Podocyte-specific expression of a novel *trans*-Golgi protein Vear in human kidney

**ANSSI M. POUSSU, ISMO VIRTANEN, HELENA AUTIO-HARMAINEN, and VELI-PEKKA LEHTO**

Department of Pathology, University of Oulu, Oulu, and Department of Anatomy, University of Helsinki, Helsinki, Finland

**Podocyte-specific expression of a novel *trans*-Golgi protein Vear in human kidney.**

**Background.** Vear is a recently identified Golgi apparatus-associated protein. It has been suggested to be involved in vesicular trafficking between the Golgi and the vacuolar/lyosomal system. Proteins similar to Vear have also been shown to interact with activated ARF proteins (ADP ribosylation factor), and they are probably involved in membrane trafficking from the *trans*-Golgi network (TGN). We have previously shown that Vear is widely distributed in human tissues, with an especially high level of mRNA in the kidney. This study further characterizes the distribution and subcellular localization of Vear in normal adult kidney and shows its association with glomerulogenesis in fetal kidney.

**Methods.** Immunofluorescence and immunoelectron microscopy were used to study the expression of Vear in fetal and adult kidney. The expression of Vear in isolated glomeruli was shown by immunoblotting. The distribution of its mRNA was analyzed by using in situ and Northern hybridization.

**Results.** In situ hybridization and immunofluorescence microscopy showed that in the kidney, Vear is present in glomerular structures. By fluorescence microscopy, the immunoreactivity for Vear was found only in podocytes, as judged by its distinct localization with podocalyxin and vimentin, well-established marker proteins of podocytes. Its specific expression in the glomeruli versus other compartments of the kidney was also verified by Western blotting. By using immunogold electron microscopy, Vear was seen in the Golgi apparatus, tubulovesicular structures, and membranes adjacent to the Golgi complex. In fetal kidney, expression of Vear coincided with the formation of segmental structures of the glomeruli. It was first seen close to the undifferentiated luminal cells at the vesicular stage and increasingly in the differentiating podocytes at the more advanced stages of glomerulogenesis.

**Conclusions.** In the kidney, Vear shows a distinct, specific, and developmentally regulated expression in glomerular podocytes. This suggests that Vear has a specific function in podocytes. It could be associated with the known high secretory and synthetic activity of the podocytes, especially the production of the basement membrane components, which are critically involved in the glomerulogenesis and the maintenance of the glomerular function.

Vesicular transport between the Golgi complex and the different membrane compartments of the cell is a multifactorial event regulated by many different systems. One of the principal regulatory stages is the formation of the intermediate vesicles at the budding site in the Golgi and the selection of the cargo. This is partially mediated by the cytosolic proteins called “coat proteins.” Five types of coat complexes have been characterized so far: clathrin and its adaptors (AP-1 and AP-2), the adaptor-related AP-3 complex, the adaptor-related AP-4 complex, COPI, and COPII [1].

Vear (VHS- and “ear”-domain containing protein) is a recently discovered Golgi complex-associated protein [2]. It displays structural similarities with other vesicular transport-associated proteins and is closely associated with the Golgi complex [2]. As an indication that Vear could have cell-type–specific functions, we have recently shown that in skeletal muscle, Vear is expressed at a high level in type I fibers but not in type II fibers [3]. Recently, proteins similar or identical to Vear have been shown to associate with ARF (ADP ribosylation factor) and to be involved in ARF-regulated vesicle transport from the *trans*-Golgi network (TGN) [4–6]. Recent results also suggest that Vear and Vear-like proteins also can be present in membranous structures/locations other than Golgi [4–6].

Our previous study used Northern hybridization to show that Vear mRNA is prominently expressed in human kidney [2]. However, the cell-type–specific and subcellular distribution of Vear in the kidney is not known. In our current study, we examined the expression of Vear in normal human fetal and adult kidney by using in situ hybridization (ISH), Northern hybridization, fluorescence, and immunoelectron microscopy (IEM) and Western blotting. We found that Vear shows a highly cell-specific expression in the podocytes in the glomeruli and that its occurrence is developmentally regulated.
The results suggest that Vear could be involved in some podocyte-specific synthetic and secretory functions, such as augmentation and maintenance of the structurally unique basement membranes of the filtration unit.

METHODS

Antibodies

Affinity-purified rabbit polyclonal antibodies to Vear (anti-Vear) [2] and anti-vimentin antibody (mouse monoclonal) are characterized elsewhere [7]. Mouse monoclonal anti-podocalyxin antibody against a human antigen was kindly provided by Dr. Dontscho Kerjaschki (University of Vienna, Vienna, Austria). For IEM, colloidal gold (10 nm)–protein A complexes were prepared as described by Slot and Geuze [8].

Tissues

Fetal kidneys (N = 2; 14 gestational weeks) were obtained from autopsies carried out at the Department of Pathology, Oulu University Hospital, Oulu, Finland. Specimens representing normal adult kidney cortex and medulla were obtained from kidneys (N = 3) that were surgically removed (at the Department of Surgery, Oulu University Hospital) because of a renal carcinoma.

Immunofluorescence stainings and fluorescence microscopy

For immunofluorescence microscopy, the following protocol was used. Five-micrometer thick cryostat sections were cut and fixed in acetone at −20°C for 10 minutes. After washing in phosphate-buffered saline (PBS), the slides were incubated in fetal bovine serum (FBS; 20% for 30 min) and then overlaid with anti-Vear, diluted in PBS, for two hours at room temperature. For double-staining experiments, the sections were overlaid with a solution containing either mouse monoclonal antibody to vimentin or to podocalyxin along with anti-Vear. After washing, the slides were incubated with the Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) and Oregon Green-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for another 60 minutes. As controls, incubations with the primary antibodies only, with secondary antibodies only, or by using anti-Vear preabsorbed with the bacterially produced Vear-protein were used. For conventional immunofluorescence microscopy, the slides were viewed under Olympus BH2 fluorescence microscope equipped with appropriate filters.

Immunoelectron microscopy

Immunoelectron microscopy on ultrathin sections of normal human kidney was performed as follows. Sections made of the freshly obtained tissue were fixed in 4% paraformaldehyde, 2.3 mol/L sucrose for 24 hours, immersed in 2.3 mol/L sucrose, and frozen in liquid nitrogen. Cryosections were cut with Leica Ultracut UCT microtome. In order to block nonspecific binding of antibodies, the sections were then incubated in 5% bovine serum albumin (BSA; Aurion, Wageningen, The Netherlands) with 0.1% gelatin in PBS for 10 minutes. Thereafter, the sections were overlaid with anti-Vear diluted in 0.1% BSA. After washing in 0.1% BSA in PBS, protein A-gold complex (size 10 nm) was added for 30 minutes. The sections were washed, embedded in methylene cellulose, and examined in a Philips 410 LS transmission electron microscope.

In situ hybridization

The cDNA corresponding to the first 192 NH2-terminal amino acids of Vear [2] was cloned into the BamHI/HindIII cloning site of the pGEM-4Z-vector (Promega, Madison, WI, USA). The construct was then linearized by BamHI or HindIII, and the sense and anti-sense cRNA probes were generated by using a Riboprobe Transcription Kit (Promega) and α[35S]-UTP (Amersham Corporation, Arlington Heights, IL, USA). The protocol used for ISH was as described by Autio-Haromain et al, with minor modifications [9, 10]. Five micrometer cryosections fixed in 4% paraformaldehyde in PBS were incubated in 0.2 mol/L HCl for 20 minutes and then washed in diethylpyrocarbonate (DEPC)-treated water for five minutes. For proteolysis and unmasking the RNA, the sections were incubated with proteinase K (10 μg/mL; Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C, and the reaction was stopped with 0.2% glycine in PBS. The sections were then washed twice in PBS for 30 seconds, followed by postfixation with 4% paraformaldehyde in PBS for 20 minutes and washing in PBS. Acetylation was done in 0.25 to 0.5% acetic anhydride in 0.1 mol/L triethanolamine for 10 minutes. The sections were then washed in PBS, dehydrated, and air dried for one to two hours at room temperature. This was followed by incubation in the prehybridization solution [10 mmol/L dithiothreitol (DTT), 10 mmol/L Tris-HCl, 10 mmol/L NaPO4, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.3 mol/L NaCl, 1 mg/mL yeast tRNA, 50% deionized formamide, 10% (wt/vol) dextran sulphate, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone, 0.02 mg/mL BSA] for two hours, washing in PBS, and dehydration. For the hybridization, the probes were denaturated by boiling for one minute and were then placed on ice. The 35S-labeled antisense or sense probe (3 × 106 cpm) in 40 μL of the prehybridization solution was applied on each section, and the hybridization was carried out overnight at 50°C. Posthybridization washes were done as follows: first, twice in a solution similar to the prehybridization solution (except that dextran sulphate and tRNA were omitted) for one hour at 50°C; second, in 0.5 mol/L NaCl in TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA) for 15 minutes at 37°C; third, in 0.5 mol/L NaCl in TE containing...
40 μg/mL RNAase A (Sigma, St. Louis, MO, USA) for 30 minutes at 37°C; fourth, in 0.5 mol/L NaCl in TE for 15 minutes at 37°C; fifth, in 2 × standard saline citrate (SSC) twice for 15 minutes at 50°C; and sixth, in 1 × SSC twice for 15 minutes at 50°C. The sections were then dehydrated in ethanol containing 300 mmol/L ammonium acetate and air dried for one hour at room temperature. For autoradiography, the slides were dipped in NTB-2 film emulsion (Kodak, New York, NY, USA) and were then placed in light-tight boxes for 7 to 14 days. The slides were developed in D-19 developer (Konica, Japan) and were then counterstained with hematoxylin and eosin (HE). Consecutive sections of same tissue blocks were used for experimental and control hybridization.

**Northern hybridization**

Isolation of total RNA from the freshly obtained specimens representing renal cortex and medulla was performed using the SV Total RNA Isolation System (Promega). The cDNA corresponding to the nucleotides 501 to 950 of Vear [2] was labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) and used as a probe. Fifteen micrograms of the total RNA per each sample was separated by formaldehyde-agarose gel electrophoresis and transferred to a Hybond-N nylon filter (Amersham). Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA, USA) as recommended by the manufacturer. The blot was stripped and reprobed with the control β-actin cDNA (Clonetech).

**Isolation of the glomeruli**

Glomeruli were isolated from the adult kidney tissue essentially according to the method of Striker and Striker [11]. Briefly, the cortical tissue was first finely minced with the razor blade. It was then forced, with a spatulum, through steel sieves of a pore size of 250, 150, and 100 μm. The purified glomeruli were collected by rinsing them from the surface of the third sieve of the 100 μm pore size. The preparation obtained was examined under light microscopy for purity. Usually, nearly 100% pure glomeruli were obtained. The homogenate passing the 100 μm pores was found to represent cortical nonglomerular structures. It was collected and used for immunoblotting and considered to represent a cortical fraction devoid of glomeruli.

**Western blotting**

Western blotting was carried out as described earlier [12]. Briefly, the samples representing both renal cortex and medulla were obtained from surgical nephrectomy samples. For Western blotting, equal amounts of tissue lysates and lysate of COS-7 cells overexpressing Vear [2] were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard protocol as described by Sambrook, Fritsch, and Maniatis [13]. For Western blotting, the electrophoretically separated polypeptides were transferred from the polyacrylamide gel to nitrocellulose filters. The filters were blocked with 3% milk in Tris-buffered saline (TBS). They were then incubated with anti-Vear in TBS for two hours at room temperature and washed several times with TBS. The blots were overlaid with the anti-rabbit IgG secondary antibodies, washed, and developed by the ECL method (Amersham) as recommended by the manufacturer.

**RESULTS**

**Immunofluorescence stainings and fluorescence microscopy**

In the adult kidney, a distinct reactivity with anti-Vear was seen only in the glomerular structures (Fig. 1 A, B). No or only a very weak and restricted staining was seen outside the glomeruli in some tubules and in the interstitial compartment. At a closer examination, the positivity in glomeruli was located within the cell body and the foot processes (pedicles) of the podocytes. For more definitive localization of the immunoreactivity, double-staining experiments by using antibodies to podocalyxin (Fig. 1D) and vimentin (Fig. 1F), both widely used markers for podocytes [7, 14–17], were carried out. A close colocalization was seen between Vear and podocalyxin (Fig. 1 C, D, respectively) and also between Vear and vimentin (Fig. 1 E, F, respectively) in the glomeruli. No Vear-positive staining was seen in the endothelial or mesangial cells of the glomeruli, and also, the cells of the Bowman’s capsule remained negative. The lack of Vear in the mesangial cells was also verified by carrying out a double-staining experiment involving antibodies to a mesangial cell marker α-smooth muscle actin (α-SMA) [18]. No anti-Vear reactivity was seen in the SMA-positive mesangial cells (data not shown). From these observations, we conclude that Vear is expressed exclusively in the podocytes in the glomeruli.

The expression of Vear in the fetal kidney (14 gestational weeks) was also studied. In the cryosections, glomeruli representing various developmental stages extending from the vesicle stage to the mature juxtamedullary glomeruli could be discerned by conventional light microscopy after staining with HE. In immunofluorescence microscopy of the same sections, no or only a faint staining was seen in the luminal cells of the vesicle phase, which represent the earliest stages of glomerulogenesis (Fig. 2 A, B). At the S-shaped body stage, a clear immunoreactivity for anti-Vear was found in a row of tall cuboidal cells representing early podocytes (Fig. 2C, white arrow, Fig. 2D). At this stage, there was some anti-Vear reactivity also in the cells of the evolving proximal tubular structures (Fig. 2C, white arrowhead). No staining of...
the tubuli was seen at the later stages of the tubular development. At the capillary loop stage, a strong immunoreactivity for Vear was detected in closely packed podocytes that appeared cubic, cylindrical, or flattened (Fig. 2 E, F). The staining was especially distinct in the cellular compartments closest to the capillary basement membranes (Fig. 2E). With the advancement of the glomerular maturation, a strong anti-Vear-reactivity was found in those podocytes that now started to display well-developed cytoplasmic processes (Fig. 2G).

The expression of Vear in the fetal kidney was also compared with that of podocalyxin. From double-immunofluorescence stainings, it could be concluded that Vear is expressed earlier than podocalyxin during glomerulogenesis; a faint but clear Vear-positivity was already present at the vesicular stage (Fig. 2A), while reactivity for anti-podocalyxin started to be faintly present at the S-shaped body stage. Clear expression of podocalyxin was seen at the capillary loop stage (data not shown).

In control experiments employing the secondary antibodies alone or the preabsorbed anti-Vear as primary antibody, no staining was seen.

**Immunoelectron microscopy**

The subcellular localization of Vear was studied in greater detail by using IEM on ultrathin cryosections of the normal adult kidney. In concordance with the immunofluorescence microscopy, labeling for Vear was only seen in the podocytes, while other glomerular cell types remained negative. The gold particles were seen to decorate both the pedicles of the podocytes (Fig. 3A) and the Golgi membranes and as yet uncharacterized tubulovesicular structures adjacent to the Golgi complex (Fig. 3B). No labeling was seen when PBS was substituted for anti-Vear (data not shown).

**Western blotting analysis**

Western blotting analysis with anti-Vear of the solubilized fractions corresponding to the glomeruli, medullary region of the kidney, and cortical structures devoid of glomeruli is seen in Figure 4. The lysate prepared from COS-7 cells expressing exogenously introduced full-length cDNA of Vear was used as a positive control. A distinct band of 75 kD corresponding to Vear was seen in protein lysate prepared from the isolated glomeruli.
Fig. 2. Vear in fetal kidney. Immunofluorescence localization of Vear in developing glomeruli: (A) vesicle stage, (C) S-shaped body, (E and F) capillary loop stage, and (G) mature juxtamedullary glomeruli. (B) and (D) are the HE-stained images of the same sections as in (A) and (C). Vear-positive cells representing early podocytes are marked with white arrows (C and D). The cells of the evolving proximal tubular structures are marked with white arrowheads (C and D). Magnifications ×200.

(lane 1), while no detectable bands were present in the lysates prepared from the renal medulla (lane 2) or the cortical fraction devoid of glomeruli (lane 3). No bands were detected when a preimmune serum was substituted for anti-Vear (data not shown).

In situ hybridization

We also used ISH to determine the site of synthesis of Vear in the kidney. Figure 5A shows a bright field image (magnification ×100) and Figure 5B a dark field image (magnification ×100) of an adult kidney probed for the presence of Vear mRNA. A distinct accumulation of grains was seen over the glomeruli (Fig. 5A, B, white arrows). At a higher magnification, the labeling is seen in a pattern compatible to that of podocytes (Fig. 5C, black arrows), while no specific labeling above the background level (seen in the section hybridized with the sense probe; Fig. 5D) was seen in any other cellular component of the glomeruli or in the extraglomerular structures. No hybridization over the background level was seen in the glomeruli in the negative controls (sense probe), attesting to the specificity of the reaction (Fig. 5D).
Northern hybridization

Northern analysis of human kidney was performed using total RNAs extracted from renal cortex and medulla. In the cortical tissue, messages of 3.5 and 3.9 kb were present. There was also a faint band corresponding to the size of 5.2 kb (Fig. 6, upper panel, lane 2). These messages correspond in size to those seen in various combinations also in other tissues [2]. No bands were detected in the total RNA extracted from the renal medulla (Fig. 6, upper panel, lane 1), attesting to the distinctly compartmentalized presence of the Vear mRNA in the renal cortex. Hybridization for β-actin showed that roughly similar amounts of RNA were loaded in the lanes 1 and 2 (Fig. 6, lower panel).

DISCUSSION

This work studied the localization of Vear, a novel trans-Golgi protein [2, 4–6], in normal adult kidney by using immunofluorescence stainings in fluorescence microscopy, IEM, Western blotting, Northern hybridization, and ISH. Additionally, the expression of Vear at
Fig. 4. Western blotting analysis of Vear in isolated glomeruli. Western blotting of the lysates of isolated glomeruli (lane 1), renal medulla (lane 2), renal cortical fraction devoid of glomeruli (lane 3), and COS-7 cells transfected with cDNA for Vear (lane 4). Immunoblotting was carried out by using anti-Vear, secondary antibodies, and an ECL-detection system. The sizes of the molecular weight markers are indicated on the left-hand side.

Fig. 5. In situ hybridization. Bright field image (A) and dark field image (B) of adult kidney hybridized with the antisense probe to Vear are shown. In (C), a higher magnification of a heavily labeled region of the glomeruli is shown. (D) Bright field image of adult kidney hybridized with the sense probe for Vear. Magnification ×100 (A and B) and ×250 (C and D).

Fig. 6. Northern hybridization. Total RNAs isolated from renal medulla (lane 1) and cortex (lane 2) are shown, and hybridization with a probe for Vear (upper panel) and a probe for β-actin (lower panel) is shown.
different stages of glomerulogenesis in fetal kidney was analyzed by using immunofluorescence microscopy. This study was motivated by our earlier results based on Northern hybridization, which showed a high expression level of Vear in the kidney [2].

The results show that in the kidney, Vear is distinctly and, at least at a level of immunofluorescence microscopy, exclusively located to podocytes. This was inferred from a typical staining pattern and from the codistribution with the well-known podocyte markers vimentin and podocalyxin [7, 14–17]. Moreover, Vear could be unequivocally localized to podocytes by using IEM in which podocytes are easily recognizable by their typical ultrastructural features. Its distinctly compartmentalized expression in the glomeruli and in the renal cortex related to the renal medulla was also demonstrated by in situ and Northern hybridization. Vear was also seen in developing fetal glomeruli in which its occurrence correlated with the maturation of the podocytes and their cytoplasmic processes. At an ultrastructural level, Vear was seen in the foot processes and was also associated with the Golgi apparatus and vesicular structures adjacent to the Golgi complex. As a conclusion, in both adult and fetal kidney, Vear is distinctly and specifically expressed in glomerular podocytes.

Besides podocytes, the visceral epithelial cells of glomeruli, there are three other resident cell types in the glomeruli: endothelial cells, mesangial cells, and parietal epithelial cells [19]. No apparent Vear positivity was seen in any of these other cell types. The lack of staining in mesangial cells was also verified by using antibodies to the mesangial cell marker α-SMA [18] in double-labeling setting.

Podocytes are highly specialized epithelial cells in which three architecturally and functionally different segments can be discerned: the cell body, the major processes, and the foot processes [19]. Podocytes cover the exterior surface of the basement membranes, which abut the capillary tuft and are interconnected by the slit diaphragms [19, 20]. They have been proposed to be involved in many glomerular functions, including the turnover of the glomerular basement membrane, maintenance of the filtration barrier, support of the capillary tuft, regulation of the glomerular filtration, and some immunological functions [19, 20]. Podocytes are also characterized by a high endocytic capacity and a high level of synthesis and post-translational modification of proteins. The structural attribute of these functions is the presence of a well-developed endoplasmic reticulum and a massive Golgi complex in podocytes, along with a large number of endocytic vesicles, multivesicular bodies, and lysosomes [19]. Recently, Simons et al showed that a constant transport of the vesicles to the specific docking sites and an intact Golgi apparatus is required for a normal formation of the pedicles in podocytes [21]. This demonstrates the pivotal role of the post-Golgi transport vesicles in these cells. Thus, the abundant expression of Vear in podocytes conforms well with the current view of podocyte as an actively synthesizing and secreting cell type.

One way to explain the podocyte-specific expression of Vear in kidney is to attribute it to the presence of a high number of prominent Golgi structures in podocytes. However, some other cells, most notably the mesangial cells and the tubular epithelial cells, also possess well-developed Golgi complexes [22, 23], but appear Vear-negative. Thus, the distinctive expression of Vear only in podocytes is most probably due to a genuine cell-type–specific expression rather than a reflection of a size of the organelle. In this regard, the expression of Vear during glomerulogenesis is intriguing. An increase in the intensity in the staining for Vear was seen in podocytes as they progressed to form the well-developed segmental structures typical of mature glomeruli. This coincides with the appearance of laminin α5 chain and disappearance of laminin α1 chain in the glomerular basement membrane, which underlies and is synthesized partly by the podocytes [24]. This raises the possibility that the high level of Vear expression may be involved in the transport and secretion of the podocyte-specific basement membrane components and that it could play an important regulatory role in early glomerulogenesis.

ACKNOWLEDGMENTS

This work was supported by the Finnish Academy, the Emil Aaltonen Foundation, the Cancer Society of Northern Finland, the Finnish Cultural Foundation, the Finnish Cancer Society, and the Oulu University Hospital. The authors thank Ms. Annikki Huhtela, Ms. Anna-Liisa Oikarainen, Ms. Mirja Vahera, Ms. Marjaana Vuoristo, Mr. Hannu, Wäänäinen, and Mr. Tapio Leinonen for skillful technical assistance, and Dr. Raija Sormunen for valuable advice.

Reprints requests to Veli-Pekka Lehto, M.D., Ph.D., Department of Pathology, P.O. Box 5000 (Aapistie 5), FIN-90014 University of Oulu, Oulu, Finland.
E-mail: lehto@csc.fi

REFERENCES

ate filaments and synaptophysin show neuronal properties and lack of glial characteristics in Y79 retinoblastoma cells. Lab Invest 59:649–656, 1988