A Novel Protein, Densin, Expressed by Glomerular Podocytes

HEIKKI AHOLA,* EJIA HEIKKILÄ,* EVA ÅSTRÖM,* MASAKI INAGAKI,† ICHIRO IZAWA,‡ HERMANN PAVENSTÄDT,‡ DONTSCHO KERJASCHKI§, and HARRY HOLTHÖFER*

*Haartman Institute, Department of Bacteriology and Immunology, University of Helsinki, and Helsinki University Central Hospital, Helsinki, Finland; †Division of Biochemistry, Aichi Cancer Center Research Institute, Aichi, Japan; ‡Department of Medicine, Division of Nephrology, University Hospital of Freiburg, Freiburg, Germany; and §Department of Pathology, University of Vienna-Allgemeines Krankenhaus, Vienna, Austria.

Abstract. With the recent molecular findings, the podocyte is emerging as a key cell type involved in glomerular damage, but protein complexes involved remain poorly understood. To systematically search for additional podocyte molecules interacting with nephrin, a key structural molecule of the interpodocyte filtration slit, precipitation of glomerular lysates was set out with anti-nephrin antibodies to identify members of the nephrin-associated protein complex. Proteins of the precipitate were subsequently identified with MALDI-TOF mass analysis. One of the proteins thus obtained showed identity with densin, a protein originally purified from rat forebrain postsynaptic density fraction and so far shown to be highly brain-specific. The expression of densin appeared distinctly in the glomerulus and cultured podocytes by RT-PCR. Immunoblotting studies revealed a specific band of 185 kD in brain and cultured podocytes; in human glomerulus, densin appeared as a 210-kD band. By immunocytochemistry, densin localizes in glomeruli in a podocyte-like pattern. Electron microscopic studies revealed densin localization in the slit diaphragm area. Due to its known involvement in the synaptic organization, maintenance of cell shape and polarity in nerve cells, together with its demonstrated interactions with α-actinin-4, densin may share the same functions in podocytes by associating with the nephrin interacting protein complex at the slit diaphragm.

The ability of glomerular epithelial cells, podocytes, to form foot processes with unique intercellular slit diaphragms (SD) reflects a special organization with possible direct consequences for glomerular filtration. The protein complex forming the SD has been poorly known until the milestone discovery of NPHS1 (1), the gene encoding nephrin, which apparently forms the backbone of the SD (2–4). Thereafter, additional molecules with possible role in the maintenance of the functional filtration barrier have been rapidly found. These include e.g. the CD2-associated protein (CD2AP) (5), α-actinin-4 (6), podocin (7), P-cadherin (8), FAT (9), NEPH1 (10), and filtrin (11). Two of these proteins, CD2AP and podocin, reportedly interact with nephrin (12–16), probably mediating the connection of the SD protein complex to the actin cytoskeleton (14,17). Other interactions of this complex remain to be shown.

The NPHS1 gene is characteristically mutated in the congenital nephrotic syndrome of the Finnish type (CNF) (1), resulting in the absence of nephrin. Clinically CNF presents with heavy, treatment-resistant proteinuria at or even before birth (18,19). CNF and nephrin thus offer a useful model disease and key molecule, respectively, to study molecular mechanisms of proteinuria and to search for additional components of the molecular complex.

Nephrin and podocin are associated with lipid rafts and mediate outside-in signaling (16,20). The resulting specific phosphorylation events most likely are involved in fine-tuning the functional properties of the SD (20). Through the binding of nephrin to podocin and CD2AP, specific signaling has been described suggesting that a proper molecular interaction network at the SD domain is essential to control the dynamic passage of fluids, electrolytes, and particularly proteins through the glomerular filtration barrier.

To search for additional molecules interacting with nephrin, we took an alternative approach by systematically co-precipitating glomerular lysates with anti-nephrin antibodies and identified the protein yields with mass spectrometry. Surprisingly, we detected a protein, densin, previously only found in the central nervous system (21). Densin belongs to a family of proteins known to function in cell adhesion and polarity (22). In addition to its first identification in the kidney, we here describe densin expression in glomerular podocytes as verified by RT-PCR, immunofluorescence, Western blotting, and immunoelectron microscopy. The results suggest that densin is an important component of a larger complex of proteins localized at the SD area with a proposed role in glomerular filtration.
Materials and Methods

Tissue Samples and Cell Culture

Renal tissue of CNF patients and cadaver kidneys unsuitable for transplantation for vascular anatomic reasons (Department of Surgery, University of Helsinki) or normal poles of kidneys removed because of Wilms tumor were used as described earlier (2). The cortical tissue was separated and frozen in liquid nitrogen and stored at −70°C until processed for RNA isolation or immunohistochemistry. Glomeruli were isolated with the sieving method as described earlier (23).

A sample of normal brain tissue adjacent to a tumor in the temporal lobe was from a young adult taken during tumor surgery.

All the procedures described were approved by the ethics committee of the Helsinki University Central Hospital.

Human podocytes were isolated from normal human kidney as earlier described extensively (24). The cultured cells showed an epithelial morphology and stained positively for Wilms tumor antigen (WT1) and nephrin in indirect immunofluorescence. These cells were negative for the endothelial cell marker, factor VIII related antigen.

Production of Antibodies to Nephrin and Densin

The whole intracellular sequence of rat nephrin (GenBank AF125521) was cloned to pGEX-2T expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and transformed into the Escherichia coli strain BL-21 (Amersham Pharmacia Biotech) followed by culture in 2 × YTAg medium (yeast extract 10 g/L, tryptone 16 g/L, NaCl 5 g/L, glucose 2%, and 100 μg/ml ampicillin) at 37°C. After isopropyl β-D-thiogalactosidase (IPTG; Amersham Pharmacia Biotech) induction expression was allowed for 1 to 2 h before purification as described earlier (25). Shortly, the cells were lysed in STE buffer (150 mM NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA) in the presence of 100 μg/ml lysozyme, 3% sarcosyl, and 5 mM dithiothreitol following clearing with centrifugation at 7000 × g. Supernatant was adjusted to 2% with Triton X-100 before fusion protein collection with glutathione sepharose 4B (Amersham Pharmacia Biotech). The eluted protein was used for immunizations. The respective number of the antiserum produced with this antigen is #050.

To produce antibodies against soluble nephrin, human nephrin sequence (GenBank AF035835) 3091–3645 lacking the membrane-spanning exon 24 (GenBank AF126957) was first inserted into donor vector pUni/V5-His-TOPO (Invitrogen, Carlsbad, CA) followed by fusion to acceptor vector pBAD/Thio-E Echo (Invitrogen) according to manufacturer’s protocol. This thioredoxin fusion construct was transformed to TOP10 Escherichia coli strain for further culturing in LB medium with added kanamycin 50 μg/mL. Expression was induced with 0.02% arabinose followed by production at 37°C for 4 h. Collected cells were lysed with 100 μg/ml lysozyme, sonication, and flash freezing in the presence of the Complete Mini EDTA-free protease inhibitor mix (Roche, Mannheim, Germany). Purification was performed with HisTrap Kit (Amersham Pharmacia Biotech) using nickel as binding cation for coupling the fusion protein. Bound protein was eluted using an imidazole step gradient from 40 to 300 mM. The antiserum against this human antigen used is #1109.

To obtain antiserum, rabbits were immunized with standard protocol as earlier described (2). Briefly, the suitable antigen was injected in Freund complete adjuvant (Difco Laboratories, Detroit, MI) at multiple sites in the back, and the booster immunizations were given at 4-wk intervals. The serum was collected and tested for specificity after each booster injection.

Production and characterization of the nephrin antipeptide (covering amino acids 1101 to 1126; numbered according to AF035835) antibody #409 has been earlier described in detail (2,26).

Production and characterization of the polyclonal, affinity-purified, anti-densin antibody directed to the putative extracellular part (amino acids 475 to 754; numbered according to human densin sequence AF434715) of densin has been described by Izawa et al. (27).

Immunoprecipitations

Normal rat or human glomerular lysates were prepared using the same lysis buffer as for isolation of detergent-resistant membranes (DRM) known to enrich lipid rafts (16,20). Lysate was centrifuged and supernatant precleared by Protein A Sepharose 4B (Zymed, San Francisco, CA). After overnight incubation at +4°C with anti-nephrin antiserum Protein A, Sepharose 4B was added followed by collection and washes with homogenization buffer. Finally, Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue) was added to the washed beads and the mixture was boiled for 10 min at 100°C.

Sample Preparation for MALDI-TOF-MS Analysis

Immunoprecipitated samples were separated on 8% sodium dodecyl polyacrylamide gels (SDS-PAGE) and silver-stained according to Blum et al. (28). A stained protein band with an approximate size of 200 kD was excised, cut into pieces, and further processed as described by O’Connel and Stults (29). Briefly, the stain was removed with 0.2 M NH₄HCO₃/ACN solution followed by additional ACN treatment and drying in vacuum centrifugation. Disulfide bonds were blocked using 55 mM iodoacetamide. The gel pieces were washed using 0.1 M NH₄HCO₃ and H₂O with ACN shrinking between and final drying in vacuum. Trypsin digestion was performed overnight at 37°C with 0.05 μg/μL sequencing-grade modified trypsin (Promega, Madison, WI) followed by peptide extraction from the gel by 0.1% TFA/60% ACN. After additional 0.1% TFA treatment, peptides were desalted and saturated with equal volume of β-cyan4-hydroxy cinnamic acid. This mixture was used for the mass analysis.

MALDI-TOF-MS Analysis

Fingerprinting for trypsinized peptides was performed with a BiFlex matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (2 GHZ digitizer; Bruker, Rheinstetten, Germany) at a local protein chemistry unit (Biomedicum, University of Helsinki). Positive ion reflector mode was used with an accelerating voltage of 19,000 V and delayed extraction of 2 ns. Internal peptide calibration standards (Bruker Daltons, Bremen, Germany) were applied to obtain higher peptide mass accuracy.

RT-PCR

The reverse transcription and semiquantitative RT-PCR was performed as described earlier from this laboratory (30).

PCR for densin cDNA was performed with primers (sense 2485U 5’-ATGCTTTCCCTGAACTGG-3’, antisense 2692L 5’-GTGTTGAGTGTTGGGACTG-3’) designed according to the rat densin sequence (Gen Bank U66707). The same primers were used for human and rat cDNA due to only two-base difference inside the primer regions with no changes at the 3’ end of the primers. To verify the amplification results, another human sequence (AF434715) specific primer pair (sense 3754U 5’ GAC AAG CCA TCA GAT AAC A 3’; antisense 4641L 3’ AGT TGC ACT CGA ATG ACA G 3’) were used. Amplification program included denaturation at 94°C followed by 35 cycles with 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. After the last cycle, elongation at 72°C for 10 min was allowed.
**Immunoblotting**

Western blotting and semiquantitation of the densin protein was done essentially as described earlier from this laboratory (31). Affinity-purified polyclonal rabbit anti-densin antibody directed to the putative extracellular part of densin was used at 1:1000 dilution followed by an incubation with the horseradish peroxidase–conjugated affiniPure goat anti-rabbit IgG (H+L; Jackson Immuno Research Laboratories Inc., West Grove, PA) at 1:50000. Visualization of the bound antibodies was done using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL). Broad-Range Prestained SDS-PAGE Standard (Catalog 161–0318; Bio-Rad Laboratories, Hercules, CA) was used as a molecular size marker.

**Immunofluorescence**

Normal human and CNF kidney cortical tissue samples were prepared for immunofluorescence as described earlier (32). Briefly, 5-μm frozen cortical sections were fixed in acetone and incubated with the primary antibodies diluted in 10% normal human serum. The affinity-purified tetramethylrhodamine (TRITC)–conjugated goat anti-rabbit Ig (Jackson Immuno Research Laboratories Inc.) was used as second antibody for anti-densin antibodies and FITC-conjugated AffiniPure goat anti-mouse Ig (Jackson Immuno Research Laboratories Inc.) for mouse monoclonal podocalyxin antibody PHM5 (Dakopatts, Glostrup, Denmark) (used for internal control of tissue reactivity and preservation). Immuno-Mount embedding medium (Shandon, Pittsburgh, PA) covered sections were analyzed with an Olympus OX50 microscope equipped with appropriate filter systems for TRITC and FITC (Olympus Optical GmbH, Hamburg, Germany) fluorescence. The semiquantitative analysis was done by two independent observers.

**Immunoelectron Microscopy**

Immunoelectron microscopy was done as described earlier (33). After fixation of normal cortex in 4% formaldehyde and embedding with Lowicryl K4M (Chemische Werke LOW1, Waldkraiburg, Germany), 1-micron-thick sections were cut. The sections were blocked with ovalbumin followed by incubation with affinity-purified anti-densin antibodies and the relevant 10-nm gold conjugate (1:50). Cryo EM was performed with the same antibodies.

**Results**

**Characterization of Nephrin Antibodies**

Antisera #050 and #1109 were tested for specificity to nephrin by immunoblotting and immunoprecipitation using lysates of isolated glomeruli (Figure 1, A and B). A clear 185-kD band was detected in immunoblotting studies and in immunoprecipitations followed by immunoblotting with another anti-nephrin antibody. Furthermore, preincubation with the corresponding immunizing antigen blocked the blotting reaction. Antibodies were also tested on kidney cortical sections with immunofluorescence assay revealing distinct reactivity exclusively within glomeruli in a typical manner (data not shown).

**Peptide Fingerprint and Data Bank Search**

The list of peptide masses obtained in MALDI-TOF-MS analysis was used to search the NCBI database using ProFound software (Version 4.10.5, Rockefeller University). Of the peptide masses obtained, 11 matched with rat densin covering 10% of the 1495-long amino acid sequence.

**RT-PCR**

RT-PCR analysis for densin revealed specific mRNA expression in glomeruli as well as in cultured human podocytes (Figure 2). Amplification of the cDNA prepared from rat brain tissue gave a band of the same size when the samples were run in 1.5% agarose gel. Results were confirmed by sequencing the amplified PCR products of brain and podocytes revealing 100% identity with densin. Semiquantitative RT-PCR study revealed increased densin mRNA expression in CNF kidney cortex when compared with normal human cortex (Figure 4A).

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*Figure 1.* Reactivity of the anti-nephrin antibodies #050 and #1109 shown by immunoblottings and immunoprecipitations. (A) Immunoblotting of rat glomerular lysate with (1) #050 antiserum shows distinct reactivity with a protein band of nephrin size, whereas (2) blotting after preincubation with corresponding recombinant nephrin antigen remains negative. (3) Crossreactivity with human glomerular lysate with size of 185 kD. Immunoprecipitation of rat glomerular lysate with #050 antiserum and further blotting using nephrin-specific #409 antiserum (see Materials and Methods) shows a clear band at the expected size. The same procedure with preimmune serum fails to show precipitation product. (B) (1) Human glomerular lysate immunoblotted with #1109 shows a specific band of 185 kD, whereas the (2) preimmune serum remains negative. Immunoprecipitation with #1109 using human glomerular lysate reveals nephrin precipitation when immunoblotted with #050 antiserum. Preimmune serum does not show any precipitation product.

*Figure 2.* Expression of densin mRNA in rat brain, cultured human podocytes, normal human cortex, and normal human glomerulus. RT-PCR results (marked with +) were verified by direct sequencing of the PCR products of rat brain and human podocyte. Amplification remains negative in the controls without reverse transcription (marked with −).
Immunoblotting

In immunoblotting experiments, the appropriate cell and tissue lysates were blotted with affinity purified anti-densin antibodies raised against the extracellular part of human densin (Figure 3A). These experiments revealed the typical band of 210 kD in human glomerular lysate, while a 185-kD band was obtained in the lysate of human podocytes. Also, blotting of human brain lysate revealed the same 185-kD band as was described earlier in rat brain lysate (27). Increased densin protein expression was detected in CNF glomeruli by semiquantitative immunoblotting (Figure 4B).

Co-Immunoprecipitations

After precipitation with anti-nephrin antibody #1109 of human glomerular lysate, the resulting precipitate was separated on SDS-PAGE followed by Western blotting using affinity-purified anti-densin antibody. This revealed a densin-specific band of the size of 210 kD, while no such band was observed from glomerular lysate incubated with Protein A Sepharose 4B only (Figure 3B).

Immunofluorescence and Electron Microscopy

Immunofluorescence studies of semi-thin frozen section revealed intensive glomerular staining of podocytes (Figure 5, A and B) and faintly also in proximal tubuli (Figure 5C). In CNF glomeruli, anti-densin antibodies showed reduced staining. Staining intensity with anti-podocalyxin antibodies was equal in both CNF and normal tissues (Table 1). In immunoelectron microscopy (Figure 6, A, B, and C), gold particles were seen in close association with the slit diaphragm domains.

Discussion

Here we report of a new molecular component, densin, in the kidney glomerular podocytes. This molecule was discovered in the systematic search for new nephrin-associated molecules from human glomeruli using precipitation with anti-nephrin antibody and followed by mass spectrometric identification. In addition to previously reported expression in the brain, our results here show densin mRNA and protein distinctly in the kidney glomeruli and cultured podocytes. According to the amino acid sequence, the calculated molecular weight of densin is 167 kD. However, in immunoblotting experiments the size of densin appeared to be 185 kD in the human and rat brain as well as in human podocytes. In the human glomerulus the molecular size of densin, however, was 210 kD. The differences in the molecular weights observed are most likely due to tissue-specific posttranslational modifications, because densin belongs to the O-sialoglycoprotein family abundant in sialic acids (21). Another postsynaptic density (PSD) protein, synaptopodin, is similarly shared by the podocytes and the brain with slightly differing molecular weight in these tissues due to tissue-specific glycosylation patterns (34). In immunofluorescence studies, anti-densin antibody shows strong reactivity in podocytes and faintly also in the proximal tubular brush border area. Whether the observed tubular expression reflects true in situ functional densin or is due to leakage of soluble densin into urine much like nephrin (35) followed by tubular reabsorption remains to be studied in detail.

Immunoelectron microscopic studies revealed densin, particularly at the slit diaphragm domain (Figure 6). Interestingly, our results showed increased densin mRNA and protein expression by semiquantitative RT-PCR and Western blotting in
We were able to precipitate densin with anti-
main pool of nephrin is also seen in the high-sucrose fractions
after sucrose gradient centrifugation of glomerular lysate. The
densin association in the detergent-resistant membrane
suggest of some yet unidentified shared functions. Here we did
preferentially to SD and particularly in the lipid rafts like in many
directly supported by recent findings (31). Nephrin association
functional domains has been proposed previously (37) and is
its nephrin backbone (36). This kind of compartmentalization to
cytoplasmic proteins of podocytes converge to form the SD with
overproduction and dispersed localization within the podocyte
and functional association with nephrin.

The transmembrane nature of nephrin with an extracellular and
a characteristic intracellular domain has been shown to reflect
functionality. The extracellular domain most likely mediates out-
side in signaling (20). The intracellular part of nephrin has no
marked similarity to other known proteins and, according to
recent evidence, mediates binding to the cytoskeleton via CD2AP
(12,13). Association with podocin (16) suggests that the many
cytoplasmic proteins of podocytes converge to form the SD with
its nephrin backbone (36). This kind of compartmentalization to
functional domains has been proposed previously (37) and is
directly supported by recent findings (31). Nephrin association
preferentially to SD and particularly in the lipid rafts like in many
proteins of the central nervous and immunologic systems may
suggest of some yet unidentified shared functions. Here we did
not find densin association in the detergent-resistant membrane
fractions, e.g., lipid rafts, but instead in the high-sucrose fractions
after sucrose gradient centrifugation of glomerular lysate. The
main pool of nephrin is also seen in the high-sucrose fractions
(data not shown). We were able to precipitate densin with anti-
nephrin antibodies from rat glomerular lysates as well as from
human glomerular lysates (Figure 3B). The absence of densin
from the lipid rafts raises the question whether the interaction of
nephrin and densin is due to interaction via a larger protein
complex or with the non-lipid raft associated form of nephrin. The
first proposal is supported by the fact that in the lysate most of the
membrane proteins are still insoluble and larger membrane com-
plexes remain in the supernatant allowing precipitation of proteins
without direct interactions. However, the second possibility cannot
be ruled out, as it is known that a low detergent concentration
preserves most of the protein-protein interactions.

Densin belongs to the LAP (leucine-rich repeats [LRR]
and PDZ domains) protein family (21). LAP proteins have thus
far been found in specialized epithelia and postulated to
be involved in the maintenance of cell shape and the apical-
basal polarity (22). These would be the prime candidate
functions of densin also in the podocytes. Podocytes can
similarly be divided into distinct apical and basal compart-
ments along the division plane of the slit diaphragm. Densin
may thus be one of the important molecules needed for the
maintenance of podocyte polarity. In neuronal cultures,
densin localizes mainly at the site of cellular synapses (21).
The proposed function of densin in bridging presynaptic to
postsynaptic membranes in the glutamatergic synapses (21)
also remains intriguing; the physical dimensions match
closely with the interpodocyte distance separated by the slit
diaphragm. Homotypic adhesion found in some LRR-conta-
aining proteins also resembles the proposed functions of
nephrin as an interpodocyte spacer. Typical function for the
type I PDZ domains is binding to distinct C-terminal se-
quences of other proteins. It is of particular interest that the
newly proposed additional component of the slit domain,
FAT (9), contains the typical three C terminal amino acids
(TEV) interacting with the PDZ I type domains (9,38,39).
Also podocalyxin and megalin, abundant proteins of podo-
cytes, have this sequence at their C terminus.

\(\alpha\)-actinin-4 has been shown to directly interact with densin
PDZ domain (40). The intermediary role of this molecule
appears likewise important for the cytoarchitecture of podo-
cytes (41) and could be an important mediator of appropriate

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*+++, +++*, and *++++* denote staining intensity ranging from weak to strong. Staining with secondary antibody alone was
negative (control).

Figure 6. Immunoelectron microscopy with anti-densin antibody shows specific localization at the slit diaphragm domains. (A and C, Lowicryl; B, cryoEM).
linking of densin to the actin-cytoskeleton. Interestingly, mutations in the ACTN4 gene lead to familial focal segmental glomerulosclerosis (FSGS) (6), showing its importance in maintaining podocyte stability.

One of the most widely used methods in protein interaction studies is yeast two-hybrid screening. However, this method has obvious limitations, including autoactivation for some target molecules. For nephrin intracellular domain, repeated experiments either with the full-length intracellular domain or after using only the known interaction domains of it constantly resulted in autoactivation and thus did not produce reliable results. Here the alternative to yeast two-hybrid screening, the coprecipitation method to pull down interaction partners appeared highly useful. For the subsequent molecular identification mass spectrometry was used. The data show that true in vivo protein interactions can be detected with this approach, without cDNA cloning and recombinant protein production steps.

The choice of tissue lysis conditions, including the choice of buffer system, plays a critical role in co-precipitation studies. High salt content and choice of strong detergents will release transmembrane proteins, but most in vivo interactions may be lost. On the other hand, weak buffer conditions may not reach proper solubilization and precipitation of many membrane-bound proteins. Thus, a compromise in the lysis conditions is inevitable. With the chosen conditions combined with the MALDI-TOF analysis, we could identify ten potential interactors, however, not including the thus far reported proteins interacting with nephrin, including podocin and CD2AP. In addition to densin, our conditions revealed distinctly typical adhesion proteins and protein kinases highly relevant for cell-cell interaction (Ahola et al., manuscript in preparation).

On the basis of the data presented, we propose that densin belongs integrally to the complex of nephrin-associated molecules of the SD actively involved in maintaining the podocyte cytoarchitecture and connections to the cytoskeleton.

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