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Presence of fructose transporter GLUT5 in the S3 proximal tubules in the rat kidney

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Background. Fructose is a nutrient as well as a potent agent for the formation of advanced glycation end product in diabetes. GLUT5 is a facilitated-diffusion fructose transporter expressed in the small intestine and kidney. Previous reports on the localization of GLUT5 by *in situ* hybridization and immunohistochemistry were controversial.

Methods. The expression of GLUT5 was checked by reverse transcription-polymerase chain reaction and Southern blotting and immunoblotting analyses. Localization of GLUT5 was visualized by high-resolution immunofluorescence and immunogold electron microscopy.

Results. We were able to confirm the expression of GLUT5 in the kidney. GLUT5 was predominantly present in the outer stripe of the outer medulla, where it was localized in the S3 proximal tubule cells. Double labeling with phalloidin showed that GLUT5 was localized in the brush border of the S3 proximal tubule cells. Ultrastructural examination revealed that GLUT5 was present along the plasma membrane of the apical microvilli.

Conclusion. GLUT5 is present at the apical plasma membrane of S3 proximal tubule cells and may serve as the transporter of fructose.

Fructose is a monosaccharide commonly found in food and its intestinal hydrolysis products. Hyperglycemia in diabetes mellitus leads to the activation of the polyol pathway, which results in the accumulation of sorbitol and fructose. Fructose was shown to be one of potent agents for the formation of advanced glycation end product, the nonenzymatic modification product of proteins by sugars [1, 2]. Glycation is considered to be one of major factors for the complications associated with diabetes mellitus.

Key words: transport, glucose, GLUT5, renal proximal tubule, advanced glycation end product, diabetes, hyperglycemia.

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Glucose transporters are membrane proteins that transport sugars such as glucose, galactose, and fructose. Two types of glucose transporters, Na^+-dependent active glucose transporter (SGLT family) and facilitated-diffusion glucose transporter (GLUT family) are known to exist in mammalian cells [3–7]. A variety of glucose transporters of the SGLT family (SGLT1, SGLT2, and pSGLT2/SGLT3) [8] and GLUT family (GLUT1, 2, 4, 5) [9, 10] have been reported to be expressed in the kidney. Immunohistochemical and *in situ* hybridization studies have revealed family type-specific and isoform-specific expression in specific cells in the kidney [8, 9, 11, 12]. These observations suggest that glucose transporters play diverse roles such as absorption of sugars from the glomerular filtrate and supplying glucose to the glycolytic cells in the kidney.

GLUT5 is an isoform of the facilitated-diffusion glucose transporter family [13], which transports fructose and is expressed in the small intestine, kidney, and testis in the human [14]. In the rat, GLUT5 is mainly expressed in the small intestine and kidney but not in the testis [15, 16]. In the small intestine, GLUT5 is present in the absorptive epithelial cells, where it is localized along the apical brush borders [17–19]. It is proposed that GLUT5 plays an important role in the uptake of fructose from the intestinal lumen. Transepithelial transfer of the dietary fructose seems to be carried out by the combined action of apical entry of fructose via GLUT5 and subsequent basolateral exit via GLUT2 [20, 21].

In the kidney, *in situ* hybridization studies show that GLUT5 is expressed in the outer stripe of the outer medulla, especially in the proximal straight tubules [9, 10]. The results of immunohistochemical studies, however, are controversial. Asada et al reported that GLUT5 was localized not in the medulla but in the cortex, that is, at the brush border of the proximal convoluted tubules in the cortex and at the glomerular mesangial cells in the normal rat kidney [22]. In our current study, we show that GLUT5 is localized in the S3 segment of the

proximal tubules by immunofluorescence and laser confocal microscopy. Immunogold electron microscopy reveals that GLUT5 resides in the microvilli of these cells.

METHODS

Animals and tissue preparations

Male four-week-old Wistar rats were obtained from the Animal Breeding Facility, Gunma University (Gunma, Japan). The animals were killed under anesthesia with sodium pentobarbital, and kidney specimens were removed and cut into pieces.

RNA extraction and reverse transcription-polymerase chain reaction

Kidney slices were cut into two parts, cortex plus outer medulla and inner medulla, and were homogenized with TRIzol Reagent (GIBCO BRL, Gaithersburg, MD, USA) [23]. RNA was prepared in a similar fashion from the rat jejunum. After homogenization, RNA was separated by chloroform extraction. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the GeneAmp RNA PCR Core Kit (Perkin-Elmer, Foster City, CA, USA) according to the manufacturer's instruction. PCR specific primers for GLUT5 were purchased from GIBCO BRL. The sense and antisense primers corresponded to bases 57 to 80 (5′-AACTTTCC TAGCTGCCTTTGGCTC-3′) and 1495 to 1472 (5′-TAG CAGGTGGGAGGTCATTAAGCT-3′), respectively. In addition, primers were designed outside of the open reading frame to amplify the full length of the rat GLUT5. For PCR, the reaction tubes were incubated in the Program Temp Control System PC-800 (Astec, Fukuoka, Japan) as follows: initial denaturation at 94°C for five minutes, 30 cycles of denaturation at 94°C for one minute, annealing at 57.5 or 65.0°C for two minutes, extension at 72°C for three minutes, and enhancement at 72°C for five minutes. The PCR products (10 μl) were separated by 1% agarose gel electrophoresis. After ethidium bromide staining, DNA fragments were visualized with an ultraviolet transilluminator. Southern blot analysis was performed using ECL random labeling kit (Amersham Pharmacia Biotech, Uppsala, Sweden). DNA fragments were transferred to the nylon filter (Biodyne B; Pall, Port Washington, NY, USA) and hybridized with a DNA probe including the full length of rat GLUT5 cDNA [24].

Antibodies

Anti-GLUT5 antibody raised in rabbit against the amino acids 490 to 502 of rat GLUT5 was used as previously described [24, 25].

Immunoblotting

Immunoblotting was carried out as previously described [26]. In short, tissue specimens of the rat kidney were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors [50 mM ethylenediaminetetraacetic acid (EDTA), 3 μM pepstatin A, 4 μM leupeptin, 120,000 kIU/ml aprotinin, 2 mM phenylmethanesulfonyl fluoride]. Protein content was measured by the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane filters. The blotted membranes were sequentially incubated with 3% bovine serum albumin (BSA) in rinse buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), rabbit anti-GLUT5 antibodies diluted at 1:5000, and [^{125}I]-protein G (3.7 kBq/ml; New England Nuclear, Wilmington, DE, USA). After washing with 1 M NaCl in rinse buffer, autoradiography was performed overnight with imaging plates and was processed with a BAS2000 bioimage analyzer (Fuji Film, Tokyo, Japan).

Glycosidase treatment

Glycosidase digestion was carried out basically as described earlier [27]. Aliquots of each homogenate (20 μg) were incubated with 0.3 unit of N-glycosidase F (Boehringer-Mannheim, Mannheim, Germany) in a solution containing 20 mM sodium phosphate buffer, pH 7.4, 10 mM EDTA, 0.5% Triton X-100, and 0.5% mercaptoethanol at 37°C overnight. The mixtures were subjected to SDS-PAGE and immunoblotting.

Immunofluorescence and laser confocal microscopy

Fresh specimens were rapidly embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Cryostat sections (6 μm thickness) were cut, immersed in ethanol for 30 minutes at −20°C, followed by fixation in 3% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB) for 30 minutes on ice. Then sections were covered with 5% normal goat serum in PBS for 10 minutes and incubated for one hour with rabbit anti-GLUT5 antibodies diluted at 1:500. After washing with PBS, the sections were incubated with lissamine rhodamine sulfonyl chloride (LRSC)-labeled donkey antirabbit IgG (Jackson Immunoresearch, West Glove, PA, USA) for one hour. They were washed with PBS and incubated with a mixture of 2 μM 4′,6-diamidino-2-phenylindole (DAPI; Boehringer-Mannheim) and fluorescein-phalloidin (Molecular Probes, Eugene, OR, USA) for nuclear DNA and F-actin staining, respectively [28]. For laser confocal microscopy, 2 μM TO-PRO-3 iodide (Molecular Probes) was used for nuclear DNA staining instead of DAPI [29]. Specimens were washed with PBS and mounted with antibleaching mounting medium [30]. Conventional fluorescence images were observed with an AX-70 microscope equipped with epifluorescence and Nomarski differential interference-contrast optics (Olympus, Tokyo, Japan). Microscopic images were recorded with a cooled-CCD camera (PXL 1400; Photometrics, Tucson, AZ, USA) and analyzed with IPLab Spectrum software (Signal Analytics,

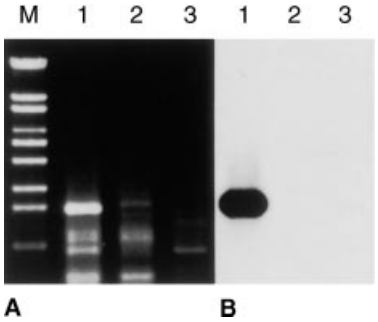

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR; *A*) and Southern blot analysis (*B*) of GLUT5 in the rat kidney. Kidney slices were divided into two parts, cortex plus outer medulla and inner medulla. RT-PCR was carried out using sense and antisense primers corresponding to bases 57 to 80 and 1495 to 1472, respectively. Bands of amplified products are recognized at the location corresponding to the 1440 base pairs of GLUT5 in the cortex plus outer medulla (lanes numbered 1) but are not in the inner medulla (lanes 3). No products are seen without reverse transcriptase (lanes 2). M:λ-DNA StyI digests.

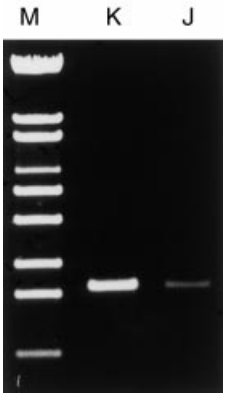

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) of GLUT5 in the rat kidney and jejunum. The kidney sample was prepared from the cortex plus outer medulla (K) and the jejunum sample from the whole jejunum (J). RT-PCR was performed using primers outside the open reading frame of GLUT5 cDNA. Bands corresponding to the 1578 base pairs for the full length of GLUT5 are seen in the kidney and the jejunum. M:λ-DNA StyI digests.

Vienna, VA, USA). Laser confocal microscopic observation was carried out with a BX-50 microscope (Olympus) equipped with a laser confocal system (MRC-1024; Bio-Rad Laboratories, Hercules, CA, USA). Images were captured and processed with Bio-Rad Laser Sharp software. As immunohistochemical controls, sections were incubated with normal IgG or the primary antibody in the presence of the peptide (50 μg/ml) used to generate the antibody.

Electron microscopic immunohistochemistry

Fresh specimens were rapidly embedded in O.C.T. compound and frozen in liquid nitrogen. Cryostat sections (10 μm thickness) were cut, immersed in ethanol for 30 minutes at −20°C, and fixed in 3% paraformaldehyde in PB for 30 minutes on ice. These sections were first covered with 5% normal goat serum in PBS for one hour and then incubated with rabbit anti-GLUT5 antibodies diluted at 1:500 for one hour at room temperature. After a rinse with PBS, the sections were incubated with 1.4 nm gold particles conjugated to Fab′ fragments of antirabbit IgG (Nanogold; Nanoprobes, Stony Brook, NY, USA), diluted at 1:50 for 1.5 hours at room temperature. After washing with PBS, the sections were refixed with 1% glutaraldehyde in PB for 30 minutes. They were subjected to silver enhancement and gold toning as previously described [31]. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Reverse transcription-polymerase chain reaction and Southern blot analysis

Reverse transcription-polymerase chain reaction (RT-PCR) and subsequent Southern blot analysis detected a band of amplified product corresponding to the 1440 bp fragment from GLUT5 in the cortex plus outer medulla (Fig. 1), whereas it was not detected in the inner medulla. These results show that GLUT5 is expressed in the cortex and/or the outer medulla, but not in the inner medulla. In addition, the RT-PCR using the primers designed to amplify the full length of the rat GLUT5 demonstrated an expected GLUT5 band of 1578 bp in the kidney. Exactly the same band was detected in the jejunum (Fig. 2).

Immunoblotting

Immunoblotting was carried out with the homogenate of the kidney and the small intestine. A 58 kDa protein was detected in the jejunum using rabbit anti-GLUT5 antibodies. In the kidney, a 51 kDa protein was detected (Fig. 3), showing that GLUT5 protein is present in the kidney. To examine whether or not glycosylation of GLUT5 contributes to the difference in the apparent relative molecular mass (M_r), glycosidase treatment was performed (Fig. 3). After N-glycosidase F treatment, the apparent M_r of the GLUT5 in both the kidney and jejunum was reduced to 40 kDa.

Immunofluorescence localization of GLUT5

Positive staining for GLUT5 was found mainly in the outer stripe of the outer medulla (Fig. 4a), but was hardly seen in the cortex. The double-immunofluorescence staining with GLUT5 and F-actin (Fig. 4 a–c) revealed that brush borders of the S3 segments of proximal tubules were highly positive for GLUT5, and the S1 and S2 segments were at a very low level if any. The signal was negative in the inner stripe of outer medulla (Fig. 4 a–c) and inner medulla (data not shown).

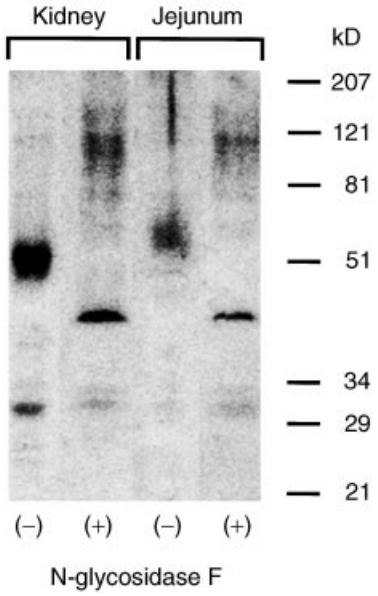

Fig. 3. Immunoblotting of the kidney and jejunum with antibody to GLUT5. Twenty micrograms of protein were applied to 10% sodium dodecyl sulfate-polyacrylamide gel and were electrophoresed and subjected to immunoblotting with rabbit anti-GLUT5 antibody. A 51 kDa protein is detected in the kidney, whereas a 58 kDa protein is detected in the jejunum. After N-glycosidase F treatment, the apparent M_r of GLUT5 in the kidney and jejunum changes to 40 kDa.

To see the localization of GLUT5 in detail, kidney specimens were examined with a laser confocal microscope. The double immunofluorescence staining with GLUT5 and F-actin clearly showed that brush borders of the proximal tubule cells of the S3 segment were positive for GLUT5 (Fig. 4 d, e). The basolateral membrane and the cytoplasm of the proximal tubules were negative for GLUT5. The signal of the brush borders of S1 and S2 segments in the cortex was hardly seen (Fig. 4 a–c, f, and g). The cells of glomeruli and tubules lacking brush borders were negative for GLUT5 (Fig. 4 d–g). Blood vessel cells, including endothelial cells and smooth muscle cells, were also negative for GLUT5.

Ultrastructural localization of GLUT5

Ultrastructural localization of GLUT5 was demonstrated by a pre-embedding method using ultrasmall gold probes. The immunolabel representing GLUT5 was seen in the microvilli of the apical brush border of S3 proximal tubule cells (Fig. 5). The lateral membrane and cytoplasmic organelles were negative (data not shown).

Immunohistochemical controls

When the primary antibody was replaced with normal IgG or a mixture of the primary antibody and the antigen peptide, no positive labeling was observed (Fig. 4 h, i), confirming the specificity of the immunostaining in this study.

DISCUSSION

This study demonstrated that fructose transporter GLUT5 is expressed in the kidney, where it is mainly localized in the S3 segment of the proximal tubule cells. RT-PCR followed by the Southern blotting clearly showed that the GLUT5 transcript is present in the cortex and/or the outer medulla in the rat kidney. On the other hand, no signal was detected in the inner medulla. This observation is in accord with the results obtained by *in situ* hybridization histochemistry showing that GLUT5 mRNA was concentrated in the medullary ray and outer stripe of the outer medulla in the rat kidney [9, 10]. By immunoblotting, we detected a 51 kDa band in the kidney homogenate, whereas a 58 kDa band was detected in the jejunum. Apparently, the M_r of GLUT5 varies depending on the glycosylation [14], and the observed M_r is within the range of the previous studies of 42 kDa [32] and 60 kDa [22] in the rat kidney, 60 kDa in the rat intestine [15], and 50 kDa in the human intestine [14]. Moreover, N-glycosidase F digestion gave a single 40 kDa band in the kidney and the jejunum. These observations show that mRNA for GLUT5 is present in the outer medulla and that GLUT5 protein is present in the rat kidney.

Detailed examination by *in situ* hybridization indicated that hybridization signals for GLUT5 were restricted to the proximal straight tubules [9]. Our double-fluorescence examination revealed that GLUT5 was abundant in the S3 proximal tubule cells, an observation that is in good agreement with the results of *in situ* hybridization. In addition, we detected a trace amount in the other segments (S1 and S2) of the proximal tubules. As was clearly demonstrated by the double-fluorescence labeling with fluorescein-phalloidin, GLUT5 was localized in the brush border of the S3 proximal tubule cells. By immunoelectron microscopy, GLUT5 was shown to be clearly localized to the apical microvillus membrane. Apical localization of GLUT5 was observed in the intestinal absorptive epithelial cells [17–19] and human colonic epithelial cell line Caco-2 [18, 33]. Apical localization of GLUT5 was also observed by expressing GLUT5 in the cultured polarized epithelial cells, such as Caco-2 cells of the intestinal origin [24] as well as Madin-Darby canine kidney cells of the kidney tubule origin [34]. GLUT5 may be present in the apical membrane in the same mechanism in the intestine and kidney, although their ontogenic lineages are quite different.

By immunoperoxidase and immunofluorescence histochemistry, Asada et al reported that GLUT5 was predominantly localized in the brush border membrane of the proximal convoluted tubules of the cortex and the glomerular mesangial cells in the normal rat kidney [22]. This observation is in marked contrast to our present immunohistochemical examination and previous *in situ*

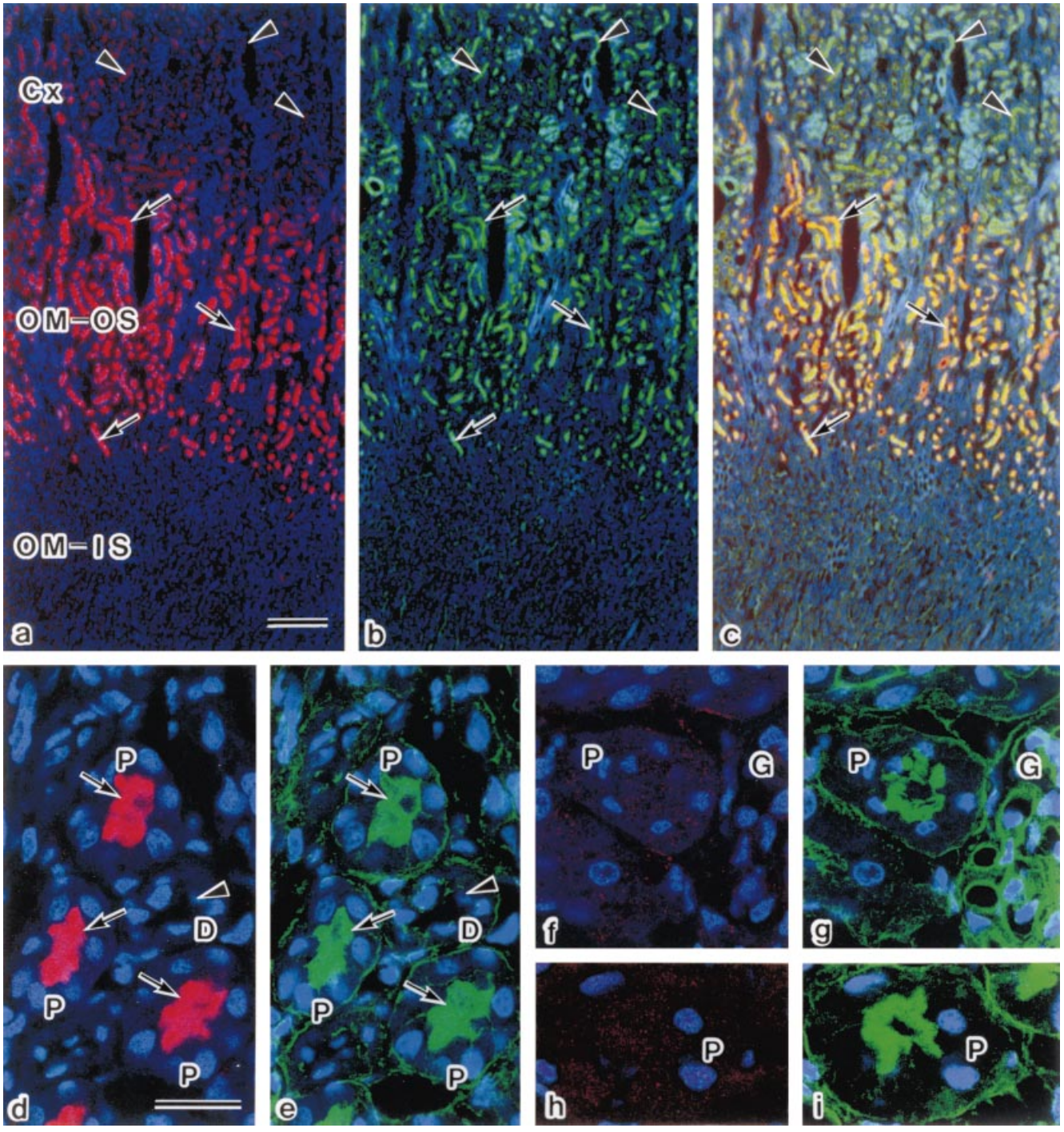

Fig. 4. Immunofluorescence localization of GLUT5 in the rat kidney. Cryostat sections were triple stained for GLUT5 by the LRSC labeling (red), F-actin with fluorescein-phalloidin (green), and nuclear DNA with DAPI (*a–c*) or TO-PRO-3 (*d–i*; blue). (a–c) Survey views around cortex and outer medulla. Abbreviations are: Cx, cortex; OM-OS, outer stripe of the outer medulla; OM-IS, inner stripe of the outer medulla. Double-exposure images for GLUT5 and nuclei (a), for F-actin and nuclei (b), and triple-exposure images for GLUT5, F-actin, and nuclei (c) are shown. The brush borders of S3 proximal tubule cells in the outer stripe are positive for GLUT5 (arrows). Note that most of the GLUT5 positive sites (red) correspond to brush borders (F-actin) of proximal tubules in OM-OS. In the cortex, GLUT5 is hardly seen in the F-actin–positive sites (arrowheads) (bar, 200 μm). (d–i) Confocal images in the outer stripe of the outer medulla (d, e, h, and i) and cortex (f, g). Abbreviations are: D, distal tubule; G, glomerulus; P, proximal tubules. Double-exposure images for GLUT5 and nuclei (d, f, h) and for F-actin and nuclei (e, g, i) are shown. The brush borders of S3 proximal tubule cells in the outer stripe of the outer medulla are positive for GLUT5 (arrows in d, e). GLUT5 signal of proximal tubule cells in the cortex (S1 and S2) are hardly seen (f, g). Tubules lacking brush borders are negative for GLUT5 (arrowheads in d, e). The glomerulus is also negative for GLUT5 (f, g). GLUT5 staining in the S3 proximal tubule is completely abolished when immunostained in the presence of antigen peptide (h, i) (bar, 20 μm).

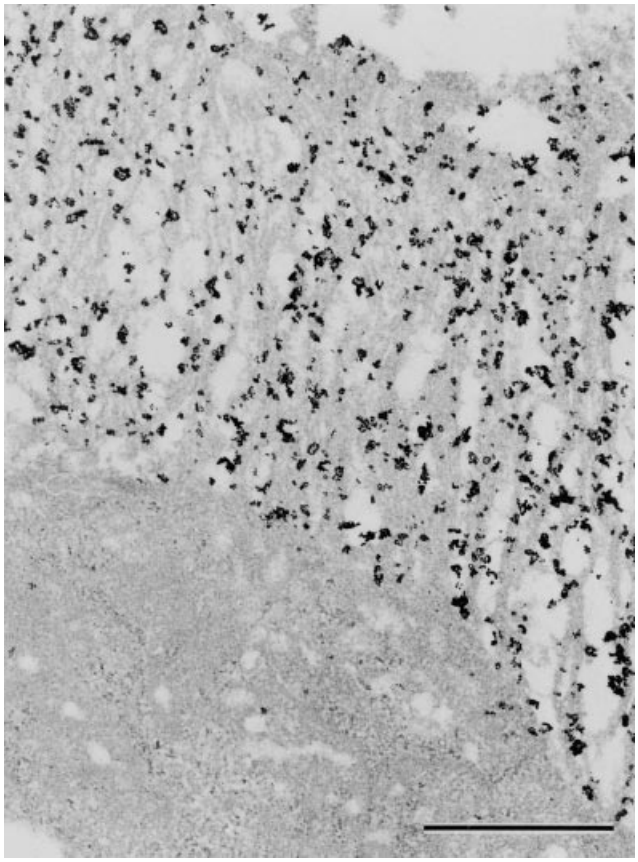

Fig. 5. Ultrastructural localization of GLUT5 in the rat kidney by the Nanogold-labeling method. GLUT5 is localized at the plasma membrane of the microvilli in the apical brush border of a S3 proximal tubule cell (bar, 1 μm).

hybridization studies [9, 10], where GLUT5 is localized in the S3 proximal tubules but not in the proximal convoluted tubules nor glomerulus. The discrepancy might be due to the anti-GLUT5 antibody used by the authors and/or different specimen processing procedures [22].

GLUT5 is a fructose transporter [14, 16] that is considered to play an important role in the transepithelial transport of fructose in the small intestine [21, 35, 36]. In fact, GLUT5 in the intestine is specifically up-regulated by the dietary fructose [15, 32]. Apical GLUT5 in combination with basolateral GLUT2, which transports fructose as well [37], seems to serve as a key molecule for the transepithelial transfer of fructose in the intestinal absorptive epithelial cells [21]. Little is known about the roles of renal GLUT5, but it is present at the apical plasma membrane of the S3 segment of the proximal tubule cells, where a Na^+-dependent active high-affinity glucose transporter SGLT1 resides [12, 38]. SGLT1 is responsible for the complete reabsorption of glucose from the glomerular filtrate using the chemical gradient of Na^+ generated by Na^+,K^+-ATPase. Such apical colocalization of GLUT5 and SGLT1 is the same as seen in the intestinal absorptive epithelial cells. In the S3 proximal tubule cells, however, GLUT1 instead of GLUT2 is localized at the basolateral membrane [11, 12]. GLUT1 at the basolateral membrane seems to serve as the exit of the reabsorbed glucose to the extracellular matrix [35] or for the uptake of glucose to provide cells with a source of metabolic energy [21, 35]. Because GLUT1 does not transport fructose and no other glucose transporter isoforms have yet to be found in the basolateral membrane, the role of GLUT5 in the transepithelial transport of sugars remains elusive.

Expression of GLUT5 is regulated by various factors. Developmental studies showed low levels of GLUT5 mRNA in the rat kidney during the prenatal period, with a rapid induction of GLUT5 expression postnatally [16]. Renal GLUT5 is up-regulated by the dietary administration of fructose or sucrose [32], although the normal blood fructose level and the subsequent elevated level by fructose feeding are lower than the reported Km values of GLUT5 for fructose (6 to 11 mM) [14, 21, 39]. In diabetic conditions, GLUT5 is also up-regulated in the kidney [10, 22]. Hyperglycemia leads to the activation of the polyol pathway for the production of fructose. Up-regulation of renal GLUT5 might be related to the handling of fructose in the kidney, such as the renal uptake or secretion of fructose. Further study is needed to clarify the physiological role of GLUT5 in the kidney.

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