Mouse Glucose Transporter 9 Splice Variants Are Expressed in Adult Liver and Kidney and Are Up-Regulated in Diabetes

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A novel glucose transporter (GLUT), mouse GLUT9 (mGLUT9), was recently cloned from mouse 7-d embryonic cDNA. Several splice variants of mGLUT9 were described, two of which were cloned (mGLUT9a and mGLUT9a
def209–316). This study describes the cloning and characterization of another splice variant, mGLUT9b. Cloned from adult liver, mGLUT9b is identical to mGLUT9a except at the amino terminus. Based on analysis of the genomic structure, the different amino termini result from alternative transcriptional/translational start sites. Expression and localization of these two mGLUT9 splice variants were examined in control and diabetic adult mouse tissues and in cell lines. RT-PCR analysis demonstrated expression of mGLUT9a in several tissues whereas mGLUT9b was observed primarily in liver and kidney. Using a mGLUT9-specific antibody, Western blot analysis of total membrane fractions from liver and kidney detected a single, wide band, migrating at approximately 55 kDa. This band shifted to a lower molecular mass when deglycosylated with peptide-N-glycosidase F. Both forms were present in liver and kidney. Immunohistochemical localization demonstrated basolateral distribution of mGLUT9 in liver hepatocytes and the expression of mGLUT9 in specific tubules in the outer cortex of the kidney. To investigate the alternative amino termini, mGLUT9a and mGLUT9b were overexpressed in kidney epithelium cell lines. Subcellular fractions localized both forms to the plasma membrane. Immunofluorescent staining of polarized Madin Darby canine kidney cells overexpressing mGLUT9 depicted a basolateral distribution for both splice variants. Finally, mGLUT9 protein expression was significantly increased in the kidney and liver from streptozotocin-induced diabetic mice compared with nondiabetic animals. (Molecular Endocrinology 20: 686–697, 2006)

GLUCOSE HOMEOSTASIS depends on regulation of glucose transport across the cell membrane down a concentration gradient and is crucial for cell survival, growth, and tissue functions. Imbalance of tissue glucose regulation caused by diabetes mellitus leads to chronic tissue damage and organ failure. Membrane-associated glucose transporters (GLUTs) are required to allow hexose transport across the plasma membrane (PM) and down a concentration gradient. Two families of hexose transporters have been described in mammalian tissues: active Na+/glucose cotransporters (SLC5A family-SGLTs) and facilitative GLUTs (SLC2A family-GLUTs). GLUTs accelerate the transport of glucose along the gradient by facilitative diffusion across the cell membrane (1, 2). Fourteen mammalian GLUTs have been identified and cloned to date. They are expressed in a tissue-specific manner, have intrinsic or inducible glucose transport activity, and show variable affinity to specific hexoses (1, 3). These transporters have 12 putative transmembrane-spanning helices and share conserved domains as signature patterns of GLUTs. GLUTs are classified into three major classes, based on sequence homology (4, 5).

GLUT9 was recently cloned from human kidney cDNA (6), and our laboratory identified and cloned mouse GLUT9 from a d-7 embryo cDNA library (7). mGLUT9 shares 85% homology with human (h) GLUT9. Sequence analysis has shown that GLUT9 is most similar to GLUT11, and both are categorized into the class II (subgroup) that also includes GLUT5 and GLUT7. mGLUT9a and hGLUT9 exhibit glucose transport activity as demonstrated by 2-deoxyglucose uptake in Xenopus oocytes (7, 8).

A unique feature of mGLUT9 is the existence of multiple splice variants: a long form having 12 transmembrane segments (mGLUT9a; GenBank accession no.
AF469480); and two short forms each having a (107-amino acid) deletion resulting in the loss of two transmembrane-spanning domains [mGLUT9a (mGLUT9a), GenBank accession no. AF490463; and mGLUT9b (mGLUT9b), GenBank accession no. BC006076 (7)]. A recently cloned long form, mGLUT9b (GenBank accession no. AY776155), is presented here. mGLUT9b is identical to mGLUT9a except that it utilizes an alternative transcriptional/translational start site resulting in a different amino terminus. hGLUT9, hGLUT11, and hGLUT14 have also been reported to have different splice variants, which are differentially expressed in various tissues (8-10).

Initial characterization of mGLUT9 showed that this transporter is expressed predominantly in adult liver and kidney, tissues critical for maintaining whole-body glucose homeostasis (7). Defining the GLUT expression pattern in these tissues and how this pattern may be altered by diabetes mellitus could help to elucidate the diabetic pathophysiology of these organs. Previous studies have shown that diabetes affects the expression level of GLUTs in a variety of tissues. For example, GLUT2, expressed in the proximal tubules of the kidney, shows an increase in expression under diabetic conditions (11-13), whereas GLUT1 expression has been shown to decrease (14, 15). Additionally, GLUT1, GLUT2, and GLUT3 have all been shown to be down-regulated in the diabetic mouse embryo (16). Finally, GLUT8, a class III transporter expressed predominantly in embryonic tissue and testis, has shown altered expression patterns in different diabetes models (17). These observations suggest that the GLUTs may play a role in the pathology of the diabeticogenic processes associated with these organs and chronic tissue damage.

This study evaluates the localization of the different splice variants, mGLUT9a, and mGLUT9b, in overexpression systems as well as endogenous tissue. In addition, this paper investigates the expression of GLUT9 in adult mouse kidney and liver from control and diabetic animals.

RESULTS

Cloning of mGLUT9b

Both mGLUT9a and the newly identified mGLUT9b were mapped within the mouse genome using the www.ucsc.edu genome browser program. Blast search results mapped this gene to the minus strand of chromosome 5 spanning 371 kb (between 36707748 and 37078747). mGLUT9a was found to have 12 exons and 538 amino acids, with the start ATG present in exon 1, whereas mGLUT9b was shown to have 13 exons with the start ATG located in the second exon, identifying alternative translational start sites for the two mGLUT9 isoforms (Fig. 1). mGLUT9b consists of 523 amino acids and is identical to the previously cloned GLUT9 isform, mGLUT9a, except for the presence of a shorter amino terminus (19 amino acids vs. 34 amino acids). An amino acid sequence alignment of mGLUT9b with the other mouse and human GLUT9 isoforms was performed using the Megalign DNASTAR program (Fig. 2).

RT-PCR Analysis

Expression of these two splice variants was studied in mouse tissues by RT-PCR. The mGLUT9a splice form was detected in heart, skeletal muscle, lung, brain, liver, and kidney (Fig. 3A), whereas mGLUT9b was found expressed predominantly in liver and kidney with a lower level of expression detected in heart (Fig. 3B). PCR products obtained with mouse GAPDH-specific primers confirmed the integrity of the cDNA and ensured that equal amounts of cDNA were amplified for each tissue (Fig. 3C).

GLUT Activity of GLUT9b Expressed in Xenopus laevis Oocytes

We have previously shown that mGLUT9a transports glucose using a X. laevis model system (1). To test the transport ability of mGLUT9b splice variant, cDNA was in vitro transcribed, and the resulting RNA was injected into Xenopus oocytes. mGLUT9b transports glucose in comparison with water-injected controls. mGLUT9b did not transport galactose (Fig. 4).

Expression Analysis

mGLUT9 was identified in total membrane (TM) extracts from liver and kidney by Western blot analysis, as a single highly glycosylated band migrating at about 55 kDa using an antibody raised against the C-terminal peptide of the protein (Fig. 5A). The mGLUT9 band corresponded with a positive control sample obtained from Madin Darby canine kidney (MDCK) cells overexpressing mGLUT9. After digestion and deglycosylation with peptide-N-glycosidase F (PNGase F), this band shifted to a lower molecular mass of about 49 kDa (Fig. 5B), consistent with the predicted molecular mass for mGLUT9. To resolve the mGLUT9 splice
variants, which differ in size by only 4 kDa, deglycosylated tissue extracts from liver and kidney were separated using a standard (20 x 20 cm) SDS-PAGE gel apparatus. Using this technique, two distinct bands were identified migrating at approximately 49 and 45 kDa. These bands corresponded with bands present in positive control PM fractions generated by overexpressing mGLUT9a and mGLUT9b in MDCK cells.
These results confirm that both splice forms of mGLUT9 are expressed in liver and kidney.

Next, to investigate the presence of a putative N-glycosylation site in the first exofacial loop of mGLUT9, the sequence, $\gamma_1$NGT, was mutated using site-directed mutagenesis. Mutation of this site resulted in a protein that migrated at a lower molecular mass, similar to the deglycosylated protein (Fig. 5B), thus confirming that this site does serve as an N-glycosylation motif in mGLUT9.

**Immunohistochemistry and Immunofluorescence Staining**

Immunostaining of paraffin-embedded liver sections demonstrated localization of mGLUT9 in hepatocytes predominantly along the basolateral or sinusoidal membrane surface (Fig. 6, A and B). GLUT2 has previously been localized to the sinusoidal membrane (18). Therefore, to further characterize the location of mGLUT9 in the liver, consecutive sections were stained with both GLUT2 and GLUT9. Dual fluorescence staining confirmed the colocalization of these transporters, demonstrating the basolateral distribution of both GLUT2 and mGLUT9 (Fig. 6, C–E).

Next, the localization of GLUT9 was investigated in the kidney. Immunostaining of these sections revealed that GLUT9 localized to specific tubules of epithelial cells in the kidney, revealing a pattern consistent with basolateral distribution (Fig. 7, A and B). Unlike the findings in the liver tissue, however, 3,3'-diaminobenzidine (DAB) staining of consecutive sections with GLUT2 and GLUT9 showed that GLUT9 existed in a distinct location from GLUT2, which is known to be...
expressed in the proximal tubules (Fig. 7C). Dual immunofluorescent staining confirmed this distinct localization of GLUT9 from GLUT2 (Fig. 7, D–F).

To further characterize the expression of GLUT9, three markers known to stain specific tubules in the kidney cortex were colocalized with GLUT9. These markers included *Lotus tetragonolobus* lectin, which labels proximal tubules, Tamm-Horsfall antibody, which is specific to the thick ascending limb of Henle’s loop and *Dolichos biflorus* lectin, which stains the collecting ducts (data not shown). Localization of these markers was compared with those tubules labeled with mGLUT9 (Fig. 8, A–D). No colocalization was seen with any of these markers and mGLUT9.

**Cell Culture Studies**

Because previous work from our laboratory has shown that the alternate amino termini in hGLUT9 results in differential targeting of this transporter, we next wanted to determine whether this effect is conserved in the mouse. Immunofluorescent staining of polarized MDCK cells overexpressing mGLUT9a or mGLUT9b showed that both splice variants are targeted to the basolateral surface (Fig. 9A). Western blot analysis of subcellular fractions of these MDCK cells confirmed mGLUT9a and mGLUT9b expression in PM fractions only (Fig. 9B). These results are in contrast to what we found in the human, in which the two splice variants targeted to different surfaces in polarized MDCK cells, one to the basolateral surface and one to the apical surface. The purity of the membrane preparations was confirmed by immunoblotting with an antibody to the α-subunit of Na/K-ATPase to identify the PM fraction. Na/K-ATPase protein was only seen in PM fractions. The membranes were also immunoblotted with GM130, a Golgi matrix protein and Calnexin, and integral endoplasmic reticulum membrane protein. Neither of these proteins were detected in the PM fraction (data not shown).

**Comparison of mGLUT9 Expression of Control and Diabetic Tissues**

Previous work in both embryonic and adult tissues has shown that hyperglycemic conditions can alter the expression pattern of GLUTs. To examine the effect of diabetes on GLUT9 expression, Western blot analysis was performed on the TM fractions of the liver and kidney from diabetic and control mice. A significant increase in the expression of mGLUT9 was seen in both tissues from the diabetic animals (Fig. 10, A–D). mGLUT9 expression in the liver TM fraction was nearly 2-fold greater in diabetic samples compared with controls ($117 ± 29$ vs. $231 ± 32$ relative OD units; $P < 0.04$) (% normalized to actin control ± sem). Similarly, mGLUT9 expression in the kidney TM fraction was 1.3-fold increased in diabetic samples ($331 ± 12$ vs. $440 ± 33$ relative OD units; $P < 0.05$). Immunostaining of paraffin-imbedded tissues showed that the local-
The localization of GLUT9 was not altered under diabetic conditions (data not shown).

In addition, real-time PCR was performed on control vs. diabetic tissues to determine whether both mGLUT9a and b are up-regulated or whether this effect is specific for one splice variant. As shown in Fig. 11, mGLUT9b was significantly increased in expression in both kidney and liver from diabetic mice as compared with control mice (P = 0.003, kidney; P = 0.008, liver). With mGLUT9a, there is a tendency for increased expression with diabetes, but this difference did not achieve statistical significance. Skeletal muscle samples were examined from diabetic and nondiabetic mice for control levels of expression of mGLUT9a and b forms by real-time PCR.

**DISCUSSION**

Several splice variants of mGLUT9 have been identified in embryonic (7) and now adult tissue. mGLUT9b, described in this paper, has an alternative N terminus and otherwise is identical to mGLUT9a. These murine isoforms are homologous to hGLUT9 wild-type and alternative N terminus isoform, described previously in our laboratory (8). hGLUT9 transports glucose but this transporter is not inhibited by cytochalasin B, and the transporter does not bind to cytochalasin B. Both mGLUT9 splice variants map to the reverse strand of mouse chromosome 5, with mGLUT9a having 12 exons and mGLUT9b having 13 exons including an alternative start site for transcription (Fig. 1). This finding suggests the possibility of transcriptional regulation by different promoter elements for each of the splice forms. mGLUT9 is the first of the mGLUTs shown to have multiple splice forms. Among hGLUTs, hGLUT9, hGLUT11, and hGLUT14 (a duplcon of GLUT3) have all been found to have alternative splice forms. A similar pattern of exon organization was described for these transcripts leading to multiple splice variants (8–10).

RT-PCR analysis of mRNA expression reveals that mGLUT9a is present in most tissues, whereas mGLUT9b is predominately expressed in liver and kidney. These data are consistent with Northern blot analysis shown previously for mGLUT9, which suggested the presence of two transcripts in liver and kidney (7). Similar findings were also observed with hGLUT9 splice forms demonstrating selective distribution in different tissues and multiple transcripts on Northern blot (6, 8). This differential expression suggests that these alternative forms may serve selective functions in these particular tissues to maintain glucose homeostasis. Deg-
lycosylation of the PM fractions from kidney and liver revealed the presence of two bands, confirming the protein expression of both transcripts in these tissues. Both of these tissues play a key role in glucose homeostasis by transepithelial transport: the liver by regulating blood glucose levels via glucose storage vs. glucose synthesis and the kidney by reabsorption of glucose from the urine and by gluconeogenesis.

Immunohistochemical staining of liver tissue sections localizes mGLUT9 in polarized, epithelial hepatocytes predominantly along the sinusoidal membrane. Hepatocytes are exposed to blood vessels at this sinusoidal side, which is considered the basolateral surface. The alternative or apical side of the polarized hepatocytes faces the bile canalicular membrane domain (19). GLUT2 is also expressed in hepatocytes and is similarly localized to the basolateral surface (1, 19). We confirm the basolateral expression of both GLUT proteins in the liver. The question remains as to why three GLUTs, GLUT2 and the two forms of GLUT9, would be expressed at the same cell surface. GLUT2 is a low-affinity, high-capacity transporter, which is sensitive only to high concentrations of cytochalasin B. GLUT9 may serve as an alternative bidirectional transporter when the intracellular concentration of glucose in the hepatocytes drops below the $K_m$ of GLUT2. For example, experiments have shown that hepatocytes from GLUT2-null mice transport glucose into the blood and this transport is not inhibitable by cytochalasin B (20, 21). Information reported so far on mGLUT9 transport properties (7) and the present observations on localization suggest the possibility that GLUT9 is responsible for transport activity in GLUT2-null hepatocytes. Further studies, however, are needed to address this possible explanation.

Localization of mGLUT9 in the kidney is restricted to specific tubules in the outer cortex of the kidney. Extensive studies have shown that GLUT2 is expressed in proximal convoluted tubules of the kidney and co-localizes with $L.~tetragonolobus$, whereas GLUT1 is expressed in the proximal straight tubules (18). Unlike the findings in hepatocytes, the immunohistochemical staining in kidney indicates that mGLUT9 expression in tubules is entirely distinct from GLUT2 expressing tubules. Several markers were used in this study for...
colocalization with mGLUT9 to identify the location of this transporter. *L. tetragonolobus* lectin was used to stain proximal tubules. Tamm-Horsfall antibody was used to label the thick ascending limb of Henle’s loop, and *D. biflorus* lectin was used to stain collecting ducts. No colocalization was seen with any of these markers, suggesting that mGLUT9 localizes to the basolateral surface of the distal convoluted tubules or connecting tubules of the kidney cortex. No other GLUTs have been identified in this region of the adult nephron; however, one recent report places GLUT12 in the distal tubules and collecting ducts of embryonic d 19 mouse fetuses (22). GLUT8 has also recently been localized to podocytes in murine glomeruli (23). It is possible that mGLUT9 serves to supply the distal tubule with glucose from the interstitium as an energy source critical for ion transport. The presence of another facilitative GLUT on the basolateral surface of the distal tubule is therefore teleologically reasonable to meet the demands of this region of the nephron.

Protein expression of mGLUT9 was, on average, 2-fold higher in membrane fractions from diabetic mouse kidney and liver vs. that from control animal tissue. mRNA expression also was increased in diabetic tissues as compared with controls. Similarly, GLUT2 protein expression has been shown to increase in both tissues in response to induction of hyperglycemia, whereas GLUT1 protein expression decreases (1, 15, 24). By real-time PCR, mGLUT9b is up-regulated to a greater extent in diabetic kidney and liver compared with control and compared with skeletal muscle, which expressed very low levels of both GLUT9 isoforms and did not increase with diabetes. It is not clear why the b form would be preferentially up-regulated. Both isoforms transport glucose, which had been shown previously by us for GLUT9a and now, in this study, for GLUT9b. A recent study with GLUT11, another GLUT with splice variants, reported that all three isoforms, which differ only in their N-terminal sequences, transport glucose with similar affinity (25). Thus it is unlikely that these two isoforms would have different transport characteristics. It is possible that, although the overexpression data did not show a difference, the two isoforms localize to...
different regions of the cell or organ in vivo, and up-regulation occurs more with GLUT9b for a physiological reason. Further studies are necessary to test this hypothesis as well as understand the factors regulating the mGLUT9a and mGLUT9b expression.

Pericentral regions of the liver and the tubules of the kidney are vulnerable to hypoxia during periods of increased glucose uptake due to increased glucose consumption (26). Therefore, not only blood glucose, but also oxygen content, insulin/glucagon ratio, and ATP/AMP ratio are altered in these two tissues in diabetic animals. Changes in GLUT9 expression may be due to adaptive responses to variations in metabolic and environmental conditions. Analysis of the mGLUT9 promoter regions will elucidate possible regulatory mechanisms likely to be modulated in diabetes. In conclusion, this suggests that the increase in GLUT9 expression in our diabetic animal model may be due to differential regulation of the splice variants of mGLUT9 in response to hyperglycemia or other physiological conditions known to be altered by diabetic conditions. The pathophysiology of liver and kidney changes, in diabetic mice or humans, may be a result of this adaptive response, and thus further investigation of the mechanisms responsible for this increase in mGLUT9 expression in diabetes is warranted.

MATERIALS AND METHODS

Cloning mGLUT9b

mGLUT9b was cloned from mouse liver cDNA using specific primers designed based on sequence information obtained from GenBank accession no. BC 007076. The mGLUT9b coding sequence was amplified using the following primers: forward, 5'-GGATCAAGCTTGCCACCATGAAGCTCAGTGAA-3'; and reverse, 5'-GGCCGGTCTAGATTAGACAATTTT-GTTTTGGCC-3'. The PCR primers included a HincIII and an XbaI site, respectively, for subsequent cloning into pcDNA3.1+ (Invitrogen, Carlsbad, CA). Klentaq LA Polymerase (a high-fidelity enzyme from Washington University, St. Louis, MO) was used for the PCR. Selected clones were sequenced using the big-dye method (Applied Biosystems, Foster City, CA) to confirm sequence fidelity.

RNA Extraction

Total RNA was extracted from mouse tissues using Trizol (Invitrogen) and digested with DNase (Ambion, Inc., Austin, TX). cDNA was synthesized from 5 μg of total RNA using
RT-PCR Analysis

Tissue expression of mGLUT9a and mGLUT9b was studied by RT-PCR analysis. Primers were designed using the Primer Select program in DNASTAR to differentially recognize the mGLUT9a splice variants. To identify mGLUT9a, the following primers were used: forward, 5' -GGGTCCACCCACAGGAGGAG and reverse, 5' -TTGACCAAGCAGGACGCA, which generated a band of 696 bp. The mGLUT9b-specific PCR was performed with the following primers: forward, 5'-TGAACAAGACACCAGAGAACTCAGCA, and reverse, 5'-CAGAAGCTCAGCACAGACACCAG, which generated a band of 811 bp. One twentieth of the cDNA reaction was used as template for PCR. Hot start Taq DNA polymerase (Invitrogen) was used, and the reaction was amplified utilizing the PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA). PCR cycle conditions were: 94°C for 1 min, and 35 cycles of 94°C for 30 sec, 57°C (for mGLUT9a) and 60°C (for mGLUT9b) for 30 sec, and 72°C for 55 sec. PCR amplicons were sequenced in a 1.5% agarose gel. These fragments were sequenced to verify their identity. Quality and initial amounts of cDNA template in each reaction were assessed by a control PCR with primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH gene; forward, 5'-TTGACCTCAACTCATGG-3'; and reverse, 5'-ATGAGTGCCACCCCTG-3').

Real-Time Quantitative PCR

Total RNA was extracted using the RNeasy RNA isolation kit (QIAGEN, Chatsworth, CA). RNA was reverse transcribed according to the Superscript II (Invitrogen) protocol. Quantitative PCR assays were performed using Applied Biosystems 7000 Sequence Detection system. Taqman gene expression assays (Applied Biosystems) Mm01211146_m1 and Mm00455117_m1 were used to examine expression of 7000 Sequence Detection system. Taqman gene expression assays were performed using Applied Biosystems according to the Superscript II (Invitrogen) protocol. Quantitative PCR was performed using the following primers: forward, 5’-TGAACAAGACACCAGAGAACTCAGCA, and reverse, 5’-CAGAAGCTCAGCACAGACACCAG, which generated a band of 811 bp. One twentieth of the cDNA reaction was used as template for PCR. Hot start Taq DNA polymerase (Invitrogen) was used, and the reaction was amplified utilizing the PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA). PCR cycle conditions were: 94°C for 1 min, and 35 cycles of 94°C for 30 sec, 57°C (for mGLUT9a) and 60°C (for mGLUT9b) for 30 sec, and 72°C for 55 sec. PCR amplicons were sequenced in a 1.5% agarose gel. These fragments were sequenced to verify their identity. Quality and initial amounts of cDNA template in each reaction were assessed by a control PCR with primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH gene; forward, 5’-TTGACCTCAACTCATGG-3'; and reverse, 5’-ATGAGTGCCACCCCTG-3').

Protein Isolation and Subcellular Fractionation

Protein from pooled tissue samples was extracted into ice-cold 10 mM HEPES buffer supplemented with 250 mM sucrose, 1 mM EDTA, and protease inhibitor mix (PI mix; 10 µ g aprotinin, 10 µ g leupeptin, 10 µ g antipain, 10 µ g benzamidine, 50 µ g trypsin inhibitor, 10 µ g chymostatin, 10 µ g pepstatin A, and 0.87 mg phenylmethylsulfonylfluoride/ml of buffer (Sigma). Tissues were homogenized on ice using a Potter Elvehjem homogenizer (Kimblenotes, Vineyard, NJ). The tissue homogenate was spun down at 1500 g for 10 min at 4°C to separate the tissue debris. The supernatant was centrifuged at 200,000 g for 45 min at 4°C to isolate the TM fraction. The TM pellet was resuspended in HEPES buffer with PI mix and stored at −20°C until analysis.

To further define the localization of mGLUT9, subcellular fractionation was performed to separate PM, high-density microsome (HDM), and low-density microsome fractions (LDM). Briefly, the homogenate was spun down at 14,000 g for 15 min. The pellet was resuspended in HEPES buffer with PI mix and overlaid on an equal volume of 38% sucrose and spun at 100,000 g for 1 h in a swinging bucket rotor. The PM fraction was isolated at the interface as a white fluffy band and pelleted at 50,000 g for 30 min. The supernatant from the first spin was centrifuged at 50,000 × g for 30 min to obtain the HDM fraction. Finally, the resultant supernatant was recentrifuged at 200,000 × g for 75 min to isolate the LDM fraction. PM, HDM, and LDM fractions were homogenized in HEPES buffer with PI mix and stored frozen until analysis. Protein was quantified using the BCA reagent (Pierce Chemical Co., Rockford, IL). Purity of the subcellular fractions was tested using antibodies to Na+/K+-ATPase protein (a generous gift from Dr. Robert Mercer, Washington University School of Medicine, St. Louis, MO), GM130, a Golgi matrix protein (Pharmingen, San Diego, CA), and Calnexin, an integral endoplasmic reticulum membrane protein (Stressgen, Victoria, British Columbia, Canada).

Radiotracer Flux Assays

The influx experiments were performed at 20°C using pools of 10–12 oocytes for each condition with 14C-labeled hexose at a specific activity of 1 µ Ci/ml. Oocyte pools were washed with ice-cold MBM to stop the incubation, after which individual eggs were placed in vials and dissolved in 0.5 ml 5% SDS for 30 min. Finally, scintillation fluid (5 ml) was added to each vial and radioactivity measured using a Beckman liquid scintillation counter LS6500 (Beckman Coulter, Inc., Fullerton, CA). All experiments were performed in triplicate, and the results were compared with the influx values obtained with water-injected oocytes.

Antibodies and Markers

Protein expression was analyzed using mGLUT9-specific antibodies generated in sheep against a C-terminal 20-amino acid peptide (SOTEPDSSSTLDGYQNKW) (1). Immune sera were purified using a HiTrap protein G column (Amersham Biosciences, Piscataway, NJ) used for Western blots and fluorescent staining. Protein G purified preimmune sera was...
used as the negative control. Horseradish peroxidase-conju-
gated preabsorbed goat antischis secondary antibody and
mouse antiacin antibody were from Chemicon Inc. (Te-
mecula, CA). Alexa fluor 488 and 568 conjugated secondary
antibodies and TO-PRO-3 iodide nuclear marker were ob-
tained from Molecular Probes (Eugene, OR). The GLUT2 an-
tibody was a generous gift from Dr. B. Thorens (University of
Lausanne, Lausanne, Switzerland). L. tetragonalobus (Vector
Laboratories, Inc., Burlingame CA), D. biflorus (Vector Lab-
atories), and Tam-m-Horsfall polyclonal antibody (Biomedical
Technologies, Stoughton, MA, and gift of Dr. Jeffrey Minor,
Washington University School of Medicine) were used to
stain different parts of the mouse nephron.

Western Blot Analysis

TM fraction (30 μg) from liver and kidney were separated by
10% SDS-PAGE and transferred to nitrocellulose using the
miniprotein system (Bio-Rad Laboratories, Hercules, CA). TM
fractions isolated from MDCK cells overexpressing mGLUT9
were used as a positive control for Western blots (for over-
expression methods, see below). Blots were blocked with 5%
milk for 1 h and probed with sheep anti-mGLUT9 (1:1000)
overnight at 4 C. Horseradish peroxidase-conjugated goat
antisheep antibody (1:20,000) was used as the secondary
antibody. Signal was detected by West Dura chemilumines-
cence detection system (Pierce Chemical Co.). To further
characterize mGLUT9, 10 μg (PM) were stained with antibodies from different tissues and sizes were compared.

Tissue Staining for Endogenous mGLUT9 Expression

and Colocalization Studies

Bouin’s fixed paraffin-embedded liver and kidney tissues
were used for the immunohistological staining procedure
using the Vector staining kit specific for sheep primary anti-
body (Vector Laboratories). Sections were precoated with
citrate buffer (pH 5.2) and blocked with rabbit serum. Sec-
tions were incubated with mGLUT9 antibody (10 μg/ml) over-
night at 4 C and secondary antibody for 1 h at room temper-
ate. 3,3’-DAB substrate was used to identify the mGLUT9 and
counter stained with hematoxylin. Negative controls
were stained with preimmuno sera (10 μg/ml).

Frozen tissues mounted in Optimal Cutting Temperature
(O.C.T.) compound (Electron Microscopy Sciences, Wash-
ington PA), were sectioned using a cryostat and used for
immunofluorescence staining. Sections were fixed with 5%
paraformaldehyde and stained with anti-mGLUT9 (10 μg/ml),
anti-mGLUT2 (15 μg/ml), L. tetragonalobus (1:200), D. bifo-
rus (1:1000), or Tam-m-Horsfall antibody (1:400). Sections
stained with preimmuno sera or normal species-specific sera
were used as the negative control. Secondary antibody
specific for appropriate host species conjugated with Alexa
fluoro 488 and TO-PRO-3-iodide nuclear staining was used for
the fluorescence staining. Dual staining was performed sequentially incubating with appropriate secondary antibo-
dies. Fluorescence images were obtained using a confocal
microscope operated with Nikon EZ.T.1 software (Nikon
ECLIPSE E800; Nikon Instruments Corp., Melville, NY). Dual
fluorescence signal collected by sequential exposure to each
laser using the channel series control reduced bleaching be-
tween channels.

Cell Lines and in Vitro Expression Studies

MDCK cells were grown in DMEM supplemented with 10% fetal
calf serum, 1% Penicillin/Streptomycin 2% l-glutamine,
and 1% sodium pyruvate (Fisher Scientific, Hanover Park, IL).
Cells were transfected when they were 50–70% confluent
using Fugene 6 reagent (Roche, Indianapolis, IN) according
to the manufacturer’s instructions. After 48 h, transfection
media supplemented with G418 (0.8 μg/ml) was added to select
the transfected cells. A bulk stable population was used for protein extraction. Subcellular fractionation was per-
dored as described above (10 μg). Protein was subjected to
SDS-PAGE and Western blot assays. PNGase F digestion
was also conducted as explained for the tissues.

Next, to investigate the presence of a putative N-glycosyl-
ation site in the first exofacial loop of mGLUT9, the sequence,
N1GNT was mutated to NGT using the QuikChange Site-
Directed Mutagenesis kit (Stratagene, La Jolla, CA). Mutated
protein expression was analyzed by overexpression these
constructs in MDCK cells.

Immunocytochemical Studies

Transfected cells were grown for 48 h and selected for stable
expression using G418. Monoclonal cell lines were generated
by serial dilution. Three monoclonal MDCK cell lines expres-
sing mGLUT9a and mGLUT9b were grown on polyethylene
terephthalate (PET) membranes (0.4-μm pore size, from BD
 Biosciences, Franklin Lakes, NJ). Monolayer cultures were
polarized for 5 d. Cells were fixed in 3% paraformaldehyde
for 15 min and permeabilized with 0.1% Triton X100 for 5 min
and blocked with 2% BSA for 30 min. mGLUT9 antibody (10
μg/ml) staining was conducted overnight at 4 C. For fluores-
cence detection, antishis secondary antibody conjugated
with Alexa fluoro 488 and nuclear staining with TO-PRO-3
iodide dye were used. mGLUT9 expression in polarized
MDCK cells was observed by confocal microscopy to ana-
lize the vertical distribution.

Comparison of Normal and Diabetic Tissues

mGLUT9 expression was compared in diabetic vs. control
using 30 μg of TM fractions for both liver and kidney. Experi-
ments were performed in triplicate. For quantitative analysis,
respective blots were stripped and reprobed with an actin
antibody (1:5000). mGLUT9 expression was normalized to
actin. Real-time PCR was used to quantify the relative levels
of gene expression in diabetic tissue as compared with con-
trol. The comparative threshold cycle (Ct) was measured
and then normalized to 18S rRNA levels. All data was expressed
differences in the change in threshold cycle (∆ΔCt) between
control and diabetic tissues normalized to skeletal muscle.
Diabetic and nondiabetic skeletal muscle was used as a negative
control. Both isoforms were expressed at relatively low
levels in skeletal muscle, and no increase was seen in the
diabetic tissue. All real-time experimental values are ex-
pressed as change in cycle threshold compared with skeletal
muscle. All values are expressed as means ± SEM.

Statistical Analysis

Protein expression was quantitated using densitometry, uti-
lizing National Institutes of Health (NIH) Image software (Be-
thesda, MD). mGLUT9 expression was normalized to the
benchmark actin band. Data were expressed as a percent
normalized to control ± SEM. Statistical significance of the
means was calculated in triplicate groups, and P < 0.05 was
considered significant. Student’s t test was used for statisti-
cal analysis of diabetic vs. control tissue. ANOVA with Fish-
er’s post hoc test was used for the comparison of diabetic vs.
control mRNA levels by real-time PCR.
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