GAPDH binds GLUT4 reciprocally to hexokinase-II and regulates glucose transport activity

Hilal ZAID*, Ilana TALIOR-VOLODARSKY*, Costin ANTONESCU*†, Zhi LIU* and Amira KLIP*†

*Program in Cell Biology, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8, and †Department of Biochemistry, University of Toronto, Toronto, ON, Canada M5S 1A8

Dietary glucose is taken up by skeletal muscle through GLUT4 (glucose transporter 4). We recently identified by MS proteins displaying insulin-dependent co-precipitation with Myc-tagged GLUT4 from L6 myotubes, including GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HKII (hexokinase-II). In the present paper we explored how GAPDH and HKII interact directly with cytoplasmic regions of GLUT4 and their possible inter-relationship. Endogenous and recombinant GAPDH and HKII bound to a chimeric protein linearly encoding all three cytosolic domains of GLUT4 [GST (glutathione-transferase)–GLUT4-cyto]. Both proteins bound to a lesser extent the middle cytosolic loop but not individual N- or C-terminal domains of GLUT4. Purified GAPDH and HKII competed for binding to GST–GLUT4-cyto; ATP increased GAPDH binding and decreased HKII binding to this construct. The physiological significance of the GAPDH–GLUT4 interaction was explored by siRNA (small interfering RNA)-mediated GAPDH knockdown. Reducing GAPDH expression by 70% increased HKII co-precipitation with GLUT4–Myc from L6 cell lysates. GAPDH knockdown had no effect on surface-exposed GLUT4–Myc in basal or insulin-stimulated cells, but markedly and selectively diminished insulin-stimulated 3-O-methyl glucose uptake and GLUT4–Myc photolabelling with ATB-BMPA [2-N-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(o-mannos-4-yloxy)-2-propylamine], suggesting that the exofacial glucose-binding site was inaccessible. The results show that GAPDH and HKII reciprocally interact with GLUT4 and suggest that these interactions regulate GLUT4 intrinsic activity in response to insulin.

Key words: ATP, glucose transporter 4 (GLUT4), glucose uptake, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), insulin, intrinsic activity.

INTRODUCTION

Skeletal muscle is the largest site for glucose disposal in the mammalian body, and GLUT4 (glucose transporter 4) is the major glucose carrier in muscle [1,2] mediating most of the glucose influx [3]. Insulin activates signalling cascades that ultimately lead to GLUT4 translocation to the plasma membrane (reviewed in [4]), where it can mediate glucose influx into the cells. However, it is still debated whether GLUT4 is activated further at the plasma membrane [5–7]. The basis of the debate has been the non-equivalence between the gain in surface GLUT4 and the expected gain in glucose uptake under a variety of conditions (reviewed in [5]). However, unlike the ‘housekeeping’ GLUT1 isofrom for which a molecular mechanism regulating its activity has been proposed based on conformational changes [8,9], there is no molecular information on how the activity of GLUT4 could potentially be regulated. GLUT4 consists of 12 α-helical transmembrane segments. Facing the cytosol are N- and C-terminal chains and a large middle loop [2,10], which therefore are the most likely regions to interact with cytosolic proteins. Indeed, a handful of proteins can bind to a construct encoding the C-terminal region of GLUT4 such as aldolase [11], Ubc9 [12], Daxx [13] and glypican3 [14] but there is no evidence that insulin regulates such interactions (reviewed in [15]). On the other hand, insulin causes release of the protein TUG (tethering protein containing a UBX domain for GLUT4) from adipocytes, a process deemed to be required to free up the transporter for insulin-dependent mobilization to the plasma membrane [16].

Using SILAC (stable isotope labelling of amino acids), we previously identified a small number of proteins that co-purify with full-length GLUT4 in an insulin-dependent manner, upon immunoprecipitation of GLUT4–Myc via its exofacially facing Myc epitope from L6 myotubes [17]. Interestingly, two glycolytic proteins were found in this group, which behaved oppositely in response to insulin: the hormone increased association of GLUT4–Myc with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and conversely diminished association of HKII (hexokinase-II). These two enzymes are particularly attractive because each has functions additional to their catalytic activity in glycolysis. Indeed, GAPDH can catalyse insulin-containing granule fusion with the plasma membrane coupled to glycolytic flux and associates with cytoskeletal elements [18–20]. Moreover, GAPDH associates with synaptic vesicles where production of ATP fuels neurotransmitter reuptake [21]. HKII is rather selectively expressed in the insulin-sensitive muscle, fat and heart tissues [22] and its expression is transcriptionally induced by insulin and muscle contraction [23,24], yet its enzymatic activity does not always parallel the protein expression levels. Insulin rapidly rises HKII activity in mitochondria [25,26], and this protein has additional functions in dictating apoptosis [27]. The aim of the present study was to determine whether GAPDH and/or HKII directly interact with GLUT4, and whether their interaction with the transporter might regulate glucose uptake into muscle cells. We found that both proteins bind directly to cytosolic regions of GLUT4 and compete with each other for such interaction in vitro. KD (knockdown) of GAPDH expression

Abbreviations used: ATB-BMPA, 2-N-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(o-mannos-4-yloxy)-2-propylamine; CB, cytochalasin B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, glucose transporter-4; GST, glutathione transferase; hGAPDH, human GAPDH; HK, hexokinase; hHKII, human HKII; KA, konicic acid; KD, knockdown; LS, Laemmli sample buffer; 3-OMG, 3-O-methyl-D-glucose; RT–PCR, reverse transcriptase–PCR; siRNA, small interfering RNA; TUG, tethering protein containing a UBX domain for GLUT4.

1 To whom correspondence should be addressed (email amira@sickkids.ca).
in muscle cells promoted HKII co-precipitation with GLUT4, suggesting that the two enzymes experience reciprocal association with GLUT4 in vivo. GAPDH KD completely abolished the insulin-evoked 3-OMG (3-O-methyl-D-glucose) uptake without affecting GLUT4 translocation to the plasma membrane, suggesting that GAPDH might play a significant role in regulating GLUT4 intrinsic activity.

**EXPERIMENTAL**

**Materials**

Bio-LC-ATB-BMPA (2-N-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-iloxy)-2-propylamine) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Antibodies to GAPDH (sc-25778) and to HKII (sc-6521) and polyclonal (A-14) anti-α-Myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). AK (konzid acid) was a gift from Dr K. Hasumi (Department of Applied Biological Science, Tokyo Noko University, Tokyo, Japan). Immobilized streptavidin was from Thermo Scientific (Rockford, IL, U.S.A.). Fetal bovine serum, α-MEM (modified Eagle's medium) standard culture medium and other tissue culture reagents were purchased from Wisconsin (Toronto, ON, Canada). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Recombinant human HKII was a gift from Dr H.E. Park (Department of Biochemistry, University of Toronto, Toronto, ON, Canada). siRNAs (small interfering RNA) targeted against GAPDH (siGAPDH, CGGGAAGCUCUCAUGCAUG) or non-related control (siNR, AUCUAUACUCAUCGGAGCUUU) were from Qiagen (Valencia, CA, U.S.A.). All other reagents, including purified hGAPDH (human GAPDH), were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). GLUT4-cyto, a chimera of the N-terminal tail, the major intracellular loop and the C-terminal tail of GLUT4, was provided by Dr Lynn M. Kozma and Dr Thomas A. Gustafson of Metabolex, Hayward, CA, U.S.A. (Dr Lynn M. Kozma is currently with Cepheid, Sunnyvale, CA, U.S.A.).

**Cell culture and siRNA transfection**

Parental L6 muscle cells or L6 muscle cells stably expressing Myc-tagged GLUT4 (L6-GLUT4–Myc) were maintained in myoblast monolayer culture or differentiated into multinucleated myotubes as previously described in [28]. Transfection of 100 nM of non-related siRNA or siRNA targeting GAPDH was performed 60 h prior to the experiment, using the CellPhect kit (GE Healthcare Biosciences, UK) as specified by the manufacturer.

**Determination of 3-OMG uptake and surface GLUT4–Myc**

3-OMG uptake measurements were carried out as described previously in [29]. Briefly, cells were serum-deprived for 3 h and left untreated or stimulated with 100 nM insulin for 20 min or 0.5 mM 2,4-dinitrophenol for 30 min in selective experiments). After 3 washes with PBS, cells were exposed to Hepes-buffered saline (140 mM NaCl, 20 mM Hepes-Na, pHi 7.4, 5 mM KCl, 2.5 mM MgSO4 and 1 mM CaCl2) containing 10 μM [3H]OG (10 μCi/ml) for 1 min, then the extracellular medium was rapidly removed in the presence of the GLUT4 inhibitor CB (cytochalasin B) and intracellularly trapped [3H]OMG was determined. Non-specific uptake was determined in the presence of 10 μmol/l CB and subtracted from all experimental values. Surface Myc-tagged GLUT4 was measured in intact, non-permeabilized cells as previously described [30] using anti-Myc antibody followed by secondary antibody conjugated to horseradish peroxidase.

**Glycolytic protein co-immunoprecipitation with GLUT4–Myc**

Co-immunoprecipitation was performed as previously validated [17]. L6-GLUT4–Myc myoblasts were seeded in one 25 cm dish, allowed to reach 50-60% confluence, then transfected with GAPDH-specific siRNA or non-related siRNA. After 60 h, the cells were deprived of serum for 3 h at 37°C, then treated without or with 100 nM insulin for 20 min at 37°C. The cells were washed with cold PBS, scraped with PBS containing 1% (w/v) Triton X-100 and protease inhibitor cocktail (Sigma–Aldrich, P8340), passed 5 times through a 27.5 gauge syringe and incubated on ice for 1 h. The lysates were centrifuged (13,000 g) for 40 min at 4°C and the protein concentration in the supernatants was quantified using the BCA (bicinchoninic acid) method. Equal amounts of protein (1.5 mg of protein/reaction) from each sample were immunoprecipitated with 40 μl of anti-Myc antibody conjugated to Sepharose beads, with overnight rotation at 4°C. Beads were collected by centrifugation for 1 min at 1000 g at 4°C and washed 5 times with PBS containing 1% Triton X-100 and protease inhibitor cocktail by gentle rotation for 20 min at 4°C. Beads were then resuspended in 20 μl of X SDS/PAGE LSB (Laemmli sample buffer) containing 5% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.005% (w/v) Bromophenol Blue and 125 mM Tris/HCl, pH 6.8, and heated at 80°C for 5 min. Proteins were separated by SDS/10% PAGE, transferred onto PVDF membranes and blotted with the indicated antibodies. Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Perkin Elmer, Waltham, MA, U.S.A.).

**GST (glutathione transferase)–GLUT4 DNA construct preparation, expression and purification**

Rat GLUT4 DNA constructs were inserted into the pGEX-2TK expression vector (Amersham Biosciences, Piscataway, NJ, U.S.A.) and expressed in Escherichia coli (DH5α). GST–GLUT4-cyto is a linear fusion protein encompassing the main cytosolic parts of GLUT4, i.e. N-terminal amino acids 1–24, large central loop amino acids 222–287, and C-terminal amino acids 476–509, linked one to another through two 14 amino acid linkers. The sequence of the first peptide linker (between the N-terminal and loop regions) is AAGGMPAAAGGM and the second linker (between the loop and C-terminal region) is AAGGMPAAAGGM. GST–GLUT4-N-term (amino acids 1–24), GST–GLUT4-loop (amino acids 222–287) or GST–GLUT4-C-term (amino acids 476–509) were prepared from GST–GLUT4-cyto using restriction enzyme sites introduced by PCR. GST was bound to the N-terminus of each construct. All constructs were verified by sequencing.

Expression and purification of GST–GLUT4 constructs was done according to [31] with few modifications. E. coli Rosetta-DE3 (Novagen) cells transformed with GST-polypeptide constructs in pGEX-2TK vector were grown at 37°C in LB medium containing 50 μg/ml ampicillin until they reached 0.4 units of attenuation at 600 nm. Cells were harvested 3 h after induction with 0.2 mM IPTG (isopropyl β-D-thiogalactoside), spun down and washed in PBS. Cells were resuspended in 20 ml PBS per litre of culture, supplemented with bacteria protease inhibitor cocktail (Roche) and passed 3 times through a French press at 1000 psi (1 psi = 6.9 kPa). Triton X-100 was added to a final concentration of 1% and the lysate was incubated at 4°C under rotation for 1 h. Cellular debris were removed by 3 to 4 times of centrifugation at 12000 g for 30 min until no pellet was seen. Supernatant (100 mg) was added to 1 ml of washed (with PBS plus 1% Triton X-100) glutathione–Sepharose beads (Amersham Biosciences) and...
incubated for 1 h at 4°C under rotation. Beads were washed at least 20 times with PBS supplemented with 1% Triton X-100 and 1 to 4 M NaCl, then stored in PBS plus 1% Triton X-100 at 4°C.

**GST pull-down and gel-overlay assays**

L6-GLUT4–Myc lysates (0.5–2 mg), purified hGAPDH (1 μg) or recombinant hHKII (human HKII; 1 μg) were added to 5 μg GST–GLUT4-loop or GST–GLUT4-cyto conjugated to glutathione–Sepharose beads for overnight incubation at 4°C. Beads were washed, proteins eluted with 2 × LSB and analysed by SDS/10% PAGE and immunoblotting. Blots were probed with rabbit polyclonal anti-GAPDH (1:2000) or goat anti-HKII (1:100) followed by either goat anti-rabbit or donkey anti-goat HRP-conjugated secondary antibodies.

For gel overlay assays, GST–GLUT4 constructs were eluted from the glutathione beads by incubation in 10 mM reduced glutathione (in 50 mM Tris/HCl pH 8.0) for 30 min on ice. Equal amounts of each construct (50 ng) were loaded on a PVDF membrane, blocked with 5% milk and incubated with 10 μg/mL hGAPDH or purified recombinant rat HKII for 2 h at room temperature (24°C), followed by anti-GAPDH or anti-HKII antibodies, secondary antibodies and detection by enhanced chemiluminescence.

**GAPDH activity assay and PCR**

GAPDH activity was measured as described in [32] with slight modifications. Briefly, the reaction mixture volume was 1 ml and contained 0.5 mM glyceraldehyde 3-phosphate, 1 mM NAD+, 50 mM sodium arsenate, 2.5 mM glutathione and 50 mM Tris/HCl at pH 7.5. The reaction was initiated by the addition of 50 μg of L6-GLUT4–Myc lysate pre-incubated with or without KA for 10 min and GAPDH activity was followed from the change in 340 nm absorbance. To determine the relative ratio of GAPDH to HKII, semi-quantitative RT–PCR (reverse transcriptase–PCR) was performed on 200 ng of total RNA using the Qiagen One-step RT–PCR Kit (Qiagen) as follows: reverse transcription (50°C, 30 min), heat inactivation (95°C, 15 min), denaturation (94°C, 45 s), annealing (56°C, 45 s), and extension (72°C, 45 s). RT–PCR products were subjected to 2% agarose gel electrophoresis in Tris/acetate/EDTA buffer, ethidium bromide stained and photographed under UV light. The number of PCR cycles was titrated for each gene-specific primer pair target to ensure linearity. The primers used were: HKII, F-CAC-TCCAGATGGCCACAGACA and R-TAACCTCCTGTGGGATG; GAPDH, F-TGCCACTCAGAAGACTTGGG and R-TTACGCTCTGGGATGACCTT, where F is forward and R is reverse.

**GLUT4 photo-affinity labelling**

Bio-LC-ATB-BMPA photolabelling of GLUT4 was performed according to ([33], adapted from [34]). Briefly, L6-GLUT4–Myc myotubes were washed 3 times with Heps buffer pH 7.4 at 18°C, then exposed to Bio-LC-ATB-BMPA (0.2 mM in Heps buffered saline) and irradiated 3 times for 40 s at 350 nm in a Rayonet photochemical reactor, with manual rotation during the intervals. Cells were then washed and lysed in PBS containing 2% C12E8 and protease inhibitor cocktail, passed 5 times through a 27.5 gauge syringe and centrifuged at 13000 g for 40 min. Supernatants were incubated with immobilized streptavidin on 6% agarose beads for 20 h at 4°C, beads were pelleted and washed twice with PBS containing 1% C12E8 and twice with PBS containing 0.1% C12E8. Precipitated proteins were solubilized in LSB containing 8 M urea and 1 mM EDTA, boiled for 30 min, loaded on to SDS/PAGE and GLUT4–Myc was detected by anti-Myc antibody. Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence.

**Statistical analysis**

Results are expressed as means ± S.E.M. (n ≥ 3). The statistical significance of the difference of the means was analysed by t test. A P < 0.05 was considered statistically significant.

**RESULTS**

Insulin stimulation of L6 myotubes led to a 1.6-fold increase in co-precipitation of GAPDH with GLUT4, and concomitantly to a 0.6-fold decrease in co-precipitation of HKII [17]. Here we explore the hypothesis that GAPDH and HKII interact directly with GLUT4, whether these interactions are mutually exclusive, and whether the glycolytic proteins regulate the transporter.

**GAPDH interacts directly with GLUT4**

To determine whether the intracellular-facing regions of GLUT4 interact with GAPDH or HKII, we established an in vitro pull-down assay using chimeric GST-fusion proteins containing the linear sequences of the N-terminal region (N-term), the central cytoplasmic loop (loop) and the C-terminal region (C-term), individually and as a linearly linked fusion protein (termed GLUT4-cyto), each bound to Sepharose beads (Figure 1A and Supplementary Figure S1 at http://www.BiochemJ.org/bj/419/bj4190475add.htm). The purified GST–GLUT segments, attached to Sepharose beads, were added to L6 cell lysates and incubated overnight at 4°C under rotation. After bead sedimentation and protein solubilization with LSB, the amount of endogenous GAPDH was assayed by immunoblotting. GAPDH bound robustly to GST–GLUT4-cyto, less so to GST–GLUT4-loop, and not at all to GST–GLUT4 C-term or N-term (Figure 1B). This result suggests that the middle cytoplasmic loop of GLUT4 is required, but not sufficient, for maximal GAPDH binding. A similar result was observed when purified hGAPDH was used instead of cell lysate for the pull-down assay (Figure 1C). This result suggests that GAPDH can bind directly to GLUT4. In this experimental set-up using recombinant proteins, using 1 μg hGAPDH (equivalent to 28 pmol) and 5 μg GST–GLUT4-cyto (equivalent to 150 pmol), the pulled-down complex had a molar ratio of 0.12 ± 0.05 hGAPDH to GST–GLUT4-cyto.

When increasing amounts of cell lysate (Figure 1D, left panel) or of hGAPDH added to the cell lysate (Figure 1D, right panel) were present in the pull-down assay, GAPDH co-sedimentation with GST–GLUT4-cyto was correspondingly increased (Figure 1D). These results suggest that GAPDH in the cell lysate, and not another lysate factor, is the determining factor for co-sedimentation.

To confirm further the direct interaction between GAPDH and GLUT4, a gel overlay assay was performed (Figure 1E). Consistent with the results obtained in the pull-down assay, hGAPDH bound to GST–GLUT4-cyto and GST–GLUT4-loop, but not to GST–GLUT4 N-term or C-term, or to GST alone. Finally, the insulin-dependent co-precipitation of GAPDH with GLUT4 was confirmed by immunoprecipitating full-length GLUT4–Myc with anti-Myc antibody from basal and insulin-stimulated cells, and this response was shown to be sensitive to inhibition of phosphatidylinositol 3-kinase with LY294002 (Figure 1F). As positive control, this Figure shows the insulin-dependent co-precipitation of α-Actinin4 with GLUT4–Myc [54] and its expected sensitivity to inhibition of phosphatidylinositol
GAPDH KD did not alter the amount of surface-exposed GLUT4–Myc, either in the basal (unstimulated) state or upon insulin stimulation (Figure 2A). Insulin (100 nM) caused a 1.8-fold increase in surface-exposed Myc-epitope regardless of whether GAPDH expression was normal or markedly depleted by its cognate siRNA. Stimulation with 100 nM insulin also nearly doubled the uptake of the non-phosphorylatable glucose analogue 3-OMG. In L6-GLUT4–Myc myoblasts, GLUT4–Myc mediates the majority of glucose uptake in both basal-state and insulin-dependent hexose uptake [3]. The basal-state level of 3-OMG influx was similar in cells with normal and reduced GAPDH expression (Figure 2B). Remarkably, however, the insulin-induced gain in glucose influx was lost in GAPDH KD cells. This effect was seen whether the cells were stimulated with 10 or 100 nM insulin. Taken together, the results in Figures 2(A) and 2(B) suggest that the activity of the insulin-recruited GLUT4 transporters requires GAPDH, or that events associated with the loss of GAPDH reduces GLUT4 activity selectively in the insulin-stimulated state. Of note, and in contrast with the insulin response, the ratio of glucose uptake to surface GLUT4 achieved by mitochondrial uncoupling with 2,4-dinitrophenol [55] was not reduced by silencing GAPDH (0.75 relative units compared to 0.87 in cells with normal GAPDH expression). This observation highlights the selectivity of GAPDH towards the insulin-dependent gain in GLUT4 activity.

3-kinase. As negative control, the Figure shows the lack of precipitation of GAPDH or α-Actin4 when the same anti-Myc antibody and immunoprecipitation protocol were applied to wild-type cells not expressing GLUT4–Myc.

GAPDH regulates GLUT4 transport activity

Insulin causes GLUT4 translocation to the surface of myoblasts, to mediate an increase in glucose uptake. To examine the contribution of GAPDH to GLUT4 localization or function, the expression of the glycolytic protein was reduced by RNA interference. By transfecting GAPDH siRNA, a 70% KD of GAPDH expression was achieved (Figures 2D and 3C). The amount of surface-exposed GLUT4 is easily detectable with anti-Myc antibodies presented to non-permeabilized L6-GLUT4–Myc myoblasts [30].
lysates with anti-GAPDH antibody. NT, non-transfected; NR, non-related siRNA sequence.

GLUT4 in this activity of purified hGAPDH might be affected by the presence of constructs (Figure 1G). We also examined whether the catalytic activity of GAPDH is required for the presentation of the protein(s) can regulate GLUT4 transport activity.

To explore further the ability of surface GLUT4 to interact with hexose substrate, we used a photo-activatable sugar derivative Bio-LC-A TB-BMPA [34] that covalently binds to the exofacial glucose-binding site in GLUT family members, which are then identified upon purification via immobilized avidin and immunoblotting. The amount of Bio-LC-ATB-BMPA binding to GLUT4 in intact cells would be indicative of surface-exposed active glucose transporters. In response to insulin, there was a clear (1.5-fold) increase in the amount of photolabelled GLUT4. GAPDH KD had no significant effect on basal-state GLUT4 photolabelling, but notably prevented the increase in GLUT4 photolabelling in response to insulin (Figure 2C). These results suggest that GAPDH is required for the presentation of the active site of GLUT4 to the cell exterior. As under the same circumstances the Myc-epitope of GLUT4 was fully exposed (Figure 2A), these results suggest that GAPDH or other associated protein(s) can regulate GLUT4 transport activity.

We next examined whether the enzymatic activity of GAPDH is relevant to the regulation of glucose transport. KA is a covalent and specific inhibitor of GAPDH activity [35]. When KA (10 or 50 µg/ml) was applied to L6 myoblasts for 1 h, it did not affect 3-OMG uptake in either basal or insulin-stimulated states. Conversely, KA markedly decreased GAPDH activity in cell lysates by approx. 90% (results not shown). KA also failed to affect the amount of GAPDH pulled-down by GST–GLUT4 constructs (Figure 1G). We also examined whether the catalytic activity of purified hGAPDH might be affected by the presence of GLUT4 in this in vitro assay. None of the GST–GLUT4 peptides affected hGAPDH activity (results not shown). Taken together, these results suggest that the interaction of GAPDH with GLUT4 may regulate glucose transport but not the catalytic activity of GAPDH.

HKII binds to GLUT4 and competes with GAPDH for binding

The results in Figure 1 showed that GAPDH interacts directly with GLUT4. We next tested if other glycolytic proteins also co-precipitate with the transporter, potentially as part of a glycolytic metabolon. Binding of HKII was first tested, since we had previously shown by MS analysis that this protein can co-precipitate with GLUT4–Myc from cell lysates, and such co-precipitation decreased when cells were previously stimulated with insulin [17]. This behaviour was confirmed here by immunoblotting the GLUT4–Myc immunoprecipitates with a HKII-specific antibody (Figure 3A). Interestingly, the level of HKII co-precipitation with GLUT4–Myc was markedly augmented in cells depleted of GAPDH (Figure 3B). The overall expression of HKII and GLUT4 was not affected by GAPDH KD (Figure 3C). We next tested whether this phenomenon is specific for HKII or if other glycolytic proteins might interact with GLUT4 and be affected by GAPDH KD. Among the proteins tested, aldolase, pyruvate kinase and lactate dehydrogenase were not detected in the co-IP with GLUT4–Myc. On the other hand, phosphofructokinase co-immunoprecipitated with GLUT4; however, neither insulin nor GAPDH KD affected this behaviour (Supplementary Table S1 at http://www.BiochemJ.org/bj/419/bj4190475add.htm).

GLUT4 and HKII are predominantly expressed in skeletal muscle, and insulin increases the expression of both proteins [23,36]. Yet a direct interaction between HKII and GLUT4 has never been documented. Such interaction, however, was shown for HKI with human erythrocyte GLUT1 [9,37], and HKI colocalizes with GLUT1 and GLUT3 in rat spermatogenic cells, where those isoforms are highly expressed [38]. Therefore we tested whether HKII interacts directly with GLUT4 in L6 muscle cells. Endogenous HKII was effectively pulled-down from cell lysates by GST–GLUT4-cyto and to a lesser extent by GST–GLUT4-loop. No pull-down was observed with GST alone, GST–GLUT4-C-terminal or -N-terminal (Figure 4A). Similar results were obtained when recombinant hHKII was pulled-down with the same GST–GLUT4 constructs (Figure 4B), indicating that the bimolecular HKII–GLUT4 interaction is direct. Most interestingly, increasing amounts of GAPDH or GLUT4 competed against each other for binding to GST–GLUT4-cyto in the pull-down assay (Figures 4C and 4D). Moreover, when HKII was laid over PVDF membranes containing GLUT4 constructs, it bound to GLUT4-cyto and -loop but not to GLUT4-C-terminal, -N-terminal or GST alone as detected by anti-HKII antibody (Figure 4E). These results confirm that HKII directly interacts with GLUT4 and that the interaction is mutually exclusive with GAPDH. Of note, the relative mRNA expression in L6 myotubes was found to be 2.9 ± 0.3 GAPDH to HKII.

Since GAPDH KD decreased insulin-dependent glucose uptake via GLUT4, and HKII competed with GAPDH binding to GLUT4, we explored the consequence of HKII KD on 3-O-MG uptake. We hypothesized that HKII KD would increase GAPDH binding to GLUT4 and thereby potentiate insulin-dependent glucose uptake. Unexpectedly, however, GLUT4–Myc protein levels rose by 2- to 3-fold upon HKII KD, potentially as an adaptive response due to the compromised generation of glucose-6-phosphate that occurs during the course of HKII KD. This encumbered the calculation and precluded further analysis of the effect of HKII KD. Similarly, it would have been attractive to test whether overexpressing the cytosolic domain of GLUT4

![Figure 3](http://www.BiochemJ.org/bj/419/bj4190475add.htm)
would alter its transport activity, presumably through interfering with regulatory mechanisms involving protein binding to this domain on the full-length transporter. However, overexpression of the cytosolic loop itself caused a rise in surface GLUT4–Myc (Supplementary Figure S2 at http://www.BiochemJ.org/bj/419/bj4190475add.htm), presumably due to competition with retention mechanisms such as those involving the proteins TUG and α-Actinin4 [4]. Moreover, the low transfection efficiency experienced by muscle cells precluded assessing the consequence on glucose uptake activity, as the assay requires cell populations.

ATP enhanced GAPDH-GLUT4 and diminished HKII–GLUT4 interactions

GLUT4 displays two putative ATP-binding motifs between amino acids 127–134 and 348–360 and another potential ATP binding site in its cytosolic loop between amino acids 240–247 (amino acid numbers in rat GLUT4) [39]. Since GAPDH binding to GLUT4 involves this loop, we examined the effect of ATP on hGAPDH binding to GLUT4. ATP enhanced the amount of hGAPDH pulled-down by GST–GLUT4-cyto in vitro, in a dose-dependent manner (Figure 5, left panel). The steepest effects occurred around 1 mM ATP, a concentration that approximates the cytosolic levels of the nucleotide. This result suggests that cytosolic ATP levels may modulate the GLUT4 interaction with GAPDH. As mentioned earlier, GAPDH and HKII compete for GLUT4 binding (Figures 3C, 3D and 4B). Consequently, we asked next whether ATP impinges on HKII binding to GLUT4. As predicted, ATP diminished the amount of recombinant hHKII pulled-down by GLUT4-cyto, in a dose-dependent manner (Figure 5, right panel). Taken together, these results suggest that ATP binds to GLUT4 and that this binding changes GLUT4 affinity for the associated proteins GAPDH and HKII.

DISCUSSION

GLUT4 is the major glucose transporter in skeletal muscle, and most of the glucose enters this tissue through GLUT4,
whether in the resting or insulin-stimulated states [3]. Insulin increases the abundance of GLUT4 at the muscle surface, and this phenomenon is reproduced in muscle cells in culture [4]. The increase in surface GLUT4 enables the increased glucose influx, but it has long been debated whether the increase in transporter number suffices or whether there is additional regulation at the level of GLUT4 activity [5], as it occurs for numerous other membrane transporters [40,41]. In an attempt to explore the diverse modalities of GLUT4 regulation, we embarked on a comprehensive search for proteins that co-purify with GLUT4 in an insulin-dependent manner [17]. From that analysis we identified not only proteins that regulate vesicle traffic and the cytoskeleton, but also glycolytic proteins. Specifically, insulin stimulation of L6 myotubes produced an increase in GAPDH and a decrease in HKII co-precipitating with GLUT4–Myc. Because the Myc tag is in the first exofacial loop of GLUT4 (facing either the extracellular or intravesicular milieu), it is unlikely to affect binding of proteins to the cytosolic regions of the transporter. Until then, only a handful of proteins (Ubc9, Daax, aldolase) had been reported to co-purify with a C-terminal fragment of GLUT4, and none of them in an insulin-responsive manner [15]. Neither GAPDH nor HKII were identified by such searches. Two other proteins (glypican3 and TUG) were found to coprecipitate with full-length GLUT4. TUG is interesting since it binds to the middle cytosolic loop of the transporter and is required for GLUT4 translocation [16,42].

**Reciprocal binding of GAPDH and HKII to GLUT4**

The insulin-dependent increase in co-precipitation of GAPDH with GLUT4–Myc from L6 myotubes was confirmed further by immunoblotting [17], and in the present study we confirm the insulin-dependent decrease in HKII co-precipitation by the same approach (Figure 4A). The identification of two glycolytic proteins with opposite behaviour in such co-precipitation prompted a more detailed investigation of their interaction with the transporter and the significance to its function. First, we observed that purified GAPDH and HKII could each directly bind to a chimeric protein of GLUT4 encompassing all the major cytosolic regions, GST–GLUT4-cyto. Binding was shown to be direct by in vitro binary pull-down assays using only GST–GLUT4-cyto and either purified GAPDH or HKII. Strikingly, in ternary assays, GAPDH and HKII competed each other out for binding to GST–GLUT4-purified GAPDH or HKII. Strikingly, in ternary assays, GAPDH and HKII could each directly bind to a chimeric protein of GLUT4 encompassing all the major cytosolic regions, GST–GLUT4-cyto. Binding was shown to be direct by in vitro binary pull-down assays using only GST–GLUT4-cyto and either purified GAPDH or HKII. Strikingly, in ternary assays, GAPDH and HKII competed each other out for binding to GST–GLUT4-cyto (Figures 3C and 3D). Interestingly, physiologically relevant and HKII competed each other out for binding to GST–GLUT4-purified GAPDH or HKII. Strikingly, in ternary assays, GAPDH and HKII could each directly bind to a chimeric protein of GLUT4 encompassing all the major cytosolic regions, GST–GLUT4-cyto. Binding was shown to be direct by in vitro binary pull-down assays using only GST–GLUT4-cyto and either purified GAPDH or HKII. Strikingly, in ternary assays, GAPDH and HKII competed each other out for binding to GST–GLUT4-cyto (Figures 3C and 3D). Interestingly, physiologically relevant concentrations of ATP in the in vitro pull-down assay promoted GAPDH binding and conversely decreased binding of HKII to GST–GLUT4-cyto (Figure 5). These results suggest the intriguing possibility that, in addition to insulin and in a manner akin to it, cytosolic ATP levels might regulate the reciprocal binding of these proteins. Conversely, although not known if insulin-dependent, GAPDH also binds to purified human erythrocyte GLUT1 reconstituted into vesicles [37], to GLUT1 in the intact erythrocyte or purified tetrameric GLUT1 [43]. Interestingly, physiological levels of ATP (2 mM) reduced such interaction [43], whereas they increased GAPDH interaction with GLUT4. These results suggest that the paradigm of GAPDH binding may extend to other transporters, and that ATP might exert GLUT isoform-specific regulation of such binding. Also suggesting selectivity, GLUT1 colocalizes with the HKI isoform in rat spermatogenic cells [38], raising the possibility that HKI binds to GLUT1 and HKII to GLUT4. The regulation by insulin of glycolytic enzyme binding may be particular to GLUT4, potentially linked to the environment of the transporter in the recruited vesicles. In this regard, it is interesting that insulin causes dissociation of HKII from GLUT4, and association of the enzyme with the outer membrane of the mitochondria [25,26]. The latter has been proposed to allow more efficient phosphorylation of glucose by placing the enzyme near the site of ATP production [44]. One might speculate that the intracellular complement of GLUT4 serves as a reservoir of HKII, and that the enzyme is freed up to migrate to mitochondria, concomitant with the translocation of GLUT4 vesicles to the membrane.

**Impact of GAPDH on GLUT4**

To begin to explore the possible regulation by GAPDH of HKII binding to GLUT4 in intact cells and its possible consequence on glucose uptake, we decreased the cellular content of GAPDH by gene silencing. Reducing GAPDH levels by 70% markedly increased the amount of HKII co-precipitating with full-length GLUT4–Myc (Figure 3B). This result is in agreement with the converse decreased pull-down of recombinant hHKII with GST–GLUT4-cyto by increasingly elevated concentrations of GAPDH in the assay (Figure 4C). Hence a 70% reduction in GAPDH allowed us to probe the consequence on glucose uptake under conditions where GLUT4–Myc binds more HKII and less GAPDH. L6–GLUT4–Myc myotubes are ideal cells for this purpose as glucose uptake is vastly mediated by GLUT4–Myc in both the basal and insulin-stimulated states [3], avoiding confounding contribution by other transporters. When uptake of 3-O-MG is assayed, there is also no contribution of phosphorylation by HKs, and hence only the transport rate through the transporter is assayed. Strikingly, a 70% reduction in GAPDH precluded the increase in glucose uptake elicited by insulin (Figure 2B), without preventing the gain in surface-exposed GLUT4–Myc assessed from the availability of its Myc epitope to extracellular antibodies (Figure 2A). The basal rate of glucose uptake and Myc-exposed epitope were not affected by GAPDH gene silencing. These results argue for a selective decrease in GLUT4 transport activity in the insulin-stimulated state, without affecting GLUT4 translocation. Additional support for this model can be derived from the reduction in GLUT4–Myc photolabelling by ATB-BMPA observed selectively in the insulin-stimulated state of cells depleted of GAPDH (Figure 2C).

**Regulation of GLUT4 activity**

Bio-LC-ATB-BMPA is an affinity photolabel that, when presented to intact cells, binds to the exofacial glucose-binding site of GLUT transporters. Because the Myc epitope on GLUT4–Myc was exposed to the outer medium but not so the glucose-binding site, we propose that the glucose-binding site is not available at the exofacial side of the membrane. This is compatible with the transporter being locked in the ‘inward’ facing conformation, according to the ping-pong kinetic model of glucose transport that proposes the glucose-binding site switches from an ‘outward’ and an ‘inward’ conformation in the course of transport activity of GLUTs [45,46]. The model is supported by the fact that the intramolecular ligand CB, which locks the transporter in the ‘inward’ conformation, precludes reaction of GLUT4 with ATB-BMPA [34]. Since insulin does not change the affinity of GLUT4 for glucose [47], the loss of photolabelling may be related to ‘on’ or ‘off’ states of the availability of the glucose-binding site as described above. This is reminiscent of the regulation of ion channels, which are either opened or closed, with the number of units being in each state creating a versatile regulation of ion influx into cells. Supplementary Table 2 (at http://www.BiochemJ.org/bj/419/bj4190475add.htm) lists a number of conditions where exposure of the Myc epitope on GLUT4–Myc does not correlate with glucose uptake, and the latter instead correlates with...
Bio-LC-ATB-BMPA photolabelling. Among these, kinetic analysis showed that acute exposure to the compound SB203580 locks GLUT4 in the ‘inward’ conformation in insulin-stimulated adipocytes [48]. SB203580 reduces glucose uptake and photolabelling with Bio-LC-ATB-BMPA but not Myc-epitope surface exposure in L6-GLUT4–Myc myotubes, selectively in the insulin-stimulated state [49]. These results are consistent with SB203580 recognizing an insulin-dependent conformation on GLUT4, and locking it in the ‘inward’ mode, preventing glucose influx. This conformation could potentially be the same one that binds GAPDH and loses HKII, a prediction that requires future verification.

Activation, there is attractive information on GLUT1 activity and its structural correlates. Indeed, ATP can block GLUT1 transport activity via intramolecular interaction of its central cytoplasmic loop and C-terminal domain [8]. Interestingly, when the central loop of GLUT1 was replaced with the corresponding GLUT4 loop, ATP no longer effected the conformational change reducing glucose uptake. Those results support our findings and indicate that ATP acts as an inhibitor for GLUT1 but may be an activator for GLUT4 potentially through enhancing GAPDH and decreasing HKII binding to GLUT4. Indeed, both proteins interact with the loop region of GLUT4, in conjunction with the participation of both N- and C-terminal domains. With the extensive mapping of GLUT1 helical organization and the amino acid side chains underlie the non-equivalence between both parameters.

The above results suggest that GAPDH is required for the full activity of GLUT4 recruited to the membrane in response to insulin, possibly by making the glucose-binding site exofacially available to the hexose. This could in turn be due to GAPDH binding to intracellular sites on GLUT4, and/or to its consequences on other proteins such as the dissociation of HKII from the transporter. The latter is an intriguing possibility, since insulin stimulation causes unmasking of cytosolic epitopes on GLUT4 in both adipocytes and muscle cells [50–52], which might arise from removal of blocking constraints exerted upon exchange of the tetrameric GAPDH for the smaller HKII. In either case, the effect is not linked to reduced GAPDH activity itself, as inhibiting GAPDH activity with KA did not affect basal or insulin-stimulated glucose uptake through GLUT4. Because the inhibitory effect on glucose uptake caused by GAPDH silencing is only manifest in the insulin-stimulated state, it is possible that insulin-dependent GAPDH binding takes place selectively on the newly recruited GLUT4, whether in endomembranes en route to the membrane or at the plasma membrane itself. Future studies should explore some of the predictions of this model, illustrated in Figure 6.

Figure 6 A model for GAPDH and HKII interaction with GLUT4

GAPDH and HKII primarily bind to the central loop of GLUT4. ATP and insulin increases GAPDH but decreases HKII binding to GLUT4. HKII leaves GLUT4 and moves to the mitochondria upon insulin stimulation. These interactions may occur at the cell surface or in endomembranes containing GLUT4, and we propose they are part of the mechanism regulating glucose transport activity via GLUT4.

An examination of conditions where changes in glucose uptake are not paralleled by commensurate changes in GLUT4 exposure at the surface is beyond the scope of this study, but have been previously summarized [5,49]. While earlier studies suffered from discrepancies in the techniques used to document each parameter precluding valid comparisons, discrepancies between the change in exposed surface GLUT4–Myc and glucose uptake in intact cells (discussed above) are not prone to this criticism. Similarly, a gain in surface-exposed GLUT4 evoked by acutely activated Akt failed to produce a commensurate increase in glucose uptake in 3T3-L1 adipocytes [53]. We propose that changes in GLUT4 conformation linked to selective binding of cytosolic proteins may underlie the non-equivalence between both parameters.

A link to regulation by ATP?

Whereas there has been a dearth of molecular detail on GLUT4 activation, there is attractive information on GLUT1 activity and its structural correlates. Indeed, ATP can block GLUT1 transport activity via intramolecular interaction of its central cytoplasmic loop and C-terminal domain [8]. Interestingly, when the central loop of GLUT1 was replaced with the corresponding GLUT4 loop, ATP no longer effected the conformational change reducing glucose uptake. Those results support our findings and indicate that ATP acts as an inhibitor for GLUT1 but may be an activator for GLUT4 potentially through enhancing GAPDH and decreasing HKII binding to GLUT4. Indeed, both proteins interact with the loop region of GLUT4, in conjunction with the participation of both N- and C-terminal domains. With the extensive mapping of GLUT1 helical organization and the amino acid side chains involved in glucose transport [56], it should become possible in the future to produce a similar map of GLUT4 and test some of the predictions of this study.

ACKNOWLEDGMENTS

We are indebted to Dr Philip J. Bilan for insightful comments on this manuscript, and to Dr Jonathan Schertzer for advice on RT–PCR measurements. We thank Dr K. Hasumi and Dr H. E. Park for generous supply of koningic acid and recombinant hexokinase-II respectively.

FUNDING

This work was supported by grants to A. K. from the Canadian Institutes of Health Research [grant numbers MOP-7307 and MOP-12601]. H. Z. was supported by a postdoctoral fellowship from the Research Training Centre, The Hospital for Sick Children (Toronto, ON, Canada).

REFERENCES


Received 1 July 2008/7 January 2009; accepted 14 January 2009
Published as BJ Immediate Publication 14 January 2009, doi:10.1042/BJ20081319
SUPPLEMENTARY ONLINE DATA

GAPDH binds GLUT4 reciprocally to hexokinase-II and regulates glucose transport activity

Hilal ZAID*, Ilana TALIOR-VOLODARSKY*, Costin ANTONESCU*†, Zhi LIU* and Amira KLIP*††

*Program in Cell Biology, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8, and †Department of Biochemistry, University of Toronto, Toronto, ON, Canada M5S 1A8

Table S1 Co-immunoprecipitation of endogenous glycolytic proteins with GLUT4–Myc

L6-GLUT4–Myc muscle cells treated with or without GAPDH siRNA or insulin (100 nM) for 20 min were lysed and immunoprecipitated with 40 μl of Myc antibody conjugated to Sepharose beads as described in the Experimental section. The precipitated proteins were separated on SDS/PAGE and detected with specific antibody. NA, not available; NP, No co-immunoprecipitation; + and ++, indicates relative co-immunoprecipitation amounts; =, no change; −, decreased.

<table>
<thead>
<tr>
<th>Co-immunoprecipitation with GLUT4–Myc</th>
<th>Basal</th>
<th>Insulin</th>
<th>Non-related siRNA</th>
<th>GAPDH siRNA</th>
<th>Protein levels in response to GAPDH KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKII</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>=</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>=</td>
</tr>
<tr>
<td>Aldolase</td>
<td>NP</td>
<td>NP</td>
<td>NA</td>
<td>NP</td>
<td>NA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>NP</td>
<td>NP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>NP</td>
<td>NP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>GLUT4–Myc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>=</td>
</tr>
</tbody>
</table>

Figure S1 GST–GLUT4 constructs

SDS/PAGE gel stained with Commassie Blue (A) and Western blot probed with anti-GST (B) demonstrating the purification of GST–GLUT4 fusion proteins expressed in bacteria and purified on glutathione beads.

† To whom correspondence should be addressed (email amira@sickkids.ca).
Table S2  GLUT4 translocation to the plasma membrane versus ATB-BMPA photo-labelling and glucose uptake

<table>
<thead>
<tr>
<th>Treatment and stimulation</th>
<th>Myc signal</th>
<th>Photo-labelling</th>
<th>3-OMG or 2-deoxy-D-glucose uptake</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[1] and the present paper</td>
</tr>
<tr>
<td>Ins</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[1] and the present paper</td>
</tr>
<tr>
<td>GAPDH KD, B</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>The present paper</td>
</tr>
<tr>
<td>GAPDH KD, Ins</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>The present paper</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P3, B</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>[2]</td>
</tr>
<tr>
<td>SB203580, B</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>[1]</td>
</tr>
<tr>
<td>SB203580, Ins</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>[1]</td>
</tr>
</tbody>
</table>

Figure S2  Expression of GFP (green fluorescent protein)–G4-loop increases both basal and insulin-stimulated cell surface GLUT4–Myc in L6-GLUT4–Myc myoblasts

L6-GLUT4–Myc myoblasts were transfected with cDNA encoding either GFP–G4-loop (WT) or GFP only. Following serum starvation, cells were stimulated with 100 nM insulin or left unstimulated (basal), followed by measurement of cell surface GLUT4–Myc by immunofluorescence microscopy. Transfected cells were identified by GFP fluorescence, as indicated. (A) Stacked projections of z-plane images obtained by confocal microscopy of GLUT4–Myc at the cell surface and GFP fluorescence. Images are representative of at least eight independent experiments. Scale bar, 10 μm. (B) Graphic representation of (A), showing means ± S.E.M. from eight independent experiments. * and #, P < 0.05 relative to GFP-expressing basal and insulin-stimulated cells respectively.

REFERENCES
