View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Kidney International, Vol. 52 (1997), pp. 1469–1477

GENETIC DISORDERS - DEVELOPMENT

P_{CD}Amp1, a new antigen at the interface of the embryonic collecting duct epithelium and the nephrogenic mesenchyme

RAIMUND STREHL, SABINE KLOTH, JOACHIM AIGNER, PAT STEINER, WILL W. MINUTH

Department of Anatomy, University of Regensburg, Regensburg, Germany

P_{CD}Amp1, a new antigen at the interface of the embryonic collecting duct epithelium and the nephrogenic mesenchyme. In the neonatal rabbit kidney nephrogenesis is not yet terminated. The ampullar collecting duct epithelium acts as an inducer that generates the nephron anlagen, however, to date the morphogenic mechanisms involved are unknown. A presupposition for successful nephron induction is the close tissue interaction between the basal aspect of the ampullar collecting duct epithelium and the surrounding mesenchyme. To gain new insights in this area we raised monoclonal antibodies (mabs), to identify specific structures localized at the tissue interface. With the generated mab $_{CD}$Amp1 we found an intensive immunohistochemical reaction between the basal aspect of the ampullar collecting duct epithelium and the mesenchyme. The label was most concentrated at the ampullar tip and continuously decreased in the shaft region. In the maturing collecting duct of the neonatal kidney and in the adult renal collecting duct no immunohistochemical reaction was found. The binding pattern of mab $_{CD}$Amp1 is different from that of all known collecting duct cell markers and from antibodies against known basement membrane compounds such as laminin or collagen type IV. Under *in vitro* conditions immunoreactivity with mab $_{CD}$Amp1 was obtained using embryonic collecting duct epithelia and perfusion culture. The antigen was present in specimens treated with Iscove's modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum. Omittance of serum or hormonal treatment with aldosterone, insulin or vitamin D_3 led to the disappearance of the newly detected antigen, while characteristics of the differentiated collecting duct cells were up-regulated. We conclude that the expression of P_{CD}Amp1 is a characteristic feature of the embryonic parts of the collecting duct epithelium. It may play a pivotal role during nephron induction.

Many organs such as salivary glands, liver and lung develop in a process named 'branching morphogenesis.' The parenchymal tissue develops from single epithelial cells that appear early in development and coordinate the three dimensional expansion of the organ through their high proliferation and branching activity. In contrast, the kidney develops in a two-stage mechanism and only follows this pattern in the primary phase of its development. After the ingrowth of the ureter bud into the nephrogenic blastema, an apical pole and a basal pole are formed by dichotomous branching, and then two ventral and dorsal parts, the so-called ductus renculares, arise [1]. The second phase of kidney development starts after several further branchings. The initial collecting ducts with their blindly ending ampullae appear and the induction of the nephrons begins. The collecting duct ampullae induce competent cells in the surrounding mesenchyme that condensate to form the comma-shaped bodies, the first visible stages of the immature nephron [2]. By further dichotomous branching and a successive elongation of the collecting duct the ampullae are pushed further into the uninduced mesenchyme towards the capsule where the next generation of nephrons is generated. Thus far the nature of the stimulus by which the mesenchyme is induced is unknown.

The kidney of neonatal rabbits serves as an excellent model for tissue development [3]. The organ exhibits fully embryonic, half mature and fully differentiated tissues in a single cortico-medullar section. The embryonic collecting duct ampullae are found in the outer cortex. According to earlier experiments the epithelium of the embryonic collecting duct is not a homogenous epithelium, but consists of several cell types. Supposedly, nephron induction is triggered by the cells in the ampullar tip. In the neck region the cell proliferation for tubular elongation takes place, while in the shaft development into the functional principal and intercalated cells is observed [4]. The induction stimulus for nephron generation is believed to be secreted at the basal aspect of the ampullar collecting duct epithelium [5], where it needs to penetrate a basement membrane and a thin layer of endothelial cells [6] before reaching the competent cells in the mesenchyme.

Histochemical labeling of the embryonic collecting duct ampulla shows a distribution of proteins that is quite different from that in the maturing or adult collecting duct [3]. The basement membrane of the mature collecting duct contains laminin, for example, which is not found in the tip of the ampulla [7, 8]. Furthermore, the basal aspect of the collecting duct ampulla is positive for peanut lectin (PNA), while in the maturing collecting duct this particular staining is lost and an apical β-type IC cell reaction becomes visible [9, 10]. These findings indicate that the molecular composition of the epithelial cells in the embryonic collecting duct ampulla differs from that of the adult collecting duct epithelium. To date, localization of the ampullar cells could only be performed by PNA and cytokeratin 19 expression [9] or by the lack of individual cell markers [11].

In the following experiments our interest was to determine whether the ampullar collecting duct epithelium exhibits specific features showing the embryonic character of the cells. Special interest was directed towards the interface between the collecting duct ampulla and the nephrogenic mesenchyme. We generated

Key words: collecting duct, antigen, development, monoclonal antibodies, perfusion culture.

Received for publication February 21, 1997
and in revised form July 17, 1997
Accepted for publication July 17, 1997

© 1997 by the International Society of Nephrology

monoclonal antibodies against embryonic renal tissue and selected hybridoma clones according to their immunohistochemical reaction profile. By immunohistochemical methods we showed the presence of a novel antigen, which we named P_{CD}Amp1. It surrounds the basal aspect of the ampullar collecting duct epithelium in the cortex of neonatal rabbit kidney. Expression of P_{CD}Amp1 under *in vitro* conditions could be obtained when the embryonic collecting duct epithelia were kept under perfusion culture conditions in medium containing fetal bovine serum.

METHODS

Tissue preparation

One- to three-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed immediately and frozen in liquid nitrogen. Mature organs were prepared from rabbits older than six months.

Scanning electron microscopy

For scanning electron microscopy (SEM) pieces of neonatal rabbit kidney cortex were fixed in 3% glutaraldehyde, dehydrated in a graded series of ethanols, critical point dried with CO_2 and spatter-coated with gold (Polaron, Watford, UK). The specimens were examined in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany) as described earlier [4].

Production of monoclonal antibodies

The production of monoclonal antibodies has already been described in detail [9, 11]. Homogenate from neonatal kidneys was used for the immunization of Balb/c mice. Primary immunization and two further booster injections were applied intraperitoneally. Monoclonal antibodies were produced according to the method of Koehler and Milstein [12]. Spleen cells were fused with myeloma cells of the x63Ag8.653 line. The cells were cultured in RPMI 1640 (Gibco-BRL Life Technologies, Eggenstein, Germany) with 2 mM L-glutamine (Gibco-BRL Life Technologies), 10% fetal bovine serum (Boehringer, Mannheim, Germany), 1×10^{-5} M 2-mercaptoethanol (Serva, Heidelberg, Germany) in an incubator (37°C, 95% air/5% CO_2 atmosphere; Heraeus, Hanau, Germany). Hybridomas were cloned using a limiting dilution procedure. Culture supernatants were tested for tissue specific antibodies by immune incubation of renal tissue sections.

Biochemical analysis of the antigen

For the biochemical analysis of the $_{CD}$Amp1-antigen, samples were prepared by SDS-extraction of cultured collecting duct epithelia. Fractionation was performed by SDS-PAGE according to Laemmli [13], and antigen was detected by Western blot analysis [14].

Five renal explants were cultured in a perfusion container with IMDM + 75 mM HEPES + 10% FCS for 14 days as described below. The explants were then mechanically disrupted and extracted with SDS sample-buffer (containing 130 mM Tris/HCl, 20% 2-mercaptoethanol, 12% SDS and 40% glycerine) for 15 minutes.

Following a centrifugation spin at $43,000 \times g$ for 30 minutes the samples were fractionated in discontinuous 4.5%/10% acrylamide gels. As a molecular weight standard broad range electrophoresis molecular weight standard (Bio-rad, Munich, Germany) was used. The gels were blotted onto Immobilon-P membrane (Millipore GmbH, Eschborn, Germany) by semi-dry blot in a continuous borate-buffer system. The blots were incubated and developed according to the immunoperoxidase method [15, 16].

Light microscopical techniques

Cryosections (8 μm) of neonatal and adult rabbit kidneys or cultured epithelia were cut with a cryomicrotome (Microm, Heidelberg, Germany). Detection of bound primary antibody was carried out by the indirect immunofluorescence method [15] or by the indirect immunoperoxidase method [16] as described earlier. Tissue sections were fixed in ice-cold ethanol and washed. The sections were then incubated in blocking-solution (PBS + 1% BSA + 10% HS) for 30 minutes to saturate unspecific binding sites. Primary antibodies were applied for 90 minutes. Fluorescein-isothiocyanate conjugated (diluted 1:200) or biotinylated species-specific antisera (diluted 1:600) (Dianova, Hamburg, Germany) served as detecting antibodies and were applied for 45 minutes. Following the final washing step the sections were mounted in FITCguard (Testoc, Chicago, IL, USA) embedding medium and analyzed using an Axiovert microscope (filter I: 450 to 490, FT 510, LP 520; Zeiss). For documentation TriXPan film (Kodak, Hemel-Hepstead, UK) was used.

Co-incubation experiments

To analyze whether antibody labeled or unlabeled areas belonged to the peanut agglutinin (PNA) binding sites co-incubation experiments were carried out. Sections were incubated according to the protocol for the indirect immunofluorescence method and were subsequently treated with rhodamine-conjugated PNA (1:2000; Vector, Burlingame, VT, USA) for 30 minutes. The sections were embedded as described above and analyzed using the Axiovert microscope (filter II, 510 to 560, FT 580, LP 590; Zeiss).

Irrelevant monoclonal antibodies and pre-immune sera from mouse served as general controls. As a control for unspecific binding of the detecting antibody, sections were incubated omitting the primary antibody. Neither of these controls revealed any labeling of the collecting duct epithelium.

Generation of an embryonic collecting duct epithelium in vitro

Cortical explants from the kidneys of newborn New Zealand rabbits were isolated according to methods described earlier [11, 17]. They consisted of a piece of capsula fibrosa with adherent collecting duct ampullae, S-shaped bodies and nephrogenic blastema. The explants were mounted in sterile tissue carriers (Minucells and Minutissue, Bad Abbach, Germany; Fig. 1A), which were placed in 24-well culture dishes (Greiner, Nürtingen, Germany). During the culture of these explants in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL Life Technologies) and 10% fetal bovine serum (Boehringer) an outgrowth of cells from the collecting duct ampullae was observed (Fig. 1B). Within 24 hours of the initiation of culture the entire surface of the explant, 6 mm in diameter, was covered by a monolayer of collecting duct epithelium cells (Fig. 1C). Culture for the first 24 hours was carried out in a tissue incubator (Heraeus, Hanau, Germany) at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Perfusion culture of embryonic collecting duct epithelia

To optimize culture conditions the isolated epithelia were transferred into a special container (Fig. 1D; Minucells and Minutissue, Bad Abbach, Germany) 24 hours after the initiation

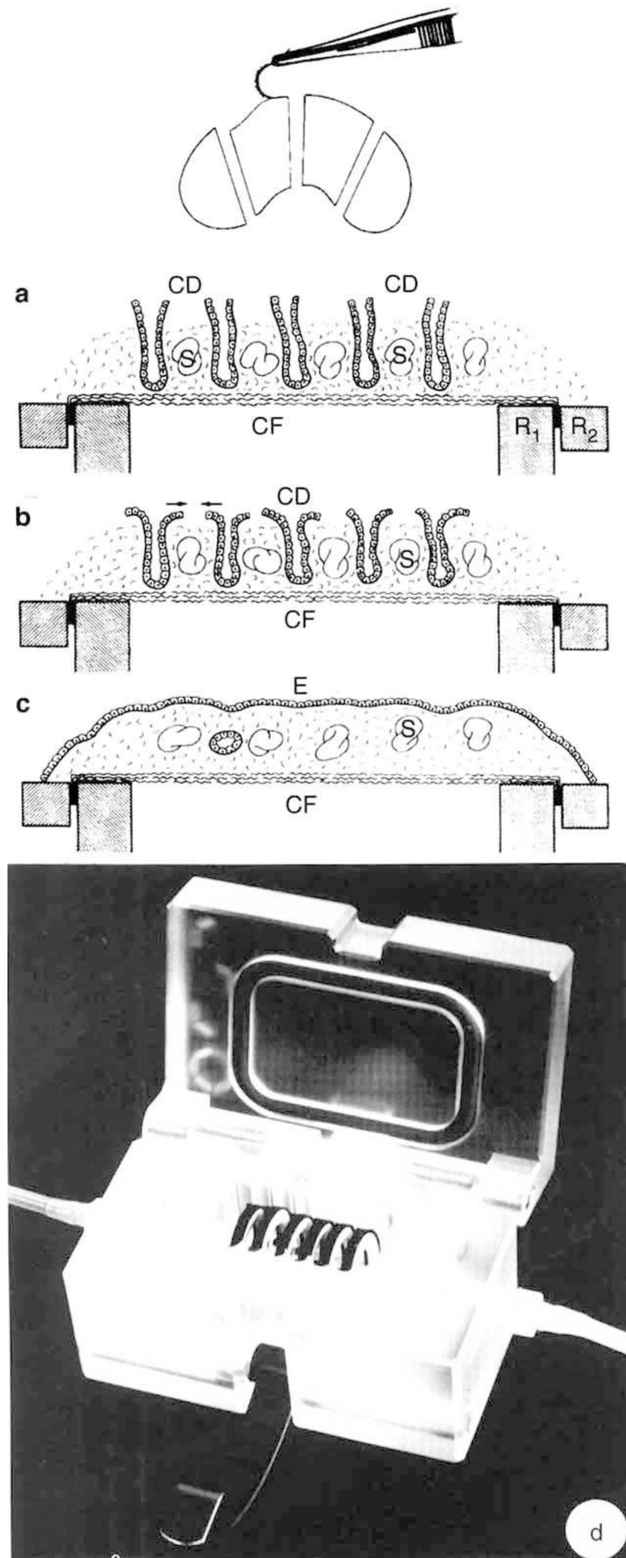

Fig. 1. Isolation of a cortical explant from neonatal rabbit kidney and mounting on a specific tissue carrier (R1,R2). (*a*) The isolated explant consists of capsula fibrosa (CF) with adherent collecting duct ampullae, S-shaped bodies (S) and mesenchyme. (*b and c*) An outgrowth of collecting duct cells (CD) during the first day of culture leads to a polar-differentiated epithelium (E) covering the whole surface of the explant. (*d*) Container for perfusion culture. Culture medium is perfused through the container to guarantee a constant supply of nutrients to the cells and to swiftly remove toxic metabolites from them. In the perfusion system it is possible to culture tissue under improved conditions and to control differentiation of the cells by addition of hormones or other factors.

Fig. 2. Scanning electron microscopy of the outer cortex of neonatal rabbit kidney. No cell boundaries are visible on the collecting duct ampullae (A) due to a cover of consistently developed basement membrane embracing the ampullae-like stockings. Bar = 10 μm.

of culture and exposed to a permanent superfusion with fresh medium [18]. The container was placed onto a 37°C heating plate (Medax, Kiel, Germany). IMDM without serum was used as the control medium and continuously perfused at a rate of 1 ml/hr with an IPC N8 peristaltic pump (ISMATEC, Wertheim, Germany) for 13 days. Total culture time was 14 days (1 days preculture, 13 days perfusion culture). Aldosterone (1×10^{-7} M), insulin (1×10^{-9} M), 1,25 dihydroxyvitamin D_3 (1×10^{-6} M) or 10% fetal bovine serum (all Sigma-Aldrich-Chemie, Deisenhofen, Germany) were added in different experimental series for the whole culture period.

RESULTS

Scanning electron microscopy of the collecting duct ampulla

According to previous experiments [19, 20] it is uncertain whether a consistently developed basement membrane is present around the collecting duct ampulla. For that reason, scanning electron microcopy of the outer cortex of neonatal rabbit kidneys was performed in a first set of experiments (Fig. 2). Underneath the fibrous capsule, the blindly ending collecting duct ampullae embedded in the nephrogenic mesenchyme were found. On the surface of the ampullae no cell boundaries could be observed due

to a cover of consistently developed basement membrane embracing the ampulla like a stocking.

Generation of a monoclonal antibody detecting the collecting duct ampulla

The aim of the second set of experiments was to generate monoclonal antibodies against epitopes in the basement membrane area of the embryonic collecting duct ampulla. Screening the multiply generated clones according to their immunohistochemical labeling pattern on cryosections of the neonatal kidney, we found one clone with striking features; the hybridomas produced an antibody of IgG 1 subclass, reacting at the basal aspect of the ampullar collecting duct epithelium (Fig. 3). We named it mab $_{CD}$Amp1, and the recognized epitope P_{CD}Amp1.

Immunohistochemical pattern of P_{CD}Amp1

On a cortico-medullar tissue section of the neonatal rabbit kidney mab $_{CD}$Amp1 immunolabel was present at the basal aspect of the collecting duct epithelium in the ampulla. In the outer cortex the mab $_{CD}$Amp1-label was most prominent in the embryonic tip region and continously diminished in the shaft-region (Fig. 4A). In the inner cortex of the neonatal kidney neither maturing collecting ducts nor other tubuli, glomeruli or blood vessels were labeled by the antibody (Fig. 3).

PNA is known to intensively label the basal aspect of the collecting duct ampulla [9]. Present coincubation experiments were performed to show similarities or dissimilarities between the labeling pattern of mab $_{CD}$Amp1 (Fig. 4A) and PNA (Fig. 4B). The lectin was bound in high concentrations in the ampullar tip (Fig. 4b) while concentrations were found to decrease towards the neck and shaft. As described for mab $_{CD}$Amp1 (Figs. 3 and 5) the lectin label was absent in the basement membrane area of the maturing or adult collecting duct. In this region it labeled the β-type IC cells at the luminal cell pole as described in detail earlier [10]. The basal aspect of the whole S-shaped body was intensively labeled with PNA while mab $_{CD}$Amp1 label was restricted to the lower cleft of the S-shaped body (not shown). According to our light microscopical observations, the PNA label and mab $_{CD}$Amp1 immunostaining completely colocalized in the tip but only partly colocalized in the shaft region of the collecting duct ampulla.

Immunohistochemistry was further performed on sections of adult rabbit kidney (Fig. 5). Neither in the cortex (Fig. 5A), nor in the medulla (Fig. 5B) was immunoreactivity found. These results demonstrated that mab $_{CD}$Amp1 exclusively reacts in the embryonic region of the developing kidney.

The mab $_{CD}$Amp1 labeling patterns showed that in the course of nephron differentiation and collecting duct development striking changes occur at the basal aspect of the epithelium.

P_{CD}Amp1 expression in cultured embryonic collecting duct epithelia

The mechanisms triggering the appearance and disappearance of the $_{CD}$Amp1-antigen during kidney development are unknown. To investigate P_{CD}Amp1 expression under *in vitro* conditions, embryonic collecting duct epithelia were isolated and brought into perfusion culture on a special tissue carrier (Fig. 1, A–C). The tissue was exposed to a constant medium flow in a perfusion container for 13 days (Fig. 1D). After time period a polar-differentiated collecting duct epithelium resting on the kidney

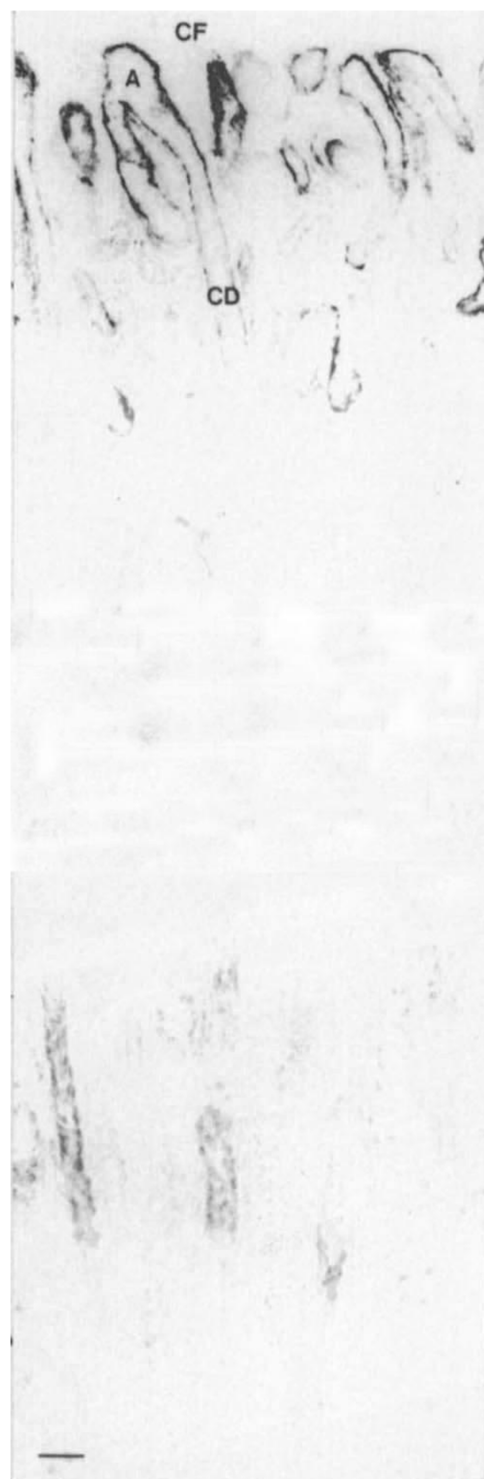

Fig. 3. Mab $_{CD}$Amp1 reaction in the cortex of neonatal rabbit kidney. Indirect immunoperoxidase method. The embryonic collecting duct ampullae (A) show a strong immunolabel that decreases in the maturing collecting duct. No reaction is found in the inner cortex of the neonatal rabbit kidney. Abbreviations are: CD, collecting duct; CF, capsula fibrosa; A, ampulla. Bar = 40 μm.

specific matrix could be analyzed. The epithelium showed no mab $_{CD}$Amp1 immunolabel in experimental series where hormonal supplements such as aldosterone (Fig. 6A), vitamin D_3 (Fig. 6B)

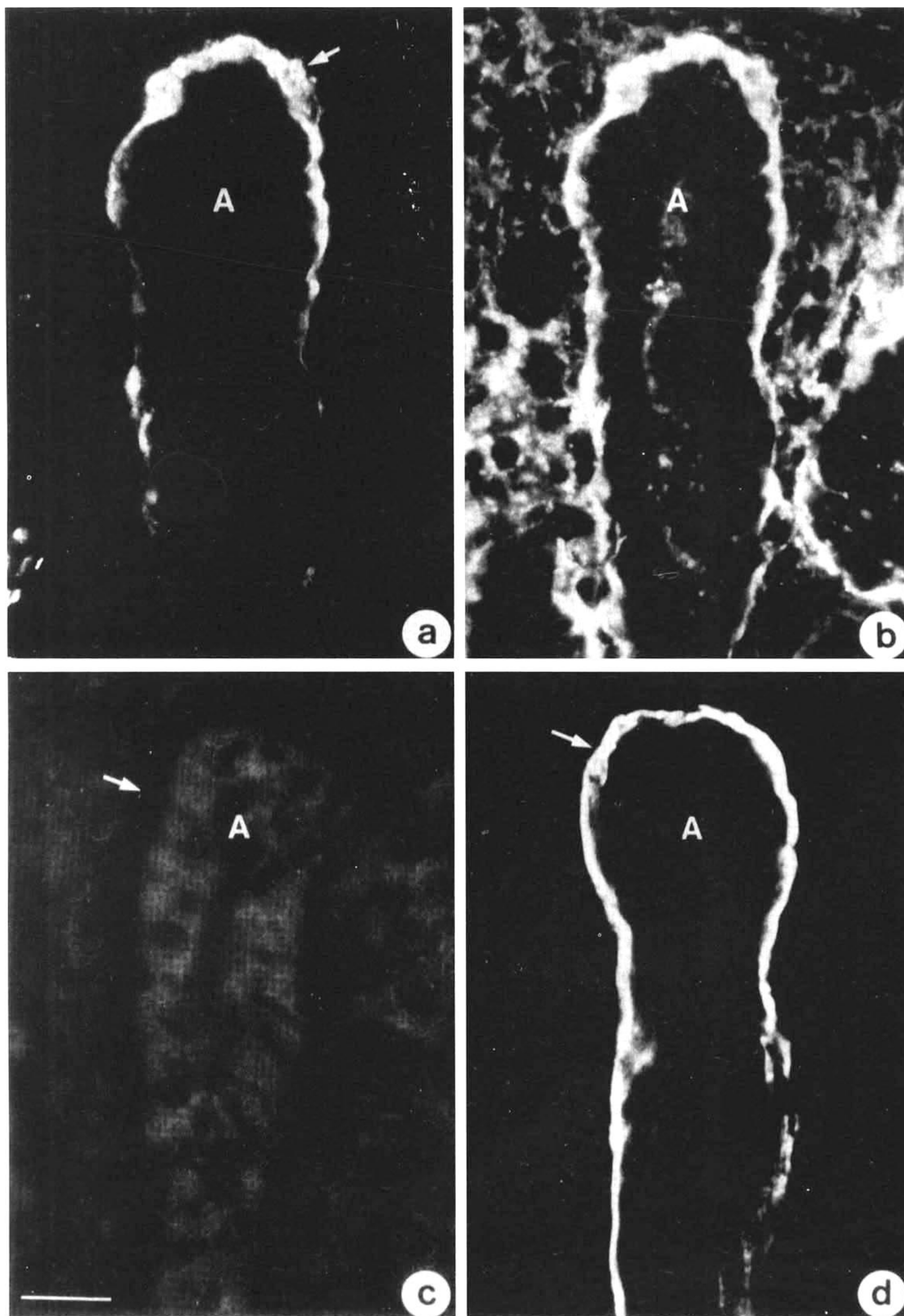

Fig. 4. Neonatal kidney, collecting duct ampulla (A). Indirect immunofluorescence. (*a*) Mab $_{CD}$Amp1-label. (*b*) PNA-label. (*c*) Negative control without primary antibody. (*d*) Mab $_{CD}$Amp1-label. A confocal laser scanning microscope was used to show 2 μm optical sections. The mab $_{CD}$Amp1 label and PNA label are not identical. Mab $_{CD}$Amp1 immunolabel is most prominent in the tip region of the collecting duct ampulla. Symbol (→) shows basement membrane area. Bar = 40 μm.

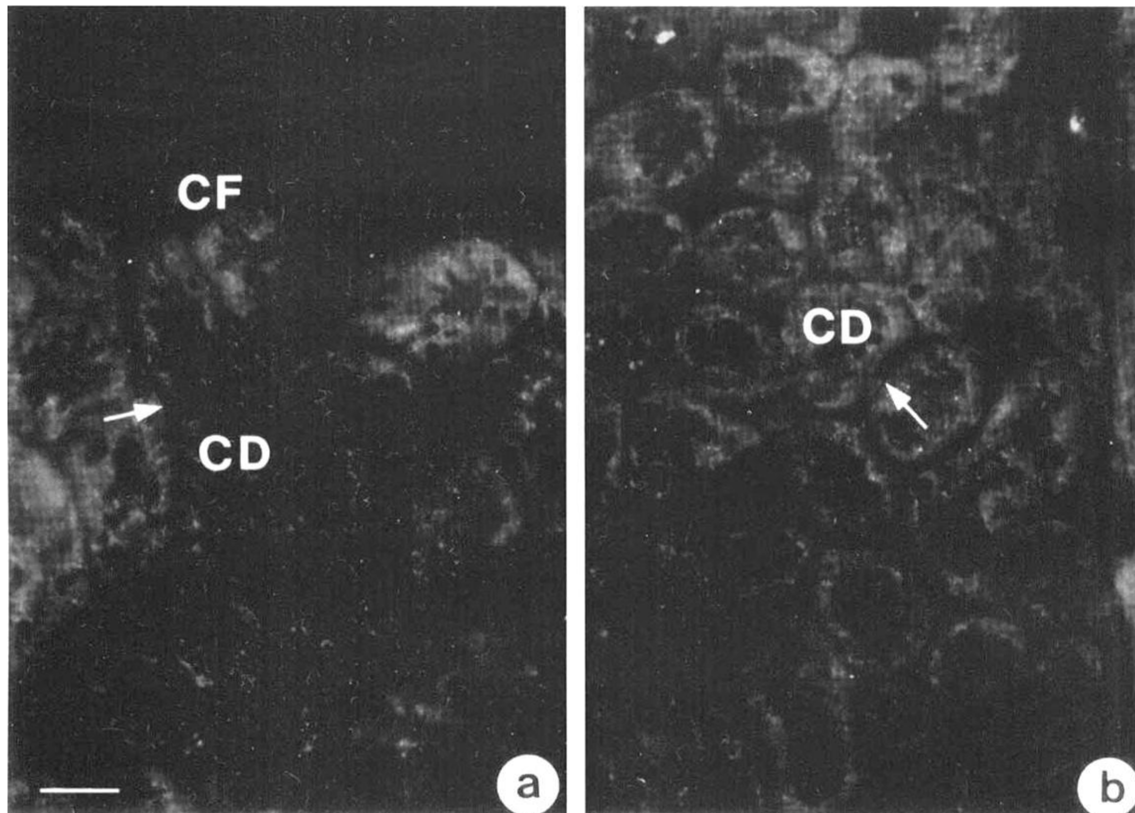

Fig. 5. Adult rabbit kidney. Indirect immunofluorescence with mab $_{CD}$Amp1. (*a*) Cortex. (*b*) Medulla. No specific mab $_{CD}$Amp1 reaction was found in any part of the basement membrane area (→) of the collecting duct (CD) in adult rabbit kidney. Abbreviation CF is capsula fibrosa. Bar = 25 μm.

or insulin (Fig. 6C) were added to IMDM. In contrast, intensive labeling was obtained when 10% fetal bovine serum but no hormonal supplements were added to IMDM (Fig. 6D). In that case the complete basal aspect of the cultured epithelium was positive for mab $_{CD}$Amp1. As controls, embryonic collecting duct epithelia were cultured without serum and hormonal supplements (Fig. 6E). Omitting the serum resulted in a completely negative immunopattern. Thus, serum supplementation of IMDM is necessary for the maintenance of P_{CD}Amp1 expression under *in vitro* conditions.

Biochemical features of P_{CD}Amp1

Extraction of the mab $_{CD}$Amp 1-antigen from neonatal kidneys proved very difficult due to a low antigen per volume ratio. Therefore cultured explants were used for extraction. In these thin explants the concentration of P_{CD}Amp 1 is high. Fractionation in discontinuous SDS-PAGE and immunoblot of extracts revealed a clear protein band at an apparent molecular weight of approximately 39 kDa and a very faint second band at approximately 32 kDa (Fig. 7).

DISCUSSION

Multiple processes at the collecting duct ampulla during nephrogenesis

In the outer cortex of the neonatal rabbit kidney not only the induction of the nephrons [19], but also the conjunction of the connecting tubule, the elongation of the collecting duct [21], the development of P and IC cells [4], and the vascularization [6] take place. All of these developmental events occur in a close proximity to the collecting duct ampulla.

Involvement of the antigen in nephron development

The mab $_{CD}$Amp1 immunolabel seems to appear in conjunction with four different developmental events.

(1.) Nephron induction. It is known that the collecting duct ampulla and the surrounding mesenchyme interact during nephrogenesis [19]. It remains unclear though, whether the induction stimulus is a unidirectional signal from the ampullar tip towards the mesenchyme or if a permissive effect involving a crosstalk of morphogenic signals leads to nephron generation [2]. In either case the induction signal has to be exchanged at the basal aspect of the ampullar collecting duct epithelium [5]. It has to penetrate a surrounding basement membrane (Fig. 2) and has to pass neighboring endothelial cells [9] before reaching the competent cells in the mesenchyme. P_{CD}Amp1 is found in the highest concentrations in this specific area (Figs. 3 and 4 A, D).

(2.) Connecting tubule formation. The ampullar basement membrane has to be dissolved when the connecting tubule is formed. From its primary stages the primitive nephron develops apart from the ampulla. Thus, the presumtive connecting tubule has to make contact with the collecting duct system later on [21], in the neck region of the ampulla. P_{CD}Amp1 is found in decreased concentrations at this site (Fig. 4 A, D).

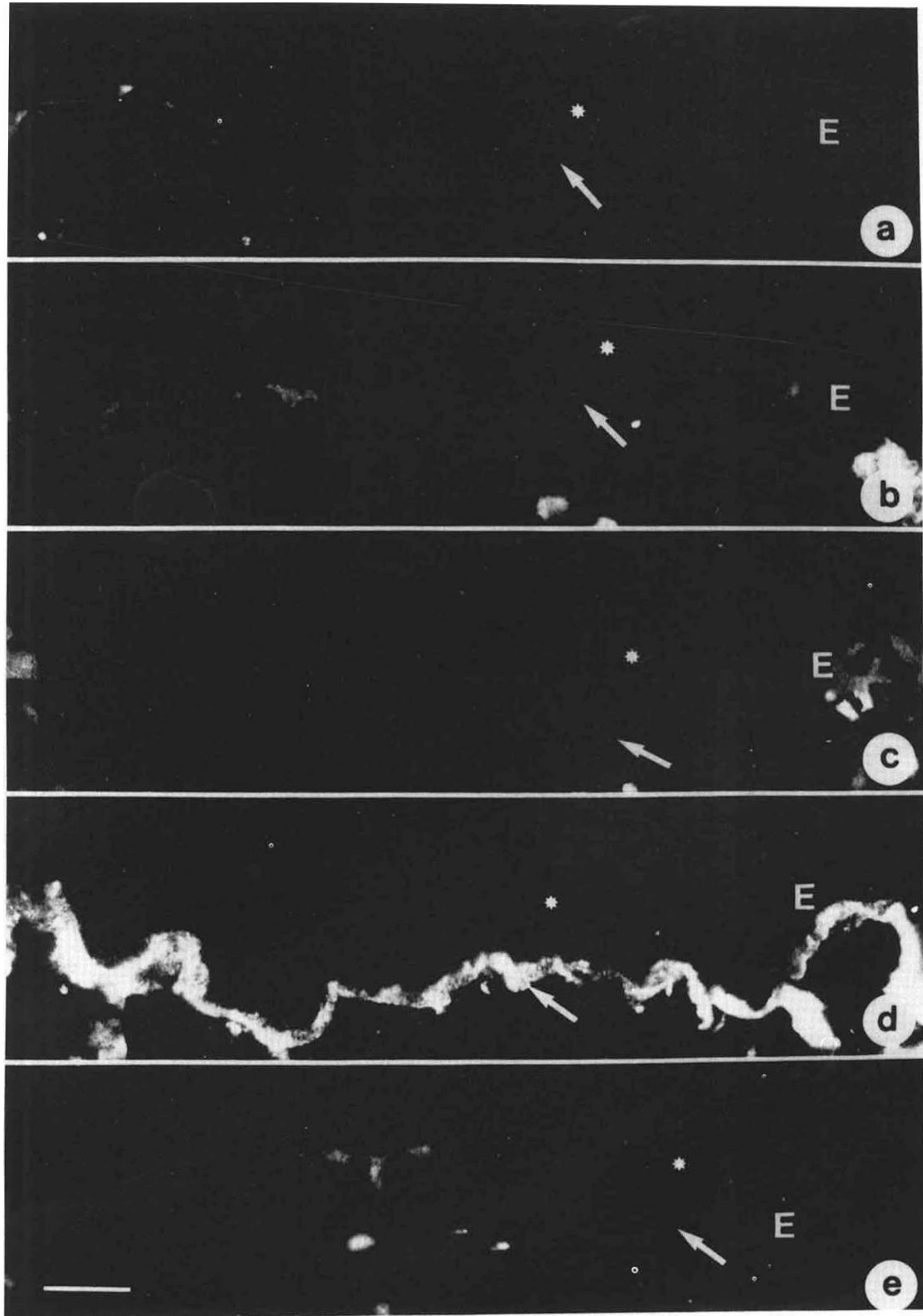

Fig. 6. Cultured neonatal collecting duct epithelia. Indirect immunofluorescence with mab $_{CD}$Amp1 in a 14 day perfusion culture in IMDM containing: (*a*) 1×10^{-7} M aldosterone, (*b*) 1×10^{-9} M vitamin D_3, (*c*) 1×10^{-6} M insulin, (*d*) 10% FCS, (*e*) no supplements. Culture in medium containing serum leads to an up-regulation of P_{CD}Amp1 on the basal aspect (→) of the epithelium (E). (*) = luminal. Bar = 20 μm

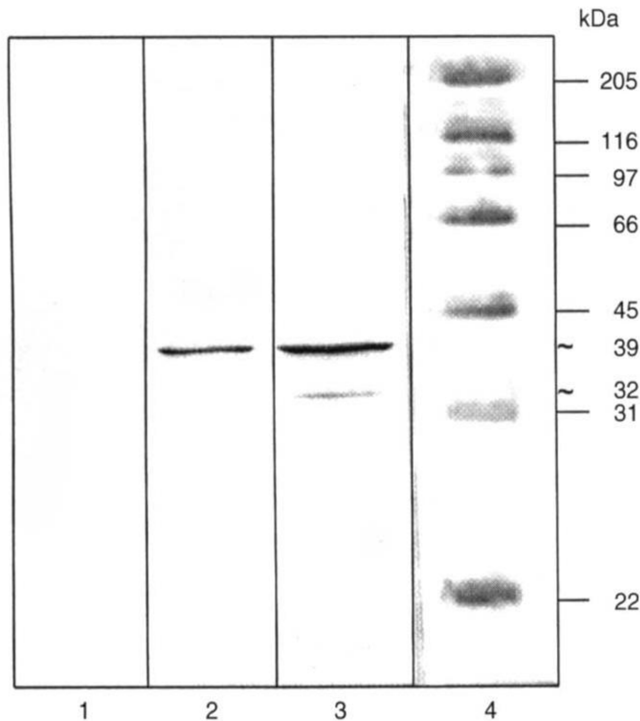

Fig. 7. SDS-PAGE fractionation and Western blot analysis of cultured neonatal collecting duct epithelia. Lane 1. Negative control without primary antibody (15 μg total protein). Lane 2. Mab $_{CD}$Amp1 immunolabel (15 μg total protein). Lane 3. Mab $_{CD}$Amp1 immunolabel (30 μg total protein). Lane 4. Broad range molecular weight standard, Ponceau-S stained. Control reveals no unspecific reaction. Mab $_{CD}$Amp1 detects a clear protein band at an apparent molecular weight of 39 kDa. At increased protein concentrations a faint second band becomes visible at 32 kDa.

(3.) Collecting duct elongation. Besides the nephron induction process the ampullar collecting duct epithelium is involved in the elongation growth of the tubular element. Telescopic growth is caused by a large number of proliferating cells in the ampullar neck region. By this mechanism the tip is pushed further into uninduced areas of mesenchyme [21]. The collecting duct permanently increases in length, new nephrons are successively induced and the organ expands in volume. In areas where elongation growth takes place P_{CD}Amp1 concentration is low (Figs. 3 and 4 A, D).

(4.) Development of the embryonic collecting duct cells. Beyond the elongation-zone the development of embryonic collecting duct cells into the functional P and IC cells is observed [4]. At this particular site P_{CD}Amp1 is not found.

The results suggest that mab $_{CD}$Amp1 outlines a developmental gradient. High immunoreactivity is found where the induction of nephrons takes place. In contrast, the immunolabel decreases where the formation of the connecting tubule or the transdifferentiation into P and IC cells take place.

P_{CD}Amp1 exclusively frames ampullar collecting duct cells

By comparing earlier and present experiments we obtained indications that P_{CD}Amp1 is found on embryonic and not on maturing collecting duct cells. In addition to functional proteins, such as Na,K-ATPase [22], Na channels [23] and aquaporins [24], three further groups of proteins are synthesized by collecting duct cells (Table 1). The newly detected P_{CD}Amp1 belongs to the first group. The concentration of the antigen is high in the ampullar tip (Fig. 4 A, D) and decreases in the shaft region (Fig. 4 A, D). The second group contains collecting duct proteins described earlier, such as P_{CD} 1, 2, and 3 [25], and 5V [11] or others [34, 35]. In contrast to P_{CD}Amp1 all of these proteins are not present in the embryonic collecting duct ampulla, but are first synthesized in the maturing part of the neonatal collecting duct. P_{CD}Amp1 disappears, while the proteins of group 2 are up-regulated. This shows on the one hand the loss of embryonic features and on the other hand the development of differentiated collecting duct cell features. The third group contains proteins such as P_{CD}9 [26], P_{CD} 4V [11] and cytokeratin 19 [9]. These proteins are present in the embryonic collecting duct ampulla as well as in the maturing collecting duct of the neonatal kidney. Except for PNA-binding sites (Fig. 4B), RET [27] and WNT-11 [28], only P_{CD}Amp1 has to date been described to be exclusively present in the embryonic collecting duct ampulla and to be down-regulated during further nephron development. A similar transient expression is known for the cystic fibrosis transmembrane conductance regulator (CFTR) [29].

Table 1. Protein expression in the embryonic, maturing and adult renal collecting duct cells of the rabbit kidney

Antigen	Neonatal kidney collecting duct ampulla	Neonatal kidney maturing collecting duct	Adult kidney mature collecting duct	[Reference]
Group 1				
P_{CD}Amp1	+	–	–	Stre 97
Group 2				
GP_{CD} 1	–	+	+	[32]
P_{CD} 1	–	+	+	[25]
Na, K-ATPase	–	+	+	[22]
P_{CD} 2	–	+	+	[25]
P_{CD} 3	–	+	+	[25]
RCT-30	–	+	+	[33]
P_{CD} 5V	–	+	+	[11]
503/703	–	+	+	[34, 35]
P_{CD} 7	–	+	+	[9]
Group 3				
P_{CD} 9	+	+	+	[26]
P_{CD} 4V	+	+	+	[11]
CK 19	+	+	+	[9]

Symbols represent immunolabeling: (−) not present; (+) present.
Group 1, proteins were exclusively present in the embryonic ampulla.
Group 2, proteins were not present in the ampulla but were up-regulated during development.
Group 3, proteins were present throughout development.

Modulation of P_{CD}Amp1 in cultured collecting duct epithelia

In vitro experiments confirm that P_{CD}Amp1 is found only on embryonic, but not on differentiated collecting duct cells. In earlier studies [30] and in the present experiments we found that in perfusion culture the embryonic collecting duct epithelia develop individual P and IC cell features. The up-regulation of P_{CD} 2, 3 [3] or PNA binding [31] in cultured collecting duct cells is dependent on the administration of aldosterone. In the present experiments, however, we found that treatment with aldosterone (Fig. 6A), vitamin D_3 (Fig. 6B) or insulin (Fig. 6C) was always

correlated with a loss of mab $_{CD}$Amp1 immunolabeling. Thus, the cultured embryonic collecting duct epithelia lose P_{CD}Amp1 expression during differentiation as it is observed in the maturing collecting duct of the neonatal kidney (Figs. 3 and 5). In contrast, culture of the embryonic epithelia in medium containing serum and no hormonal supplements led to an intense mab $_{CD}$Amp1 immunoreaction (Fig. 6D). Since modulation of the mab $_{CD}$Amp1-antigen is possible under *in vitro* conditions, we will be able to investigate the signals involved more closely in future experiments.

ACKNOWLEDGMENTS

This investigation was supported by the Deutsche Forschungsgemeinschaft (Mi 331/4-2). The technical assistance of Mrs. Elfriede Eckert is gratefully acknowledged.

Reprint requests to Will W. Minuth, University of Regensburg, Department of Anatomy, Universitätsstrasse 31, D-93053 Regensburg, Germany.
E-mail: will.minuth@vkl.uni-regensburg.de

REFERENCES

1. PERANTONI AO, WILLIAMS CL, LEWELLYN AL: Growth and branching morphogenesis of rat collecting duct anlagen in the absence of metanephrogenic mesenchyme. *Differentiation* 48:107–113, 1991
2. SOROKIN L, KLEIN G, MUGRAUER G, FECHER L, EKBLOM M, EKBLOM P: Development of kidney epithelial cells, in *Epithelial Organisation and Development*, London, Chapman & Hall, 1992, pp 163–190
3. MINUTH WW, GILBERT P, GROSS P: Appearance of specific proteins in the apical plasma membrane of cultured renal collecting duct principal cell epithelium after chronic administration of aldosterone and arginine vasopressin. *Differentiation* 38:194–202, 1988
4. AIGNER J, KLOTH S, MINUTH WW: Transitional differentiation patterns of principal and intercalated cells during renal collecting duct development. *Epithelial Cell Biol* 4:121–130, 1995
5. BARASCH J, PRESSLER L, CONNOR J, MALIK A: A ureteric bud cell line induces nephrogenesis in two steps by two distinct signals. *Am J Physiol* 271:F50–F61, 1996
6. KLOTH S, AIGNER J, SCHMIDBAUER A, MINUTH WW: Interrelationship of renal vascular development and nephrogenesis. *Cell Tissue Res* 277:247–257, 1994
7. MINUTH WW, GILBERT P, LAUER G, AKTORIES K, GROSS P: Differentiation properties of renal collecting duct cells in culture. *Differentiation* 33:156–167, 1986
8. MINUTH WW, AIGNER J, KUBAT B, KLOTH S: Improved differentiation of renal tubular epithelium in vitro: Potential for tissue engineering. *Exper Nephrol* 5:10–17, 1997
9. KLOTH S, AIGNER J, BRANDT E, MOLL R, MINUTH WW: Histochemical markers reveal an unexpected heterogenous composition of the renal embryonic collecting duct epithelium. *Kidney Int* 44:527–536, 1993
10. LEHIR M, KAISSLING B, KOEPPEN BM, WADE JB: Binding of peanut lectin to specific epithelial cell types in kidney. *Am J Physiol* 242:C117–C120, 1982
11. MINUTH WW, GILBERT P, RUDOLPH U, SPIELMAN WS: Successive histochemical differentiation steps during postnatal development of the collecting duct in rabbit kidney. *Histochemistry* 93:19–25, 1989
12. KÖHLER G, MILSTEIN C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495–497, 1975
13. LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
14. KYHSE-ANDERSEN J: Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 10:203–209, 1984
15. JOHNSTONE A, THORPE R: *Immunochemistry in Practice.* London, Blackwell Scientific Publications, 1982
16. KUJAT R, ROSE C, WROBEL KH: The innervation of the bovine ductus deferens: Comparison of a modified acetylcholinesterase-reaction with immunoreactivities of cholintransferase and pan-neuronal markers. *Histochemistry* 99:231–239, 1993
17. MINUTH WW: Neonatal rabbit kidney cortex in culture as a tool for the study of collecting duct formation and nephron differentiation. *Differentiation* 36:12–22, 1987
18. KLOTH S, EBENBECK C, KUBITZA M, SCHMIDBAUER A, RÖCKL W, MINUTH WW: Stimulation of renal microvascular development under organotypic culture conditions. *FASEB J* 9:963–967, 1995
19. SAXÉN L: Organogenesis of the kidney, in *Developmental and Cell Biology Series*, edited by BARLOW PW, WYHI CC, Cambridge, Cambridge University Press, 1987
20. LEHTONEN E: Epithelio-mesenchymal interface during mouse kidney tubule induction in vivo. *J Embryol Exp Morphol* 34:695–705, 1975
21. NEISS WF: Morphogenesis and histogenesis of the connecting tubule in the rat kidney. *Anat Embryol* 165:81–95, 1982
22. MINUTH WW, GROSS P, GILBERT P, KASHGARIAN M: Expression of the α-subunit of Na/K-ATPase in renal collecting duct epithelium during development. *Kidney Int* 31:1104–1112, 1987
23. BENOS DJ, AWAYDA MS, BERDIEV BK, BRADFORD AL, FULLER CM, SENYK O, ISMAILOV II: Diversity and regulation of amiloride-sensitive Na^+-channels. *Kidney Int* 49:1632–1637, 1996
24. KNEPPER MA, WADE JB, TERRIS J, ECELBARGER CA, MARPLES D, MANDON B, CHOU C, KISHORE BK, NIELSEN S: Renal aquaporins. *Kidney Int* 49:1712–1717, 1996
25. GILBERT P, MINUTH WW, BACHMANN S: Microheterogeneity of the collecting duct system in rabbit kidney as revealed by monoclonal antibodies. *Cell Tissue Res* 248:611–618, 1987
26. KLOTH S, GMEINER T, AIGNER J, JENNINGS ML, MINUTH WW: Transitional stages in renal collecting duct development. (submitted for publication)
27. PACHNIS V, MANKOO B, COSTANTINI F: Expression of the C-RET proto-oncogene during mouse embryogenesis. *Development* 119:1005–1017, 1993
28. KISPERT A, VAINIO S, SHEN L, ROWITCH DH, MCMAHON AP: Proteoglycans are required for maintenance of WNT-11 expression in the ureter tips. *Development* 122:3627–3637, 1996
29. DEVUYST O, BURROW CR, SCHWIEBERT EM, GUGGINO WB, WILSON PD: Developmental regulation of CFTR expression during human nephrogenesis. *Am J Physiol* 271:F723–F735, 1996
30. AIGNER J, KLOTH S, KUBITZA M, KASHGARIAN M, DERMIETZEL R, MINUTH WW: Maturation of renal collecting duct cells in vivo and under perifusion culture. *Epith Cell Biol* 3:70–78, 1994
31. MINUTH WW, FIETZEK W, KIOTH S, AIGNER J, HERTER P, RÖCKL W, KUBITZA M, STÖCKL G, DERMIETZEL R: Aldosterone modulates PNA binding cell isoforms within renal collecting duct. *Kidney Int* 44:537–544, 1993
32. MINUTH WW, LAUER G, BACHMANN S, KRIZ W: Immunocytochemical localization of a glycoprotein ($gp_{CD}1$) synthesiszed by cultured collecting duct cells. *Histochemistry* 80:1717–182, 1984
33. BURNATOWSKA-HLEDIN MA, SPIELMAN WS: Immunodissection of mitochondria-rich cells from rabbit outer medullary collecting tubules. *Am J Physiol* 254:F907–F911, 1988
34. TAUC M, KOECHLIN N, JAMOUS M, OUAGHI M, GASTINEAU M, LE MOAL C, POUJEOUL P: Principal and intercalated-cells in primary cultures of rabbit renal collecting tubules revealed by monoclonal antibodies. *Biol Cell* 76:55–65, 1992
35. JAMOUS M: Evolution of a cortical collecting tubule primary culture. *Eur J Cell Biol* 61:392–399, 1993