Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leucocytes

Holger DOEGE, Andreas BOCIANSKI, Hans-Georg JOOST[1] and Annette SCHÜRMANN

Institut für Pharmakologie und Toxikologie, Medizinische Fakultät der RWTH Aachen, Wendlingweg 2, D-52057 Aachen, Germany

The *GLUT9* gene encodes a cDNA which exhibits significant sequence similarity with members of the glucose transporter (GLUT) family. The gene is located on chromosome 9q34 and consists of 10 exons separated by short introns. The amino acid sequence deduced from its cDNA predicts 12 putative membrane-spanning helices and all the motifs (sugar-transporter signatures) that have previously been shown to be essential for transport activity. A striking characteristic of GLUT9 is the presence of two arginines in the putative helices 7 and 8 at positions where the organic anion transporters harbour basic residues. The next relative of GLUT9 is the glucose transporter GLUT8/GLUTX1 (44.8 % amino acid identity with GLUT9). A 2.6-kb transcript of *GLUT9* was detected in spleen, peripheral leucocytes and brain. Transfection of COS-7 cells with *GLUT9* produced expression of a 46-kDa membrane protein which exhibited reconstitutable glucose-transport activity and low-affinity cytochalasin-B binding. It is concluded that GLUT9 is a novel member of the family of sugar-transport facilitators with a tissue-specific function.

Key words: cytochalasin B binding, glucose-transporter protein, reconstituted glucose-transport activity, sugar transporter signature.

INTRODUCTION

Hexose transport into mammalian cells is catalysed by a small family of 45–55-kDa membrane proteins (GLUT1–GLUT5) which belong to the larger family of transport facilitators [1–3]. The GLUT isoforms differ in their expression in different tissues, in their kinetic characteristics, i.e. K_m values, and in their substrate specificity [4]. Two findings suggest the possibility that additional sugar-transport facilitators exist. First, in some tissues only low levels of mRNA of the known isoforms were detected [5]. Secondly, GLUT4 knock-out mice exhibited almost normal glucose transport in muscle, although no compensatory increase in the expression of the *GLUT1* or *GLUT3* gene was detected [6]. Thus we took advantage of the conserved 'hexose-transporter signatures' and searched the expressed sequence tag (EST) databases in order to identify additional glucose-transporter-like sequences. This approach led to the identification and characterization of the novel glucose transporter GLUT8 [7]. Here we describe the identification and characterization of human GLUT9, a transport facilitator with glucose-transport activity and a tissue-specific gene expression.

MATERIALS AND METHODS

RNA preparation, PCR cloning and sequencing

Total RNA was prepared by homogenization in guanidine thiocyanate and centrifugation through caesium chloride. Rapid amplification of cDNA ends (RACE) was performed with a kit from Gibco BRL (Eggenstein, Germany) according to the instructions of the manufacturer. PCR-generated DNA fragments were isolated and subcloned into pUC18 with the Sureclone® kit (Pharmacia, Freiburg, Germany).

All cDNA clones and PCR products were sequenced in both directions by the method of Sanger (Thermo sequenase sequencing kit: Amersham Life Science, Little Chalfont, Bucks., U.K.).

Northern-blot analysis

Northern blots with RNA from different human tissues were purchased from Clontech (Palo Alto, CA, U.S.A.). Probes were generated with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP by random oligonucleotide priming. The nylon membranes were hybridized at 42 °C, and blots were washed twice with 0.8 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS.

Expression of GLUT9 in COS-7 cells

A fragment of the GLUT9 cDNA comprising the 5′ untranslated region and the full reading frame was amplified by PCR, and was subcloned into the mammalian expression vector pCMV, which harbours a simian virus 40 origin, a cytomegalovirus promoter and a polyadenylation site. COS-7 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in Dulbecco's modified Eagle's medium (pH 7.4) containing 10 % fetal-calf serum, 20 mM Hepes and 2 mM glutamine. Transfection of COS-7 cells with pCMV-GLUT9 was performed with calcium phosphate/DNA co-precipitates as described previously [8].

Preparation of membrane fractions from transfected cells

Cells were homogenized and fractionated 48 h after the transfection as described previously [8]. Most of the glucose-trans-

Abbreviations used: EST, expressed sequence tag; RACE, rapid amplification of cDNA ends.

[1] To whom correspondence should be addressed (e-mail joost@rwth-aachen.de).

The nucleotide sequence of GLUT9 has been deposited in the EMBL, DDBJ, GenBank® and GSDB Nucleotide Sequence Databases under the accession number Y17803.

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                                                        *    *  *
GLUT8      1 MTP.....EDPE.ETQPLLGPPG.GSAPR.....GRRVFLAAFAAALGPL
             |       | |: :| |   ||  |   |      :|||||  ||| ||
GLUT9      1 MQEPLLGAEGPDYDTFPEKPPPSPGDRARVGTLQNKRVFLATFAAVLGNF
               *                                     *
GLUT8     39 SFGFALGYSSPAIPSLQRAAPPAPRLDDAAASWFGAVVTLGAAAGGVLGG
             |||:|| |.|| ||.|:|.  |   |  . |||||.| ||||||||.
GLUT9     51 SFGYALVYTSPVIPALERSLDPDLHLTKSQASWFGSVFTLGAAAGGLSAM
               **                                  *   *   *
GLUT8     89 WLVDRAGRKLSLLLCSVPFVAGFAVITAAQDVWMLLGGRLLTGLACGVAS
              | |  |||||::  .||  ||:|..  |  .|||| || ||| | |. .
GLUT9    101 ILNDLLGRKLSIMFSAVPSAAGYALMAGAHGLWMLLLGRTLTGFAGGLTA
               * *  *      ** **   **   * *     *   *  *
GLUT8    139 LVAPVYISEIAYPAVRGLLGSCVQLMVVVGILLAYLAGWVLEWRWLAVLG
              |||:|||| | ||| ||.  ||| | | |  |  | .| ||||||
GLUT9    151 ACIPVYVSEIAPPGVRGALGATPQLMAVFGSLSLYALGLLLPWRWLAVAG
               *       * *  **  *       *   *  * *
GLUT8    189 CVPPSLMLLLMCFMPETPRFLLTQHRRQEAMAALRFLWGSEQG..WEDPP
              |  :|:||: ||| .|||||.. | :||: || .| |.:     ||
GLUT9    201 EAPVLIMILLLSFMPNSPRFLLSRGRDEEALRALAWLRGTDVDVHWEFEQ
                                *                ** *     *
GLUT8    237 IG...AEQSFHL..ALLRQPGIYKPFIIGVSLMAFQQLSGVNAVMFYAET
             |      ||  .  |  | | : :|  : . :   |||.|:  :: | :.
GLUT9    251 IQDNVRRQSSRVSWAEARAPHVCRPITVALLMRLLQQLTGITPILVYLQS
             *   *           *                 **  *
GLUT8    282 IFEE.AKFKDSSLASVVVGVIQVLFTAVAALIMDRAGRRLLLVLSGVVMV
             ||:  |        . :|| :..|   :||| || |||:.|| .|  :|
GLUT9    301 IFDSTAVLLPPKDDAAIVGAVRLLSVLIAALTMDLAGRKVLLFVSAAIMF

GLUT8    331 FSTSAFGAY..FKLTQGGPGNSSHV.AISAPVSAQPVDASVG.LAWLAVG
              .   | |  |      | ... . . |    |||. |  | |  . .
GLUT9    351 AANLTLGLYIHFGPRPLSPNSTAGLESESWGDLAQPLAAPAGYLTLVPLL
                    *  **       *          *        *   *
GLUT8    377 SMCLFIAGFAVGWGPIPWLLMSEIFPLHVKGVATGICVLTNWLMAFLVTK
             .  ||| |:|||||| |||||: ||  :|||.|:||| .|| ||..||
GLUT9    401 ATMLFIMGYAVGWGPITWLLMSEVLPLRARGVASGLCVLASWLTAFVLTK
              *          *                ****          *
GLUT8    427 EFSSLMEVLRPYGAFWLASAFCIFSVLFTFSCVPETKGKTLEQITAHFE.
              |  ..     |. .| |: |..||  |||||||:.|||| . |
GLUT9    451 SFLPVVSTFGLQVPFFFFAAICLVSLVFTGCCVPETKGRSLEQIESFFRM

GLUT8    476 GR*.....
             ||
GLUT9    501 GRRSFLR*
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Figure 1 Alignment of GLUT9 with its next relative (GLUT8) in the GLUT family

Positions of presumed membrane-spanning helices are underlined, and residues conserved in GLUT1–GLUT5 are marked by asterisks above the sequence. The alignment was performed with the GAP program.

porter protein was found in plasma membranes (13000 ***g***) and high-density microsomes (45000 ***g***); these fractions were used for the subsequent assays.

Assay of cytochalasin B binding

Equilibrium cytochalasin B binding in membranes from transfected cells was assayed by a method established with fat-cell membranes [9] with modifications described in detail elsewhere [10]. Scatchard plots were evaluated graphically as described previously [11,12].

Reconstitution of glucose-transport activity from membrane fractions

Glucose-transporter protein was solubilized and reconstituted into lecithin liposomes as described previously [13,14]. Initial uptake rates of D-[U-^{14}C]glucose were assayed after 10 s at a substrate concentration of 1 or 5 mM. The data were corrected for non-carrier-mediated uptake with tracer L-[1-^{3}H]glucose.

RESULTS

Isolation of the GLUT9 cDNA

In order to identify unknown glucose-transporter-like sequences, we performed a search of the EST databases with the sequences of the known GLUT isoforms (tBLASTx program). Two murine sequence tags (GenBank accession numbers AA050622 and W80060) identified in this search exhibited significant similarity to the GLUT family, but differed from the known GLUT isoforms. Additional sequence information was obtained by RACE and PCR cloning. Further searches of the databases with the connected sequences revealed that they were identical with parts of a known genomic sequence (BAC clone HSAC1644, accession number AC001644). The similarity of the deduced amino acid sequence of GLUT9 to the recently identified GLUT8 was used to locate tentatively the translation start of the transporter on the genomic sequence. Thereafter, a cDNA fragment comprising the full coding region was isolated by reverse-transcriptase PCR with RNA from human leucocytes and primer oligonucleotides derived from the putative 5′ and 3′ untranslated regions; the sequence (from two independent PCR

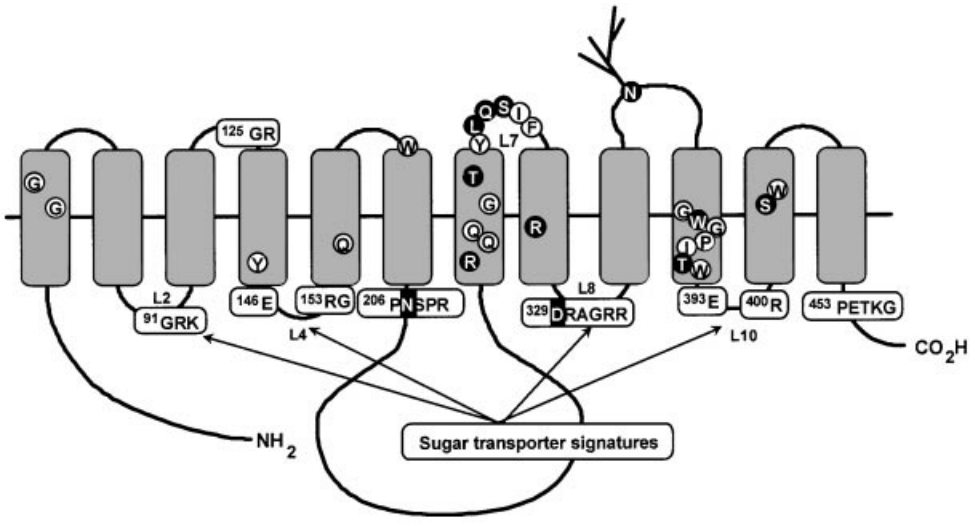

Figure 2 Putative membrane topology of GLUT9 and sequences of its sugar-transporter signatures

The model is based on structural predictions obtained with the HELIXMEM program, and is drawn according to that introduced by Mueckler et al. [1]. Residues of GLUT9 that correspond to the sugar-transporter signatures (determined by sequence comparisons of GLUT1–GLUT5) are highlighted. White letters on black background indicate substitutions of these residues within the GLUT9 sequence. The Figure depicts a presumed glycosylation site in loop (L) 9.

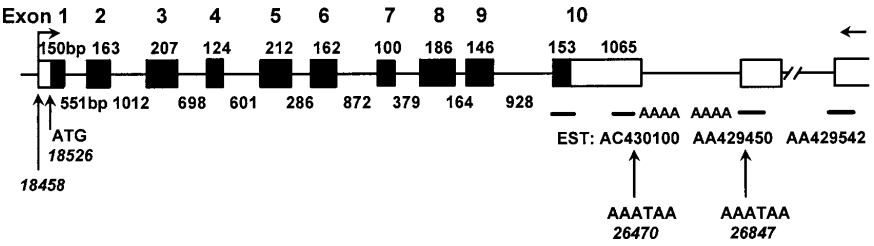

Figure 3 Genomic organization of the *GLUT9* gene

Exons are shown as black bars. ESTs mapping polyadenylation sites and the adjacent gene are given as accession numbers. Italic numbers depict the positions in the genomic clone HSAC1644.

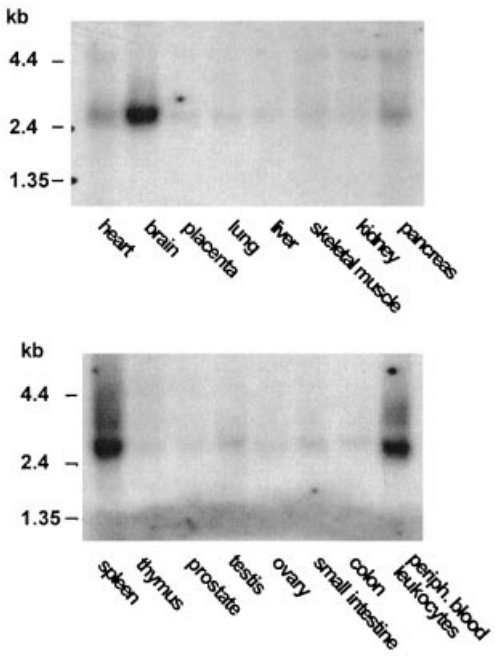

Figure 4 mRNA levels of GLUT9 in different human tissues

Northern blots with mRNA from the indicated tissues were hybridized as described with human GLUT9 cDNA.

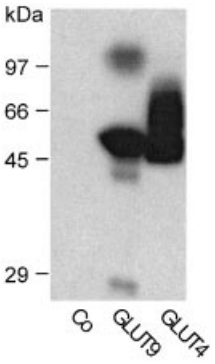

Figure 5 Expression of GLUT9 in COS-7 cells transfected with GLUT9 cDNA

COS-7 cells were transfected with blank vector (Co), human GLUT9 cDNA fused with a GLUT4 tag (12 codons of the GLUT4 C-terminus), or rat GLUT4 cDNA. Plasma membranes were prepared as described in the text, separated by SDS/PAGE and transferred on to nitrocellulose. GLUT4 and the tagged GLUT9 protein were detected immunochemically with antiserum against the C-terminus of the GLUT4.

clones) was 99.9% identical with the corresponding parts of the genomic sequence. The translation start in the cDNA was assigned to the first AUG following a stop codon; there is no alternative to this translation initiation site.

Sequence characteristics of GLUT9

The protein with the highest similarity with GLUT9 (Figure 1; 44.8% identical amino acids) was the transport facilitator GLUT8/GLUTX1, which was cloned recently [7,15]. Together with GLUT8, GLUT9 is located on a separate branch of the sugar-transporter family. The next relatives are the GLUT1 (28.5% identical residues), the yeast inositol transporter (26.4% identical residues) and the *Escherichia coli* arabinose and xylose transporters (28.4 and 25.7% identical amino acids, respectively).

Analysis of the sequences with the HELIXMEM program reveals the presence of 12 putative membrane-spanning helices, consistent with the presumed tertiary structure of a transport facilitator. The sequences contain all the motifs that are characteristic of the family of sugar transporters (Figure 2), in particular motifs corresponding with the PESPR/PETKGR motifs after helices 6 and 12, the GRR motifs in loops 2 and 8, and glutamate and arginine residues in the intracellular loops 4 and 10. Furthermore, tryptophan residues corresponding to Trp-388 and Trp-412 in GLUT1 are present. As in GLUT8, loop 1 in GLUT9 is short and lacks a glycosylation site; instead, a glycosylation site appears to be located in the larger loop 9. A striking difference from the other members of the GLUT family is the presence of two arginine residues in the helices 7 and 8, and the lack of the QLS motif [16] in helix 7.

Genomic organization of the *GLUT9* gene

The organization of the *GLUT9* gene was obtained by a comparison of the cDNA with the genomic sequence (BAC clone HSAC1644). As illustrated in Figure 3, the *GLUT9* gene is approx. 8 kb in size and consists of 10 exons separated by short introns. The transcription start was mapped to nucleotide 18458 of clone HSAC1644 with the aid of 5′-RACE products amplified from leucocyte cDNA. The translation start is defined by a stop codon 35 bp before the first AUG of the open reading frame,

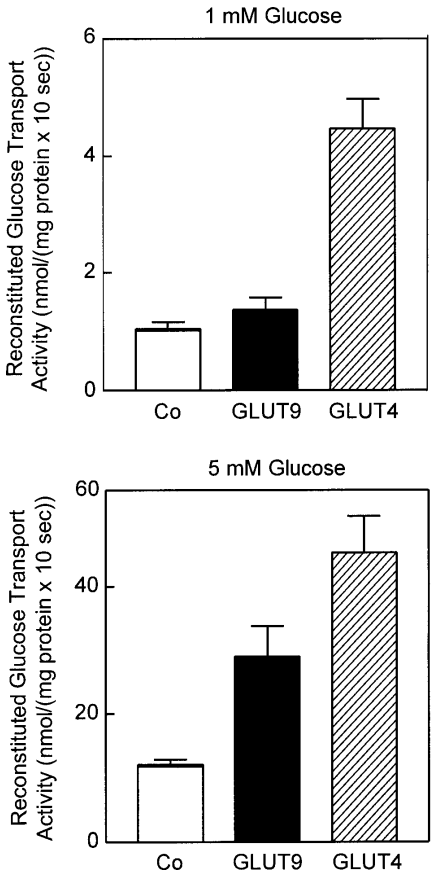

Figure 6 Glucose-transport activity reconstituted from membranes of cells transfected with GLUT9 cDNA

COS-7 cells were transfected with blank vector (Co), human GLUT9 or rat GLUT4 cDNA. Membrane fractions were prepared, and plasma membranes were assayed for reconstitutable glucose-transport activity as described in the Materials and methods section. The concentration of D-glucose in the transport assay was 1 (upper panel) or 5 mM (lower panel). The data represent means ± S.D. from four different reconstitution experiments with membranes from independent transfections, each assayed with triplicate samples.

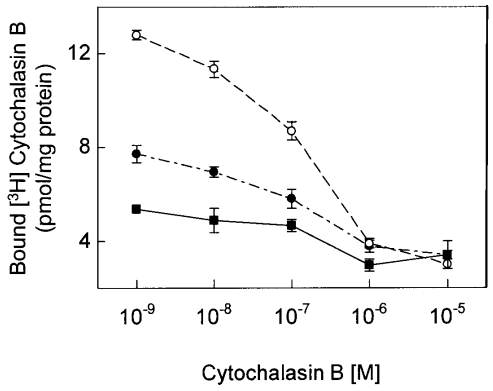

Figure 7 Specific binding of cytochalasin B to GLUT9 expressed in COS-7 cells

COS-7 cells were transfected with human GLUT9 (●), GLUT4 (○) or blank vector (■), and membranes were prepared and assayed for specific binding of [^{3}H]cytochalasin B. Data represent means ± S.D. from triplicate samples from a representative experiment. K_d values derived from the binding curves were 210 ± 30 nM for GLUT9 (three independent transfections) and 93.6 ± 28 nM for GLUT4 (eight transfections).

which starts in exon 1. The region flanking exon 1 contains a CAAT box 36 bp upstream from the transcription start, a GC-rich region and several consensus motifs for binding of lymphocyte-specific transcription factors (results not shown), consistent with the assumption that it may represent the promoter region. The 3′ end of the GLUT9 mRNA could be mapped with a polyadenylated 3′ EST (HS1229757); a polyadenylation signal (AAATAA) precedes the presumed polyadenylation site. With the aid of several ESTs corresponding to the 3′-flanking sequence of the *GLUT9*, the 3′-end of the 3′-neighbouring gene could be located 400 bp downstream of *GLUT9* (Figure 3).

Tissue distribution of GLUT9 mRNA

By Northern-blot analysis (Figure 4), mRNA of GLUT9 was exclusively found in brain and lymphoid tissue (spleen and peripheral leucocytes). The blots showed the presence of a single transcript with the expected size of 2.6 kb in these tissues.

Reconstituted glucose-transport activity and cytochalasin B binding in membranes from COS-7 cells transfected with GLUT9 cDNA

In order to show that a transporter-like protein is expressed after transfection of cells with GLUT9, we used a tagged construct of the GLUT9 cDNA. As illustrated in Figure 5, transfection of COS-7 cells with this construct produced the specific expression of a 48-kDa protein. A second specific band was detected at approx. 100 kDa. Since glucose transporters tend to aggregate even under denaturing conditions, this band might represent a homodimer of GLUT9. A similar band was observed after expression of the GLUT8 in COS-7 cells [7].

Membranes from cells transfected with GLUT9 cDNA (untagged construct) were solubilized, and proteins were reconstituted into lecithin liposomes for assay of their glucose-transport activity (Figure 6). At 5 mM glucose, overexpression of GLUT9 produced a 2–3-fold increase in D-glucose transport activity as compared with membranes from cells transfected with blank vector. In contrast, overexpression of GLUT9 produced a less than 2-fold increase when the reconstituted transport activity was assayed at 1 mM glucose. The overexpression of GLUT4 in parallel samples produced a larger effect at both glucose concentrations. It should be noted, however, that at present we have no reliable method to compare the abundance of the different transporters in the COS-7 cells. Thus the glucose-transport activities of GLUT9 and GLUT4 cannot be normalized with respect to molarity of transporter protein.

Membranes from transfected cells were incubated with [^{3}H]cytochalasin B and increasing concentrations of unlabelled agent in order to determine specific binding of this ligand. As is illustrated in Figure 7, overexpression of GLUT9 produced a significant increase in specific binding of cytochalasin B. The K_d value derived from Scatchard plots of the binding curves (results not shown) was 210 ± 30 nM (mean ± S.D. from three independent transfections) and is significantly higher than the K_d value of GLUT4 (93.6 ± 28 nM, mean ± S.D. of eight independent transfections).

DISCUSSION

The sequence of GLUT9 presents all the elements (glucose-transporter signatures) characteristic of the GLUT family and

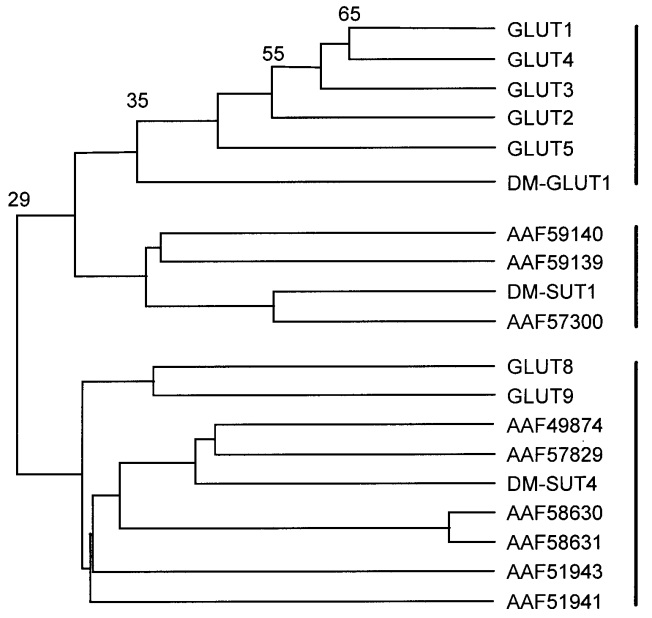

Figure 8 Dendrogram of a multiple alignment of the mammalian and *Drosophila* sugar transporters

The *Drosophila melanogaster* proteins with the highest similarity to the human GLUT1 were identified with a BLASTp search, and an alignment was performed with the CLUSTAL program (open gap cost 10, unit gap cost 10). Numbers at the branches of the tree indicate percentage identity. GLUT proteins represent the human isoforms. AAF codes, DM-GLUT1, DM-SUT1 and DM-SUT4 represent *Drosophila* proteins.

which are required for their function as sugar transporters. Specifically, the sequence predicts a tertiary structure that includes 12 membrane-spanning helices [1]. In addition, it presents conserved residues that have previously been shown to be essential for transporter function in mutagenesis experiments. These include several conserved arginine and glutamate residues on the cytoplasmic surface [10], the tryptophan residue in helix 11 corresponding to Trp-412 in GLUT1 [17,18], tyrosine residues in helices 4 and 7 corresponding to Tyr-146 and Tyr-292/293 [19,20], and glutamines in helices 5 and 7 corresponding to Gln-161 and Gln-282 in GLUT1 [21,22]. Thus the gene represents a novel member of the family of sugar-transport facilitators and has been designated accordingly.

The present data indicate that the GLUT9 protein has glucose-transport activity. This finding is consistent with the previously shown glucose-transport activity of its closest relative, GLUT8/GLUTX1 [7,15]. However, it should be noted that GLUT8 and GLUT9 form a separate branch of the GLUT family (Figure 8), and exhibit several marked differences from GLUT1–GLUT5. Most strikingly, two arginine residues are present in helices 7 and 8 of the GLUT9, a region that has been thought to determine the substrate specificity of the transport facilitators [3,16]. Interestingly, the renal anion transporter harbours arginines at a similar position to GLUT9 in putative transmembrane helices 7 and 8 [23]. Thus it might be speculated that the GLUT9 is a sugar anion transporter, and further experiments are needed to define the exact substrate specificity of this novel transport facilitator.

Another striking difference between GLUT9 and GLUT1–GLUT5 is the lack of a glycosylation site in its outer loop 1, and the presence of a putative glycosylation site in loop 9. Like the GLUT8, GLUT9 harbours a dileucine motif in its N-terminus. The STS motif that is present in the outer loop 7 of GLUT1-4 is exchanged for AET; this motif has previously been suggested to be important for the conformational alteration during the transport process [24]. Furthermore, glucose-transport activity of GLUT9 transfected into COS-7 cells was detectable against the background of constitutive GLUT1 at 5 mM rather than at 1 mM glucose. This finding suggests that GLUT9 transports glucose with a high K_m, but needs re-evaluation in a different expression system. Our finding that GLUT9 binds cytochalasin B with low affinity is consistent with the assumption that it is a high-K_m transporter, given that low-affinity ligand binding is correlated with high-K_m glucose transport, as is the case for the GLUT2 [25,26].

A comparison of the human and *Drosophila* glucose-transporter-like sequences indicates that GLUT8 and GLUT9, together with seven *Drosophila* sequences, are located on a separate branch of the dendrogram (Figure 8). So far, the mammalian GLUT family comprises nine members [GLUT1–5, 8 and 9, and the unpublished sequences GLUT10 and GLUT11 (H. Doege, A. Bocianski, H.-G. Joost and A. Schürmann, unpublished work)]. In the *Drosophila* genome, there are at least 12 genes showing significant similarity ($> 29\%$ identical amino acids) to the GLUT family. Alignment of these sequences indicates that the GLUT family may be divided into three branches (Figure 8). It remains to be shown that this distinction reflects diverse substrate specificities and functions of the family members.

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Received 30 May 2000; accepted 11 July 2000