Identification and Characterization of Human Glucose Transporter-like Protein-9 (GLUT9)

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The recently cloned human GLUT9 gene, which maps to chromosome 4p15.3-p16, consists of 12 exons coding for a 540-amino acid protein. Based on a sequence entry (NCBI accession number BC18897) and screening of expressed sequence tags, we have cloned an alternative splice variant of GLUT9 from human kidney cDNA. The RNA of this splice variant consists of 13 exons and codes for a putative protein of 512 amino acids (GLUT9AN). The predicted proteins differ only in their N terminus, suggesting a different subcellular localization and possible physiological role. Screening human tissue RNA by reverse transcription-PCR showed that GLUT9 is expressed mainly in kidney, liver, placenta, and leukocytes, whereas GLUT9AN was detected only in kidney and placenta. The GLUT9 protein localized by immunohistochemistry to human kidney proximal tubules, and subcellular fractionation of human kidney revealed the GLUT9 protein in plasma membranes and high density microsomal membranes. Treatment of kidney membrane proteins with peptide N-glycosidase F showed that GLUT9 and GLUT9AN are expressed in vivo. Localization of GLUT9 and GLUT9AN in three kidney-derived cell lines revealed a plasma membrane distribution for GLUT9 in COS-7 and H9004 cells, whereas GLUT9AN showed a perinuclear pattern and plasma membrane staining in COS-7 and HEK293 cells, respectively. In polarized Madin-Darby canine kidney cells, GLUT9 trafficked to the basolateral membrane, whereas GLUT9AN localized to the apical membrane. Using heterologous expression of GLUT9 in Xenopus oocytes, GLUT9 appears to be a functional isoform with low affinity for deoxyglucose. Deoxyglucose transport mediated by GLUT9 was not inhibited by cytochalasin B. GLUT9 did not bind cytochalasin B as shown by a cytochalasin B binding assay, indicating a similar behavior of GLUT9 compared with GLUT5.

Transport of hexoses across plasma membranes of mammalian cells is mediated by active as well as passive mechanisms, represented by the protein families of Na+ glucose symporters (1) and facilitative glucose carriers (2, 3), respectively. The family of facilitative transporters currently comprises 14 isoforms that differ in their tissue distribution, kinetic properties, and substrate specificity. A common structural feature shared among all members of the glucose transporter family (SLC2A family) is the presence of 12 transmembrane helices with N and C termini facing the cytoplasm and an N-linked glycosylation site. Highly conserved motifs, also referred to as sugar transporter signatures, are located in helices 6 and 12 (PESPR/PETK) and loops 2 and 8 (GRR/GRK) and further specify the sequence characteristics of SLC2A family members. Amino acid residues that are required for glucose transport/function as well as binding of the specific transport inhibitor cytochalasin B have been identified by site-directed mutagenesis analysis of GLUT1, the most intensively characterized isoform (4).

Previous studies suggested the presence of additional isoforms (5, 6), and the availability of expressed sequence tag data bases and the sequencing and mapping of the human genome allowed the identification of several new members during the last few years, leading to a rather complex picture of glucose/hexose transport and whole body sugar homeostasis. To date, only few functional data are available for the new isoforms. However, based on sequence characteristics and phylogenetic alignments, three subclasses in the family can be distinguished (7). This suggests a broader range of possible substrates and transport characteristics among these new isoforms that is not restricted to hexoses, as shown by the cloning of the H’inositol transporter isoform or GLUT13 (8). Here, we describe the characterization of human GLUT9 (9) and demonstrate that alternative splicing leads to differential targeting, suggesting possible new mechanisms in the regulation of hexose transport in mammalian cells.

Experimental Procedures

RNA Extraction, Reverse Transcription-PCR, and PCR Cloning

Total RNA from human tissues was prepared using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions. DNase (DNA-free™, Ambion Inc., Austin, TX)-treated total RNA was primed with random hexamers (Roche Applied Science) and reverse-transcribed using SuperscriptII (Invitrogen).

The human GLUT9 splice variant (GLUT9AN) was cloned by reverse transcription-PCR using KlenTaq LA polymerase (Wayne Barnes, Washington University) from human kidney cDNA based on the expressed sequence tag clone IMAGE:3949549 and NCBI accession number BC018897. The GLUT9AN coding sequence was amplified using the following primers: 5′-CCCGTGTACCCGCCATGAAAGCAGC-3′ and 5′-GCCCTCTAGATTTAGGCGCTCATTTACTAAC-3′. The PCR primers included the restriction sites for KpnI and XbaI for subsequent cloning into pCDA3.1 (Invitrogen). The resulting GLUT9AN clone was confirmed by bidirectional sequencing.
To study the expression pattern of GLUT9 splice variants in human tissues, cDNAs were amplified with GLUT9- and GLUT9ΔN-specific forward primers (5′-ACT GAC ACC CAT GGC AAG A-3′ and 5′-ATG AAG CTC AGT AAA AAG GAC-3′, respectively). A common reverse primer for both splice variants was used (5′-GAG TGT CTG GGT CTA TTG GA-3′), resulting in PCR amplicons of 326 and 229 bp for GLUT9 and GLUT9ΔN, respectively. To ensure that an equal amount of DNA was used for PCR amplification from different tissues, the housekeeping gene actin was amplified as an internal standard at a linear range of the PCR (forward primer, 5′-TCC GTG ACA TTG AGG AGA AG-3′ and reverse primer, 5′-CTG CAT CCT GTC GGC AAT G-3′).

Preparation of Membrane Fractions from Human Kidney and Cell Lines

Human kidney biopsy samples were homogenized in buffer A (20 mM Tris-HCl, 1 mM EDTA, and 255 mM sucrose, pH 7.4) containing a protease inhibitor mixture (Sigma) using a Potter-Elvehjem tissue grinder (Kimble/Kontes, Vineland, NJ). Nuclei, mitochondria, and plasma membranes were obtained by centrifugation at 14,000 × g for 15 min (Beckman JA-20, Beckman Coulter, Fullerton, CA). The resulting pellet was resuspended in buffer A, homogenized, and layered on a 38.7% sucrose cushion in Tris/EDTA (Sigma). After centrifugation at 100,000 × g for 1 h, the plasma membrane containing interphase was pelleted by centrifugation at 20,000 × g for 1 h. High density microsomes were obtained by centrifugation from the first supernatant at 50,000 × g for 1 h. The second supernatant was centrifuged at 200,000 × g for 75 min to sediment low density microsomal membrane fraction. Membrane pellets were resuspended in homogenization buffer and stored at -80 °C prior to further analysis. For preparation of membrane fractions from cell lines stably expressing GLUT9, cells were washed twice with phosphate-buffered saline (PBS). After rinsing in cold (4 °C) buffer A, cells were resuspended into buffer A containing proteinsase inhibitors. All subsequent steps were carried out as described above.

Western Blot Analysis

Plasma membrane and high and low density microsomal protein fractions were separated on 10% polyacrylamide gels, transferred onto nitrocellulose, blocked with 5% dry milk in Tris-buffered saline/Tween 20, and probed with an antibody raised against a C-terminal peptide of human GLUT9 (KIDSATVDKINGRP). Detection of GLUT9 in human kidney membrane extracts, IgG-purified antiserum (HiTrapTM IgG, Amersham Biosciences) was applied at a concentration of 5 μg/ml (in 1% dry milk in Tris-buffered saline/Tween 20). Western blots were probed with a monoclonal anti-peroxidase labeled goat anti-rabbit secondary antibody (Pierce) and developed using the SuperSignal Dura Western kit (Pierce). For detection of human GLUT9 in overexpressing cell lines, anti-human GLUT9 serum (1:1000) and enhanced chemiluminescence (ECL, Amersham Biosciences) were used.

To determine the extent of glycosylation and the molecular masses of human GLUT9 and GLUT9ΔN, 10 μg of plasma membrane extracts from transfected HEK293 cells were treated for 1 h at 37 °C with peptide N-glycosidase F (New England Biolabs Inc., Beverly, MA) following the manufacturer's instructions. After enzyme incubation, the reaction was stopped adding SDS-PAGE loading buffer, and Western blot analysis was carried out as described above. To show in vivo expression of GLUT9 and GLUT9ΔN, 20 μg of human kidney high density microsomes were treated with peptide N-glycosidase F, and the protein samples were separated on a standard 10% SDS-polyacrylamide gel (20 × 20 cm). Western blotting was carried out as described above, and combined plasma membrane fractions from GLUT9- and GLUT9ΔN-transfected HEK293 cells were used as positive controls.

Cell Culture and Transfections

Three kidney-derived cell lines were used to investigate the localization of human GLUT9 and GLUT9ΔN. COS-7, HEK293, and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium with glutamine, sodium pyruvate, penicillin/streptomycin, and 10% fetal calf serum. Prior to transfection, cells were split and seeded for 24 h, and transfection was carried out using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Transiently transfected cells were analyzed for expression by immunocytochemistry and Western blotting 48 h after transfection. Stable expression was achieved by splitting the cells 24 h after transfection into medium supplemented with 1 or 0.8 μg/ml G418 for HEK293 and MDCK cells, respectively. Clonal GLUT9- and GLUT9ΔN-expressing cell lines were obtained by serial dilution. To investigate the localization of GLUT9 and GLUT9ΔN in polarized epithelial cells, clonal expressing MDCK cells were plated at high density (1.6 × 10^6 cells) onto 0.4-μm Falcon cell culture inserts (catalog number 3090, BD Biosciences) and cultured for 5 days under daily media renewal.

Immunohistochemistry/Immunofluorescence in Human Kidney Sections

After rehydration, paraffin-embedded human kidney sections (3 μm) were cooked in 10% citrate buffer, pH 5.2, for antigen retrieval using a pressure cooker. After washing with PBS, nonspecific antibody binding was blocked for 1 h with 2.5% goat serum and 0.1% bovine serumalbumin (BSA) in PBS. IgG-purified human GLUT9 antiserum (10 μg/ml) diluted in the blocking buffer was applied overnight at 4 °C. After washing with PBS, sections were developed using an alkaline phosphatase-conjugated secondary antibody (ABC-AP kit AK-5001, Vector Labs, Inc., Burlingame, CA). Nuclei were counterstained with Mayer's hematoxylin. IgG-purified rabbit preimmune serum was used as a negative control.

Connections of human kidney were used for immunofluorescent staining of GLUT9. Sections stored at ~80 °C were brought to ~20 °C for 20 min, followed by fixation at room temperature with 3% paraformaldehyde in PBS. After washing with PBS, sections were blocked with 2% BSA in PBS for 30 min and subjected to IgG-purified anti-GLUT9 antibody (10 μg/ml) for 1 h at room temperature. GLUT9 staining was visualized using a cross-absorbed goat anti-rabbit Alexa 488 antibody (Molecular Probes, Inc., Eugene). Topro-3 iodide (Molecular Probes, Inc.) and labeled phallloidin (Sigma) were used to counterstain nuclei and actin TRITC-labeled filaments, respectively. Sections were mounted in Vectashield (Vector Labs, Inc.) and examined by confocal microscopy using a Nikon C1 confocal microscope equipped with an Eclipse E800 upright microscope.

Immunocytochemistry and Confocal Microscopy

Transient or stably transfected cells expressing GLUT9 and GLUT9ΔN were grown on coverslips or, in the case of polarized MDCK cells, on transfectant polyethylene terephthalate membranes. Cells were washed twice with PBS, fixed for 10 min in 3% paraformaldehyde, and quenched by three washes with 50 mM NH4Cl in PBS. Cells were permeabilized with 0.1% Triton X-100 for 4 min, followed by three washes with PBS, and nonspecific antiserum binding was blocked with 2% BSA in PBS for 30 min. Cells were incubated for 1 h with GLUT9 antiserum diluted 1:500 in 2% BSA, washed with PBS, and probed with goat anti-rabbit Alexa 488 antibody (1:200 in 2% BSA in PBS). Cells were washed with PBS, and nuclei were stained with Topro-3 iodide for 10 min. After a final PBS wash, cells were mounted using Vectashield. Polarized MDCK cells grown on polyethylene terephthalate membranes from cell culture inserts were mounted using Secure Seal imaging spacers (Sigma). Specimens were examined by confocal microscopy using a Bio-Rad MRC-600 or Nikon C1 confocal microscope.

Functional Characterization of GLUT9

Glucose Uptake into Xenopus laevis Oocytes—Glucose transport by GLUT9 was determined as uptake of 2-deoxy-D-[3H]glucose into Xenopus oocytes. Transport measurements were carried out as described in detail by Keller et al. (10). Briefly, stage V and VI oocytes were injected with 50 ng of RNA obtained from in vitro transcribed GLUT4 and GLUT9 cDNAs cloned into the pXOV vector (the GLUT4 construct was a gift from Dr. Mike Muesing). High-speed pellets were performed 3 days after injection with groups of 8–10 oocytes incubated in 25 μl unlabeled deoxyglucose and 1 μCi/ml 2-deoxy-D-[3H]glucose in modified Barth's solution for 30 min at room temperature in the presence or absence of 100 μM cytochalasin B. Uptake was stopped by three washes with ice-cold modified Barth's solution with 100 μM phloretin. Individual oocytes were blotted dry, mixed with scintillation liquid, and radioactivity was counted. Uptake rates were determined as pico moles of deoxyglucose transported per oocyte/30 min and compared with water-injected (control) oocytes. GLUT9 expression in Xenopus oocytes was determined by Western blot analysis of total membrane fractions. Total membrane proteins were obtained by homogenizing 10–20 oocytes in 1 ml of buffer A by 10 passages through a 25G needle on a 1-ml syringe; and after an initial centrifugation at 1000 × g for 10 min, membranes were pelleted from the supernatant at 100,000 × g for 45 min. To determine the localization of GLUT9 in Xenopus oocytes, whole mount immunocytochemistry was carried out.
The dileucine motif in GLUT9 is human wild-type GLUT9 and GLUT9/H9004. An alignment of the different N termini of sites are indicated by arrows. B, shown is an alignment of the different N termini of human GLUT9 and GLUT9N. The dileucine motif in GLUT9 is underlined. TM, transmembrane region.

Immunofluorescent staining of paraformaldehyde-fixed oocytes was performed as described for cell lines. Oocytes were mounted on slides using Secure Seal spacers to keep the cells intact.

Cytochalasin B Binding Assay—Binding of cytochalasin B to GLUT9 was determined by a competitive cytochalasin B binding assay. Plasma membrane extracts from GLUT9-, GLUT4-, and empty vector-transfected HEK293 cells were processed as described (11). Briefly, binding was initiated by adding 15 μg of plasma membranes (total volume of 30 μl) to a reaction mixture consisting of 100 μl of 20 mM Tris-HCl, pH 7.4, 50 μl of unlabeled cytochalasin B, and 20 μl of radiolabel mixture (2.5 μCi/ml [3H]cytochalasin B and 2.0 μCi/ml [14C]urea). Samples were placed on ice for 10 min, and membranes were pelleted at 18,000 × g for 30 min. The supernatant was discarded, and proteins were dissolved in 500 μl of BTS-450 tissue solubilizer (Beckman Coulter). After adding 10 ml of Ready Safe liquid scintillation mixture (Beckman Coulter), samples were counted in a scintillation counter for 3H and 14C. Nonspecific counts for cytochalasin B were corrected using [14C]urea. Data were fitted by nonlinear regression to determine Kd values using GraphPAD Prism Version 4.0 software.

RESULTS

Genomic Organization and Structural Features of Human GLUT9—The human GLUT9 gene, which maps to chromosome 4p15.3-p16, codes for two alternative RNAs (Fig. 1A). The original GLUT9 gene described by Phay et al. (9) consists of 12 exons spanning 195 kb of the GLUT9 gene and codes for a 540-amino acid protein. Based on a sequence entry (NCBI accession number BC018897) and screening of human expressed sequence tags, we cloned an alternative splice variant of GLUT9 from human kidney cDNA, here referred to as GLUT9N. The GLUT9N splice variant consists of 13 exons spanning 215 kb of the GLUT9 gene and codes for a shorter putative protein of 512 amino acids. The two predicted proteins differ only in their N termini (Fig. 1B). The alternative splicing of the GLUT9 gene suggests that GLUT9 and GLUT9N are transcriptionally regulated by different promoters that are upstream of the corresponding exon 1 (12). Multiple protein alignments of the currently known GLUT protein sequences showed that GLUT9 belongs to the class II family of facilitative sugar transporters (see Fig. 8). Analysis of the deduced protein sequence for GLUT9 showed several motifs characteristic of the glucose transporter family such as the PESPR/PETK and GRR/GRL motifs in helices 6/12 and loops 2/8, respectively. Like the other members of the class II glucose transporter family, GLUT9 lacks the QLS motif in helix 7 (positions 279–281 of GLUT1) and the tryptophan residue corresponding to Trp285 in GLUT1. Furthermore, GLUT9 does not contain serine and threonine at the positions corresponding to amino acids 294 and 295 in GLUT1, respectively. A unique structural feature of GLUT9 is the replacement of two leucine residues in the large cytoplasmic loop with phenylalanine (positions 228 and 231 in GLUT1) and the replacement of leucine with threonine (position 278 in GLUT1). As for GLUT11, serine (position 285 in GLUT1) and phenylalanine (position 385 in GLUT1) are changed to cysteine and glycine, respectively. A specific motif of the class II family transporters is a PFI motif (PS1 in GLUT7) in the last extracellular loop corresponding to amino acids 423–425 in GLUT1. Amino acids that were characterized to be involved in glucose transport and cytochalasin B binding by GLUT1 (4) differ in GLUT9 as well as in GLUT7 and GLUT11: Gin161, Val165, Gin282, Asn317, Glu329, Thr321, and Glu380 (Table I).

Expression of GLUT9 RNA and Protein in Human Tissues—GLUT9 RNA was expressed mainly in liver, kidney, and placenta and to a lesser extent in lung, leukocytes, and brain. In contrast, mRNA for GLUT9ΔN was detected only in kidney and placenta (Fig. 2). To demonstrate endogenous expression of the GLUT9 protein in human tissues, we performed Western blot analysis of kidney membrane fractions using IgG-purified GLUT9 antisera. A specific immunoreactive band was detected in plasma membrane and high density microsomal fractions in the range of 48–55 kDa (Fig. 3A). No signal was detected upon probing membranes with preimmune serum. Western blot analysis of kidney membrane fractions treated with peptide N-glycosidase F showed two bands at the molecular masses of deglycosylated GLUT9 and GLUT9ΔN (Fig. 3B), indicating that both forms of GLUT9 are expressed as proteins in human tissue.

To determine the localization of human GLUT9 in human tissues, we performed immunohistochemistry on paraffin-embedded as well as frozen kidney sections using an alkaline phosphatase detection system and immunofluorescence, respectively. We were able to show that GLUT9 is expressed in the proximal tubules (Fig. 4, A and B). Furthermore, immunofluorescent staining revealed that GLUT9 predominantly localized to the basolateral membrane in proximal tubule epithelial cells (Fig. 4B).

Expression and Localization of Human GLUT9 and GLUT9ΔN Proteins in Cell Lines—Using three different kidney-derived cell lines (COS-7, HEK293, and MDCK), we studied the effect of the two different N termini of human GLUT9 and GLUT9ΔN on their subcellular localization. We first determined the immunoreactivity of our GLUT9 antisera with the GLUT9 and GLUT9ΔN proteins overexpressed in HEK293 cells. As shown in Fig. 5A, GLUT9 and GLUT9ΔN were detected in plasma membrane and high density microsomal fractions of likewise transfected cells, whereas empty vector-trans-
fected cells did not show immunoreactivity in the corresponding area. Treatment of plasma membrane fractions with peptide N-glycosidase F revealed that GLUT9 and GLUT9/H9004N were highly glycosylated as shown by the shift in their molecular masses from a 55–60-kDa band to 45 kDa for GLUT9 and a from 48–55-kDa band to 42 kDa for GLUT9/H9004N (Fig. 5B).

To study the subcellular localization of GLUT9 and GLUT9/H9004N, we analyzed transiently transfected cells by confocal microscopy. In all three cell lines, GLUT9 localized to the plasma membrane (Fig. 6A), whereas a cell type-specific pattern was seen for GLUT9/H9004N. In COS-7 cells, GLUT9/H9004N remained intracellular, whereas in HEK293 cells, GLUT9/H9004N localized to the plasma membrane (Fig. 6A). In contrast, in transiently transfected non-polarized MDCK cells, GLUT9/H9004N showed a predominant vesicular pattern and as well as weak plasma membrane staining.

Because GLUT9 is localized in human kidney proximal tubule epithelial cells, we investigated whether GLUT9 and GLUT9/H9004N are targeted differentially in polarized MDCK cells, a well established epithelial cell model for the study of basolateral versus apical sorting of proteins. Staining of polarized MDCK cells stably expressing GLUT9 or GLUT9/H9004N showed a striking difference in localization for the two proteins. Whereas GLUT9 was found exclusively in the basolateral membrane, GLUT9/H9004N trafficked to the apical membrane of polarized MDCK cells (Fig. 6B).

Fig. 2. Expression of GLUT9 and GLUT9/H9004N mRNAs in human tissues. Human GLUT9 and GLUT9/H9004N were amplified by reverse transcription-PCR from cDNA obtained from total RNA isolated from various human tissues. Actin PCR was performed to control for RNA quality and to ensure that equal amounts of cDNA were used for amplification.

Fig. 3. Detection of the GLUT9 protein in human kidney membrane fractions. A, expression of the GLUT9 protein was detected in plasma (PM) and to a lesser extent high density microsomal (HDM) membrane fractions from human kidney by Western blot analysis. PI, preimmune; LDM, low density microsomes. B, high density microsomal membrane proteins prepared from human kidney treated with peptide N-glycosidase F were subjected to Western blot analysis. The deglycosylation shifted the immunoreactive band and resulted in two bands with the molecular masses of GLUT9 and GLUT9/H9004N. As positive controls, plasma membrane proteins from GLUT9- and GLUT9/H9004N-transfected HEK293 cells were pooled and treated with peptide N-glycosidase F.

Table I

| Class | Isoform | Residue(s) |
|-------|---------|------------|
| I     | GLUT1   | Val165     |
|       | GLUT2   | Gln161     |
|       | GLUT3   | Gln290     |
|       | GLUT4   | Glu380     |
|       | GLUT5   | T          |
|       | GLUT7   | E          |
|       | GLUT9   | A          |
|       | GLUT11  | A          |
|       | GLUT6   | RLL        |
|       | GLUT8   | MAF        |
|       | GLUT10  | E          |
|       | GLUT12  | E          |
|       | GLUT13  | T          |

Functional Characterization of GLUT9—Heterologous expression of GLUT9 in Xenopus oocytes resulted in a 2–3-fold increase in deoxyglucose uptake compared with water-injected control oocytes. The transport mediated by GLUT9 was not inhibited by cytochalasin B. Compared with GLUT4-expressing oocytes, GLUT9 showed a lower overall uptake of deoxy-
glucose that was not inhibited by cytochalasin B (Fig. 7A).

Immunofluorescent staining of *Xenopus* oocytes showed plasma membrane localization of GLUT9 (Fig. 7B).

To further characterize the functional properties of GLUT9, we performed a cytochalasin B binding assay to determine

Fig. 4. **Localization of GLUT9 in human kidney.** Immunohistochemistry carried out with paraffin-embedded (A) and frozen (B) human kidney sections revealed GLUT9 expression in the proximal tubules as detected by alkaline phosphatase (A) and fluorescence (B) staining. The immunofluorescent staining of human kidney (B) showed that GLUT9 localized mainly to the basolateral membranes of proximal tubules (panel e, arrowhead) compared with the apical membrane (arrow). The green channel indicates GLUT9; the nuclei are stained blue. The red channel shows phalloidin staining of actin filaments. A, paraffin-embedded human kidney sections stained with the IgG-purified anti-GLUT9 antibody (panel a) or preimmune serum (panel b); B, immunofluorescent staining of human kidney using anti-GLUT9 antibody (panel c) and preimmune serum (panel d) and proximal tubules stained with GLUT9 showing all three channels (panel e) or GLUT9 and nuclei staining only (panel f). PT, proximal tubules; gl, glomerulus. Bars = 20 μm.

Fig. 5. **Expression of GLUT9 and GLUT9ΔN in HEK293 cells.** A, membrane fractions were prepared as described under “Experimental Procedures,” separated by SDS-PAGE, and blotted onto nitrocellulose. GLUT9 was detected immunohistochemically in the plasma and high density microsomal fractions. PM, plasma membrane; HDM, high density microsomes; LDM, low density microsomes. B, plasma membrane proteins prepared from human GLUT9- and GLUT9ΔN-transfected HEK293 cells were either treated or not with peptide N-glycosidase F, separated by SDS-PAGE, and immunoblotted. HEK293 cells transfected with empty vector, GLUT9, and GLUT9ΔN.
whether GLUT9 binds cytochalasin B. Plasma membrane proteins from empty vector-, GLUT9-, and GLUT4-transfected HEK293 cells were incubated with radiolabeled cytochalasin B, and binding was assayed by competing with increasing concentrations of the unlabeled ligand. As illustrated in Fig. 7C, GLUT9-containing plasma membranes did not bind cytochalasin B and showed no difference in the binding curve compared with membranes from empty vector-transfected cells. The binding curve obtained for GLUT4 by nonlinear regression revealed a $K_d$ value of 105.2 nM (mean $\pm$ S.D. from three replicates with $r^2 = 0.94$), which is in the range of previously reported data (13).

**DISCUSSION**

GLUT9 is a novel transporter protein that belongs to class II of the glucose transporter family. GLUT9 shows structural features that are conserved among all glucose transporter isoforms. These include the PESPR and PETK motifs after helices 6 and 12 and the GRK and GRR motifs in loops 2 and 8, respectively. A dendrogram aligning all human glucose transporter isoforms known today shows that the closest relatives of GLUT9 are GLUT5 and GLUT11 (Fig. 8). Comparison of amino acid residues in all isoforms that were found to be essential for GLUT1 function, glucose transport, and cytochalasin B binding (4) reveals that members of the same class share homologies for those residues (Table I).

Our characterization of GLUT9 shows that GLUT9 is a functional isoform, transporting deoxyglucose with a lower affinity compared with GLUT4. This transport was not inhibitable by cytochalasin B; and using a competitive binding assay, we showed that GLUT9 did not bind cytochalasin B. Because GLUT9 is targeted to the plasma membrane in *Xenopus* oocytes and is not retained intracellularly, uptake rates reflect maximum GLUT9-mediated transport. The absence of the QLS motif in GLUT9 might be responsible for the low transport rate for deoxyglucose, similar to its well characterized relative, GLUT5 (14). As described for GLUT5, the transport of deoxyglucose is not inhibited by cytochalasin B (14), and the transporter is not capable of binding cytochalasin B (15). Another recently characterized member of the class II transporter family (GLUT11) transports glucose only at high glucose concentrations measured by reconstitution of the protein, and the transport is competed by fructose, suggesting that GLUT11 mediates fructose transport (13). GLUT11 shows a remarkably lower capacity for cytochalasin B binding compared with GLUT4 (13). These data indicate that class II transporters share similar functional characteristics and lead us to suggest that another hexose than glucose might be the preferred substrate of GLUT9. Additional studies will focus on further characterizing the transport properties of GLUT9.

A specific feature of GLUT9, reported thus far only for two other isoforms, GLUT11 (16, 17) and GLUT14 (18), is the presence of alternative splice variants expressed in a tissue-specific pattern. As for GLUT11 and GLUT14, alternative splicing of GLUT9 results in presumptive proteins that differ only in their N-terminal regions. Using an antibody directed against the C-terminal part of GLUT9 that does not distinguish between GLUT9 and GLUT9N, we showed that GLUT9 was expressed in human kidney proximal tubules, with a predominant staining of basolateral membranes.

Although the expression of GLUT9 seems to account for basolateral staining, Western blot analysis of peptide N-glycosidase F-treated human kidney membrane fractions showed
two bands resembling non-glycosylated GLUT9 and GLUT9ΔN. Therefore, the two forms are expressed as proteins in human kidney.

Studying the localization of GLUT9 and GLUT9ΔN in three kidney-derived cell lines showed that GLUT9 trafficked to the plasma membrane in COS-7, HEK293, and MDCK cells. In contrast, GLUT9ΔN was retained intracellularly in COS-7 cells, whereas plasma membrane localization was seen in HEK293 cells and, upon polarization, in MDCK cells. Whereas COS-7 cells are fibroblasts, HEK293 and MDCK cells are epithelial, indicating that GLUT9ΔN harbors a targeting signal that specifically allows plasma membrane sorting in those cells. By overexpressing both splice variants in polarized MDCK cells, we showed that GLUT9 and GLUT9ΔN were differentially targeted to apical versus basolateral membranes, respectively.

Interestingly, the N-terminal dileucine motif in GLUT9 does not retain the protein in an intracellular compartment, as seen for GLUT4, GLUT8, and GLUT12 (19–22), indicating that other targeting motifs of GLUT9 direct its plasma membrane expression. Instead, the dileucine motif in GLUT9 might be responsible for its basolateral expression in MDCK cells, as seen for other proteins harboring this motif (23–25). However, residues other than the dileucine motif alone might be involved in basolateral targeting of glucose transporters in polarized MDCK cells since GLUT4 was shown to be retained in an intracellular compartment (26).

Here, we have described for the first time that alternative splicing of a glucose transporter isoform results in differential targeting, a finding that has been described for other membrane proteins (27) and transporters (28). Based on the characteristics of the class II glucose transporter family members with respect to their tissue- and cell-specific expression as well as their functional properties, these isoforms might be specialized in mediating vectorial transport of hexoses, preferentially fructose, in polarized cells and in particular epithelial cells. Our data suggest a novel mechanism of regulation of hexose transport in epithelial cells by alternative splicing, resulting in differential targeting.

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Characterization of Human GLUT9
