Expression and Subcellular Distribution of Novel Glomerulus-Associated Proteins Dendrin, Ehd3, Sh2d4a, Plekhh2, and 2310066E14Rik

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The glomerular capillary tuft is a highly specialized microcapillary that is dedicated to function as a sophisticated molecular sieve. The glomerulus filter has a unique molecular composition, and several essential glomerular proteins are expressed in the kidney exclusively by glomerular podocytes. A catalog of >300 glomerulus-upregulated transcripts that were identified using expressed sequence tag profiling and microarray analysis was published recently. This study characterized the expression profile of five glomerulus-upregulated transcripts/proteins (ehd3, dendrin, sh2d4a, plekhh2, and 2310066E14Rik) in detail. The expression pattern of these novel glomerular transcripts in various mouse tissues was studied using reverse transcriptase–PCR, Northern blotting, and in situ hybridization. For studying the distribution of corresponding proteins, polyclonal antibodies were raised against the gene products, and Western blotting, immunofluorescence, and immunoelectron microscopic analyses were performed. Remarkably, it was discovered that all five transcripts/proteins were expressed in the kidney exclusively by glomerular cells. Ehd3 was expressed only by glomerular endothelial cells. Importantly, ehd3 is the first gene ever shown to be expressed exclusively by glomerular endothelial cells and not by other endothelial cells in the kidney. Dendrin, sh2d4a, plekhh2, and 2310066E14Rik, however, were transcribed solely by podocytes. With the use of polyclonal antibodies, dendrin, sh2d4a, and plekhh2 proteins were localized to the slit diaphragm and the foot process, whereas 2310066E14Rik protein was localized to the podocyte major processes and cell body. This study provides fresh insights into glomerular biology and uncovers new possibilities to explore the role of these novel proteins in the glomerular physiology and pathology.

calyxin, and glomerular epithelial protein 1 for the glomerular filtration barrier (14–17). Importantly, many of these essential proteins are highly specific for the glomerulus and the podocyte. Nephrin, podocin, NEP1, FAT1, synaptopodin, α-actinin-4, and glomerular epithelial protein 1, for example, are expressed in the kidney only by podocytes (7,17–21). This emphasizes the unique function and molecular composition of the podocyte foot process and the slit diaphragm.

Recently, we identified >300 glomerulus-upregulated transcripts through large-scale sequencing and microarray profiling of the glomerular transcriptome (22). In our approach, we chose five novel transcripts that were identified in the microarray analyses and characterized their expression profile in detail. We show that four of the transcripts/proteins are expressed exclusively in the kidney by podocytes. Using immunoelectron microscopy, we localize two of these proteins to the slit diaphragm and the foot process. In addition, we show that one of the proteins is expressed in the kidney only by the GEC and, remarkably, not by other endothelial cells. The identification of these novel molecular components provides fresh insights into glomerular biology.

Materials and Methods
Reverse Transcriptase–PCR and Northern Blot
The expression of glomerular-enriched transcripts in a variety of mouse tissues was studied using reverse transcriptase-PCR (RT-PCR) and Northern Blot. Gene-specific oligonucleotides for PCR analysis were designed according to the predicted cDNA sequences (http://www.ensembl.org). Primer sequences and sizes of expected PCR products were as follows: Dendrin, left 5'-AATGGAAGGCTGCTGAACCT-3', right 5'-GGGAAAGCCCTAAAGTGTC-3', 502 bp; EHD3, right 5'-CAAGACAGGGTGCCACT-3', 5'-CTAATGAACGGGAGCTGAG-3', 498 bp; shdz4a, left 5'-TCTGGGCTTGCAAGTCTCT-3', right 5'-GAACTGTCCCTGTGAG-3', 573 bp; plekh2, left 5'-CTGATTTGCCACTTTCACA-3', right 5'-CGCTGGAGTTGAGAAAGTCT-3', 353 bp; and 2310066E14Rik, left 5'-GGACAGCAGTCCACATCTTCAC-3', right 5'-TGTCGATGAGCAGACTGTCC-3', 502 bp. As a template for PCR analysis, we used cDNA libraries that were generated from various adult mouse tissues (Mouse Multiple Tissue cDNA Panel I; Clontech Laboratories, Palo Alto, CA). PCR amplification was carried out with TaqDNA polymerase (Invitrogen, Carlsbad, CA), and the amplified fragments were analyzed on 1.5% agarose gel.

For Northern blots, we used cDNA probes that were obtained from amplified cDNA libraries (see previous paragraph). The probes were 32P-labeled using the Rediprime II random primer labeling system (Amersham Pharmacia Biotech, London, UK), and the probes were hybridized to the blots that contained RNA that were isolated from various mouse organs (Mouse MTN Blot; Clontech Laboratories). Hybridizations also were performed on blots that contained mRNA that was isolated from mouse kidney fractions that contained either only glomerular tufts or the kidney excluding glomeruli. Glomerular fractions were isolated as described previously (23). The hybridizations were performed according to the standard procedures. As a positive loading control, we used glyceraldehyde-3-phosphate dehydrogenase probe (Clontech Laboratories).

In Situ Hybridization
The probes for in situ hybridizations were synthesized by subcloning of the PCR products that were obtained from RT-PCR analyses (see previous section) into the pCR II-TOPO Dual Promoter Vector (Invitrogen). Antisense and sense RNA were prepared by using T7 or SP6 polymerases. In situ hybridization experiments with 35S-labeled probes were performed on snap-frozen tissue sections that were collected from newborn mouse kidneys as described previously (24).

Production of Polyclonal Antibodies Directed against Novel Glomerular Proteins
We raised antisera directed against novel glomerular proteins by purifying recombinant proteins with affinity tags and by immunizing NZW rabbits with these antigens using standard protocols (SVA, Upsala, Sweden; KTH, Stockholm, Sweden). The generation of antigens is described briefly here.

For the production of dendrin and ehd3 antigens, we generated mouse recombinant proteins. Dendrin residues 55 to 384 were cloned into the pET-28a(+) expression vector (Novagen, Madison, WI), whereas ehd3 residues 260 to 424 were cloned into the pGEX 4T-3 vector (Amersham Biosciences). The his-tagged dendrin and the GST-tagged ehd3 recombinant proteins were solubilized from inclusion bodies in 8 M urea. Then, dendrin antigen was purified using sequential S-Sepharose ion exchange and Sephadex S-200 gel filtration columns (Amersham Biosciences), whereas his-tagged ehd3 antigen was purified using G-sepharose 4B ion exchange column (Amersham Biosciences). Finally, two NZW rabbits were immunized with each prepared antigen.

Human recombinant proteins were generated for production of shdz4a, plekh2, and 2310066E14Rik antigens. Two sequences were selected for shdz4a suitable for antigen production. Residues 27 to 150 and 327 to 443. For plekh2 and 2310066E14Rik, selected sequences comprised amino acids 223 to 357 and 845 to 961, respectively. The protein fragments were expressed as recombinant proteins with a dual tag: A hexahistidine tag that enabled purification of the expressed antigen by immobilized metal ion affinity chromatography and an albumin-binding protein fragment of Streptococcus protein G with immunopotentiating capabilities (25). Antibodies were raised by immunization of NZW rabbits, and obtained sera were used for purification of monospecific antibodies. Purification was performed using a two-step purification procedure including depletion of tag-specific antibodies and subsequent affinity purification as described previously (26).

Western Blotting
In Western blotting, we compared the extracts of glomerular tufts and kidneys that lacked glomeruli. The glomeruli were isolated from 24- to 12-wk-old adult mice and from human cadaver kidneys that were unsuitable for transplantation (from the IV Department of Surgery of Helsinki, Finland). The isolation methods have been described previously (23,27). The Western blotting was done following standard procedures using polyvinyl difluoride membrane and horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences). As a positive loading control, we used polyclonal anti-β-actin antibody (Abcam, Cambridge, UK).

Immunohistochemistry
For immunofluorescence stainings, kidney samples were collected from either 4- to 12 wk-old mouse kidneys or adult human cadaver kidneys that were unsuitable for transplantation (from the IV Department of Surgery of Helsinki, Finland). The samples were snap-frozen, and the cryosections (10 μm) were postfixed with cold acetone (−20°C) followed by blocking in 5% normal goat serum. The primary antibodies were incubated overnight at 4°C, followed by a 1-h incubation with the
secondary antibody. For double-labeling experiments, the incubations were performed sequentially.

The primary antibodies used are described above in Production of Polyclonal Antibodies Directed against Novel Glomerular Proteins. For double-labeling experiments, we used anti-mouse synaptopodin (Progen, Heidelberg, Germany), anti-mouse CD31 (Pharmingen Int., San Diego, CA), anti-mouse collagen type IV α5 (H53; gift from Dr. Sado, Shiget Medical Research Institute, Yamada, Japan), anti-human vimentin (Zymed Laboratories, San Francisco, CA), and anti-human nephrin 50A9 (28) antibodies. DAPI reagent was purchased from Molecular Probe (Eugene, OR). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Microscopy was performed with standard Leica confocal laser scanning microscope.

Immunoelectron Microscopy

For immunoelectron microscopy, the samples from mouse and human renal cortices were fixed in a solution that contained phosphate-buffered 3.5% paraformaldehyde and 0.02% glutaraldehyde. After fixation, the samples were embedded in 10% gelatin, infiltrated with 2.3 M sucrose in PBS, and frozen in liquid nitrogen. Immunolabeling experiments were done as described previously (29).

Ethical Considerations

This study was approved by the ethical committees of the Karolinska Institute and The Hospital for Children and Adolescence of the University of Helsinki.

Results

Dendrin

Dendrin is an intracellular protein without known motifs or function (30,31). RT-PCR and Northern blotting experiments demonstrated the presence of dendrin transcript in brain, kidney, and lung tissues (Figure 1). The size of the transcript (approximately 3.5 kb) is in agreement with the predicted mouse dendrin cDNA (NCBI cDNA accession no. XM_912147).

Two antisera raised against the mouse dendrin protein gave similar results in our experiments, and the specificity of the antisera was confirmed by transfection of HEK293 cells with the full-length dendrin cDNA (data not shown). In the analysis of kidney fractions, both Northern and Western blotting revealed the presence of dendrin (mRNA and protein) in glomerular tufts, whereas no mRNA or protein was detected in the kidney extract that lacked glomeruli (Figure 2). Using Western blotting, anti-dendrin antisera recognized a protein that was approximately 80 kD (Figure 2). This is in line with the published rat dendrin protein size (81 kD) (30).

The glomerulus specificity of dendrin mRNA was verified using in situ hybridization. An antisense probe showed signal exclusively in the glomeruli (Figure 3). With a higher magnification, signal could be localized to the podocytes in newborn mouse kidney (Figure 3, inset). Sense probe gave only background signal (data not shown). In immunofluorescence staining, anti-dendrin antisera gave strong immunoreactivity in glomeruli, whereas the rest of the kidney remained negative (Figure 4). In glomerular tufts, dendrin protein was observed as a linear line around glomerular capillary loops. The staining for dendrin was found on the urinary side of CD31 immunoreactivity, whereas double labeling with COL4a5 revealed nearly overlapping of the two immunoreactivities. However, staining for dendrin was localized mostly on the urinary side of COL4a5 reactivity. Double staining with synaptopodin antibody revealed almost complete overlapping of the two proteins at the light microscopic level. In immunoelectron microscopy, gold

Figure 1. Expression of novel glomerular transcripts in various mouse organs using reverse transcriptase–PCR (RT-PCR; A) and Northern blot (B) analysis. Dendrin mRNA (approximately 3.5 kb) is present abundantly in brain tissue, whereas weaker expression is observed in kidney tissue. In Northern analysis, dendrin probe hybridizes also a weak band in lung mRNA. Ehkd3 mRNA is detected in all organs analyzed using RT-PCR, whereas Northern blot reveals the presence of ehkd3 mRNA (approximately 3.6 kb) only in kidney, liver, and brain tissues. Sh2d4a mRNA is detected in kidney, muscle, lung, and heart tissues using RT-PCR, whereas in Northern blotting, sh2d4a mRNA (approximately 3.3 kb) is detectable only in kidney and heart tissues. Plekhh2 mRNA (approximately 4.5 kb) is detected only in testis tissue using RT-PCR and Northern blotting. 2310066E14Rik mRNA is detected in testis, kidney, lung, spleen, and heart tissues using RT-PCR, whereas Northern blotting shows the presence of 2310066E14Rik transcript (approximately 4.4 kb) in all organs analyzed.
**Ehd3**

Ehd3 is predicted to be a cytosolic protein (32). RT-PCR and Northern blotting analyses showed that ehd3 transcripts were present in many different mouse organs (Figure 1). Using Northern blotting, the strongest expression was observed in kidney, liver, and brain tissues. The size of ehd3 mRNA was approximately 3.6 kb, which is in line with the published transcript size (32).

In the analysis of kidney fractions, both Western and Northern blotting revealed the presence of ehd3 (mRNA and protein) exclusively in the glomerular fractions, whereas no expression was detected in the kidney extract excluding glomeruli (Figure 2). With the use of Western blotting, a protein that was approximately 68 kD in size was detected, which is in line with the predicted ehd3 protein size (Ensembl protein ENSMUSP00000024860).

**Sh2d4a**

Sh2d4a is a previously uncharacterized cytosolic protein. Expression profiling using RT-PCR and Northern blotting revealed the presence of sh2d4a transcripts in kidney, liver, and heart tissues (Figure 1). The size of the transcript (approximately 3.3 kb) is in line with the predicted transcript size (XM_134197).

Two affinity-purified polyclonal antibodies raised against sh2d4a protein gave similar results in our experiments (data not shown). In the analysis of kidney fractions, sh2d4a mRNA and protein both were detected in the glomerular fraction, whereas no signal was observed in the kidney fraction that lacked glomeruli (Figure 2). In the Western analysis, the antibodies detected a protein size of approximately 46 kD.

In immunofluorescence stainings, strong sh2d4a-specific immunoreactivity was observed in glomeruli, whereas the rest of the kidney was completely negative (Figure 4). Double labeling with nephrin revealed almost complete overlapping of the two proteins in the glomerulus at the light microscopic level (Figure 4). No
overlapping was observed in double stainings with anti-vimentin antibody or DAPI stainings. In immunoelectron microscopy, gold label for sh2d4a was found diffusely distributed in the cytoplasm of podocyte foot processes (Figure 5, C and D).

Plekhh2
Plekhh2 is a previously uncharacterized intracellular protein. Using RT-PCR and Northern blotting, we detected the presence of plekhh2 transcript only in testis and kidney tissues (Figure 1). The size of plekhh2 transcript was approximately 4.5 kb.

In the analysis of kidney fractions, both Northern and Western blotting experiments showed the presence of plekhh2 (mRNA and protein) in the glomerulus fraction (Figure 2). Two plekhh2 transcripts were detected in the glomerulus (data not shown). No plekhh2 mRNA or protein was detected in the kidney fraction that lacked glomeruli. Affinity-purified polyclonal antibody recognized a protein of approximately 120 kD.

In immunofluorescence stainings, anti-plekhh2 antibody gave glomerulus immunoreactivity, and no significant signal was observed outside glomeruli (Figure 4). With higher magnification, plekhh2 was detected mainly as a linear line around glomerular capillary loops, indicating localization to the podocyte foot processes. At the light microscopic level, plekhh2 seemed to co-localize partially with nephrin. No overlapping

Figure 3. Expression of novel glomerular transcripts in newborn mouse kidneys as revealed by radioactive in situ hybridization. Insets show capillary loop stage glomerulus in which podocytes are localized as one row (arrowheads). (A) Strong signal for dendrin mRNA is detected in the glomerular tufts of newborn mouse kidneys, whereas the rest of the kidney remains negative. With a higher magnification, the signal for dendrin mRNA can be localized to the podocytes (inset). (B) Probe for ehd3 gives strong signal in the glomerular tufts, and no signal is detected outside the glomeruli. With higher magnification, ehd3 mRNA can be localized to the vascular cleft in the developing glomerulus, which indicates localization to the glomerular endothelial cells (GEC). (C) Signal for 2310066E14Rik mRNA is detected in the glomeruli, whereas no signal is observed in the rest of the kidney. With higher magnification, the signal can be localized to the podocytes (inset). Magnifications: ×50 in A through C; ×300 in insets.
was observed in double stainings with anti-vimentin antibody or DAPI stainings (Figure 4).

**2310066E14Rik**

2310066E14Rik transcript codes for an unknown intracellular protein. Using RT-PCR and Northern blotting, we observed wide expression of 2310066E14Rik transcript in mouse tissues (Figure 1). In Northern blotting, the probe for 2310066E14Rik hybridized an mRNA of approximately 4.4 kb.

In the analysis of kidney fractions, both Northern and Western analyses revealed the presence of 2310066E14Rik (mRNA and protein) in the glomerulus fraction, whereas no mRNA or protein was observed in the kidney fraction that lacked glomeruli (Figure 2). Polyclonal antibodies raised against this novel protein detected a protein of approximately 125 kD.

The exclusive glomerulus expression was confirmed using in situ hybridization; antisense probe gave strong signal in the glomeruli, and no signal was observed outside glomeruli (Figure 3).
With a higher magnification, signal for 2310066E14Rik could be localized to the podocytes in newborn mouse kidney (Figure 3, inset). Sense probe showed only background signal (data not shown). In immunofluorescence stainings, strong immunoreactivity was observed in the glomerular tufts, and no staining was observed outside glomeruli (Figure 4). Immunoreactivity was observed on the outer aspects of the glomerular tufts in which diffuse cytoplasmic staining mainly was observed. Double labeling with nephrin antibody showed that the product of 2310066E14Rik gene was localized on the urinary side of nephrin reactivity. Double labeling with anti-vimentin antibody showed partial overlapping reactivity. No overlapping was observed with DAPI stainings, although staining for 2310066E14Rik protein often was localized in close proximity of the nuclear staining. To conclude, the staining results for 2310066E14Rik protein suggest strongly that this protein localizes to the cytoplasm of the podocyte cell bodies and major processes.

Discussion

Recently, we generated a comprehensive catalogue of glomerulus-expressed transcripts (22). Through large-scale expressed sequence tag sequencing and microarray analyses, we characterized the transcriptome of the mouse glomerulus and identified >300 glomerulus-enriched transcripts. One of the transcripts discovered, a podocyte transcription factor Foxc2, was investigated further through analysis of Foxc2 knockout mice. The glomerulogenesis in the mice was arrested before the capillary loop stage of glomerular development, indicating that Foxc2 is essential for podocyte development. This example demonstrated that our large-scale approach was a feasible method to discover genes that are critical for normal glomerular function. In this report, we chose five other glomerulus-upregulated transcripts and performed a detailed expression analysis. It is interesting that we discovered that all five transcripts and their protein products were expressed in the kidney only by glomerular cells. Therefore, our results further emphasize the importance of the large-scale transcript profiling effort from the standpoint of nephrology.

The glomerulus filtration apparatus is an extremely specialized micro-organ that is dedicated to function as a sophisticated molecular sieve. The filter is composed of a variety of unique molecules that assist in the orchestration of its specialized function. Therefore, several critical glomerular proteins, such as nephrin, podocin, NEPH1, and FAT1, have very restricted expression pattern. In the kidney, these crucial proteins are expressed exclusively by podocytes. The proteins discovered in this study share this limited expression pattern. This suggests that the novel proteins identified in our study also may have a highly specific role in the glomerulus.

Dendrin is a poorly characterized cytosolic protein with no known function or homology to other proteins or protein domains. Previously, dendrin was reported to be specific for the dendritic processes of certain neuronal cells of the forebrain (30). Particularly, dendrin showed high expression in the regions of the brain that are capable of high synaptic plasticity. Because dendrin is not found in all dendritic processes, it cannot be essential for these structures. We identified dendrin in the kidney glomerulus and localized it to the cytoplasmic face of the podocyte slit diaphragm. Both dendritic processes and foot processes are long, slender cellular extensions, and they share many structural components, such as the actin-rich cytoskeleton (33), and functional features such as plasticity. Because dendrin also is shared by both structures, it could contribute to this plasticity. In the podocyte foot process, the plasticity is required to withstand the continuous filtration pressure (34). However, the localization of dendrin at the cytoplasmic face of the slit diaphragm suggests that dendrin may act as a linker molecule between the slit diaphragm and the actin cytoskeleton. In support of this idea, dendrin was shown recently to interact in vivo with two cytoskeletal components, α-actinin and membrane-associated guanylate kinase inverted (35). Recently, dendrin also was shown to interact with the CD2AP homologue CIN85 in vitro (36). We have not detected this interaction in the glomerulus by immunoprecipitating glomerulus lysates with CD2AP and dendrin antibodies (data not shown).

Ehd3 belongs to the protein family of eps15 homology domain–containing proteins, which are thought to be associated...
with endocytic vesicles (37). With the use of Northern blotting, ehd3 mRNA was found previously in mouse brain and kidney tissues (32). This is in line with our results because we detected ehd3 mRNA in brain, kidney, and liver tissues using Northern analysis. More exciting, we discovered that ehd3 was expressed in the kidney only by GEC. To our knowledge, this is the first protein shown to be expressed only by GEC and not by other endothelial cells in the kidney. This is of critical importance because the use of the ehd3 promoter enables the generation of a GEC-specific Cre mouse line. Such a mouse line could provide an important tool for future glomerulus research.

Sh2d4a encodes a novel docking protein of SH2 signaling protein family. Members of this protein family probably play a role in intracellular signaling, but no studies have been reported to elucidate their expression or function in mammals. Plekhh2 encodes for an uncharacterized cytosolic protein with pleckstrin homology, myth4, and band 4.1 domains. Pleckstrin homology domains are implicated in phosphatidylinositol phospholipid signaling (38), the band 4.1 domain is a membrane localization domain that is capable of binding integral membrane proteins (39), and the myth4 domain has no known function. However, the combination of myth4 and band 4.1 domains is found in several unconventional myosin proteins (40), which often are localized to actin-rich processes of the cells. It is interesting that we discovered that both sh2d4a and plekhh2 proteins had a markedly restricted expression pattern in mouse tissues. In the kidney, both proteins were expressed only by podocytes, and at the subcellular level, we localized these cytosolic proteins specifically to the foot processes. Sh2d4a protein may be involved in the slit diaphragm signaling. The peculiar domain structure of Plekhh2 protein suggests, however, that it may be involved in connecting the slit diaphragm to the actin cytoskeleton.

Conclusion

We have identified five novel, highly glomerulus-specific proteins. It is interesting that these proteins share the similar restricted expression pattern with several essential podocyte proteins. Therefore, it is reasonable to speculate that several of these novel glomerular proteins have dedicated roles in glomerulus physiology. This report provides new insights into the biology of the glomerulus and creates numerous possibilities to characterize the role of these novel proteins in the glomerular physiology and pathology.

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Disclosures

K.T. is co-founder of and has stock ownership in NephroGenex Inc. C.B. has stock ownership in NephroGenex Inc.

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