96 h in the presence of basal medium alone (*Control*) and basal medium with mouse IgG at a concentration of 50, 100, and 150 μg/ml, or anti-L1 mAb at a concentration of 10, 50, 100, and 150 μg/ml. Kidney morphogenesis was impaired in a concentration-dependent manner by the anti-L1 mAb. No alteration was observed in the kidney treated with mouse IgG. Bar, 160 μm.

maturation. Cleavage of the 150-kD precursor within the third fibronectin-like domain yields two chains, each containing structural motifs that could contribute to the different functions of L1 (Burgoon et al., 1995). Immunopurification experiments have shown that the resulting 100- and 50-kD fragments remain tightly associated. In addition, the 100-kD polypeptide, which lacks a transmembrane domain, may also become anchored in the cell membrane via other cell surface proteins such as integrins (Montgomery et al., 1996), GPI-linked proteins (Olive et al., 1995), or yet unidentified molecules leading to the generation of secondary response mediated by these receptors. Alternatively, the 100-kD fragment may be shed and become deposited in the extracellular matrix. Thus, increased proteolytic cleavage may stimulate a variety of autocrine or paracrine intracellular pathways. The augmented carbohydrate content of L1 when renal maturation proceeds may also play an important role since carbohydrate structures are directly involved in cell–cell interactions (Dwek, 1995). For example, carbohydrates expressed on the neuronal form of L1 determine its interactions with N-CAM and integrins (Horstkorte et al., 1993; Hall et al., 1997).

To evaluate the role of L1 in the first events of kidney morphogenesis, functional studies were performed. Addition of mAb 272 to kidney organotypic cultures induced dysmorphogenesis of the ureteric bud epithelium in a concentration-dependent manner but did not prevent mesenchyme induction or vesicle and comma-shaped body formation. The anti-L1 antibody used in this study reacts with the extracellular domain, a major adhesion site for several hetero- and homophilic ligands (Appel et al., 1993). Moreover, bivalent mAbs that bind to extracellular L1 domains have also been shown to trigger L1 association with signaling receptors (Klinz et al., 1995). Therefore, it is likely that mAb 272 perturbed interactions of L1 with its natural ligands and signaling systems, and this in turn resulted in abnormal ureteric bud development. Explant experiments were performed at 11.5–15.5 d, before appearance of the 100-kD band as a single entity, indicating that only the 150-kD precursor could be the target of mAb 272 at that time, although the 100-kD cleaved subunit might also play a developmental role later on. Involvement of L1 in bud initiation or initial inductive interactions seems unlikely since its expression follows rather than precedes these events, and T-shaped branching is not inhibited by mAb 272. Furthermore L1 is not detected at the basolateral side in the tips of the ureteric bud, where the presumptive inducing signals are believed to act. These data suggest that L1 could control ureter bud development in later stages, i.e., after intervention of the GDNF/c-ret axis, which seems to be responsible for the early steps of ureteric bud formation and extension (Sainio et al., 1997).

Generation of the arborescent collecting duct system is

a complex process requiring constant cytoskeleton rearrangements at the growing ureteric bud tips (Sakurai and Nigam, 1998). Tubulogenic growth factors and their receptors, such as HGF/c-met tyrosine kinase receptor, regulate branching morphogenesis by modulating the function of linker molecules between membrane and cytoskeleton (Cantley, 1996; Weidner et al., 1996; Crepaldi et al., 1997). A parallel can be drawn between renal tubulogenesis and the development of axon tracts, which is profoundly altered in L1 knock-out mice (Cohen et al., 1997; Dahme et al., 1997). L1-mediated guidance of axons is based on its interaction with the actin cytoskeleton via linker proteins, including ankyrin (Davis and Bennett, 1994) and a still unknown linker protein (Dahlin et al., 1997). The connection of L1 with linker proteins is reversible and controlled by a kinase-phosphatase system activated in response to specific extracellular interactions of L1 with its binding partners (Maness et al., 1996; Garver et al., 1997; Kamiguchi and Lemmon, 1997). Whether the cytoplasmic tail of L1 kidney isoform associates with the actin linker molecules in response to extracellular interaction remains to be determined. To clarify the role of L1 in these morphogenetic events, it will be essential to identify L1 ligands and signaling pathways in the ureter bud epithelium.

The other major site of L1 expression is the developing nephron. Development of renal tubules results from epithelial transdifferentiation of the mesenchyme and subsequent elongation. The late and transient L1 expression in the elongating distal tubules argues for its role in the guidance of tubule growth into the surrounding loose mesenchyme and in the arcade forming process, rather than for its implication in the maintenance of tubule segment specificity.

We have demonstrated by electron microscopy that L1 is expressed at the lateral membrane by all progenitor cells in early stages of CD development but is absent from IC cells in mature CD. Recent works have suggested that P and IC cells originate from a single precursor ureteric bud cell type (Aigner et al., 1995; Jamous et al., 1993, 1995). It has been established that cell surface receptors and their ligands participate in cell-to-cell and cell–matrix signaling events that influence cell fate decision during development (Greenwald and Rubin, 1992; Schmidt et al., 1993). Thus, L1 could also play a role in the terminal differentiation of CD epithelium. Identification of the interacting components involved in L1 mediated signaling is the next step toward understanding how this molecule could drive the generation of two distinct phenotypes in CD epithelium.

Finally, the potential of L1 to associate with ankyrin, which interacts also with the cytoplasmic domains of several ion channels and pumps (Davis et al., 1996; Dubreuil et al., 1996), could be helpful during maturation in the organization of epithelial cell polarity by the segregation of membrane components. L1 characteristic expression in the basolateral membrane of differentiated P cells is compatible with this function. In addition, in the tall cells of the IMCD and the papilla where L1 is localized laterally, the multicomplexes formed by L1, integral membrane proteins and cytoskeleton, could provide a structural support allowing distribution of shear stress throughout the tissue.

Epithelial–mesenchymal interactions drive branching morphogenesis during the development of a number of organs that fail to express L1 in the epithelial compartment. Thus, our results indicate that L1 has a kidney-specific function during morphogenesis. In addition, the involvement of L1 in multiple stages of renal epithelial morphogenesis may bear a clinical interest as in the nervous system. Mutations in *L1* gene or disturbances in posttranslational modifications might lead to developmental abnormalities affecting the integrated physiology of the CD system.

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