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Characterization of Micro-Fibers at the Interface between the Renal Collecting Duct Ampulla and the Cap Condensate

K. Schumacher R. Strehl W.W. Minuth

Department of Molecular and Cellular Anatomy, University of Regensburg, Regensburg, Germany

Key Words
Soybean agglutinin · Soybean agglutinin-positive micro-fibers · Kidney development · Two-dimensional electrophoresis · Extracellular matrix

Abstract
The development of renal histo-architecture substantially depends on the three-dimensional extension of the collecting duct (CD) ampulla, since under its influence, nephron induction takes place in the surrounding mesenchyme. Recently, micro-fibers were detected by soybean agglutinin (SBA), which line from the basal aspect of each CD ampulla through the mesenchyme towards the organ capsule in embryonic kidney. Their unique distribution suggests that they may play an important role in the control of CD ampulla growth and in forming the renal stem cell niche. A profound analysis of interstitial proteins between the CD ampulla and the nephrogenic mesenchyme is lacking. Consequently, the goal of the current investigation was to colocalize the micro-fibers detected by SBA with interstitial proteins. For this reason a detailed cell biological analysis of extracellular molecules at this site was carried out. Double labeling showed that the micro-fibers do not correspond to known collagens and other extracellular matrix molecules such as agrin, versican or MMP-9. In addition, it could be demonstrated that the micro-fibers do not contain epithelial or mesenchymal cell elements. Furthermore, two-dimensional electrophoresis with subsequent Western blotting yielded two different amino acid sequences (1: GHYADPTSPR; 2: NNGCCSSDYHA) obtained from SBA-labeled protein spots. Both amino acid sequences could not be assigned to known rodent proteins. The findings suggest that the SBA-labeled micro-fibers represent a new type of extracellular structure between the CD ampulla, the mesenchyme and the organ capsule.

Introduction

The interface between each collecting duct (CD) ampulla and the surrounding nephrogenic mesenchyme is a site of outmost importance during kidney development [1]. Morphogenic signals are exchanged between both tissues to initiate the coordinated formation of the nephrons [2, 3] followed by the successive branching and elongation of the CD ampulla [4]. The capacity to induce nephron formation is provided during the initial phase by the ureteric bud followed by the CD ampulla in further developed organ stages. However, the development of the entire organ is not based on a single morphogenic induction but must be considered as a succession of numerous and well-coordinated tissue interactions. During all these steps it must be ensured that each CD ampulla is well positioned in relation to the nephrogenic target tissue. When a comma-shaped body as the first visible sign of

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Karl Schumacher, MD
Department of Molecular and Cellular Anatomy, University of Regensburg
Universitätstrasse 31, DE–93053 Regensburg (Germany)
Tel. +49 941 9432875, Fax +49 941 9432868
E-Mail karl.schumacher@vkl.uni-regensburg.de

nephron development is formed, the CD ampulla leaves this tissue site to promote the next generation of nephrons. During that process it divides dichotomously and then elongates towards the organ capsule. After elongation the CD ampulla is in contact with a new set of competent nephrogenic cells. The process of nephron induction can start again. Both interacting tissues have to maintain the correct position to receive the inductive signals released from each other [3].

The mesenchymal cells covering each CD ampulla are more or less aggregated and form the cap condensate [5]. Although all cap condensate cells express the same molecules such as Pax-2 [6], WT-1 [7] and glial cell line-derived neurotrophic factor [8], they undergo different cell fates during kidney development. In the center, organ-specific mesenchymal stem cells are found, while at the lateral side of the cap condensate cells differentiate into epithelial cells to form the nephron [5, 9]. Part of the remaining tissue delivers the competent cells for the next nephron induction cycle [9], while some cells are involved in the development of capillaries [10], some differentiate into stromal cells [11] and some cells undergo apoptosis [12].

The tip of each CD ampulla reveals specific cell biological features. Typical protein constituents are c-ret [13], Wnt-11 [14], osteopontin [15] and P_{CD}Amp1 [16]. Furthermore, in all micrographs of embryonic kidney analyzed by other authors [1] and by our laboratory [16, 17], a clear cleft is visible between the tip of CD ampulla and the covering cap condensate. It appears that the mesenchyme is kept at a distinct distance to the CD ampulla tip. As revealed by light microscopy in neonatal rabbit kidney, the cleft shows an amorphous structure, while electron microscopy demonstrates that it is filled with a dense fiber material covering the tip of the CD ampulla [16, 17].

Recent data in developing rabbit and human kidneys further show that numerous soybean agglutinin (SBA)-positive micro-fibers originate from each CD ampulla tip and extend into the nephrogenic mesenchyme towards the organ capsule [18]. Beside the neonatal rabbit kidney, the SBA-positive micro-fibers are present in human embryonic kidney at weeks 14 and 20 of pregnancy. In mice the SBA-positive micro-fibers extending from the CD ampulla are visible at day E13.5 and in the neonatal status [unpubl. data]. The fact that the SBA-positive microfibers are present during different developmental steps in combination with their unique distribution indicates an important role during kidney development. We investigated in the present paper whether the SBA-labeled molecules are associated with a new type of fiber or whether they colocalize with known extracellular matrix proteins such as laminin [19], fibronectin [20], tenascin [21] and collagens [20]. In addition, we investigated by scanning electron microscopy the interstitial space between the CD ampulla tip and the nephrogenic mesenchyme. In a further set of experiments we analyzed whether molecules associated with the SBA-positive micro-fibers could be detected in two-dimensional electrophoresis with subsequent SBA-Western blotting.

Material and Methods

Tissue Preparation

One-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, frozen in liquid nitrogen and stored at −80°C up to the subsequent treatments.

Lectin Incubation

Corticomedullary orientated cryosections (8 μm) of neonatal rabbit kidney were prepared using a cryomicrotome (Microm, Heidelberg, Germany). The cryosections were fixed in ice-cold ethanol and then washed with phosphate-buffered saline (PBS). After incubation in blocking solution (PBS + 1% bovine serum albumin + 10% horse serum) for 30 min, the sections were exposed to fluorescein-isothiocyanate (FITC)-conjugated SBA (Vector Laboratories, Burlingame, Calif., USA) diluted 1:4,000 in blocking solution for 45 min. Following several washes in PBS, the specimens were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, Oreg., USA) and analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, Calif., USA).

Coincubation Experiments with Antibodies

FITC-conjugated SBA (Vector Laboratories) was coincubated with the following antibodies: anti-tenascin-C, anti-MMP-9, anti-collagen type XVIII, anti-Cox-1, anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA); anti-E-cadherin (Zymed, San Francisco, Calif., USA); anti-collagen type I, II, III, V, IV, IX (Medicorp, Montreal, Canada) and anti-laminin (Boehringer Mannheim, Mannheim, Germany). The mab anti-cytokeratin Endo-A TROMA-1 was developed by P. Brulet and R. Kemler. The mab anti-β-tubulin E7 was developed by M. Klymkowsky. The mab anti-collagen type II II-II6B3 was developed by Dr. T.F. Linsenmayer. The mab anti-collagen type IV M3F7 was developed by Dr. H. Furthmayr. The mab anti-agrin 6D2 was developed by Dr. W. Halfter. The mab anti-osteopontin MPIIIB10(1) was developed by Dr. M Solursh and Dr. A. Franzen. The mab anti-versican 12C5 was developed by Dr. R.A. Asher. The mabs II-II6B3, M3F7, 6D2, MPIIIB10(1) and 12C5 were obtained from the Development Studies Hydroma Bank (University of Iowa, Department of Biological Sciences, Iowa City, Iowa, USA, under contract NO1-HD-7-3263 from the NICHD). Anti-fibronectin and anti-vimentin were obtained from Sigma, Saint Louis, Mo., USA. The anti-cytokeratin 19 antibody recognizes a 40-kDa polypeptide in all CD cells in kidney and was kindly provided by Prof. Dr. R. Moll, Marburg, Germany.

Eight-micrometer-thick cryosections of 1-day-old rabbit kidneys were fixed in ice-cold ethanol. After washing with PBS the sections were blocked with PBS containing 1% BSA and 10% horse serum for 30 min. Primary antibodies were applied for 1 h in blocking solution. The specimens were incubated for 45 min with donkey-anti-mouse- or donkey-anti-goat-IgG-Texas-red-conjugated secondary antibodies diluted 1:200 in PBS containing 1% BSA (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) together with FITC-conjugated SBA (Vector Laboratories) diluted 1:4,000. In a further experiment the specimens were incubated for 45 min with FITC-conjugated SBA diluted 1:4,000 together with Alexa Fluor 568 Phalloidin 1:200 (Molecular Probes) in blocking solution. The sections were then analyzed using an Axioskop 2 plus microscope (Zeiss). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems).

Gold-Chloride Incubation and Silver Impregnation

The gold-chloride incubation of the explants isolated from neonatal rabbit kidney was performed according to a method described by Ranvier [22]. In brief, tissue explants were fixed in citric acid and thereafter exposed to 1% gold-chloride solution for 1 h. The specimens were exposed to 50% formic acid overnight, paraffin-embedded, cut in 1- or 2-μm-thick sections and analyzed using an Axioskop 2 plus microscope (Zeiss). This approach aimed to localize cellular processes at the interface between the CD ampulla tip and the surrounding mesenchyme. In a further experiment, paraffin-embedded sections of 1-day-old rabbit kidneys were silver stained according to a method described by Novotny-Gommert [22] in order to detect silver-stained extracellular fibers.

Microdissection Procedure

The embryonic area of 1-day-old rabbit kidneys was isolated as described earlier [23]. CD ampullae were microdissected from cortical explants of 1-day-old New Zealand rabbit kidneys under optical control of a KL 1500 stereomicroscope (Leica, Solms, Germany). The isolated CD ampullae were transferred to a microscope slide and fixed in ice-cold ethanol. After exposure to FITC-conjugated SBA diluted 1:2,000 together with Alexa Fluor 568 Phalloidin 1:200 (Molecular Probes) in blocking solution for 45 min, specimens were covered by a cover glass and examined with an Axioskop 2 plus microscope (Zeiss).

Scanning Electron Microscopy

For scanning electron microscopy, exactly oriented pieces of tissue were prepared, fixed in 2% glutaraldehyde in PBS under isotonic conditions for 24 h at 4°C, dehydrated in a graded series of ethanols, transferred to acetone, and critical-point dried in carbon dioxide. Finally, they were sputter coated with gold (Polaron, Watford, UK) and examined in a DSM 940-A scanning electron microscope (Zeiss).

Two-Dimensional Electrophoresis

Cortical explants of 1-day-old rabbit kidneys were homogenized in lysis buffer (pH 7.5) containing 8 *M* urea, 4% CHAPS, 40 m*M* Tris-HCl, 2 m*M* DTT and 0.5% carrier ampholytes. A 300-μg protein sample was loaded on a gel tube for isoelectric focusing. The gel tubes were run for 14 h using increasing voltage (100–1,000 V) in a model 175 Tube cell (Bio-Rad Laboratories, Hercules, Calif., USA). The focused gel tubes were equilibrated in a buffer containing 2% SDS, 10% glycerin, 125 m*M* Tris-HCl and 1 m*M* EDTA and thereafter laid on the surface of 3-mm thick 10% Laemmli gels in order to separate proteins by SDS-PAGE at 100 V and 120 mA for 7 h. The gel plates were then stained with Serva Blue R (Serva, Heidelberg, Germany).

Lectin Western Blot Experiments

In order to detect SBA-labeled molecules, two-dimensionally separated proteins were electrophoretically transferred to PVDF transfer membranes (Millipore, Bedford, Mass., USA). After exposure to PBS containing 10% horse serum and 0.02% Tween for 1 h, the membranes were incubated with SBA diluted 1:2,000 overnight at 4°C. After several washing steps, the membranes were incubated with antibody to SBA diluted 1:1,000 in PBS-Tween (anti-SBA, Vector Laboratories) followed by incubation with a horseradish peroxidase-conjugated donkey anti-goat immunoglobulin antiserum (Dianova, Hamburg, Germany). Blot development was started by addition of 0.5 mg/ml diaminobenzidine, 0.02% H_2O_2 and 0.03% cobalt chloride dissolved in citrate buffer (17 m*M*, pH 6.3). The reaction was stopped by washing the membrane in tap water. Immunoblots were documented with a Scan Jet 6200 C scanner (Hewlett Packard, Greeley, Colo., USA). Apparent molecular weight and isoelectric point proteins were performed with two-dimensional SDS-PAGE standards, which were run in parallel experiments (Bio-Rad Laboratories).

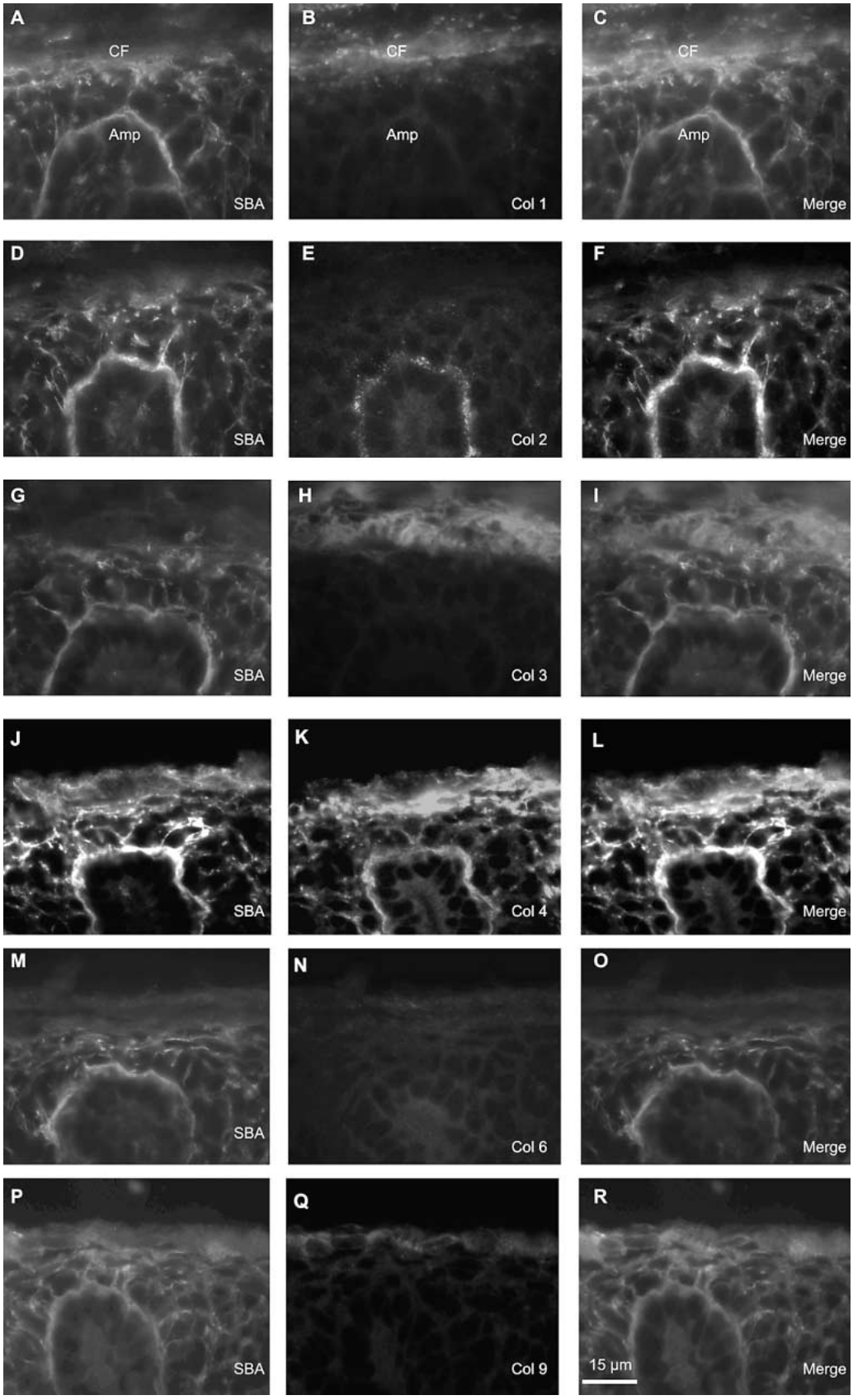
Sequence Analysis of SBA-Positive Spots

Two-dimensional gels for microsequencing were stained in Coomassie solution at room temperature for 60 min and destained until spots became visible. Single spots were excised and transported to Steinbeis-Transferzentrum (Proteome-Analyse) in Rostock, Germany. Samples were analyzed by Q-Tof-MS or QIT-Tof-MS.

Results

SBA Label in the Embryonic Cortex of Neonatal Rabbit Kidney

Cryosections of the embryonic zone of neonatal rabbit kidneys showed that the major intensity of SBA labeling was located at the basal aspect of each CD ampulla and in the organ capsule (fig. 1A, D, G, J, M, P, fig. 2A, D, G, J, M, P, fig. 3A, D, G, J, M). In addition, with minor intensity, SBA-labeled micro-fibers originate from each ampulla tip, cross the nephrogenic mesenchyme and reach the organ capsule. To assess whether the SBA-labeled structures colocalize with extracellular proteins expressed at this site, we performed a series of immunohistochemical double labeling experiments. In experimental group 1 we tested the colocalization with different collagen types (fig. 1), in group 2 non-collagenous extracellular matrix proteins (fig. 2) and in group 3 cellular molecules expressed in both epithelial and mesenchymal cells for control (fig. 3).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Colocalization of SBA Label with Collagenous Proteins

Collagen type 1 (fig. 1B) and type 3 (fig. 1H) are abundantly detected in the organ capsule. The CD ampulla with surrounding mesenchyme was negative. In contrast, collagen type II was found to be expressed only at the basal aspect of the CD ampulla in a punctate pattern, but not within the mesenchyme and not in the organ capsule (fig. 1E). Thus, not an overlapping but only a partial and very faint colocalization with SBA-positive label was found at the basal aspect of the CD ampulla. Collagen type 4 (fig. 1K) expression was conspicuous. The basal aspect of the CD ampulla and the organ capsule was labeled by the collagen type 4 antibody (fig. 1K). In the surrounding mesenchyme a partial colocalization on single SBA-positive micro-fibers was seen (fig. 1L). Collagen type 5 was not present in the neonatal kidney itself, but in the adipose tissue adherent to the capsule (no figure). Collagen type 6 was found neither in the mesenchyme nor in the CD ampulla (fig. 1N) but in capillaries. Reaction for collagen type 9 was present within the organ capsule (fig. 1Q). Collagen type 18 was absent in the mesenchyme and in the CD ampulla (no figure).

Taken together, the results show that a partial but no completely overlapping reaction of SBA-positive micro-fibers with collagenous proteins such as collagen type 4 (fig. 1L) was found.

Colocalization of SBA Label with Non-Collagenous Proteins

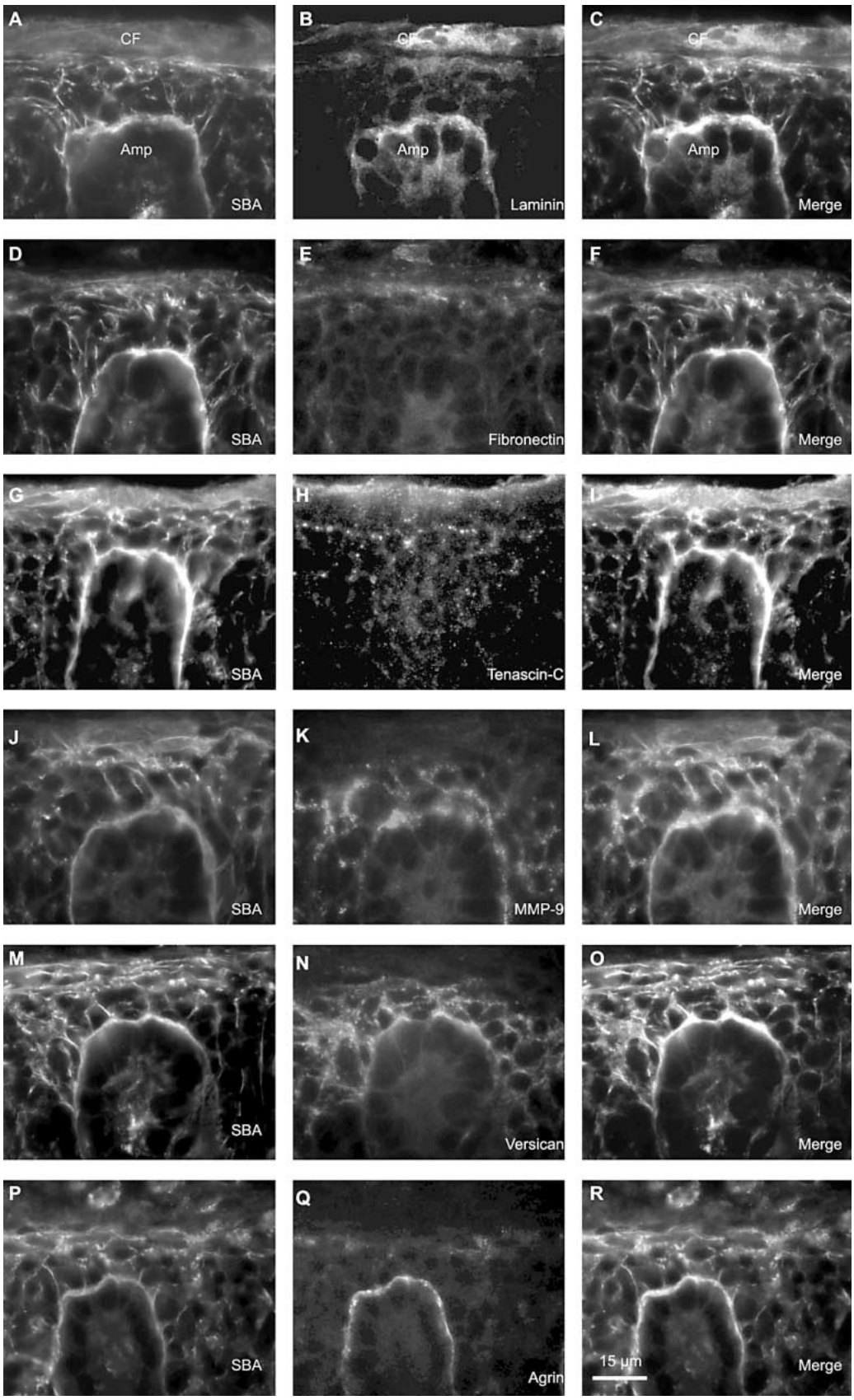
It was shown earlier that non-collagenous extracellular matrix proteins including glycoproteins and glycosaminoglycans are found between the CD ampulla and the surrounding mesenchyme [24]. To evaluate the pattern of these non-collagenous extracellular proteins in relation to the SBA-positive micro-fibers, we performed further immunohistochemical double-labeling experiments (fig. 2). Laminin was positive at the basal aspect of the CD ampulla and on parts of the capsule, while the nephrogenic mesenchyme was negative (fig. 2B). Fibronectin was not present at the CD ampulla, but within the capsule and as a faint reaction within the mesenchyme underneath the organ capsule (fig. 3E). Tenascin-C was found as a punctate pattern within the mesenchyme and in high intensity within the capsule (fig. 2H). Only in few areas the punctate pattern of tenascin-C expression was found to be colocalized with SBA-positive micro-fibers (fig. 2I). MMP-9 was detected as an intensive cluster-like reaction at the basal aspect of the CD ampulla and within the mesenchyme (fig. 2K) showing a punctate colocalization with SBA-positive micro-fibers (fig. 2L). A similar punctate colocalization was detected with the antibody directed to versican (fig. 2N). Furthermore, it is remarkable that versican was abundantly expressed in the immature cortical zone but disappeared completely in the matured cortical zone. However, expression reappeared in the kidney medulla of neonatal kidney (no figure). Finally, agrin was found to be expressed at the basal aspect of the CD ampulla as a punctate pattern, however not within the mesenchyme or within the organ capsule (fig. 2Q). In several cases, punctate agrin-positive areas at the basal aspect of the ampulla seem to be colocalized with the origin of emanating SBA-positive micro-fibers (fig. 2R). Osteopontin known as a molecule expressed at the interface of the CD ampulla and the surrounding mesenchyme showed no colocalization with the SBA-positive micro-fibers (no figure) [15].

Fig. 1. Comparison of SBA label with different collagen types in the embryonic zone of neonatal rabbit kidney. The left column reveals the SBA label (**A**, **D**, **G**, **J**, **M**, **P**), middle column shows the label of different collagen types (**B**, **E**, **H**, **K**, **N**, **Q**) and the right column displays the merge of the SBA label and the respective collagen type label (**C**, **F**, **I**, **L**, **O**, **R**). SBA is binding to molecules found in the capsula fibrosa (CF) and at the basal aspect of the CD ampulla (Amp). In the mesenchyme, SBA labels micro-fibers extending from the ampulla tip and towards the capsula fibrosa. Collagen type 1 (**B**), 3 (**H**), 4 (**K**) and 9 (**Q**) are expressed in the capsula fibrosa, while collagen type 2 (**E**) and 4 (**K**) is located at the basal aspect of the CD ampulla. Only collagen type 4 (**K**, **L**) reveals binding within the mesenchyme. The simultaneous binding of SBA and collagen type 4 is restricted only to a few fibers (**K**, **L**).

For figs 2 and 3 see pages 48/49.

Fig. 2. Comparison of SBA label with non-collagenous extracellular proteins in the embryonic zone of neonatal rabbit kidney. The left column reveals the SBA label (**A**, **D**, **G**, **J**, **M**, **P**), middle column shows the label of laminin (**B**), fibronectin (**E**), tenascin-C (**H**), MMP-9 (**K**), versican (**N**) and agrin (**Q**). The right column displays the merge of the SBA label and the respective extracellular matrix components (**C**, **F**, **I**, **L**, **O**, **R**). Some of the molecules are partly colocalized, but none of them shows an identical occurrence with SBA-positive micro-fibers.

Fig. 3. Comparison of SBA label with cellular molecules in the embryonic zone of neonatal rabbit kidney. The left column reveals the SBA label (**A**, **D**, **G**, **J**, **M**), middle column shows the label of phalloidin (**B**), vimentin (**E**), Cox-1 (**H**), cytokeratin-19 (**K**) and E-cadherin (**N**). The right column displays the merge of the SBA label and the respective cellular molecules (**C**, **F**, **I**, **L**, **O**). Some molecules such as vimentin (**E**, **F**) and Cox-1 (**H**, **I**) show partial colocalization sites with SBA, but in no case a complete colocalization is found. The results indicated that the SBA-positive micro-fibers in the mesenchyme are not formed by cellular protrusions.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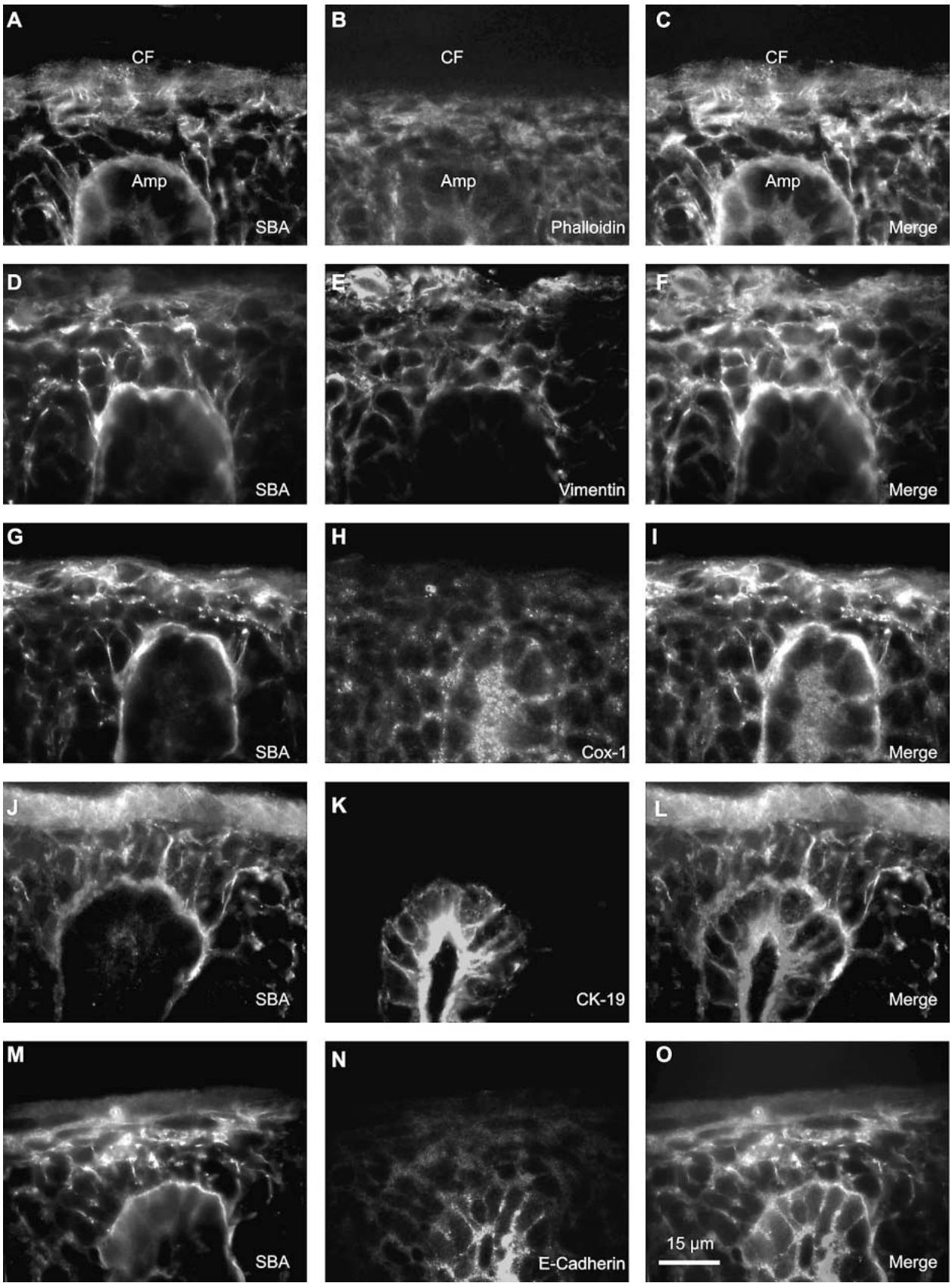
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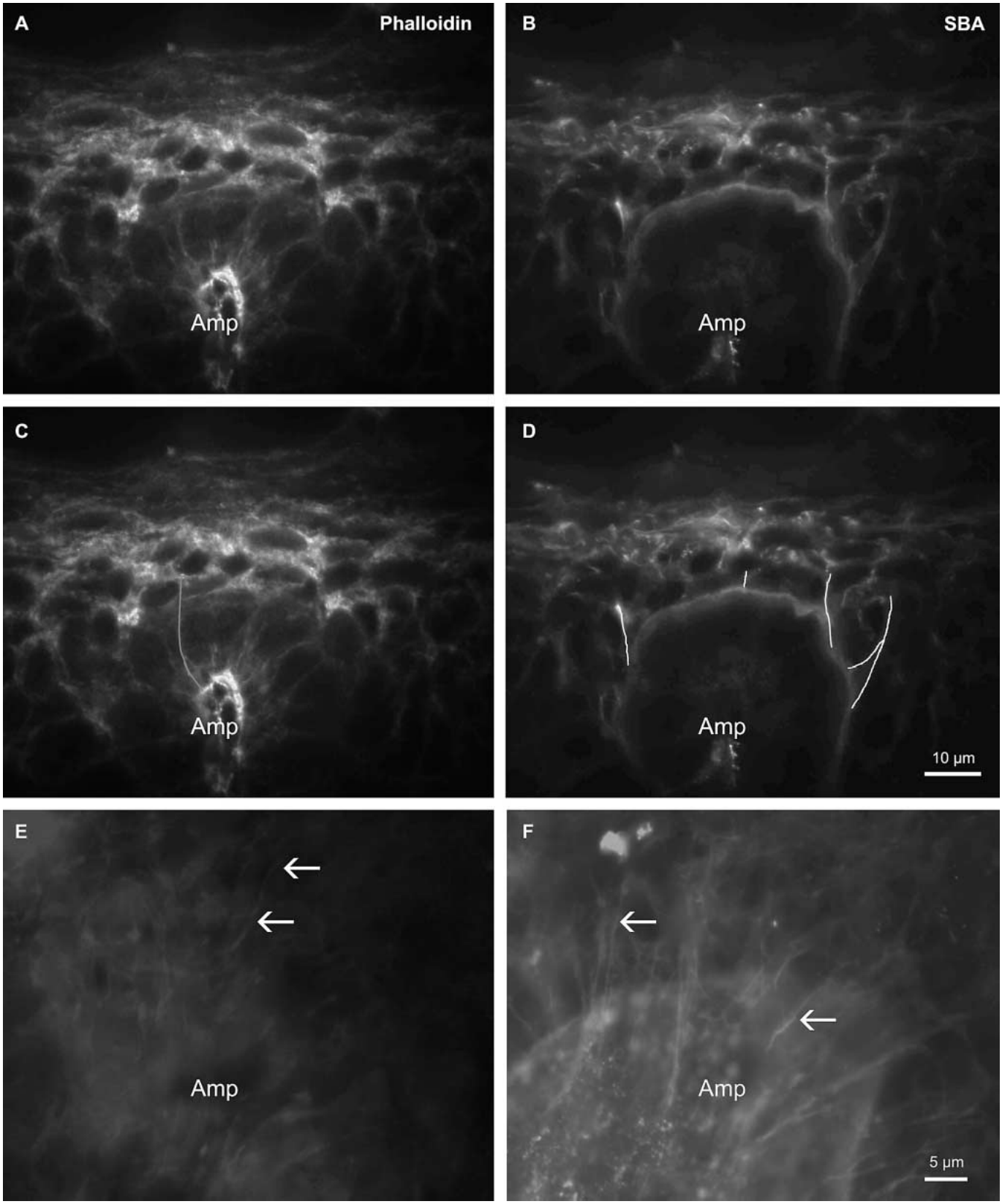
3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E
Amp
F
Amp
5 µm

These results indicate that non-collagenous molecules such as fibronectin (fig. 2F), tenascin-C (fig. 2I), MMP-9 (fig. 2K) and versican (fig. 2N) displayed a partial colocalization with SBA-positive micro-fibers. However, no completely overlapping expression was found.

Colocalization of SBA Label with Cellular Proteins

To analyze whether the SBA-positive micro-fibers correspond to cellular processes extending from the CD ampulla, we performed coincubation experiments with markers recognizing cellular proteins (fig. 3). Phalloidin was found to bind to F-actin in epithelial CD ampulla cells and in mesenchymal nephrogenic cells (fig. 3B). However, a colocalization of SBA and phalloidin label could only be detected in a few areas (fig. 3C). The same findings of partial colocalization could be obtained for vimentin (fig. 3E), which labeled mesenchymal cells and structures within the organ capsule. In addition, Cox-1 binds to all CD ampulla cells and in a punctate pattern to cells of the mesenchyme (fig. 3H), which did not colocalize with the SBA-positive micro-fibers (fig. 3I). Expression of cytokeratin-19 (fig. 3K), TROMA-1 (no figure) and E-cadherin (fig. 3N) was restricted to epithelial cells of the CD ampulla. Furthermore, the SBA-positive structures did not colocalize with α- or β-tubulin (no figure).

To elucidate further whether SBA-labeled micro-fibers belong to extracellular or cellular structures, tissue was silver stained to detect reticular fibers and gold stained to show cellular protrusions. Silver treatment showed that stained fibers are present in the embryonic zone of neonatal rabbit kidney (no figure). However, the amount and distribution of silver-stained fibers shows clear differences to SBA-labeled micro-fibers. This indicates that SBA-labeled micro-fibers do not correspond to reticular structures. In addition, a clearly visible space exists between the CD ampulla and the surrounding mesenchyme where only few cellular processes can be detected by gold-chloride incubation (no figure).

As shown in figure 4A and C, phalloidin-positive structures are detectable at the interface between CD ampulla and mesenchyme, but, as shown in figure 4B and D, they are not identical with the SBA-positive micro-fibers. Finally, while after microsurgical isolation of single CD ampullae many SBA-positive structures extending from the surface were detectable (fig. 4F, arrows), only a small number of cellular processes labeled by Alexa Fluor 488 Phalloidin were visible (fig. 4E, arrows). In these experiments no overlapping distribution pattern of both structures was observed. These results clearly show that the SBA-positive micro-fibers do not correspond to actin- or microtubule-based structures.

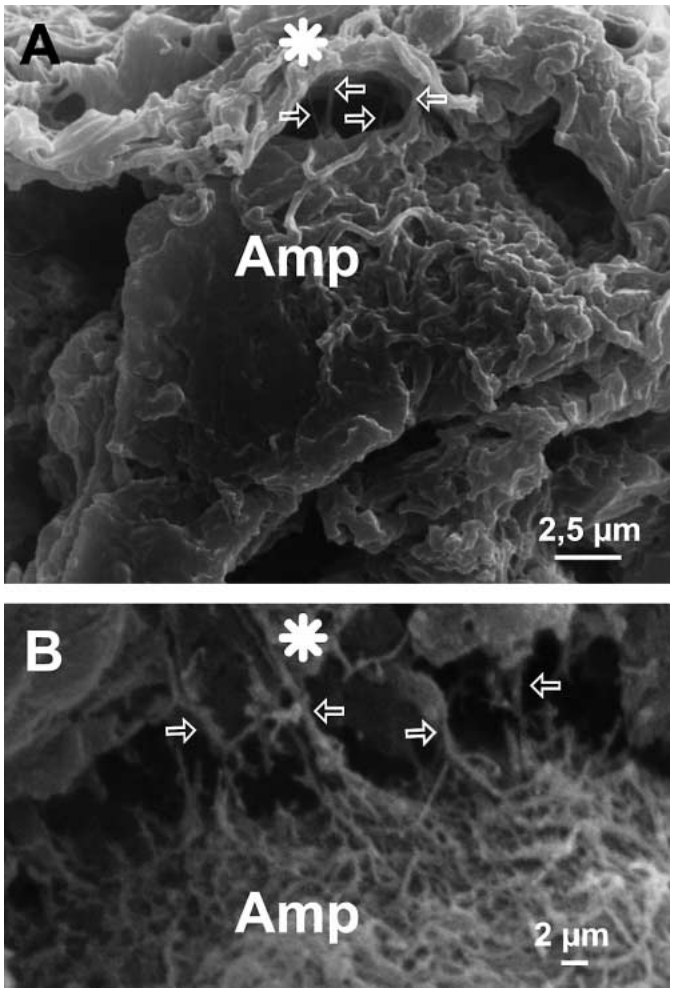
Scanning Electron Microscopy Analysis of Fibers at the Interface between the CD Ampulla and the Mesenchyme

Using exactly oriented corticomedullary tissue sections, we analyzed by scanning electron microscopy the interface between the CD ampulla and the mesenchyme. As shown in figure 5A (arrows), fibers are present emanating from the basal aspect of the CD ampulla into the surrounding mesenchyme. Higher magnification of a CD ampulla tip yielded an extended fiber network covering the CD ampulla tip as described before [16, 17]. Numerous fibers with various diameters are found between the network at the tip and the mesenchyme (fig. 5B, arrows). In part, the fibers are cross-linked to other fibers.

SBA-Labeled Proteins Analyzed by Two-Dimensional Electrophoresis

To identify the SBA-positive molecules in embryonic renal tissue, we analyzed tissue homogenates by two-dimensional electrophoresis with subsequent SBA-Western blotting experiments (fig. 6). A series of SBA-labeled spots could be detected in isolated embryonic tissue. Up to date, for two spots a sequence could be obtained which did not show any homology to known rodent proteins. The spot with pI of 6.5 and MW of 32 kDa reveals a sequence with a length of 10 amino acids with the following amino acid sequence: GHYADPTSPR. The spot with pI of 8.6 and MW of 37 kDa reveals a sequence with a length of 11 amino acids with the following amino acid sequence: NNGCCSSDYHA.

Fig. 4. Fibers at the interface between the CD ampulla (Amp) and the mesenchyme in the embryonic zone of neonatal rabbit kidney. **A** Phalloidin labeling reveals actin elements in both epithelial and mesenchymal cells. One thin phalloidin-positive structure is visible projecting from epithelial cell into the surrounding mesenchyme. The structure ends in a button. **B** SBA labeling of the same section as **A** displays numerous micro-fibers extending from the basal aspect of the ampulla into the mesenchyme and organ capsule. **C** Projecting structure as seen in **A** is illustrated by a red line. **D** SBA-positive micro-fibers are illustrated by green lines which are not identical with actin-based structures seen in **C**. **E** Phalloidin labeling shows that several phalloidin-positive structures extend from microdissected ampulla (arrows). **F** A huge bundle of SBA-positive structures extend from the ampulla (arrows) in the same section. None of them shows an identical course in comparison with the phalloidin-positive structures.



Discussion

In the present experiments we analyzed features of SBA-positive micro-fibers in the interstitial space between the CD ampulla tip, the nephrogenic mesenchyme and the organ capsule in neonatal rabbit kidney by morphological, cell-biological (fig. 1–5) and biochemical methods (fig. 6). The experiments were focused on the questions of whether the SBA-positive micro-fibers constitute a new type of fiber, whether the labeling is associated with already known extracellular matrix proteins or whether they belong to cellular processes.

Coincubation experiments showed that the SBA-labeled micro-fibers at the CD ampulla tip and in the mesenchyme are not collagen types 1, 2, 3, 5, 6, 9 and 18 (fig. 1). One exception is collagen type 4 (fig. 1K). It is found to be colocalized with SBA label along the basal aspect of the CD ampulla, within the nephrogenic mesenchyme and in the organ capsule. However, as shown in figure 1L, only two SBA-positive micro-fibers are simulta-

Fig. 5. Scanning electron microscopy showing the interface between the ampulla tip and mesenchyme. **A** Thicker fibers (arrows) bridge the cleft between the collecting duct ampulla (Amp) and the mesenchyme (asterisk). **B** Thin fibers (arrows) are abundantly detectable at the tissue interface revealing a structural connection between the ampulla (Amp) tip and the mesenchyme (asterisk). The fibers are in contact with an extracellular meshwork, which is known to cover each CD ampulla.

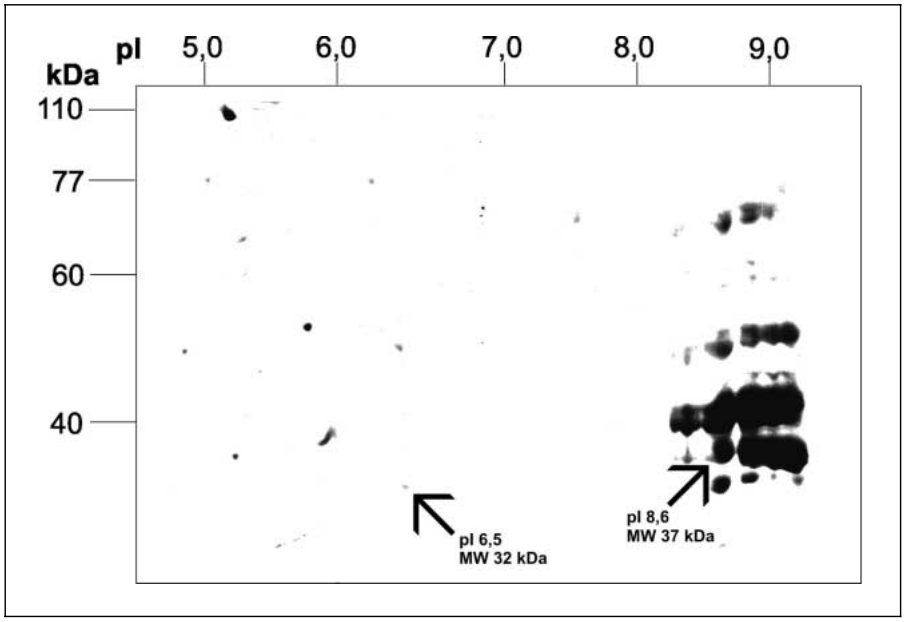


Fig. 6. Two-dimensional separation of the embryonic area with subsequent SBA-Western blotting. A series of SBA-labeled spots could be detected in isolated embryonic tissue. For two spots a sequence could be obtained which did not show any homology to known rodent proteins. The spot with pI of 6.5 and MW of 32 kDa reveals a sequence with a length of 10 amino acids with the following amino acid sequence: GHYADPTSPR. The spot with pI of 8.6 and MW of 37 kDa reveals a sequence with a length of 11 amino acids with the following amino acid sequence: NNGCCSSDYHA.

neously labeledby the collagen type 4 antibody, while the remaining SBA-positive micro-fibers fail to be labeled. Thus, the SBA-positive micro-fibers can only partially be colocalized to collagen type 4.

Colocalization experiments of SBA with non-collagenous extracellular matrix proteins further revealed that the immunohistochemical pattern of laminin (fig. 2B), fibronectin (fig. 2E), tenascin-C (fig. 2H), MMP-9 (fig. 2K), versican (fig. 2N), agrin (fig. 2Q) and osteopontin (no figure) is definitively distinct from the SBA label (fig. 2A, D, G, J, M, P), so that identity to SBA micro-fibers can be excluded.

The reciprocal exchange of signaling molecules between the CD ampulla and mesenchyme ensures normal kidney development [1–4]. It is assumed that either the morphogenic molecules reach the target sites by diffusion [2] or that both tissues communicate by the establishment of cellular contacts [4]. However, the distance between the CD ampulla cells and the surrounding mesenchyme is considerably long (fig. 4, 5). The existence of such cellular extensions during developmental processes are described as cytonemes in Drosophila and mouse [25] and as thin filopodia during sea urchin gastrulation [26]. To address the question of whether the SBA-positive micro-fibers are identical to cellular extensions such as cytonemes or thin filopodia, we analyzed whether the SBA-positive structures are associated with actin filaments (fig. 3B), vimentin (fig. 3E), Cox-1 (fig. 3H), cytokeratin-19 (fig. 3K) and E-cadherin (fig. 3N). Our present data show no complete colocalization for these cellular molecules with SBA-labeled micro-fibers. To reveal whether cellular extensions are present at the CD ampulla surface, tissue was microdissected and labeled by phalloidin (fig. 4E) or SBA (fig. 4F). The ampulla tip exhibits some cellular protrusions heading for the mesenchyme. However, the appearance of the phalloidin-positive processes (fig. 4E) was not identical to the distribution of the SBA-labeled micro-fibers (fig. 4F).

The molecular composition of the SBA-positive micro-fibers is today unclear. As revealed by two-dimensional electrophoresis with subsequent Western blotting experiments, SBA reacted with many different protein spots (fig. 6). However, the amino acid sequence for two spots could be obtained. Both sequences do not match with known protein data of rodents. Experiments are in progress to obtain longer sequences to generate antibodies against these amino acid sequences.

Summarizing, we show a detailed analysis of the area between the CD ampulla, the surrounding metanephric mesenchyme and the organ capsule in the embryonic cortex of neonatal rabbit kidney. We can provide the as yet unknown occurrence of molecules, versican and agrin, in this embryonic area. In addition, the obtained data reveal that the previously detected SBA-positive micro-fibers do not exhibit the same distribution and appearance as all of the known tested extracellular molecules. The partial identification of the amino acid sequence of two SBA-labeled protein spots does not show homology to known rodent protein data.

Although our experiments do not show a functional role of the SBA-positive micro-fibers, their unique distribution may provide a reasonable explanation for the absolutely linear growth of the CD during kidney development. When the developing kidney is expanding in volume, it is evident that the CD ampullae are connected through the SBA-positive micro-fibers with the organ capsule. As a consequence, the CD ampullae may follow this extension in a linear fashion [18]. It is further known that the embryonic kidney contains organ-specific stem cells and that every stem cell population needs a specific niche [9, 27]. We assume that the kidney-specific niche may be the compartment structured by the SBA-positive micro-fibers between the CD ampulla tip and the surrounding condensate cap. In this context, our findings described here may further contribute to the understanding of how renal stem cells are harbored in a niche during the multiple steps of nephron induction.

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