Sequence Determinants of GLUT1-mediated Accelerated-exchange Transport

ANALYSIS BY HOMOLOGY-SCANNING MUTAGENESIS

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Background: The human equilibrative glucose transporter GLUT1 catalyzes accelerated-exchange transport, but the related isof orm GLUT4 does not.

Results: Substitution of GLUT1 transmembrane helix 6 into GLUT4 permits GLUT4 to catalyze accelerated-exchange, whereas GLUT4 helix 6 prevents accelerated-exchange in GLUT1.

Conclusion: Transmembrane helix 6 sequence determines accelerated-exchange behavior.

Significance: Transmembrane helices external to the transport pathway influence transport behavior.

The class 1 equilibrative glucose transporters GLUT1 and GLUT4 are structurally similar but catalyze distinct modes of transport. GLUT1 exhibits trans-acceleration, in which the presence of intracellular sugar stimulates the rate of unidirectional sugar uptake. GLUT4-mediated uptake is unaffected by intracellular sugar. Using homology-scanning mutagenesis in which domains of GLUT1 are substituted with equivalent domains from GLUT4 and vice versa, we show that GLUT1 transmembrane domain 6 is both necessary and sufficient for trans-acceleration. This region is not directly involved in GLUT1 binding of substrate or inhibitors. Rather, transmembrane domain 6 is part of two putative scaffold domains, which coordinate membrane-spanning amphipathic helices that form the sugar translocation pore. We propose that GLUT1 transmembrane domain 6 restrains import when intracellular sugar is absent by slowing transport-associated conformational changes.

The GLUT family of glucose transporters catalyzes tissue-specific facilitative monosaccharide transport in mammalian cells (1). GLUT1 mediates sugar uptake in red blood cells, smooth muscle, and across blood-tissue barriers (2–4). GLUT4 is expressed in adipose tissue, skeletal, and cardiac muscle (5), where it is responsible for insulin-stimulated sugar uptake (6, 7).

Although GLUTs 1 and 4 exhibit similar affinities for substrates and antagonists (8, 9), their catalytic behaviors are very different. GLUT4 displays kinetic symmetry (V_{\text{max}} and K_m for net sugar uptake are indistinguishable from the corresponding parameters for net exit (10)), whereas GLUT1 kinetics are asymmetric (V_{\text{max}} and K_m for net sugar uptake are significantly lower than the corresponding parameters for net exit (11)). In addition, GLUT1 displays a behavior termed trans-acceleration, whereas GLUT4 does not (10, 12–15). Trans-acceleration (also called accelerated-exchange transport) occurs when unidirectional uptake of sugar is stimulated by the presence of intracellular sugar or, conversely, when unidirectional exit of sugar is stimulated by the presence of extracellular sugar (16). Trans-acceleration may provide a metabolic advantage to the cell because it results in a more rapid equilibration of the cytoplasm with extracellular sugar.

Trans-acceleration is one of several behaviors that distinguishes carrier-mediated from channel-mediated facilitative diffusion systems (17, 18), but the physical basis of accelerated-exchange transport is unknown. Comparative analysis of GLUT1 and GLUT4 may, therefore, permit definition of the sequence determinants and thereby the physical basis of trans-acceleration.

GLUTs 1 and 4 are structurally similar, containing cytoplasmic N and C termini, 12 transmembrane spanning a-helices (TM), and a large intracellular loop connecting TMs 6 and 7 (19–21). In the absence of GLUT crystal structures, our understanding of GLUT1 tertiary structure derives largely from scanning cysteine mutagenesis (22–25) and modeling studies (26, 27), which align and thread the GLUT1 sequence through the crystal structures of Major Facilitator Superfamily bacterial transporter homologs GlnT (28) and LacY (29). Although these homology-based threaded structures provide quite accurate descriptions of transporter topography and helix packing arrangements, they fail to accurately predict helix and amino acid side chain orientation within the active sites (30).

Some functional domains of GLUT1 have been mapped at low resolution. These include components of the GLUT1 kinetic asymmetry and transacceleration diminish but persist as temperature is raised from 4 to 37 °C.

A. Carruthers, unpublished data.

The abbreviations used are: TM, membrane spanning alpha helix; 2-DG, 2-deoxy-D-glucose; 2-DG-6-P, 2-deoxy-D-glucose 6-phosphate; 3-MG, 3-O-methylglucose; GLUT, glucose transport protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; DPBS, Dulbecco’s phosphate-buffered saline; HE, hetero-exchange; ZT, zero-trans.
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nucleotide binding domain (31–33), substrate binding sites (24, 34), inhibitor binding sites (35, 36), allosteric modulation sites (37–39) and oligomerization domains (40). However, detailed structures of these domains and the conformational changes associated with transport are not yet available. In addition, analogous modeling studies have yet to be extended to GLUT4. Thus the available data do not yet provide an explanation for substrate binding and translocation by GLUTs 1 and 4, or why GLUT1 catalyzes trans-acceleration but GLUT4 does not.

This study attempts to investigate the determinants of transporter function using homology-scanning mutagenesis of structurally related but functionally different members of a transporter family. We engineered GLUT1 and GLUT4 chimeras in which we substituted progressively smaller domains of one transporter by the corresponding domains of the other transporter. These chimeras exhibit sequence-dependent trans-acceleration gain- or loss-of-function.

We observe trans-acceleration in GLUT1-transfected HEK cells but not in cells transfected with human GLUT4. Homology-scanning mutagenesis reveals that TM6 of GLUT1 is both necessary and sufficient to confer trans-acceleration to the GLUT4 scaffold. Similarly, the replacement of GLUT1 TM6 with the corresponding region in GLUT4 ablates trans-acceleration in the GLUT1 scaffold.

These results confirm that trans-acceleration is sequence-dependent, requiring a motif within the putative scaffold region of GLUT1, rather than in the translocation pore-forming region of the protein. The implications of our findings are discussed in the context of the prevailing models for GLUT-mediated sugar transport.

EXPERIMENTAL PROCEDURES

Materials—[3H]2-Deoxy-d-glucose was purchased from MP Biomedical. HEK-293 cells were purchased from ATCC. DMEM, DPBS, penicillin/streptomycin, Lipofectamine 2000, DH5α-Subcloning cells, PCDNA 3.1(+) mammalian expression vector, BisTris gels, and MES buffer were obtained from Invitrogen. All restriction enzymes and associated buffers were obtained from New England Biolabs. All primers were purchased from Integrated DNA Technologies. Herculase polymerase, XL1-Blue Competent cells, and QuickChange Multisite-directed Mutagenesis kits were obtained from Stratagene. RNaseay, Qiashredder, One-Step RT-PCR, MinElute Gel Purification, PCR Purification, and HiSpeed Maxi kits were from Qiagen. iScript One-Step PCR kit was from Bio-Rad. PVDF membranes were obtained from ThermoFisher. 10% Bovine serum albumin was from American Bioanalytical. SuperSignal Pico West, NeutrAvidin Gel, micro-BCA kits, spin columns, and EZ-Link Sulfo-NHS-SS-Biotin were from Pierce. Protease inhibitor mixture tablets were from Bio-Rad. Polyacrylamide gel electrophoresis was performed using BisTris gels and MES buffer. All restriction enzymes and associated buffers were obtained from New England Biolabs. All point mutations and amino acid substitutions were engineered using QuikChange Mutagenesis kits and verified by sequencing.

Quantitative and End Point Reverse Transcriptase-PCR—Total RNA was isolated from HEK cells using the RNaseay kit and Qiashredder. End point RT-PCR was performed as per the One-Step RT-PCR kit instructions using GLUT-specific primers. RT-PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. Expression levels of detected GLUTs were measured by quantitative RT-PCR using the iScript One-Step PCR kit with SYBR Green. Samples were run in duplicate on an MJ Research PTC-200 Peltier Thermal Cycler with a Chromo4 real time-PCR detector running Opticon Monitor 3 software (Bio-Rad). Results were analyzed by

5. B. Levine, J. K. DeZutter, and A. Carruthers, unpublished observations.
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using the ∆ΔCt method (43) and normalized to a GAPDH control.

Transient Transfection—Cells (70–90% confluence) were transfected with 2 μg of DNA per well (12 well plates) or 5 μg of DNA per well (6 well plates), unless otherwise specified. Transfections were performed 36–48 h prior to analysis of sugar uptake or protein expression.

Western Blotting—Cells were pelleted, washed with DPBS, lysed in cell lysis buffer, and protein concentration was assessed using a micro-BCA kit. Lysates were normalized for total protein concentration and resolved by SDS-PAGE on a 10% BisTris gel in MES buffer. Gels were transferred onto PVDF membranes, blocked with 10% bovine serum albumin in TBS-T, probed with primary antibody overnight at 4 °C, probed with secondary antibody for 1 h at room temperature, and developed using SuperSignal Pico West Chemiluminescent substrate. Blots were imaged on a FujiFilm LAS-3000 and relative band densities were quantitated using ImageJ software.

Biotinylation—6-Well plates of HEK cells were washed twice with ice-cold DPBS and incubated on ice with ice-cold DPBS containing 5 mM EZ-Link Sulfo-NHS-SS-Biotin for 30 min with gentle rocking. Reactions were quenched by adjusting each well to 12.5 mM Trizma (Tris base). Cells were harvested, re-suspended in biotin lysis buffer, and lysates were bound to Neutavidin Gel in spin columns according to kit instructions. Protein concentration was determined spectrophotometrically, and normalized loads were analyzed by Western blot as described above.

2-Deoxy-d-glucose Sugar Uptake—2-Deoxy-d-glucose (2-DG) is an analog of the natural GLUT1 substrate, d-glucose. At physiologic temperature, cytoplasmic 2-DG is phosphorylated by hexokinase to form 2-deoxy-D-glucose 6-phosphate (2-DG-6-P), which is neither metabolized further nor is a GLUT1 substrate (44, 45). Imported [3H]2-DG is therefore trapped within the cell as [3H]2-DG-6-P.

2-DG uptake was measured as described previously (42). Briefly, 36–48 h post-transfection, 12-well plates of confluent HEK-293 cells were serum- and glucose-starved for 2 h at 37 °C in FBS- and penicillin/streptomycin-free DMEM lacking glucose. Cells were washed with 0.5 ml of DPBS-Mg at 37 °C, then exposed to 0.5 ml of [3H]2-DG uptake solution for 0 to 30 min at 37 °C. Uptake was stopped by addition of 1 ml of ice-cold stop solution. Cells were washed twice with ice-cold stop solution and extracted with Triton X-100. Total protein concentration was determined by using the micro-BCA kit. Lysates were normalized for total protein concentration and resolved by SDS-PAGE on a 10% BisTris gel in MES buffer. Gels were transferred onto PVDF membranes, blocked with 10% bovine serum albumin in TBS-T, probed with primary antibody overnight at 4 °C, probed with secondary antibody for 1 h at room temperature, and developed using SuperSignal Pico West Chemiluminescent substrate. Blots were imaged on a FujiFilm LAS-3000 and relative band densities were quantitated using ImageJ software.

RESULTS

GLUT1 and GLUT4 Chimeras—To identify GLUT1 domain(s) required for trans-acceleration, we swapped specific trans-membrane regions of GLUT1 with an equivalent GLUT4 sequence. This allows us to map the involvement of large regions of the transporter in trans-acceleration and thereby narrow our focus to smaller subdomains (Fig. 1). Chimera nomenclature divides the GLUTs into 4 sets of three contiguous TMs (1–3, 4–6, 7–9, 10–12). A chimera comprising the first half of GLUT1 plus the second half of GLUT4 is termed “1144,” GLUT1 TMs 1–3 and 4–6 plus GLUT4 TMs 7–9 and 10–12. If loop 6 linking TMs 6 and 7 is the focus, this is indicated in parentheses. Thus, 44(1)11 is GLUT4 TMs 1–6, GLUT1 loop 6, and GLUT1 TMs 7–12. 1411 is GLUT1 TMs 1–3 plus GLUT4 TMs 4–6 plus GLUT1 loop 6 and TMs 7–12 (Fig. 1B). Mutations involving only 1 or 2 TMs list the scaffold GLUT with substitutions from the other GLUT in parentheses, e.g. GLUT4(5,6 G1) is GLUT4 containing GLUT1 TMs 5–6.

Trans-acceleration in HEK Cells—HEK-293 cells were selected for heterologous expression of GLUT1, GLUT4, and GLUT1-Glut4 chimeras because of their very low endogenous expression of human GLUT1 and 4, as determined by quantitative PCR (Fig. 2A). Net uptake of 100 μM 2-DG from
medium containing 40 mM 3-MG increases linearly with time (0–10 min) in GLUT1-transfected cells (Fig. 2B). The experiments reported in this study employ a 5-min uptake interval, which provides an ample range of linearity to detect a 2–3-fold increase in 2-DG uptake during hetero-exchange transport catalyzed by transfected GLUTs. To characterize differences in sugar uptake in response to intracellular sugar, [3H]2-DG uptake from medium containing 40 mM 3-MG was measured in GLUT1- or GLUT4-transfected HEK cells pre-loaded with 0 to 40 mM 3-MG (Fig. 3A). GLUT1-transfected HEK cells show a dose-dependent stimulation of 2-DG uptake with increasing intracellular 3-MG, whereas GLUT4-transfected HEK cells do not. This confirms that human GLUT1 displays trans-acceleration at 37 °C, whereas human GLUT4 does not. GLUT1-mediated 2-DG uptake increases in a saturable manner with [3-MG], showing a maximal stimulation (E_{max}) of 1.72 ± 0.02-fold with 50% stimulation (K_i) at 25.6 ± 1.5 mM 3-MG. 40 mM 3-MG, was used in all further hetero-exchange experiments.

Modification of GLUT4 to Increase Surface Expression—The pre-loading experiment (Fig. 3A) indicates that zero-trans 2-DG uptake in GLUT4myc-transfected HEK cells is significantly slower than uptake in GLUT1myc-transfected cells. Total protein expression levels appear similar by Western blot (Fig. 3B), suggesting either that significantly less GLUT4Myc is expressed at the cell surface or that GLUT4Myc has lower intrinsic activity (k_cat) than GLUT1Myc. We show below that cell surface protein biotinylation analysis indicates that GLUT4Myc surface expression is less than half of GLUT1Myc.

GLUT4Myc surface expression was improved by engineering 3 GLUT4 mutations known to affect surface expression in a variety of cell types. GLUT4 N and C termini contain internalization (48, 49) and surface targeting (50, 51) motifs. GLUT4Myc mutations F5A and L489A/L490A were tested individually and together in assays of 100 μM 2-DG uptake under zero-trans conditions (Fig. 3C). In these experiments, 2-DG uptake by GLUT4Myc is only one-quarter of that catalyzed by GLUT1Myc. 2-DG uptake by GLUT4Myc F5A and GLUT4Myc L489A/L490A approaches 80% of GLUT1Myc-mediated uptake. The triple mutant (GLUT4Myc F5A/L489A/L490A) catalyzes a level of 2-DG uptake indistinguishable from that of GLUT1Myc. This mutant (GLUT4Myc-3x) was used as the GLUT4 scaffold in all further mutational analysis. GLUT1Myc typically displays a 1.8 ± 0.15-fold stimulation of sugar uptake under hetero-exchange conditions (Table 1). However, both GLUT4Myc and GLUT4Myc-3x display no trans-acceleration (Fig. 3D). This confirms that the loop 1 exofacial Myc tag and the cell surface expression mutations introduced into GLUT4 do not significantly perturb wild-type exchange-transport behavior.
Transport Rates Are Proportional to Cell Surface GLUT Expression — The absolute rate of GLUT1-mediated zero-trans (ZT) and hetero-exchange 2-DG uptake is proportional to the amount of transporter at the cell surface. However, transport behavior (GLUT1-mediated trans-acceleration) is unaffected by expression level. To illustrate this, HEK cells were transfected with a range of GLUT1myc DNA, and the relationship between cell surface GLUT1Myc expression and GLUT1Myc-mediated zero-trans and hetero-exchange transported was investigated (Fig. 4). Surface expression was quantitated by biotinylation of cell surface protein at 4 °C followed by membrane solubilization, streptavidin affinity purification of labeled proteins, and quantitation of their GLUT1Myc content by immunoblot analysis using α-Myc antibody. Although the relationship between cell surface expression and GLUT1Myc-dependent zero-trans or hetero-exchange 2-DG uptake is linear (Fig. 4), the nearly 2-fold increase in hetero-exchange over zero-trans uptake rates remains constant at every level of cell surface GLUT1Myc observed. We show below that whereas GLUT4 and its engineered variants achieve differing cell surface expression levels, their inability to catalyze trans-acceleration is independent of expression level (see Figs. 3D and 6).

Trans-acceleration (or lack thereof) is therefore independent of the amount of transporter expressed at the cell surface and is an intrinsic property of the transport protein. This is not unexpected. Endothelial cell GLUT1-mediated zero-trans and accelerated exchange 3-MG uptake (the latter being twice as fast as zero-trans uptake) are both doubled when endothelial cell surface [GLUT1] is doubled by acute metabolic stress (21, 52). Rat erythrocytes express 1,000-fold less GLUT1 than do...
human erythrocytes, but both cells display accelerated-exchange sugar transport (53, 54). Rat adipocyte GLUT4-mediated zero-trans and equilibrium exchange 3-MG uptake are both increased ~12-fold by insulin-induced GLUT4 recruitment to the cell surface, but the characteristic GLUT4 kinetic behavior (lack of trans-acceleration) is unchanged (10). Provided that heterologous expression of the transporter is sufficient to measure its function over background, parental transport, the kinetic behavior of the GLUTs (trans-acceleration or lack of trans-acceleration) is independent of cell surface expression levels. The measurement of some kinetic constants, such as \( k_{\text{cat}} \), does require specific knowledge of cell surface expression (see below).

**Analysis of Half- and Quarter-Protein Domain Chimeras for Trans-acceleration**—Zero-trans and hetero-exchange 2-DG transport were measured in HEK cells transfected with either GLUT1Myc or the half-protein domain chimeras containing GLUT1 loop 6 (11(1)44 and 44(1)11). GLUT1 TMs 7–12 are not important for trans-acceleration (Table 1). Although 2-DG uptake by GLUT1Myc and 11(1)44 is increased under hetero-exchange conditions, transport catalyzed by 44(1)11 is not (Table 1). To ascertain whether loop 6 sequence is critical, we tested an analogous set of half-domain chimeras containing the GLUT4 sequence of loop 6 (Table 1). 2-DG uptake by 44(1)11 shows trans-acceleration, whereas uptake by 11(1)44 does not (Table 1). These data show that the isoform-specific sequence in TMs 1–3 is not essential for trans-acceleration.

**Table 1**

<table>
<thead>
<tr>
<th>Chimeraa</th>
<th>Residuesb</th>
<th>ZT uptakec</th>
<th>Fold-stimulation during hetero-exchange (HE)</th>
<th>HE/ZTd</th>
<th>Trans-acceleratione</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1Myc</td>
<td>1–492</td>
<td>117.2 ± 14.9</td>
<td>1.80 ± 0.15</td>
<td>Y</td>
<td>p ≤ 0.001</td>
</tr>
<tr>
<td>GLUT4Myc</td>
<td>1–509</td>
<td>42.0 ± 6.8</td>
<td>0.96 ± 0.08</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>GLUT4Myc-3x</td>
<td>1–509</td>
<td>77.4 ± 7.4</td>
<td>1.04 ± 0.45</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>44(1)11</td>
<td>G1 1–223</td>
<td>35.4 ± 4.6</td>
<td>0.94 ± 0.17</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>11(1)44</td>
<td>G1 1–266</td>
<td>66.4 ± 7.4</td>
<td>1.90 ± 0.21</td>
<td>Y</td>
<td>p ≤ 0.001</td>
</tr>
<tr>
<td>44(4)11</td>
<td>G1 1–282</td>
<td>40.3 ± 9.0</td>
<td>0.96 ± 0.18</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>11(4)44</td>
<td>G1 1–207</td>
<td>35.3 ± 7.3</td>
<td>2.00 ± 0.31</td>
<td>Y</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>1444</td>
<td>G1 1–119</td>
<td>103.0 ± 7.0</td>
<td>1.10 ± 0.11</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4111</td>
<td>G1 1–135</td>
<td>47.3 ± 5.9</td>
<td>1.70 ± 0.21</td>
<td>Y</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>1411</td>
<td>G1 1–135; 208–492</td>
<td>146.1 ± 10.2</td>
<td>1.00 ± 0.13</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4144</td>
<td>G1 1–135; 224–509</td>
<td>61.6 ± 8.1</td>
<td>1.80 ± 0.09</td>
<td>Y</td>
<td>p ≤ 0.00001</td>
</tr>
<tr>
<td>GLUT4Myc (4,5 G1)</td>
<td>G4 1–135; 203–509</td>
<td>60.1 ± 4.5</td>
<td>0.65 ± 0.15</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>GLUT4Myc (5,6 G1)</td>
<td>G4 1–166; 224–509</td>
<td>38.1 ± 3.9</td>
<td>1.90 ± 0.14</td>
<td>Y</td>
<td>p ≤ 0.00001</td>
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<tr>
<td>GLUT4Myc (5, G1)</td>
<td>G4 1–166; 203–509</td>
<td>53.2 ± 5.7</td>
<td>0.57 ± 0.09</td>
<td>N</td>
<td></td>
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<tr>
<td>GLUT4Myc (6, G1)</td>
<td>G4 1–203; 224–509</td>
<td>25.8 ± 6.2</td>
<td>1.80 ± 0.23</td>
<td>Y</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>GLUT1Myc (6, G4)</td>
<td>G1 1–186; 208–492</td>
<td>168.3 ± 12.5</td>
<td>1.10 ± 0.11</td>
<td>N</td>
<td></td>
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<tr>
<td>GLUT1Myc SII FI 191–195 GLTVL</td>
<td>G4 1–203–223</td>
<td>162.4 ± 7.9</td>
<td>1.30 ± 0.05</td>
<td>Y</td>
<td>p ≤ 0.001</td>
</tr>
<tr>
<td>GLUT1Myc CIV 202–204 LVL</td>
<td>G4 1–208–212</td>
<td>64.1 ± 7.2</td>
<td>2.20 ± 0.18</td>
<td>Y</td>
<td>p ≤ 0.00001</td>
</tr>
<tr>
<td>4404 M yc GLTVL 208–212 SII FI</td>
<td>G4 1–207; 213–509</td>
<td>55.8 ± 4.9</td>
<td>0.59 ± 0.09</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>GLUT4Myc LVL 218–220 CIV</td>
<td>G4 1–217; 221–509</td>
<td>92.1 ± 5.1</td>
<td>0.97 ± 0.05</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

a The chimeras employed in this study were constructed using two backbones: GLUT1Myc (wt GLUT1 residues 1–492 with a c-Myc epitope [EQKLISEEDL] inserted between residues 55 and 56) and GLUT4Myc-3x (wt GLUT4 residues 1–509 in which Phe-5, Leu-489, and Leu-490 is each mutagenized to Ala, and where a c-Myc epitope is inserted c-Myc epitope.

b The sequence composition of chimeras is described as fusions of GLUT1Myc (G1) and GLUT4Myc-3x (G4) sequence in which G1 and G4 sequence numbering ignores the inserted c-Myc sequence. Chimera nomenclature is described under “Results” and in the legend to Fig. 1.

c ZT of 100 μM 2-DG (fmo1/μg of protein/min) from medium containing 40 mM 3-MG was measured in transfected HEK cells depleted of intracellular sugar. Values are reported as mean ± S.E. for a minimum of 30 assays. The range observed for HE:ZT was 1.48 ± 0.11 to 2.3 ± 0.31.

d Stimulation of 2-DG uptake observed under hetero-exchange conditions (extra- and intracellular [3-MG] = 40 μM) was determined as the ratio of hetero-exchange (HE) 2-DG uptake to ZT uptake (fmo1/μg/min). Values are reported as mean ± S.E. for a minimum of 30 assays.

e Trans-acceleration is absent (N) when HE:ZT is not significantly greater than 1. Trans-acceleration is present (Y) when HE:ZT is significantly greater than 1. Significance was determined using an unpaired, two-tailed Student’s t test.

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Zero-trans and hetero-exchange 2-DG uptake by GLUT1Myc-GLUT4Myc chimeras was measured for GLUT1Myc in every assay. This table reports the GLUT1Myc data as a global mean ± S.E. for a minimum of 30 assays. The range observed in these assays for zero-trans uptake was 39.2 ± 5.36 to 185 ± 18.8 fmol/μg/min. The range observed for HE:ZT was 1.48 ± 0.11 to 2.3 ± 0.31.
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FIGURE 4. The effect of GLUT1Myc cell surface expression on transport rates and hetero-exchange transport. A. HEK cells were transfected with varying [GLUT1Myc DNA]. Two days later, cell surface proteins were solubilized, and affinity purified on streptavidin beads, and GLUT1Myc was detected by immunoblot analysis using either α-Myc Ab or α-Ct Ab. As a loading control, the α-subunit of the Na,K-ATPase was detected using α-Na,K-ATPase Ab. The mobility of molecular weight standards is indicated. The amount of GLUT1Myc DNA added at transfection is shown above the blots. B, data obtained in the above experiment and from two similar experiments were analyzed by densitometry, background corrected, normalized to loading controls, and averaged. Ordinate, relative cell surface (GLUT1); abscissa, μg of DNA added at transfection. The curve is a section of a single rectangular hyperbola characterized by Kₐₐ = 0.47 ± 0.13 μg of DNA; maximum expression = 1.45 ± 0.12 with expression normalized to unity at 1 μg of DNA. C, rate of GLUT1Myc-catalyzed zero-trans (●) and hetero-exchange (○) 2-DG uptake as a function of cell surface [GLUT1Myc] as detected by cell surface biotinylation. Cells were transfected with GLUT1Myc DNA as described in the legend to Fig. 4A, and measurements of ZT and HE 2-DG uptake or cell surface [GLUT1Myc] were made in triplicate on 3 separate occasions. Uptake measured in mock-transfected cells was subtracted. Results are shown as mean ± S.E. The lines drawn through the points were computed by the method of least squares and have the following parameters: ZT, slope = 43.7 ± 2.8 fmol/μg/min/unit biotinylation, y intercept = 2.6 ± 0.1 fmol/μg/min, R² = 0.98; hetero-exchange, slope = 82.8 ± 8.5 fmol/μg/min/unit biotinylation, y intercept = 8.8 ± 15.3 fmol/μg/min, R² = 0.96.

Analysis of GLUT4Myc-3x scaffold (4144) produces a gain-of-function chimera characterized by robust trans-acceleration (Table 1).

Analysis of TMs 4 – 6 —We next examined paired TM substitutions in TMs 4 – 6. We tested for gain-of-function in GLUT4Myc-3x containing either GLUT1 TMs 4 – 5 (GLUT4 (4,5 G1)) or TMs 5 – 6 (GLUT4 (5,6 G1); Table 1). Our results show that GLUT4 (4,5 G1) does not show exchange stimulation. However, GLUT4 (5,6 G1) displays trans-acceleration gain-of-function. This result indicates that TMs 5 – 6 are required for trans-acceleration. Because TM5 is also present in the TMs 4 – 5 chimera, these data suggest either that TM6 alone is required for trans-acceleration, or TM6 in combination with TM5 is required. Indeed, when we substitute GLUT1 TM6 into GLUT4Myc-3x (GLUT4 (6, G1)), we observe a trans-acceleration gain-of-function (Table 1). Conversely, GLUT1 (6, G4) displays a trans-acceleration loss-of-function, indicating that GLUT4 TM6 cannot substitute for GLUT1 TM6. GLUT4 (5, G1) does not show trans-acceleration, indicating that GLUT1 TM5 alone is insufficient to produce trans-acceleration in GLUT4 (Table 1). Taken together, these data confirm that GLUT1 TM6 is both necessary and sufficient for trans-acceleration.

Analysis of Transmembrane Domain 6-Amino Acid Substitutions —Sequence alignment of GLUT1 and GLUT4 TM6 reveals two regions of sequence disparity (Fig. 5). Region A comprises GLUT1 SIIFI(191–195), corresponding to GLUT4 GLTVL(208–212). Region B is GLUT1 CIV(202–204), corresponding to GLUT4 LVL(218–220). We chose to exchange amino acids in Regions A or B between GLUT1 and GLUT4. We observe that neither Region A nor B of the GLUT1 sequence confers trans-acceleration when individually substituted into GLUT4 (Table 1). Similarly, substitution of either Region A or B of GLUT4 into GLUT1 does not produce a loss-of-function (Table 1). These results suggest that all or some of the 8 disparate amino acids within TM6 are required for trans-acceleration.

Analysis of kₐₐ/Kₐ for Wild-type and TM6 GLUT1 and GLUT4 Mutants—Despite the use of GLUT4Myc-3x to increase GLUT4 surface expression, there remains consistently lower levels of 2-DG transport among the GLUT4-based chimeras (Table 1). This may be related in part to protein stability, as we observe similar amounts of message for transfected constructs but different protein expression levels (data not shown). Due to these differences, we measured relative surface GLUT expression by cell-surface biotinylation and used this value to scale zero-trans uptake rates for constructs of interest.

Streptavidin pulldowns of biotinylated cell surface proteins confirm the presence of transfected Myc-tagged GLUTs (Fig. 6A). The identity of each Myc-tagged transporter was verified by either anti-GLUT1 or anti-GLUT4 antibodies (data not shown). Quantitation reveals that GLUT4Myc surface expression is 42 ± 2% relative to GLUT1Myc expression, whereas surface expression of GLUT4Myc-3x is only slightly improved (55 ± 18%). GLUT1 (6, G4) shows comparable surface expression to GLUT1Myc (94 ± 29%), whereas GLUT4 (6, G1) achieves only 17 ± 3% of the GLUT1Myc level. Scaling the measured zero-trans uptake rate by relative surface expression allows us to compare differences in catalytic activity (Fig. 6B).
Adjusted rates of zero-trans uptake by GLUT1Myc, GLUT4Myc, and GLUT4Myc-3x are similar (Fig. 6B). However, the trans-acceleration loss-of-function GLUT1 chimera GLUT1 (6, G4) has an adjusted rate that is 1.5-fold greater than GLUT1Myc. In contrast, the gain-of-function mutant, GLUT4 (6, G1), has an adjusted zero-trans rate that is lower than both wt GLUT4Myc and GLUT4Myc-3x.

**DISCUSSION**

Using homology-scanning mutagenesis, we demonstrate that GLUT1 TM6 is both necessary and sufficient to confer a trans-acceleration gain-of-function to the GLUT4 scaffold. Conversely, substituting GLUT4 TM6 into the GLUT1 scaffold ablates trans-acceleration. These results establish that trans-acceleration is intrinsic to GLUT1 sequence, and is not due to modulating co-factors or other cellular contexts. Although GLUT1 and GLUT4 TM6 differ by a total of 8 amino acids in two subregions, homology substitution of either region alone

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**FIGURE 5.** Sequence alignment and conservation of TM6 in GLUTs 1 and 4. A, sequence alignment of the GLUT1 TM6 subdomains critical for trans-acceleration (cyan background) in 18 mammals (amino acids 191–195PALLQ201–203). B, WebLogo plot of this alignment. Dolphin GLUT1 also displays trans-acceleration (41), but its sequence is not yet known. C, sequence alignment of the equivalent trans-acceleration subdomains (cyan background) of GLUT4 TM6 in 11 mammals (amino acids 208–212PALLQ218–220). D, WebLogo plot of this alignment.
does not materially affect the trans-acceleration profile of each transporter. This suggests that these subdomains work in concert to effect GLUT1 trans-acceleration of sugar transport.

The canonical explanation of trans-acceleration centers on two kinetic models for carrier-mediated transport: the simple carrier and the fixed-site carrier. The simple carrier (Fig. 7A) is proposed to alternate between exofacial and endofacial orientations (18, 37, 56, 57). During sugar uptake, an external sugar binds to the exofacial orientation, which then undergoes a conformational change to the endofacial state, from which the sugar dissociates into cytoplasm. For an additional round of sugar uptake to occur, the endofacial orientation of the carrier must now reorient to the exofacial state. Conformational changes (exofacial to endofacial and vice versa) are termed translocation when a sugar is bound, and relaxation when no sugar is bound (16). Trans-acceleration of sugar uptake occurs when translocation (endofacial to exofacial) is faster than relaxation. The absence of trans-acceleration is observed when translocation proceeds at the same rate as relaxation. Trans-inhibition would be observed if translocation were slower than relaxation.

The fixed-site carrier model (Fig. 7B) proposes that the carrier exposes endofacial and exofacial sugar binding sites simultaneously (11, 58–60). Transport proceeds concurrently in both directions, implying that sugars initially bound at exo- or endofacial sites exchange into a central cavity, whence they associate with the trans-binding site prior to release into the cytoplasm or interstitium, respectively. Simple exchange describes the release of a bound exo- or endofacial sugar into the central cavity when the trans-site is unoccupied by sugar. Geminate exchange describes the release of a bound exo- or...
endofacial sugar into the central cavity when the opposite site is occupied by sugar (61). Trans-acceleration is observed when geminate exchange is faster than simple exchange (61).

A hybrid model (Fig. 7C) has also been proposed, in which the transporter comprises 4 simple carriers arranged in a coupled, anti-parallel configuration. At any instant, two subunits (carriers) present exofacial orientations and two subunits present endofacial orientations (62). If one exofacial subunit undergoes a reorientation to the endofacial state, the adjacent endofacial subunit must undergo a reorientation to the exofacial state. If translocation is faster than relaxation, it is easy to see how intracellular sugar could stimulate sugar uptake.

The current study suggests that the GLUT4 TM6 sequence allows equal rates of simple carrier relaxation and translocation or equal rates of fixed-site carrier exchange and geminate-exchange. In contrast, the GLUT1 TM6 sequence inhibits simple carrier relaxation but not translocation or inhibits fixed-site carrier exchange but not geminate-exchange, thereby allowing intracellular sugar to stimulate unidirectional sugar uptake. Whichever kinetic model is correct, the following generalization is consistent with experimental evidence. In carriers containing the GLUT1 TM6 sequence, an empty endofacial sugar-binding site is inhibitory to the rate of uptake. In carriers containing the GLUT4 TM6 sequence, this inhibition is removed and the rate of uptake is unaffected by the presence of intracellular sugar.

This hypothesis is further supported by the observed differences in $k_{cat}/K_m$ ratios for GLUT1, GLUT4, and the TM6 chimeras. $V_{max}/K_m$ for enzyme-catalyzed reactions is normally obtained by measuring the rate constant, $k$, for the reaction at limiting substrate concentrations, which is converted to $k_{cat}/K_m$ by dividing $k$ by [enzyme]. $V_{max}/K_m$ is obtained from
measurements of 2-DG uptake and then normalized to cell surface GLUT expression to give $k_{\text{cat}}/K_m$. Although it is possible that TM6 mutants could alter the affinity ($\approx 1/K_m$) of GLUT1 and GLUT4 for substrate, this seems unlikely because TM6 is a putative scaffold TM quite distant to the hypothesized GLUT1 substrate-binding cavity (25, 26). Moreover, $K_{\text{m(app)}}$ for GLUT1- and GLUT4-mediated sugar uptake is similar for both 2-DG (9–10 mM (63)) and 3-MG (6 mM (10)). We therefore hypothesize that the observed changes in $k_{\text{cat}}/K_m$ (Fig. 6) largely reflect changes in $k_{\text{cat}}$.

If TM6 affects the relative rates of simple carrier relaxation and translocation, or of fixed-site carrier exchange and geminate-exchange, we predict that the “inhibitory” sequence of GLUT1 TM6 would reduce GLUT4-catalyzed zero-trans uptake. Indeed, we observe that $k_{\text{cat}}/K_m$ for GLUT4 (6, G1) is $\approx 70\%$ lower than that for either wt GLUT4Myc or the surface expression mutant GLUT4Myc:3x (Fig. 6). In contrast, substituting GLUT4 TM6 sequence into GLUT1 should increase zero-trans $k_{\text{cat}}/K_m$ relative to that of wt GLUT1, and this is observed.

GLUT1 TM6 trans- acceleration subdomains are highly conserved (Phe-194 and Cys-202 are 100% conserved among 18 mammalian species; Ser-191 and Ile-193 are 94% conserved; see Fig. 5). A homology-modeled GLUT1 three-dimensional structure (26) juxtaposes putative scaffold TMs 6 and 3 with the translocation pore-forming TM1 (Fig. 7, A and B). A study by Liu et al. (64), aimed at identifying sequences important for ATP-modulation of GLUT1, showed that a point mutation in TM3 (G111A) abolishes trans- acceleration of GLUT1 expressed in Xenopus laevis oocytes. We did not observe this effect in TMs 1–3 chimeras because this glycerine is conserved between GLUT1 and GLUT4, and is therefore present in both chimeras. Although our data suggest that Gly-111 alone is not sufficient for trans- acceleration, it does not rule out the possibility that Gly-111 makes critical contacts with TM6. The sequence of the membrane-spanning region of TM1 is invariant between GLUT1s 1 and 4, with the exception of GLUT1 Thr-30 (Fig. 8C). This position is conserved in GLUTs 1 and 3 (those carriers showing trans- acceleration), but not in GLUTs 2 and 4 (carriers lacking trans- acceleration). However, a potential role of Thr-30 in trans- acceleration is eliminated by the observation that the 4111 chimera contains substitution T30I, yet still displays trans- acceleration. It is tempting to speculate that GLUT1 TM6 residues 191–195 and 202–204 interact with partners in TM1 and/or TM3 (Fig. 8C) to stabilize endo- and exofacial orientations of the substrate-deficient carrier, thereby restraining conformational changes between exo- and endo- facial states (e.g. relaxation). We hypothesize that when sugar binds to exofacial or endofacial sites, these interactions are weakened, TM arrangements are destabilized, and conformational change is accelerated.

Although the ability to catalyze trans- acceleration has not been studied in all GLUTs, exchange transport has been measured in all four of the class I glucose transporters (GLUTs 1–4). Human GLUT3 catalyzes trans- acceleration in rat cerebellar granule neurons (65) and in transfected HEK cells, whereas rat liver GLUT2 does not exhibit trans- acceleration (55). TM6 sequence comparisons across GLUTs 1–4 (Fig. 8D) show that the same 2 subdomains responsible for trans- acceleration in GLUT1 represent the only variable TM6 sequence among all four transporters. Further homology scanning mutagenesis studies extending TM6 substitutions into GLUT2 and GLUT3 may reveal whether TM6 plays a central role in trans- acceleration in all glucose uniporers.

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