The Predicted ATP-Binding Domains in the Hexose Transporter GLUT1 Critically Affect Transporter Activity†

Qi Liu,‡,§,|| Juan Carlos Vera,||,⊥ Hong Peng,# and David W. Golde*

Program in Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021

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ABSTRACT: The glucose transporter GLUT1 has three short amino acid sequences (domains I−III) with homology to typical ATP-binding domains. GLUT1 is a facilitative transporter, however, and transports its substrates down a concentration gradient without a specific requirement for energy or hydrolysis of ATP. Therefore, we assessed the functional role of the predicted ATP-binding domains in GLUT1 by site-directed mutagenesis and expression in *Xenopus* oocytes. For each mutant, we determined the level of protein expression and the kinetics of transport under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions. Although all five mutants were expressed at levels similar to that of the wild-type GLUT1, each single amino acid change in domains I or III profoundly affected GLUT1 function. The mutants Gly_{116}→Ala in domain I and Gly_{332}→Ala in domain III exhibited only 10−20% of the transport activity of the wild-type GLUT1. The mutants Gly_{111}→Ala in domain I and Leu_{336}→Ala in domain III showed altered kinetic properties; neither the apparent K_m nor the V_{max} for 3-methylglucose transport were increased under equilibrium exchange conditions, and they did not show the expected level of countertransport acceleration. The mutant Lys_{117}→Arg in domain I showed a marked increase in the apparent K_m for 3-methylglucose transport under zero-trans efflux and equilibrium exchange conditions while maintaining countertransport acceleration. These results indicate that the predicted ATP-binding domains I and III in GLUT1 are important components of the region in GLUT1 involved in transport of the substrate and that their integrity is critical for maintaining the activity and kinetic properties of the transporter.

GLUT1 is a facilitative glucose transporter belonging to the family of integral membrane proteins that mediate the transport of hexose across the plasma membrane down a concentration gradient (*1*, *2*). Five genes have been identified encoding mammalian glucose transporter isoforms named GLUT1−5 (*3*). Although most glucose transporter isoforms show a specific and rather restricted tissue and cell distribution, human GLUT1 is widely distributed in fetal and adult tissues and cells and is therefore considered to be a constitutive transporter (*1*, *2*). The expression, subcellular distribution, and intrinsic activity of GLUT1 are altered by a variety of stimuli such as stress, growth factors, and neoplastic transformation (*4*−*9*). GLUT1 was believed to have specificity for the transport of glucose and related hexoses; however, we now know that GLUT1 is a multifunctional protein that participates in the transport of substrates other than glucose such as dehydroascorbic acid, the oxidized form of vitamin C (*10*, *11*).

GLUT1 is especially abundant in human erythrocytes (*1*). Red blood cells have therefore been used as the primary experimental system to study the functional properties of GLUT1 as intact cells and also normal and inside-out erythrocyte ghosts. GLUT1 has been highly purified from human erythrocytes and functionally reconstituted in artificial liposomes. These studies revealed that GLUT1 is a bidirectional transporter that conducts the substrate down a concentration gradient (*12*−*14*). Transport mediated by GLUT1 occurs in an ATP[1]-independent manner, and thus GLUT1 is considered a typical facilitative transporter (*1*).

Although ATP hydrolysis is not required for GLUT1-mediated transport, the intrinsic activity of GLUT1 is sensitive to the cellular content of ATP (*15*−*21*). Moreover, photolabeling experiments performed using red cell ghosts or highly purified preparations of GLUT1 reconstituted in liposomes have revealed that ATP interacts directly with GLUT1, thus establishing the fact that GLUT1 is a nucleotide-binding protein (*16*, *18*, *22*−*24*). Analysis of the primary sequence of GLUT1 revealed that GLUT1 has three short amino acid segments with homology to amino acid sequences that are part of the ATP-binding domains forming the ATP-binding sites in a number of ATPases and ATP-

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* To whom correspondence should be addressed. Tel: 212-639-8483. Fax: 212-772-8589. E-mail: d-golde@ski.mskcc.org.

‡ Clinical Scholar Biomedical Research Fellow of Memorial Sloan-Kettering Cancer Center.

§ Current address: Department of Internal Medicine, Albert Einstein College of Medicine, New York, NY.

|| The first and second authors contributed equally to this work.

⊥ Current address: Department of Pathophysiology, University of Concepcion, Concepcion, Chile.

Current address: Office of Patents & Licensing, New York Blood Center, New York, NY.

[1] Abbreviations: ATP, adenosine triphosphate; 2-deoxyglucose, 2-deoxy-D-glucose; 3-methylglucose, 3-*O*-methyl-D-glucopyranose; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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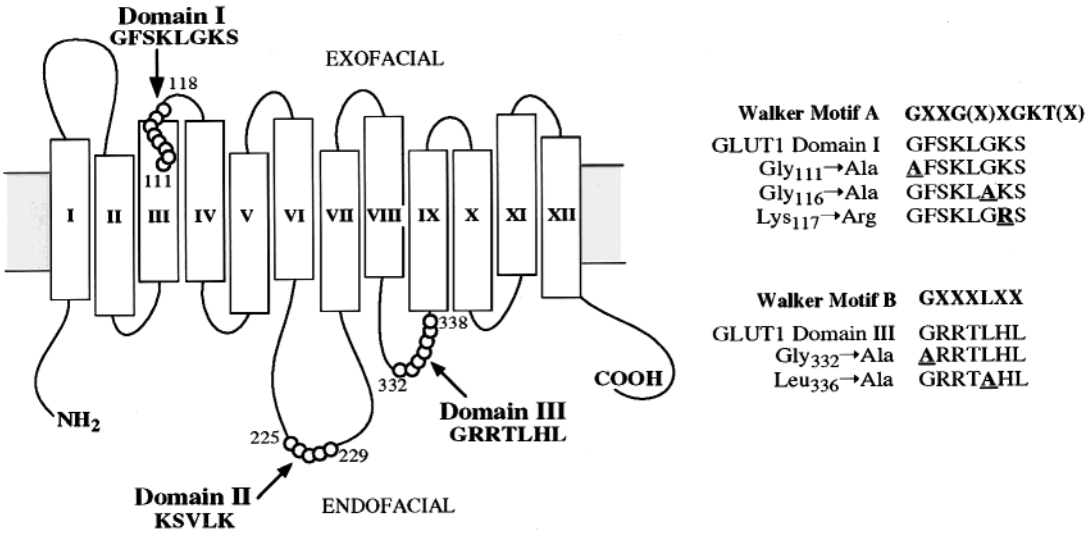

FIGURE 1: Topology of the predicted ATP-binding domains in the 12-helix model of GLUT1. The position of the predicted ATP-binding domains in GLUT1 is indicated by the strings of white circles, with each circle representing one amino acid. The conserved sequences with homology to Walker motifs A (domain I) and B (domain III) are shown. The sequences containing the mutated amino acids (three in domain I and two in domain III) are shown below the corresponding Walker motif, with the amino acid mutated indicated in bold and underlined. The 12 predicted transmembrane helices are numbered I−XII.

binding proteins (*22*, *23*, *25*, *26*). GLUT1 has three predicted ATP-binding domains: domain I, comprising amino acid residues 111−118 (Gly-Phe-Ser-Lys-Leu-Gly-Lys-Ser), which is homologous to the Walker binding motif A; domain II, comprising amino acid residues 225−229 (Lys-Ser-Val-Leu-Lys); and domain III, comprising amino acid residues 332−338 (Gly-Arg-Arg-Trp-Leu-His-Leu), which is homologous to the Walker binding motif B. In the proposed 12-helix model of GLUT1 (*27*), predicted ATP-binding domain I is located in the region encompassing the boundary of the predicted transmembrane domain 3 and the extracellular loop between predicted transmembrane domains 3 and 4; predicted ATP-binding domain II is in the large intracellular loop located between the putative transmembrane domains 6 and 7, and predicted ATP-binding domain III is located in the short intracellular loop between predicted transmembrane domains 8 and 9 (Figure 1). A recent photolabeling and peptide mapping study identified a peptide of about 10 kDa containing the domain III labeled at high specific activity with azido-ATP, supporting the notion that the predicted ATP-binding domain III may function as a true ATP-binding site (*23*).

It is known that ATP-binding domains play important roles in regulating the function of nucleotide-binding proteins. For example, mutations in the Walker binding motif A eliminated the ATPase activity and the ability of *Escherichia coli* MalK protein to transport maltose and the multidrug resistance activity of human P-glycoprotein (*28*−*30*). Although there is evidence indicating that ATP affects the functional status of GLUT1, it is unclear how the binding of ATP to putative ATP-binding domains may affect the intrinsic activity of GLUT1 in the absence of ATP hydrolysis. It is possible that ATP may exert its activity on GLUT1 in an indirect manner through the action of extrinsic proteins. Such a concept is related to the observation that GLUT1 can bind accessory proteins in an ATP-dependent fashion with a concomitant change in transport activity (*31*−*33*).

We previously reported that the isoflavone genistein, a broad-spectrum tyrosine kinase inhibitor, inhibited the GLUT1 transport of substrates in a competitive manner and the binding of cytochalasin B (*34*). We recently extended these studies to the analysis of the interaction of a large number of tyrosine kinase inhibitors with GLUT1 (*35*). We found that only those inhibitors that compete with the binding of ATP inhibit the activity of GLUT1 in a competitive manner. Taken together, this information strongly suggests that GLUT1 possesses domains with functional and structural properties typical of ATP-binding domains that may be important for regulating GLUT1 function. We addressed the role of the predicted ATP-binding domains in GLUT1 by site-directed mutagenesis followed by expression in *Xenopus* oocytes and functional analysis. Although all mutants were expressed at levels similar to that of the wild-type GLUT1, transport experiments revealed that single amino acid changes in domains I or III had a prominent effect on GLUT1 function. Our findings indicate that predicted ATP-binding domains in GLUT1 are critical for maintaining the intrinsic activity and the kinetic properties of the transporter.

EXPERIMENTAL PROCEDURES

Construction of GLUT Mutants at the Predicted ATP-Binding Domains. A 2423 base pair *Bgl*II−*Xho*I fragment from the GLUT1 cDNA cloned in pSPGT (*27*) was used to generate the mutants Gly111→Ala, Gly_{116}→Ala, Gly_{332}→Ala, and Leu_{336}→Ala that were then subcloned into the expression vector pcDNA3 (Invitrogen). The mutant Lys_{117}→Arg was derived from the same *Bgl*II−*Xho*I fragment from pSPGT and subcloned into pcDNA3 at *Bam*HI−*Xho*I sites. Site-directed mutagenesis was conducted using an overlapping PCR technique and appropriate oligonucleotides. Oligo 5′GCCGTGCTCATGGcCTTCTCGAAACTG3′ was used to generate the Gly_{111}→Ala mutant. Oligo 5′TTCTCGAAACTG-GcCAAGTCCTTTGAG3′ was used to generate the Gly_{116}→Ala mutant. In both cases, the lower case C in the oligonucleotide sequences was the mutated nucleotide which led to the substitution of alanine for glycine. Oligo 5′TCG-AAACTGGGCAgaTCTTTTGAGATGCTG3′ was used to

generate the Lys_{117}→Arg mutant, with the lower case G and A identifying the mutated nucleotides which led to the substitution of arginine for lysine. The underlined sequence indicates the introduced *Bgl*II site which corresponded to a silent mutation in the amino acid sequence and was used for the initial identification and selection of the mutant cDNA. Oligo 5′GTGGAGCGAGCAGcgCGCCGGACCTGCAC3′ was used to generate the Gly_{332}→Ala mutant. The lower case C and G are the mutated nucleotides which led to the substitution of alanine for glycine, while the underlined sequence represents the introduced *Bss*HII site which corresponded to a silent mutation in the amino acid sequence. Oligo 5′GGCCGGCGGACCgcGCACCTCATTGGCCTCGCTGGC3′ was used to generate the Leu_{336}→Ala mutant. The lower case G and C are the mutated nucleotides which led to the substitution of alanine for leucine, and the underlined sequence indicates the destroyed *Stu*I site which led to a silent mutation in the amino acid sequences. All of the mutations were confirmed by restriction enzyme digestion analysis and DNA sequencing using oligonucleotides flanking the regions of the mutations.

Preparation of RNA and Injection of Xenopus Oocytes. The plasmids containing the different constructs were linearized at the *Xho*I site, and the wild-type and mutant mRNAs were synthesized from these constructs using an in vitro transcription kit and T7 RNA polymerase (Ambion, Austin, TX). Oocytes were isolated from ovaries of female *Xenopus laevis* (Nasco, Fort Atkinson, WI) and digested with 2 mg/mL collagenase D (Sigma) in oocyte Ringer's no. 2 medium (OR2: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5, 50 μg/mL gentamicin, 10 μg/mL penicillin/streptomycin) for 1 h at room temperature. The individual oocytes were washed in OR2 medium (Bufferad, Lake Bluff, IL), and healthy stage V−VI oocytes were selected under the dissecting microscope and maintained in the same buffer at 17 °C. After an overnight incubation in OR2 medium, the healthy oocytes were injected with either 40 nL of water or 40 nL of water containing the in vitro synthesized mRNAs at a concentration of 1 mg/mL. Oocytes were maintained in OR2 media for 3 days at 17 °C before being tested for expression of the transporters.

Preparation of Oocyte Membranes and Western Blot Analysis. The membranes of oocytes injected with wild-type and mutant mRNAs were prepared according to the procedure of Garcia et al., with minor modifications (*36*). Briefly, the intact oocytes were disrupted by repeated pipetting with a Pipettman-200 micropipet in homogenization buffer (10 mM Tris, pH 7.6, 250 mM sucrose, 83 mM NaCl, 1 mM $MgCl_2$, 10 μM EDTA, 10 μM EGTA, 1 mM PMSF, and 5 μg/mL each of aprotinin, pepstatin, leupeptin, and soybean trypsin inhibitor). The total cell homogenate was centrifuged three times at 1000*g* for 10 min at 4 °C in a microcentrifuge to pellet the yolk granules and melanosomes. The final supernatant was centrifuged at 150000*g* for 1 h at 4 °C to collect the membrane fraction, and the sedimented oocyte membrane fraction was resuspended in homogenization buffer lacking sucrose. Fifteen micrograms of total membrane proteins was fractionated by SDS−PAGE in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Western blot analysis was performed using affinity-purified polyclonal antibodies raised against a synthetic peptide containing the sequence of the last 15 amino acids of the carboxy-terminal region of human GLUT1 (Alpha Diagnostics, San Antonio, TX).

Transporter Assays and Kinetic Analysis. Uptake analysis of 2-deoxy-D-[^{3}H]glucose [2.5 mM 2-deoxy-D-glucose (2-deoxyglucose) plus 0.5 μCi of ^{3}H-labeled 2-deoxyglucose] and 3-*O*-[^{3}H]methylglucose [4 mM 3-*O*-methyl-D-glucopyranose (3-methylglucose) plus 0.5 μCi of ^{3}H-labeled 3-methylglucose] was conducted as described previously (*10*). 3-Methylglucose was used as the substrate for the kinetic analysis. For zero-trans influx assays, groups of *Xenopus* oocytes injected with the synthetic mRNAs or with water were incubated in OR2 media containing a range of concentrations (1.5−35 mM) of 3-methylglucose and 1 μCi of ^{3}H-labeled 3-methylglucose for an uptake time of 2.5 min. After uptake, the oocytes were washed three times in ice-cold PBS, and each individual oocyte was solubilized in 0.1% SDS, mixed with scintillation fluid, and counted. For zero-trans efflux assays, groups of oocytes were incubated in OR2 media containing 3-methylglucose (2−90 mM) and 1 μCi of ^{3}H-labeled 3-methylglucose for 18 h at 17 °C. At the end of the incubation, the oocytes were rapidly transferred to sugar-free OR2 medium, and efflux of 3-methylglucose was measured for 12.5 min. For equilibrium exchange assays, groups of oocytes were incubated in OR2 media containing 3-methylglucose (2−90 mM) for 18 h at 17 °C. At the end of the incubation, 1 μCi of ^{3}H-labeled 3-methylglucose was added to the extracellular medium (containing cold 3-methylglucose, 2−90 mM), and uptake was measured for 2.5 min.

RESULTS

To investigate the role of the predicted ATP-binding domains of GLUT1 in transporter activity, the five amino acid residues in domains I and III, which are completely conserved in Walker motifs A and B, namely, Gly111, Gly116, Lys117, Gly332, and Leu336, were individually targeted for mutagenesis (Figure 1). The glycines at positions 111, 116, and 332 were converted to alanine, lysine 117 was converted to arginine, and leucine 336 was converted to alanine. The mutant cDNAs were confirmed by DNA sequencing, and the corresponding mRNAs were synthesized in vitro and used for functional analysis. In vitro translation using the reticulocyte lysate cell-free system with the wild-type and mutant mRNAs as templates produced a single polypeptide of 48 kDa, consistent with the expected molecular weight of unglycosylated GLUT1 (data not shown). The in vitro synthesized mRNAs were injected in *Xenopus* oocytes, and the level of expression and the functional properties of the transporters were studied by immunoblotting of oocyte membrane fractions with anti-GLUT1 antibodies and hexose transporter assays.

To examine the expression of the mutant transporters in *Xenopus* oocytes, total membrane proteins were isolated from the mRNA-injected oocytes. Equal amounts of proteins from oocytes expressing the wild-type GLUT1 and the five different mutants were fractionated by SDS−PAGE and immunoblotted with an affinity-purified antipeptide antibody against the C-terminal peptide of GLUT1. The immunoblot analysis revealed that the mRNA-injected oocytes expressed a protein giving a band with an average molecular mass of 55 kDa, reactive with anti-GLUT1 that was not present in

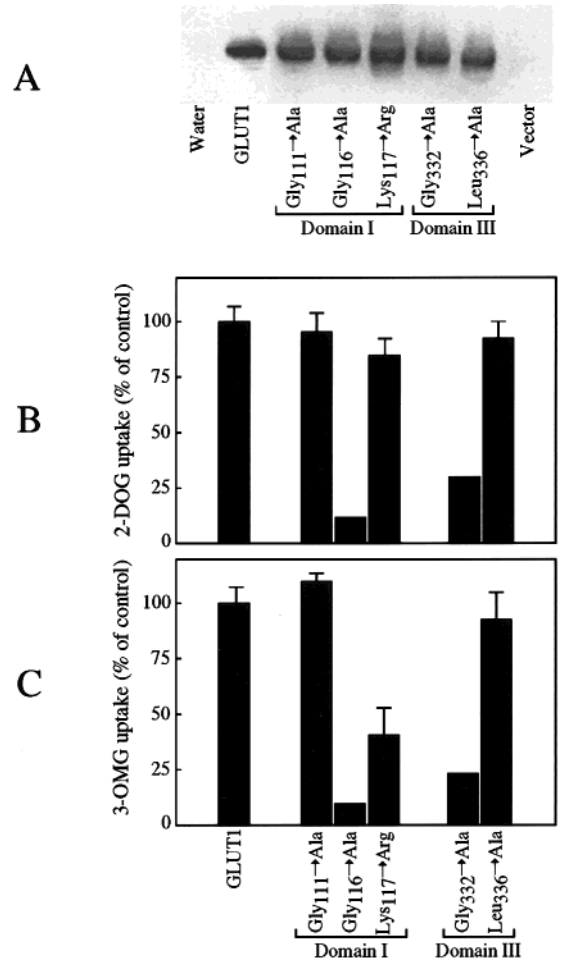

FIGURE 2: Expression of wild-type and mutant GLUT1 proteins in *Xenopus* oocytes and uptake of 2-deoxyglucose and 3-methylglucose. (A) Oocytes were microinjected with 40 ng of synthetic mRNA for wild type and the different GLUT1 mutants. Three days after injection, total membrane proteins were isolated, and 15 μg of each sample was fractionated by SDS−PAGE and subjected to immunoblot analysis using an affinity-purified anti-GLUT1 antibody. (B) Oocytes (three to five) were microinjected with either H_2O or 40 ng of synthetic mRNA from wild type and GLUT1 mutants, and uptake of 2-deoxyglucose (2-DOG) was measured 3 days after injection. (C) Uptake of 3-methylglucose (3-OMG) in oocytes treated as described in (B). Uptake was measured using a 30 min incubation period. The baseline substrate uptake by oocytes injected with H_2O was subtracted from the total uptake values. Values represent the mean ± SE from at least three independent experiments with three to five oocytes per experiment.

control oocytes injected with water or in oocytes injected with an empty vector mRNA (Figure 2A). As assessed by the intensity of the immunoblot of at least four independent experiments performed over 1 year, all five mutant proteins were expressed in *Xenopus* oocytes at levels similar to that of the wild-type GLUT1 (Figure 2A).

In parallel studies we analyzed the capacity of the different mutant proteins expressed in *Xenopus* oocytes to transport 2-deoxyglucose. These studies revealed that oocytes expressing three mutant proteins, the mutants Gly_{111}→Ala and Lys_{117}→Arg in domain I and the mutant Leu_{336}→Ala in domain III, showed a level of 2-deoxyglucose uptake activity similar to that of the wild-type GLUT1 (Figure 2B). On the other hand, oocytes expressing the mutants Gly_{116}→Ala in domain I and Gly_{332}→Ala in domain III showed a marked reduction in their capacity to take up 2-deoxyglucose (Figure 2B). Assuming a similar level of expression as indicated by the results of the immunoblotting analysis, the mutants Gly_{116}→Ala and Gly_{332}→Ala showed only about 12% and 30% of the activity of the wild-type GLUT1, respectively.

Similar results were obtained when the mRNA-injected oocytes were tested for their capacity to transport 3-methylglucose, a nonmetabolizable substrate. Oocytes expressing the mutants Gly_{116}→Ala and Gly_{332}→Ala with reduced 2-deoxyglucose transport activity had a greatly reduced capacity to transport 3-methylglucose (Figure 2C). Transport of 3-methylglucose by the mutants Gly_{116}→Ala and Gly_{332}→Ala was only about 12% and 25% of the activity of the wild-type GLUT1, respectively. This analysis also revealed that the mutants Gly_{111}→Ala and Leu_{336}→Ala exhibited 3-methylglucose transport activity similar to the activity of the wild-type GLUT1 (Figure 2C). On the other hand, the mutant Lys_{117}→Arg, which transported 2-deoxyglucose normally, showed a reduced capacity to transport 3-methylglucose at only 40% of the activity of the wild-type GLUT1 (Figure 2C).

The results of the localization studies and the transport data indicate that the observed changes in uptake of glucose by oocytes expressing the mutant transporters most likely reflected changes in the transport properties of the transporters and were not due to differences in the level of expression of the transporters. GLUT1 is kinetically characterized by its bidirectional and asymmetric transport, and therefore, a more complete analysis of the functional properties of GLUT1 requires performing transport assays under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions (*1*, *2*). Transport under zero-trans conditions may provide information on the functional characteristics of the import and export steps of the transport cycle. On the other hand, transport under equilibrium exchange conditions allows analysis of the ability of the transporter to undergo countertransport acceleration.

In initial experiments we defined the conditions for measuring transport under zero-trans influx, zero-trans-efflux, and equilibrium exchange conditions for the wild-type GLUT1 and the mutants Gly_{111}→Ala and Lys_{117}→Arg in domain I and Leu_{336}→Ala in domain III, which retained enough transport activity to be studied. The kinetic constants were obtained using uptake times of 2.5 min for zero-trans influx, 12.5 min for zero-trans efflux, and 2.5 min for equilibrium exchange conditions. These experiments showed that single amino acid mutations in either of the putative ATP-binding domains I or III of GLUT1 dramatically affected the kinetic properties of GLUT1 (Figures 3 and 4). Although the mutants Gly_{116}→Ala in domain I and Gly_{332}→Ala in domain III were expressed at levels similar to that of the wild-type GLUT1, we could not analyze their kinetics properties due to the low level of transport observed. The mutations Gly_{111}→Ala, Lys_{117}→Arg, and Leu_{336}→Ala had a profound effect on the kinetic properties of GLUT1 and segregated into two groups depending on whether they affected the transport K_m, the transport V_{max}, or both properties simultaneously (Figures 3 and 4). The wild-type GLUT1 expressed in *Xenopus* oocytes had an apparent K_m for zero-trans influx of 6.3 mM, which increased to 16.5 mM for zero-trans efflux and then showed a major increase to 52 mM associated with the countertransport acceleration observed for transport under equilibrium exchange conditions

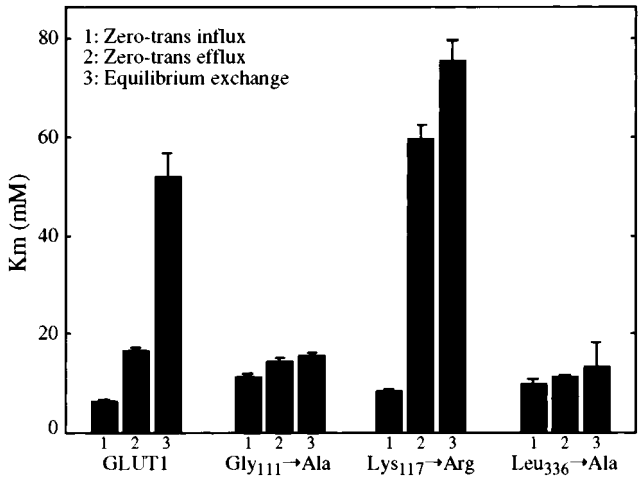

FIGURE 3: Apparent K_m for the transport of 3-methylglucose by *Xenopus* oocytes expressing wild-type and mutant GLUT1 proteins. Shown are the values of the apparent K_m determined from oocytes injected and processed as described in the legend of Figure 2. K_m for zero-trans influx was determined in uptake experiments lasting 2.5 min and using concentrations of 3-methylglucose from 1.5 to 35 mM. K_m for zero-trans efflux was measured in uptake experiments lasting 12.5 min and using concentrations of 3-methylglucose from 2 to 90 mM. K_m for influx under equilibrium exchange conditions was measured in uptake experiments lasting 2.5 min and using concentrations of 3-methylglucose from 2 to 90 mM. The values are the means ± SE from three to seven groups of independent experiments. For each experiment, the values of K_m under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions were measured in the same group of oocytes.

(Figure 3). In comparison, the mutant Gly_{111}→Ala in domain I had an apparent K_m for zero-trans influx of 11.5 mM that increased to 14.4 mM for zero-trans efflux and to 15.7 mM for equilibrium exchange (Figure 3). A similar situation was observed for the mutant Leu_{336}→Ala in domain III; the apparent K_m for zero-trans influx of 10 mM increased to 11.5 mM for zero-trans efflux and to 13.5 mM for transport under equilibrium exchange conditions (Figure 3). Although there was no major change in the ability of either mutant to transport 3-methylglucose under zero-trans influx or efflux conditions, the mutants failed to show the expected substantial increase (about 6-fold) in the value of the apparent K_m for equilibrium exchange normally associated with the occurrence of countertransport acceleration. The net result of these mutations is therefore an increased affinity for the substrate under equilibrium exchange conditions.

The situation was markedly different for the mutant Lys_{117}→Arg in domain I. This mutant had an apparent K_m for zero-trans influx of 8.4 mM that is similar to the wild-type GLUT1 and the mutants Gly_{111}→Ala and Leu_{336}→Ala (Figure 3). When transport was measured under zero-trans efflux conditions, however, there was an apparent K_m of 59.5 mM for 3-methylglucose transport, which is about 7 times greater than the K_m for zero-trans influx (Figure 3), and a K_m similar to the apparent K_m for equilibrium exchange of wild-type GLUT1. Further experiments revealed that the apparent K_m for equilibrium exchange in the mutant Lys_{117}→Arg was 75.5 mM, or about 9 times greater than that for zero-trans influx (Figure 3). Thus, the mutation Lys_{117}→Arg affected transport under zero-trans efflux by increasing the transport K_m but apparently without affecting the expected change in K_m associated with countertransport

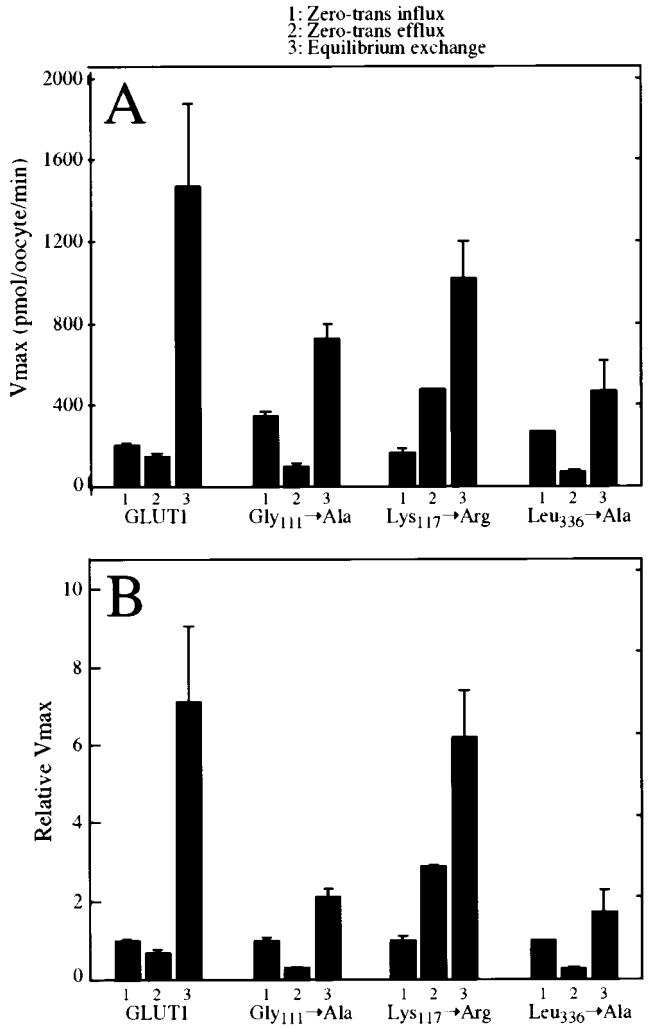

FIGURE 4: Apparent V_{max} for the transport of 3-methylglucose by *Xenopus* oocytes expressing wild-type and mutant GLUT1 proteins. (A) Shown are the values of the apparent V_{max} determined from oocytes injected and processed as described in the legend of Figure 2. V_{max} for zero-trans influx was determined in uptake experiments lasting 2.5 min and using concentrations of 3-methylglucose from 1.5 to 35 mM. V_{max} for zero-trans efflux was measured in uptake experiments lasting 12.5 min and using concentrations of 3-methylglucose from 2 to 90 mM. V_{max} for influx under equilibrium exchange conditions was measured in uptake experiments lasting 2.5 min and using concentrations of 3-methylglucose from 2 to 90 mM. The values are the means ± SE from three to seven groups of independent experiments. For each experiment, the values of V_{max} under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions were measured in the same group of oocytes. (B) Relative V_{max}. The data in panel A were normalized to the respective V_{max} under zero-trans influx that were given the relative value of 1.

acceleration. The net effect is a decreased affinity for the substrate under both zero-trans efflux and equilibrium exchange conditions.

Similar results and conclusions were obtained when the V_{max} for transport by the different mutants was studied under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions. A central property of GLUT1 is that the V_{max} of transport under equilibrium exchange conditions is greatly increased (about 7-fold) compared to the V_{max} under zero-trans influx and efflux, corresponding to the phenomenon of countertransport acceleration. *Xenopus* oocytes expressing wild-type GLUT1 had an apparent V_{max} for 3-methylglucose

Table 1: Effect of Cytochalasin B on Uptake of 3-Methylglucose by *Xenopus* Oocytes Expressing Wild-Type and Mutant GLUT1 Proteins[a]

	GLUT1	Gly111→Ala	Lys117→Arg	Gly332→Ala	Leu336→Ala
K_i (nM)	393 ± 17	287 ± 19	300 ± 41	337 ± 15	310 ± 27

[a] Oocytes were injected and processed as indicated in the legend of Figure 2. Three days after injection, uptake of 3-methylglucose was assayed in the absence or in the presence of seven different concentrations of cytochalasin B ranging from 50 nM to 5 μM. Five oocytes were used for each concentration group and measured individually. Results are the mean ± SE from three independent experiments.

transport of 207 ± 7 pmol per oocyte per minute under zero-trans influx, which increased to 1475 ± 400 pmol per oocyte per minute under equilibrium exchange conditions, a 7-fold increase in V_{max} (Figure 4A). The three mutants showed a variable degree of increase in the V_{max} of transport under equilibrium exchange conditions compared to zero-trans influx and efflux, suggesting that they were able to undergo normal countertransport acceleration (Figure 4A). A problem associated with attempting to draw conclusions regarding the functional activity of the transporter from changes in the V_{max} of transport is that V_{max} depends on the intrinsic activity of the transporters and also on the number of transporters on the cell membrane participating in transport. Although the immunoblotting data indicated that the mutants were expressed at levels similar to that of wild-type GLUT1, limitations inherent in the immunoblotting technique preclude the detection of small changes (within 1−2-fold) in expression. It is therefore difficult to interpret changes in V_{max} in terms of changes in the intrinsic activity of the transporter. Because each batch of injected oocytes was tested under the three experimental conditions (zero-trans influx, zero-trans efflux, and equilibrium exchange), the possible role of differences in the level of expression of the different mutant proteins can be minimized by normalizing the V_{max} for each mutant to the respective value of the V_{max} of transport for zero-trans influx. This analysis revealed that the mutants Gly_{111}→Ala, Lys_{117}→Arg, and Leu_{336}→Ala segregated into two groups with different kinetic properties. Wild-type GLUT1 expressed in *Xenopus* oocytes showed countertransport acceleration as revealed by a 7.1-fold increase in the normalized V_{max} of transport of 3-methylglucose under equilibrium exchange conditions compared to zero-trans influx (Figure 4B). The mutants Gly_{111}→Ala and Leu_{336}→Ala showed only a 2.1- and 1.7-fold increase, respectively, in the normalized V_{max} of transport of 3-methylglucose under equilibrium exchange conditions compared to zero-trans influx (Figure 4B). In contrast, the mutant Lys_{117}→Arg showed marked changes in both the normalized V_{max} for equilibrium exchange (6.2-fold increase) and zero-trans efflux (2.9-fold increase) compared to zero-trans influx (Figure 4B). Overall, the data indicate that the mutations Gly_{111}→Ala and Leu_{336}→Ala greatly decreased the ability of GLUT1 to undergo countertransport acceleration. The mutant Lys_{117}→Arg was able to undergo countertransport acceleration and also showed an increased capacity to transport 3-methylglucose under zero-trans efflux conditions and decreased net uptake of 3-methylglucose compared to wild-type GLUT1 shown in Figure 2C.

Our data indicate that single amino acid substitutions in the putative ATP-binding domains I and III in GLUT1 had a profound effect on the transport activity of GLUT1 as revealed by changes in the affinity for the substrate and the kinetic properties of the transporter. Two mutants (Gly_{116}→Ala in domain I and Gly_{332}→Ala in domain III) were almost nonfunctional with greatly diminished transport activity. The three mutants with transport activity similar to that of the wild-type GLUT1 (Gly_{111}→Ala and Lys_{117}→Arg in domain I and Leu_{336}→Ala in domain III) did not show substantial changes in their ability to transport 3-methylglucose under zero-trans influx conditions, a finding that could be interpreted as indicating that the exofacial (import) substrate-binding site is functional in these mutants. These mutants showed altered kinetic properties, however, that may result from changes at the level of the endofacial (export) substrate binding site. To assess the possibility that a single amino acid change could directly affect substrate binding to the endofacial region of the transporter, 3-methylglucose uptake was examined in the presence of cytochalasin B, a specific inhibitor of sugar uptake that binds to the endofacial face of GLUT1 in a region that probably overlaps the substrate-binding site. We determined the K_i of cytochalasin B for the wild-type GLUT1 and mutant transporters expressed in the oocytes. The results show that cytochalasin B inhibits the transport of 3-methylglucose by the wild-type GLUT1 and mutant transporters in a generally similar fashion (Table 1). The K_i of cytochalasin B for the wild-type GLUT1 expressed in oocytes is 393 ± 17 nM and is 287 ± 19, 300 ± 41, 337 ± 15, and 310 ± 27 nM for the Gly111→Ala, Lys117→Arg, Gly332→Ala, and Leu336→Ala, respectively. The residual activity of mutant Gly116→Ala for 3-methylglucose transport is only about 12% of the activity of the wild-type GLUT1. We were unable to measure the K_i of cytochalasin B for the mutant Gly116→Ala; however, the residual transport activity of the mutant Gly116→Ala was completely blocked in the presence of 5 μM cytochalasin B. Cytochalasin E, the inactive analogue of cytochalasin B, which was used as a control for the specificity of the inhibition, showed no effect on transport activity of wild-type GLUT1 or the mutant transporters (data not shown). These data suggest that the endofacial cytochalasin B binding site is generally functional in the mutant transporters that showed markedly lowered transport activity or altered kinetic properties.

DISCUSSION

We used site-directed mutagenesis and expression in *Xenopus* oocytes to directly address the role of predicted ATP-binding domains in GLUT1 on transporter function. The data indicate that single amino acid substitutions in the predicted ATP-binding domains I and III in GLUT1 result in either abolished transport activity or markedly altered kinetic properties of the transporter. Overall, our findings suggest that the predicted ATP-binding domains of GLUT1 are critical for transporter activity and also important in determining the kinetic properties of the transporter.

We mutated two predicted ATP-binding domains in GLUT1: domain I, which according to the 12-helix model is located in the exofacial side of the transporter at the

boundary between predicted transmembrane domain 3 and the extracellular loop between transmembrane domains 3 and 4, and domain III, which is predicted to be located at the endofacial side of the transporter in the loop between predicted transmembrane domains 8 and 9 (Figure 1). Thus, it is possible that the predicted ATP-binding domain I in GLUT1 could be part of the exofacial substrate-binding site, with domain III part of the endofacial substrate-binding site. Without the initial binding step there is no transport, as demonstrated by the inability of GLUT1 to transport L-glucose. Therefore, a simple explanation for the greatly diminished transport capacity of the mutants Gly_{116}→Ala and Gly_{332}→Ala and the markedly altered kinetic properties of the mutants Gly_{111}→Ala, Lys_{117}→Arg, and Leu_{336}→Ala is that these mutations could directly affect the topology of the substrate-binding sites, resulting in decreased substrate binding. The data from cytochalasin B inhibition studies, however, suggest that the cytochalasin B binding site is functional in the mutant transporters. Because cytochalasin B interacts with an endofacial binding site that apparently overlaps with the sugar-binding site, the results suggest that the endofacial substrate-binding site is available in the mutant transporters. This interpretation is supported by the results of an independent mutagenesis study which indicated that changing the arginine residues 333 and 334 to alanine in the predicted ATP-binding domain III of GLUT1 (next to the mutated Gly_{332}→Ala) dramatically reduced transport activity without affecting endofacial cytochalasin B binding or exofacial substrate binding (*38*). Overall, the above results suggest that both endofacial and exofacial substrate-binding sites are largely intact and functional in the mutant transporters.

For GLUT1, the values of the K_m and V_{max} of transport are a function of the rate-limiting step of the overall transport cycle, which for transport under zero-trans conditions is the relaxation step (regeneration of substrate binding sites) and for equilibrium exchange the translocation step (sugar import and export) (*1*). Thus, it is possible that the introduction of mutations in the predicted ATP-binding domains of GLUT1 resulted in alterations in the capacity of the transporters to undergo the proper conformational rearrangements that allow each transport cycle to progress, resulting in changes in both the overall substrate affinity and the kinetics of transport. The mutants in the predicted ATP-binding domains segregate in three categories depending on the overall effect on their transport activity and kinetic properties. (1) The important reduction of the transporter activity in mutants Gly_{116}→Ala and Gly_{332}→Ala could result from a change in the tertiary structure of the transporter, resulting in mutant proteins whose conformation is highly unfavorable for movement of the substrate through the transport channel. (2) The mutants Gly_{111}→Ala and Leu_{336}→Ala still retain their ability to transport substrate in and out of cells but present similar substrate affinities under zero-trans influx, zero-trans efflux, and equilibrium exchange conditions and lose most of the capacity to undergo countertransport acceleration. The limitations of the V_{max} determinations in *Xenopus* expressing the different transporters preclude an analysis of which step is most likely to be affected, but the data are consistent with the concept that the mutants may have lost part of the dynamic flexibility that allows GLUT1 to sense the presence of the substrate on both sides of the plasma membrane. (3) The mutant Lys_{117}→Arg retained normal kinetic parameters for zero-trans influx, with dramatically decreased substrate affinity and increased transport efficiency for zero-trans efflux state, and conserved the capacity to undergo countertransport acceleration.

We acknowledge that the kinetic data measured in our oocyte system are imprecise. Apparent V_{max} measurements in particular showed substantial variability as indicated by the relatively large standard errors, although we made efforts to control the number of transporters expressed in the oocytes. The apparent K_m measurements showed less variability. The V_{max}/K_m ratios for influx, efflux, and equilibrium exchange are not equal, as would be expected by the constraints of the simple carrier hypothesis (*37*). Therefore, we cannot precisely fit our results to the simple carrier hypothesis, nor can we challenge the validity of the passive transport model in the frog oocyte expression system. It is possible, however, that the simple carrier model may not be equivalently applicable for transport across the cell membranes of various living cells. In this study, we sought to address the question of the role of the predicted ATP-binding domains in GLUT1, a facilitive transporter that does not require energy derived from ATP hydrolysis for function. Taken together, our data indicate that conservation of the structural integrity of the predicted ATP-binding domains I and III in GLUT1 is essential for maintaining normal transporter function. Specific amino acid residues belonging to both domains play an important role in regulating the kinetic properties of the transporter.

Are the predicted ATP-binding domains in GLUT1 directly involved in transport? The predicted ATP-binding domains I and III are located a substantial distance from each other in the primary structure of GLUT1 (Figure 1). It is therefore possible that the introduction of the mutations in the predicted ATP-binding sites in GLUT1 may have disrupted interactions important for maintaining the tertiary structure of the transporter. These changes in tertiary structure may affect the domains in the transporter directly involved in the transport of the substrate with concomitant changes in the functional properties of the transporter. In a parallel study assessing the effect of tyrosine kinase inhibitors on GLUT1 function (*35*), we obtained independent evidence indicating that GLUT1 has domains with functional properties expected for ATP-binding sites. We found that tyrosine kinase inhibitors belonging to a subclass with specificity for ATP-binding sites blocked substrate transport through GLUT1 in a competitive manner. The complete inhibition of transporter activity and the competitive nature of the inhibition by the tyrosine kinase inhibitors suggest that putative ATP-binding domains in GLUT1 are directly involved in the transport of substrate, which is consistent with the mutagenesis results presented here. The highly specific changes in the kinetic properties of the mutants that retained substantial transport activity suggest that rather subtle conformational changes may have occurred as a result of the mutations introduced in the predicted ATP-binding sites of GLUT1. Moreover, the transport and inhibition data indicate the likelihood that both the endofacial and the exofacial substrate-binding sites are preserved in the mutants.

What is the role of ATP and the predicted ATP-binding sites in modulating the activity of GLUT1? There are several independent mechanisms by which ATP-binding domains

in proteins may participate in the regulation of protein function. (1) Typical ATP-binding proteins have an absolute requirement for ATP to function. Binding of the triphosphate nucleotide to the ATP-binding sites is coupled to ATP hydrolysis associated with the functional cycle of the protein. Point mutations in the ATP-binding domains of different ATP-binding proteins dramatically affect their function, indicating that the ATP-binding domains are essential for functional activity (*39*−*41*). The diminished functional activity of the mutants, however, results from the destruction of the ATPase activity of the protein because it is the energy liberated during the hydrolysis of ATP that is required for the functional cycle to occur. (2) GLUT1 is a facilitative glucose transporter that corresponds to an ATP-regulated protein clearly distinguishable from the typical ATP-binding proteins. Transporters of the facilitative type transport substrates down a concentration gradient; they do not require hydrolysis of ATP to function and are fully functional in the absence of ATP. Photolabeling experiments have shown that GLUT1 is a nucleotide-binding protein that binds ATP (*22*, *23*). It has been reported that intracellular ATP regulates the activity of GLUT1 in a hydrolysis-independent manner and is responsible for the observed asymmetry in the kinetic properties for sugar influx and efflux (*1*, *15*−*21*, *42*). The regulatory effect of ATP on the transport activity of GLUT1 has been observed in in vitro reconstituted systems containing highly purified preparations of the transporter. Therefore, in the absence of ATP hydrolysis, it is reasonable to propose that ATP modulates the activity of GLUT1 by directly interacting with the transporter at the predicted ATP-binding domains (*22*, *23*, *43*, *44*). (3) The results presented here reveal a novel and unexpected role for the predicted ATP-binding domains in GLUT1 in determining the functional activity of the transporter. Our data point to the predicted ATP-binding domains in GLUT1 as important components of the region in GLUT1 involved in the transport of the substrate and suggest that they participate directly in the transport cycle and regulate the kinetic properties of the transporter. We conclude that the structural integrity of the predicted ATP-binding domains in GLUT1 is essential for maintaining transporter function.

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