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ORIGINAL PAPER

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# Partial identification of the mab $_{CD}$Amp1 antigen at the epithelial–mesenchymal interface in the developing kidney

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**Abstract** The nature of the primary functional events of nephron induction is still unknown, making it impossible to completely understand the mechanism of tissue interaction between collecting duct ampulla and the surrounding nephrogenic mesenchyme. Soluble morphogenic substances are known to be exchanged in the process and it is assumed that nephron induction requires close contact between both tissues involved. Contrasting with that assumption our previous investigation revealed a thick fibrous meshwork separating nephron inducer and mesenchyme. Our present investigation focused on the molecular characterization of the mab $_{CD}$Amp1 antigen, which is found only in this meshwork. The protein was shown immunohistochemically to be located exclusively at the embryonic collecting duct ampulla and could be clearly distinguished from other extracellular matrix proteins such as collagen type IV, laminin, reticulin, and fibronectin. Two-dimensional electrophoresis of the soluble form of $P_{CD}$Amp1 showed a molecular weight of 87,000 and an isoelectric point of 4.3–4.4. Results from N-terminal sequencing indicated a partial sequence homology of $P_{CD}$Amp1 to collagen type IV α2-chain precursor but additionally yielded unknown sequences. Thus $P_{CD}$Amp1 is a novel, collagen-related protein, restricted to the fibrous meshwork at the mesenchymal–epithelial interphase, which is the site of primary epithelial–mesenchymal interaction.

**Keywords** Kidney · Development · Collecting duct ampulla · Nephrogenic mesenchyme · Extracellular matrix · $P_{CD}$Amp1

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## Introduction

Most data on nephron induction was obtained from studies of early mouse kidney development (Grobstein 1961; Saxén 1987). Nephrons are generated by an inductive interaction between the collecting duct ampulla and the surrounding mesenchyme. The mesenchyme condenses to form the comma-shaped body, which subsequently develops into the S-shaped body. Tubular development takes place in the direct neighborhood, but clearly separated from the collecting duct ampulla (Aigner et al. 1994, 1995). Molecular mechanisms involved in nephrogenesis are heterogeneous (Strehl et al. 1999), but it is still unknown whether an exchange of soluble factors is sufficient or whether additional cell–cell contact between inducer and nephrogenic mesenchyme is necessary. It is generally accepted that the initial step of nephron induction takes place at the ampullar tip of the collecting duct, while ampullar neck and shaft are the site of transdifferentiation from embryonic into functional P and IC cells (Kloth et al. 1993). The basal aspect of the collecting duct ampulla is the primary site for the exchange of morphogenic information between nephron inducer and surrounding mesenchyme (Barasch et al. 1996). Our earlier investigations demonstrated individual structural features at the basal aspect of the collecting duct ampulla (Lehtonen 1975). In order to identify specific structures localized at this tissue interface we raised the monoclonal antibody mab $_{CD}$Amp1 (Strehl et al. 1997). The mab $_{CD}$Amp1 antigen was shown to be a characteristic component of a dense coat of extracellular matrix fibers separating the nephron inducer from the nephrogenic mesenchyme (Strehl et al. 1999).

Our present paper describes the molecular characterization and partial identification of the mab $_{CD}$Amp1 antigen. A soluble form of $P_{CD}$Amp1 was isolated, purified by two-dimensional electrophoresis, and partial amino acid sequences were obtained by N-terminal sequencing. Additionally $P_{CD}$Amp1 was distinguished from other components of the extracellular matrix by immunohistochemical methods and by binding assays.

## Materials and methods

### Tissue preparation

One- to 3-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed immediately. The kidneys were then cut precisely along the corticomedullary axis.

### Indirect immunolabeling for confocal laser scanning microscopy

Cryosections (8 µm) of neonatal rabbit kidney were prepared precisely along the corticomedullary axis with a cryomicrotome (Microm, Heidelberg, Germany), fixed in ice-cold ethanol, and washed. The sections were then incubated in blocking solution (PBS + 1% BSA + 10% horse serum) for 30 min to saturate nonspecific binding sites. Primary antibodies were applied for 90 min. Fluorescein isothiocyanate-conjugated species-specific antisera (diluted 1:600; Dianova, Hamburg, Germany) were applied for 45 min. Sections were mounted in FITCguard (Testoc, Chicago, USA) embedding medium and analyzed in the confocal laser scanning microscope at 0.5-µm optical sections (Axiovert 10 with MR 500 Laserscan; Zeiss, Oberkochen, Germany).

### Primary antibodies

The monoclonal antibody recognizing $P_{CD}$Amp1 was generated as described earlier (Strehl et al. 1997, 1999). The following commercially available antibodies against extracellular matrix proteins were used: collagen type I, collagen type IV, collagen type VI, reticulin, elastin, and fibronectin (all Sigma, Deisenhofen, Germany).

### Cell culture

Capsula fibrosa explants with adherent embryonic collecting duct ampullae were isolated microsurgically from the neonatal rabbit kidneys as described earlier (Minuth et al. 1986). The explants were placed on 13-mm glass coverslips and cultured for 3 days in DMEM (Gibco Life Technologies, Karlsruhe, Germany) + 10% fetal bovine serum to allow outgrowth of cells onto the coverslips. Outgrown cells were cultured for 4 additional days in D-valine selection medium (Gibco Life Technologies) to eliminate fibroblasts and select epithelial cells from the collecting duct ampullae (Gilbert and Migeon 1977). To concentrate proteins in the supernatant, cultures were maintained for another 3–4 days in DMEM under serum-free conditions. Purity of epithelial cells was controlled immunohistochemically using an antibody against cytokeratin 19 (Sigma).

### Antigen isolation

Medium from ampullar epithelial cell cultures was pooled and centrifuged at 160 *g* for 10 min at room temperature. Supernatant aliquots (15 ml) were centrifuged at 3,000 *g* in micro concentrators with a 10-kDa cutoff (Pall Filtron, Northborough, USA) to a residual volume of 500 µl. This way a starting volume of 30 ml was decreased to an end volume of 500 µl and consequently a 60-fold end concentration was reached. The protein concentrate were stored at –20°C.

### Protein assay

Before each electrophoretic run the protein content in the samples was determined with a DC-protein assay kit according to the manufacturer's instructions (Biorad Laboratories, Munich, Germany).

### SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed according to established methods (Laemmli 1970). For separation a Mini Protean II Dual-slab cell (Biorad) with a 4.5% concentration and 10% separation polyacrylamide gel was used. A broad-range molecular weight standard (Biorad) was added to each electrophoretic run.

### Two-dimensional electrophoresis

Isoelectric focusing was performed using immobilized pH gradients (IPGs; Görg et al. 1988). IPG strips (Pharmacia Biotech, Freiburg, Germany) were incubated for 12 h with concentrated culture media supernatant + rehydration buffer according to manufacturer's instructions. Isoelectric focusing was performed using a Multiphor II unit and a 2297 Macrodrive (Pharmacia Biotech) in four steps. Current was limited to 0.05 mA per IPG strip. Voltage was set to 150 V for 1 h, 300 V for 5 h, 1,500 V for 12 h, and 3,500 V for 2 h. Marker proteins stained with Fairbanks solution served as a control. The second dimension protein separation was performed in Mini Protean II electrophoresis units (Biorad) according to methods described earlier (Laemmli 1970). Separation was performed at room temperature for 15 min at 60 V and 25 mA/gel, and 2 h at 200 V. Gels were either stained in Coomassie solution or used for western blotting.

### Western blot analysis and immunological detection of antigen

Proteins were transferred to 0.45-µm polyvinyl difluoride membranes (Millipore, Eschborn, Germany) on a Pegasus semidry blotting apparatus (Biorad) according to the manufacturer's instructions (Kyhse-Adersen 1984). Protein transfer was carried out at 1.1 mA/cm$^2$ gel surface and 25 V for 2 h. Blotted proteins were visualized with Ponceau S (Merck, Darmstadt, Germany). Membranes were incubated in a blocking solution (PBS + 1% BSA + 10% horse serum) for 12 h to saturate unspecific binding sites. Primary antibody was applied for 1 h. The secondary antibody [goat anti-mouse IgG and IgM (H+L)–peroxidase conjugate 1:2,000] was incubated for 1 h. Blots were developed using DAB (Sigma) as described earlier (Strehl et al. 1997).

### Sequence analysis of $P_{CD}$Amp1

Gels for microsequencing were stained in Coomassie solution at room temperature for 30 min and destained until bands became visible. Single bands were excised and washed successively with 0.2 M $NH_4HCO_3$, 0.2 M $NH_4HCO_3$+25% acetonitrile, 50% acetonitrile, and 100% acetonitrile for 30 min each. Samples were then incubated with trypsin in 0.2 M $NH_4HCO_3$ for 16 h at 37°C and extracted twice with 5% trifluoroacetic acid and once with 5% trifluoroacetic acid/acetonitrile (1:1). The extract was lyophilized, dissolved in 5% trifluoroacetic acid, and separated on a $C_{18}$ reversed-phase column by HPLC. Peptides were sequenced on a Procise 492A sequencer (PE Biosystems, Foster City, USA) with on-line detection of the PTH amino acids according to the manufacturer's instructions. The resulting sequences were compared with data found in the Swissprot data project. This procedure yielded several internal sequences of $P_{CD}$Amp1. Sequencing was performed with the kind help of Prof. Dr. R. Deutzmann, Universität Regensburg, Regensburg, Germany.

### Immunoblot experiments for binding evaluation to extracellular matrix proteins

Collagen type I, collagen type IV, collagen type VI, reticulin, elastin, and fibronectin (all from Sigma) were used for binding assays. Proteins were transferred to a nitrocellulose membrane (Millipore) and incubated with primary antibody and peroxidase-labeled secondary antibody. Visualization was performed according to methods described above.

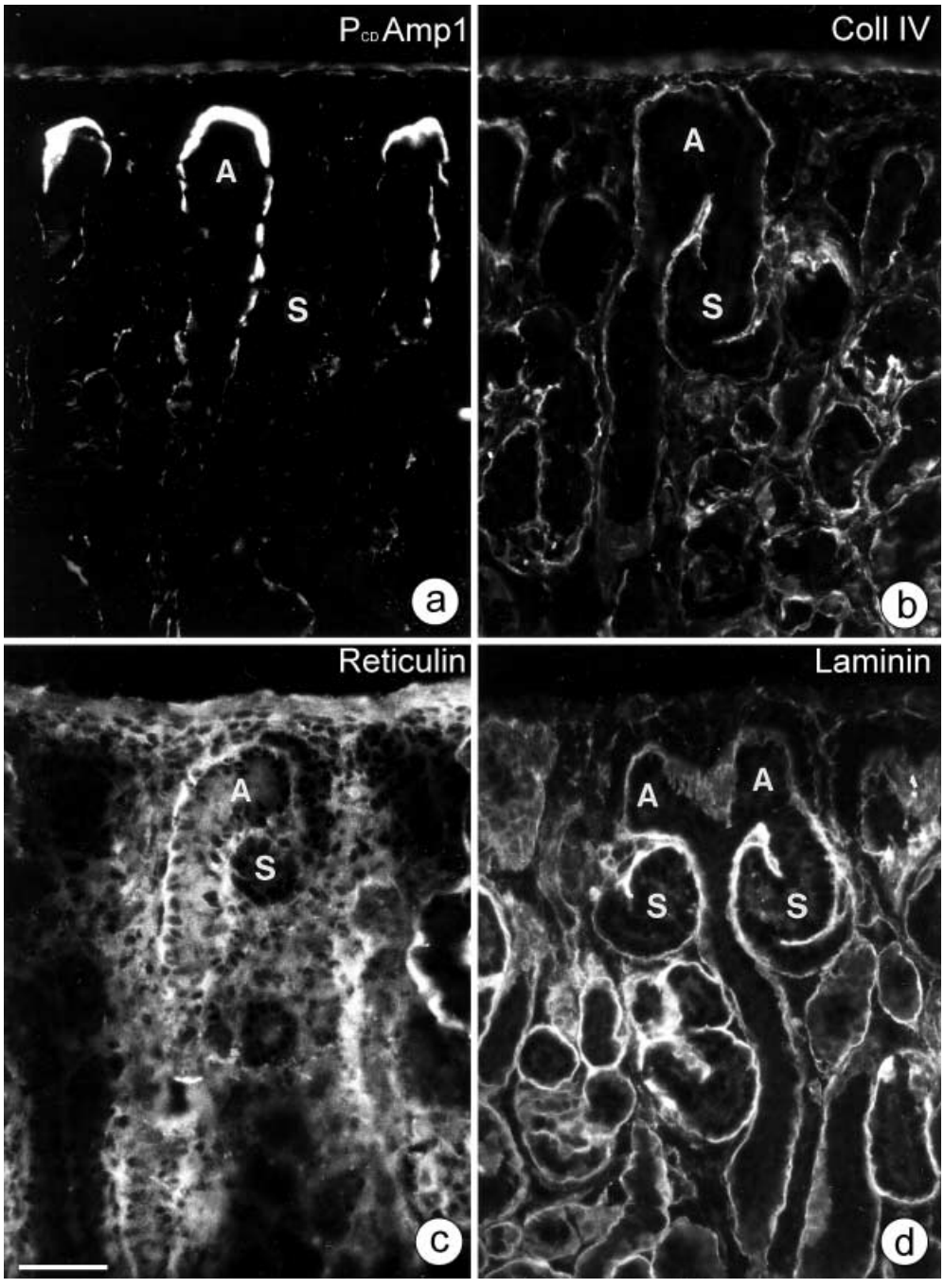


**Fig. 1a–d** Distribution of extracellular matrix components in neonatal rabbit kidney. **a** $P_{CD}$Amp1. **b** Collagen type IV. **c** Reticulin. **d** Laminin. *A* Ampulla, *S* S-shaped body. *Bar* 40 mm

## Results

In the kidney $P_{CD}$Amp1 is exclusively found as part of extracellular matrix fibers forming a dense meshwork that tightly surrounds the nephron inducer. Proteins such as laminin (Ekblom et al. 1980), collagen type IV (Wick et al. 1980), and fibronectin (Dixon and Burns 1982) are known to be widely expressed in the extracellular matrix of renal epithelia. To distinguish their occurrence from that of $P_{CD}$Amp1, parallel immunohistochemical incubations of cortex sections from neonatal kidneys were performed (Fig. 1). These corticomedullary sections display a developmental gradient from the embryonic cortex to the matured medullar region. Mab $_{CD}$Amp1 shows an exclusive reaction at the basal aspect of the embryonic collecting duct ampulla (Fig. 1a). The label is most intense at the tip and consistently decreases toward the ampullar shaft. Matured collecting duct and other tubules are not labeled. Collagen type IV, in contrast, is expressed at the basal lamina of all embryonic and matured renal epithelia including the collecting duct (Fig. 1b). Reticulin is also expressed in the basal lamina of all epithelia and is diffusely expressed in the mesenchyme (Fig. 1c). Anti-laminin shows a relatively weak immunolabel at the tip

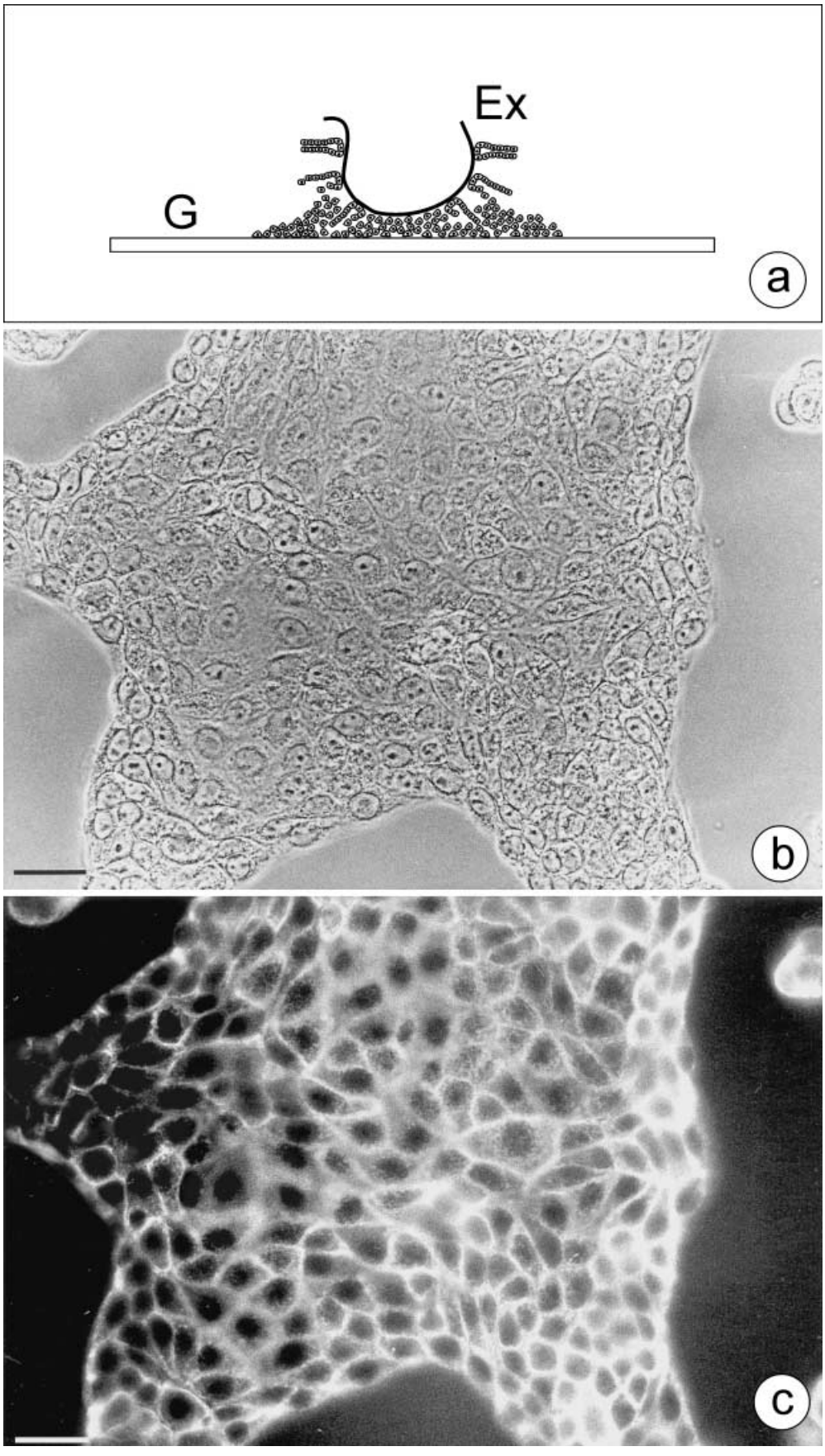


**Fig. 2a–c** Primary culture of $P_{CD}$Amp1-secreting embryonic collecting duct epithelium. **a** Schematic drawing showing a renal explant from the cortex of neonatal rabbit kidney on glass coverslip. Epithelial cells from the collecting duct ampullae migrate onto the glass. *G* Glass, *Ex* explant. **b** Light micrograph of cultures after 4 days in D-valine selection medium. **c** Immunoincubation with mab anti-cytokeratin 19. The positive reaction for mab anti-cytokeratin 19 in all cells indicates epithelial origin

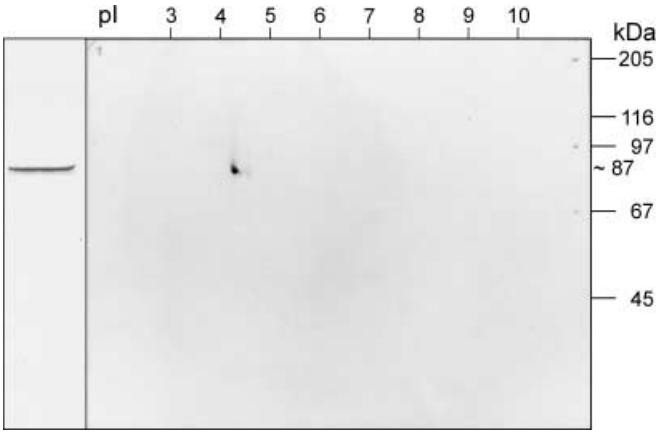


**Fig. 3** Purification of $P_{CD}$Amp1 by two-dimensional electrophoresis. $P_{CD}$Amp1 shows an isoelectric point between 4.3 and 4.4, and an apparent molecular weight of 87,000. Western blot with mab $_{CD}$Amp1

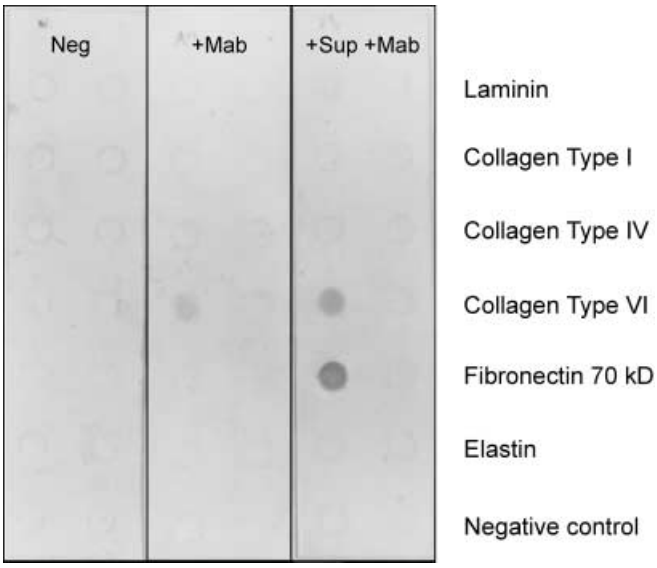


**Fig. 4** Immunoblot binding assays with different extracellular matrix proteins. *Lane 1* Negative control (*Neg*). *Lane 2* Incubation with mab $_{CD}$Amp1(*Mab*). *Lane 3* Incubation with culture supernatant (*Sup*) containing $P_{CD}$Amp1 prior to incubation with mab $_{CD}$Amp1 (*Mab*). Mab $_{CD}$Amp1 shows no crossreaction to collagen type IV. $P_{CD}$Amp1 shows a clear affinity to fibronectin

of the ampulla, while intense staining is found at the basal lamina of all other epithelial cells (Fig. 1d). Antibodies against fibronectin showed a diffuse reaction in the mesenchyme, but did not label the basal aspect of any part of the collecting duct (not shown).

Isolation of $P_{CD}$Amp1 for biochemical studies from renal tissue by enzymatic digestion did not yield reproducible results and therefore was not pursued further. Instead a soluble form of $P_{CD}$Amp1 was obtained from primary cultures of embryonic renal collecting duct epithelium. These cultures produce and secrete extracellular matrix compounds into the interstitial space in a soluble form but are unable to deposit them into a functional matrix in vitro, which in turn leads to an accumulation of the protein in the culture medium. Embryonic collecting duct cells derived from renal explants were cultured on glass coverslips. The cells of the collecting duct ampulla migrated out of the explant and could be maintained under serum-free conditions (Fig. 2a). Epithelial homogeneity of cell cultures was tested immunohistochemically with mab anti-cytokeratin 19. Only monolayers of cytokeratin 19-positive epithelial cells were used for protein isolation (Fig. 2b, c). During the culture period the embryonic epithelial cells secreted soluble $P_{CD}$Amp1 into the medium, from which it could be concentrated and isolated.

For biochemical characterization $P_{CD}$Amp1 was separated from other compounds by two-dimensional electrophoresis. The antigen was then detected with mab $_{CD}$Amp1 in western blots (Fig. 3). The soluble form of $P_{CD}$Amp1 showed an isoelectric point between 4.3 and 4.4, and an apparent molecular weight of 87,000. Further characterization of $P_{CD}$Amp1 was performed by N-terminal sequencing. For each individual experiment ten IPG strips with focused protein were used. Sites with focused $P_{CD}$Amp1 were excised, pooled, and separated by SDS-PAGE. The mab $_{CD}$Amp1-positive spot was excised and submitted to Edman degradation. The protein proved inaccessible to Edman degradation due to a blocked N-terminal amino acid sequence. Consequently, to analyze internal parts of the amino acid sequence, samples were excised from SDS gels and digested with trypsin. Fragments were separated by HPLC and submitted to sequence analysis. Three distinct sequences with a length of 17, 7, and 7 amino acids could be obtained (Table 1). Sequence 1 showed a homology of amino acid position 109–125 to collagen type IV α-chain precursor (Table 2). Sequences 2 and 3 did not show any homology to proteins known to date.

**Table 1** Internal sequences of $P_{CD}$Amp1

| | |
|---|---|
| Sequence 1 | GVXGFPGADGIPGHPGQ |
| Sequence 2 | FLQGVIG |
| Sequence 3 | FYTYERR |

Immunoelectron microscopic data showed $P_{CD}$Amp1 to be associated with extracellular fibers densely surrounding the collecting duct ampulla (Strehl et al. 1999). To clarify the nature of this association the affinity of $P_{CD}$Amp1 to proteins of the extracellular matrix was studied in immunoblot binding assays (Fig. 4). Samples of laminin, collagen type I, collagen type IV, collagen type VI, fibronectin, and elastin were applied to a nitrocellulose membrane. The membrane was then incubated with mab $_{CD}$Amp1 and developed using the indirect immunoperoxidase method. *Lane 1* shows the negative control without primary antibody. No specific reaction is noticed. *Lane 2* was incubated in the presence of mab $_{CD}$Amp1. A slight reaction with collagen type VI but no binding to any of the other proteins such as collagen

**Table 2** Sequence homologies of $P_{CD}$Amp1 isolated from culture medium (*lower line*) with rabbit collagen type IV α2-chain precursor (A61228; *upper line*). Sequence 1 of $P_{CD}$Amp1 shows homology of amino acids in positions 109–125 of collagen type IV α2-chain precursor. Sequences 2 and 3 have no homology to known proteins

| | | | | |
|---|---|---|---|---|
| 1 | | | | 50 |
| MGRERRAASG | SAMRRWLLLG | AVTAGFLAQS | VLAGVKKSDV | PCGGRDCSGG |
| .......... | .......... | .......... | .......... | .......... |
| 51 | | | | 100 |
| CQCYPEKGGR | GQPGPVGPQG | YTGPPGLQGF | PGLQGRKGDK | GERGAPGITG |
| .......... | .......... | .......... | .......... | .......... |
| 101 | | | | 150 |
| PKGDVGARGV | SGFPGADGIP | GHPGQGGPRG | PPGYDGCNGT | RGDEGPQGPS |
| ........GV | XGFPGADGIP | GHPGQ..... | .......... | .......... |
| 151 | | | | 200 |
| GTGGFPGSPG | PQGPKGQKGE | PYALSEEDRD | RYRGEIGEPG | LVGFQGPPGR |
| .......... | .......... | .......... | .......... | .......... |
| 201 | | | | 250 |
| PGPMGPMGPV | GAPGRPGPPG | PPGPKGHPGN | RGLGFYGKKG | EKGHIGAPGP |
| .......... | .......... | .......... | .......... | .......... |
| 251 | | | | 300 |
| NGIGFDTIYP | IVGPTKATFH | PDQYKGEKGS | EGDQGPQGIS | LKGEEGIMGF |
| .......... | .......... | .......... | .......... | .......... |
| 301 | | | | 350 |
| FGSRGAPGLD | GEKGSPGQKG | SRGVDGYQGP | YGPRGPKGQA | GYPGPPGAAG |
| .......... | .......... | .......... | .......... | .......... |
| 351 | | | | 400 |
| IFGLQGYRGP | PGPPGPAGLP | GSKGEDGSQG | LAGVPGSKGW | AGDPGPQGRP |
| .......... | .......... | .......... | .......... | .......... |
| 401 | | | | 450 |
| GVFGLPGEKG | PRGEQGFMGN | TGATGAAGDR | GPKGPKGDQG | FPGAPGPVGS |
| .......... | .......... | .......... | .......... | .......... |
| 451 | | | | 500 |
| PGIIGLPQRI | AVQHGPVGPQ | GKRGLPGAQG | EIGPQGPPGE | PGFRGAPGKA |
| .......... | .......... | .......... | .......... | .......... |
| 501 | | | | 550 |
| GPQGRGGVSA | VPGFRGDQGP | MGHQGPVGQE | GEPGRPGSPG | LPGMPGRSVS |
| .......... | .......... | .......... | .......... | .......... |
| 551 | | | | 600 |
| IGYLLVKHSQ | TEQEPMCPLG | MNKLWSGCSL | LYFEGPEKAH | NQEPGLAGSC |
| .......... | .......... | .......... | .......... | .......... |
| 601 | | | | 650 |
| LARFSTMPFL | YCNPGDVCYY | ASRNDKSYWL | STTAPLPMMP | VAEDEIKPYI |
| .......... | .......... | .......... | .......... | .......... |
| 651 | | | | 700 |
| SRCSVCEAPP | VAIAVHSQDV | SIPHCPAGWR | SLWIGYSFLM | HTAAGDEGGG |
| .......... | .......... | .......... | .......... | .......... |
| 701 | | | | 750 |
| QSLVSPGSCL | EDFRATPFIE | CNGGRGTCHY | YANKYSFWLT | TIPERSFQGS |
| .......... | .......... | .......... | .......... | .......... |
| 751 | | | | |
| PSADTLKAGL | IRTHISRCQV | CMKNL..... | .......... | .......... |

type IV, laminin, and fibronectin is visible. *Lane 3* was incubated with culture supernatant containing soluble $P_{CD}$Amp1 prior to incubation with mab $_{CD}$Amp1. Here a faint reaction with collagen type VI and a clear binding to fibronectin is recognizable. This result indicates that $P_{CD}$Amp1 has an affinity to fibronectin, but no affinity to other extracellular proteins. Mab $_{CD}$Amp1 shows a slight crossreaction to collagen type VI but does not crossreact with other extracellular matrix proteins and definitely not with collagen type IV.

## Discussion

$P_{CD}$Amp1 is strongly expressed in the embryonic tip region of the collecting duct ampulla and disappears as the collecting duct matures. In matured and adult structures $P_{CD}$Amp1 is not expressed. Thus $P_{CD}$Amp1 is located exclusively in the epithelial mesenchymal interphase, the site where the primary inductive events are postulated to take place (Vainio and Müller 1997) and is a unique marker for this region. Ultrastructural association with extracellular fibers as revealed by immunoelectron microscopy (Strehl et al. 1999) suggests $P_{CD}$Amp1 to be a structural component of the extracel-

lular matrix. A number of known extracellular matrix components such as collagen type IV, laminin, reticulin, and elastin are expressed in the developing kidney (Miner 1999). However, parallel immunohistochemical incubations show the distribution of $P_{CD}$Amp1 to be clearly different from that of above components of the extracellular matrix.

In monolayer cultures of ampullar epithelial cells, $P_{CD}$Amp1 is secreted into the medium in a soluble form. This demonstrates that $P_{CD}$Amp1 is synthesized and secreted by the embryonic epithelium and not the mesenchyme. The existence of a soluble form of $P_{CD}$Amp1 is homologous to known extracellular matrix components such as collagens that are known to be secreted from the cell as soluble proteins and subsequently crosslinked to form insoluble structures within the extracellular matrix (Prockop et al. 1979).

Two-dimensional electrophoresis of $P_{CD}$Amp1 isolated from culture supernatant shows an isoelectric point between 4.3 and 4.4, and an apparent molecular weight of 87,000 (Fig. 3). This clearly differs from collagen type IV, laminin, reticulin, and elastin (Miner 1999). N-terminal amino acid sequencing of $P_{CD}$Amp1 synthesized by cultured embryonic collecting duct cells reveals a partial homology to collagen type IV (Table 2) but $P_{CD}$Amp1 contains additional amino acid sequences not found in proteins known to date (Table 1).

Binding studies based on immunoblot assays demonstrate a slight crossreaction of mab $_{CD}$Amp1 with collagen type VI but no reaction with collagen type IV (Fig. 4). In addition the assays reveal an affinity of $P_{CD}$Amp1 to the fibronectin 70-kDa subunit but not to collagen type IV (Fig. 4).

The characteristic distribution of $P_{CD}$Amp1, its content of unknown amino acid sequences, the difference in molecular weight and isoelectric point, as well as the lack of crossreactivity of mab $_{CD}$Amp1 to known matrix proteins show that $P_{CD}$Amp1 is indeed a novel antigen not identical with known extracellular matrix components found in the developing kidney. On the other hand there are similarities such as the partial sequence homology to collagen type IV and the secretion as a soluble precursor that lead to the question whether $P_{CD}$Amp1 is a modified form of an extracellular matrix component from the collagen family. Extensive extracellular matrix remodeling is known to take place in the course of nephron induction (Ekblom 1996; Kuure et al. 2000). Remodeling matrix metalloproteinases such MMP-2 and MMP-9 are expressed in the region of induction (Reponen et al. 1992; Lelongt et al. 1997). The protein composition as well as the components themselves are modified during nephrogenesis (Miner and Sanes 1994; Miner et al. 1997). These processes lead to the appearance of extracellular matrix antigens exclusively found in this region. How far the extracellular matrix is functionally involved in the nephron induction process is not known. But the exclusive appearance of $P_{CD}$Amp1, a novel antigen not found anywhere else in the kidney in combination with a unique matrix morphology at the site of primary induction is definitely an important aspect that deserves further investigation.

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