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 $\text{CD Amp1}$ 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 $\text{PCD Amp1}$ 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 $\text{PCD Amp1}$ to collagen type IV $\alpha_2$-chain precursor but additionally yielded unknown sequences. Thus $\text{PCD Amp1}$ 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 · $\text{PCD Amp1}$

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 $\text{CD Amp1}$ (Strehl et al. 1997). The mab $\text{CD Amp1}$ 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 $\text{CD Amp1}$ antigen. A soluble form of $\text{PCD Amp1}$ was isolated, purified by two-dimensional electrophoresis, and partial amino acid sequences were obtained by N-terminal sequencing. Additionally $\text{PCD Amp1}$ 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 PCrAmp1 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 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/cm² 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 solution or used for western blotting.

Sequence analysis of PCrAmp1

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 NH₄HCO₃, 0.2 M NH₄HCO₃+25% acetonitrile, 50% acetonitrile, and 100% acetonitrile for 30 min each. Samples were then incubated with trypsin in 0.2 M NH₄HCO₃ 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 C₁₈ 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 PCrAmp1. 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.