Role of the C-Terminal Tail of the GLUT1 Glucose Transporter in Its Expression and Function in *Xenopus laevis* Oocytes†

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ABSTRACT: Structural determinants for the glucose transport kinetics of the erythrocyte glucose transporter have not been established. In this work the role of the cytosolic carboxy-terminal tail in the expression and function of the human GLUT1 isoform in *Xenopus* oocytes was investigated. Oocyte plasma membrane expression of GLUT1 was a saturable function of the amount of mRNA injected. Transport activity increased as a linear function of the amount of immunoreactive transporter in the plasma membrane. Transport kinetics of human GLUT1 expressed in oocytes resembled those of human erythrocyte GLUT1. Addition of up to 31 extra amino acids to the carboxy-terminal tail of GLUT1 was without effect on its function in oocytes. Removal of the carboxy-terminal 21 amino acids also did not affect GLUT1 expression or transport kinetics in oocytes. Removal of the entire carboxy-terminal tail to Phe-450 resulted in a transporter that had moderately decreased plasma membrane expression compared to that of GLUT1. However, transport activity of this construct was less than 5% of that of GLUT1, and was associated with loss of its outward-facing inhibitor binding site. When the carboxy-terminal 29 amino acids of GLUT1 were replaced with the corresponding region of GLUT4, transporter expression in the plasma membrane and the transport V_{max} fell to low levels, similar to those of native GLUT4. When the carboxy-terminal 29 or 73 amino acids of GLUT1 were swapped into the corresponding region of GLUT4, the transport V_{max} markedly increased to about one-third to one-half that of GLUT1, although the affinity for substrate was halved. These results show that the carboxy-terminal tail of the GLUT1 is not critical for targeting of the protein to the plasma membrane, but that this region is an important determinant of transport function.

The facilitated diffusion of glucose into and out of mammalian cells is mediated by one or more members of the "GLUT" family of transport proteins. These glucose transporters have all been shown to possess analogous hydropathy plots, with most similarities in the intramembrane domains (Bell et al., 1990). Thus GLUT1 and GLUT4 have 65% overall identity, but in these regions they have 74% identity (86% similarity) (Fukumoto et al., 1989). Conversely, there is little similarity in the extramembrane domains of these proteins. The conserved intramembrane domains are considered crucial for the transport reaction, whereas the variable extramembrane regions may confer differences in transport kinetics between carrier subtypes (Bell et al., 1990).

An extramembrane domain likely to contribute to the function or targeting of a transporter isoform is the intracellular carboxy-terminal (C-terminal)[1] cytoplasmic tail. Oka et al. (1990) have prepared a truncated construct of rabbit GLUT1 lacking the last 37 amino acids of the protein (leaving residues 1−455 intact). When expressed in Chinese hamster ovary cells, this construct was synthesized and inserted into the plasma membrane normally, since sodium borohydride labeling of cell-surface carbohydrate was similar to that observed for the native GLUT1. However, the truncated protein had very low rates of 2-deoxyglucose uptake. The mutant showed D-glucose-inhibitable cytochalasin B photolabeling comparable to that seen in native GLUT1, but it reacted poorly with a cell-impermeant bis-(mannose) affinity label. It was concluded that the carrier was "locked" in a nonfunctional inward-facing conformation, unable to bind the bis(mannose) affinity label outwardly. Oka's group also showed that a mutant lacking only the terminal 12 amino acids had normal transport, implicating amino acids 455−480 as the conformation-sensitive domain (Lin et al., 1992).

Chimeras involving swaps of the C-terminal tail of GLUT1 with other transporter isoforms have provided additional information regarding its targeting and function. Asano et al. (1992) swapped the C-terminal 30 amino acids of GLUT2 with the corresponding region of GLUT1. They reported that both the K_m and V_{max} of 2-deoxyglucose transport were increased in this chimera. In contrast to a plasma membrane location of the other transporter isoforms, the insulin-sensitive GLUT4 isoform is targeted to intracellular regions (James

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[1] Abbreviations: C-terminal, carboxy-terminal; EGlc, 4,6-*O*-ethylidene-D-glucose; PCR, polymerase chain reaction; OR, oocyte Ringer's; PBS-T, phosphate-buffered saline-Tween.

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et al., 1993). Studies of chimeras with GLUT4 and GLUT1 have suggested that there may be multiple domains in the transporter structure that determine where the protein will be targeted. Different groups have localized the primary targeting regions of the transporter to the N-terminal cytoplasmic region (Piper et al., 1992, 1993), to one or more internal transmembrane segments (Asano et al., 1992), or to the C-terminal tail (Marshall et al., 1993; Czech et al., 1993; Verhey et al., 1993). The most recent studies, however, have implicated the C-terminal tail as a major determinant in targeting of transporter proteins. Using different methodologies and expression systems, several groups have now reported that GLUT1/4 chimeras containing a GLUT4 C-terminal tail are targeted to the intracellular membranes. On the other hand, GLUT4/1 chimeras containing a GLUT1-C-terminal tail are targeted largely to the plasma membrane (Marshall et al., 1993; Czech et al., 1993; Verhey et al., 1993). Finally, a Leu−Leu sequence at residues 489 and 490 in GLUT4 has been implicated in targeting this protein to an intracellular as opposed to a plasma membrane location (Verhey & Birnbaum, 1994; Corvera et al., 1994).

We have evaluated the role of the C-terminal tail of GLUT1 in the expression and function of the protein in *Xenopus* oocytes by using truncations, additions, and swaps between GLUT1 and GLUT4. We also describe a new method for preparation of oocyte ghost plasma membrane and provide evidence that transport kinetics of human erythrocyte GLUT1 are retained in GLUT1 expressed in oocytes.

EXPERIMENTAL PROCEDURES

Materials. The 3-*O*-[^{3}H]methyl-D-glucose (87.6 Ci/mmol) was from New England Nuclear. Phloretin, cytochalasin B, 4,6-*O*-ethylidene glucose (EGlc) and 3-*O*-methylglucose were from Aldrich. *In vitro* RNA transcription and translation reaction kits were from Stratagene and Promega, respectively. Taq polymerase and PFU DNA polymerase were from Stratagene. Human GLUT1 and GLUT4 cDNAs that were originally cloned into the *SalI* site of pGEM4Z were a gift from Dr. Graeme Bell. Oligonucleotides were synthesized in the Vanderbilt University Diabetes Center DNA Core laboratory. Cationic silica beads were generously provided by Dr. Bruce Jacobson (Chaney & Jacobson, 1983).

Preparation of Chimeras and Truncation Mutants. The glucose transporter constructs used in this study are depicted in the diagram of Figure 1. In preparing chimeras of GLUT1 and GLUT4, advantage was taken of sequence similarities between the two transporters. Site-directed mutagenesis by the phosphorothioate method of Eckstein (Taylor et al., 1985) in M13 was performed using the "Sculptor" kit supplied by Amersham. A single nucleotide in the GLUT1 sequence was mutated to produce a restriction enzyme site corresponding to a naturally occurring site in the GLUT4 cDNA. Mutation of guanosine-1490 to a thymidine in the GLUT1 cDNA (Mueckler et al., 1985) resulted in a *BglII* site that allowed construction of a chimera having amino acids 1−463 of GLUT1 and amino acids 480−509 of GLUT4, termed G1/4-B. A second chimera was constructed that had the converse locations of GLUT1 and GLUT4, G4/1-B. A third mutation was engineered by mutating thymidine-1212 to guanosine in the GLUT1 cDNA to generate an *NcoI* site. This site was used to prepare a chimera containing amino

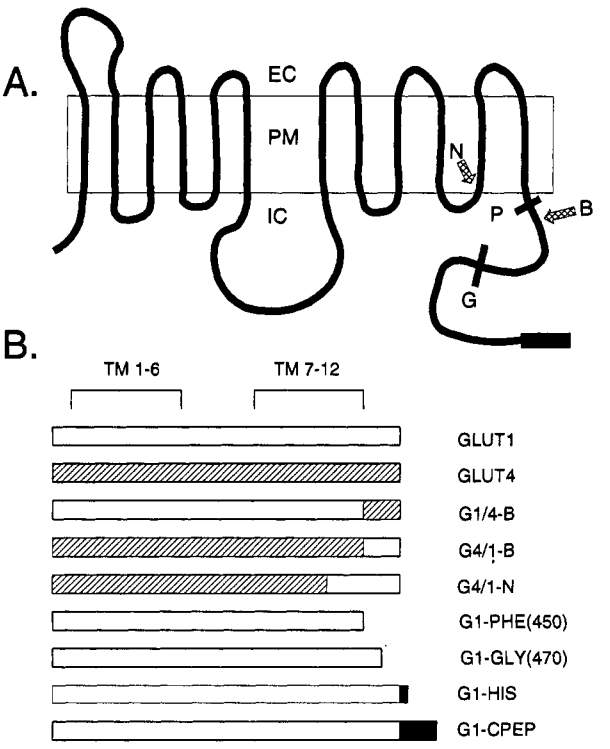

FIGURE 1: Glucose transporter constructs. (Panel A) Map of the GLUT1 transporter in the membrane (PM) showing the putative regions exposed to the intracellular (IC) and extracellular spaces (EC). Approximate locations of the sites for truncation [G, G1-GLY(470); P, G1-PHE(450)] and swapping with GLUT4 (N, G4/1-N; B, G4/1-B and G1/4-B) are shown. (Panel B) Locations and sizes of the changes made in GLUT1 and GLUT4 cDNA. GLUT1 sequence is shown by the open segments and GLUT4 by the hatched segments. The darkened extensions of GLUT1 show relative sizes of the G1-His and G1-CPEP additions.

acids 1−419 of GLUT4 and 404−492 of GLUT1, termed G4/1-N. The sequences of each chimera were confirmed by restriction enzyme digestions and by DNA sequencing by the dideoxynucleotide method.

Truncation mutants of GLUT1 were prepared by site-directed mutagenesis using the "Double-Take" method (Statagene), which is based on the use of avidin−biotin technology for DNA strand selection. The two truncation mutants of GLUT1 were constructed by making single base changes in the human GLUT1 cDNA to produce stop codons at the codons corresponding to Lys-451 and Gly-471. This resulted in constructs in which the final translated C-terminal amino acids were Phe-450 [G1-PHE(450)] and Gly-470 [G1-GLY(470)], respectively. The mutations were confirmed by DNA sequencing by the dideoxynucleotide method.

The GLUT1-His construct was prepared by the polymerase chain reaction (PCR) using primers to span the entire coding sequence of GLUT1 cDNA. The 3′ oligonucleotide primer (noncoding strand) was synthesized to produce a GLUT1 cDNA that removed the normal stop codon and added the codons for six histidines, followed by a stop codon, and lastly by a *SalI* restriction site. The polymerase chain reaction was used to amplify the construct, and the PCR fragments were purified by agarose gel electrophoresis. The appropriate fragment was eluted from the gel, digested with restriction enzymes, and ligated into the oocyte expression vector pGOV-B (see below). The sequence of the 3′ end of the insert was confirmed by dideoxynucleotide sequencing.

Part of the human insulin C-peptide protein was attached to the C-terminus of the GLUT 1 protein as has been recently described for a potassium channel (Philipson et al., 1993). Overlapping synthetic oligonucleotides, corresponding to the nucleotide sequence for amino acids 33−63 of the human proinsulin protein, were extended with Taq polymerase to encode the entire C-peptide protein. The 5′ end of this DNA fragment contained sequence complementary to the DNA sequence for the last 18 base pairs of the GLUT 1 cDNA and removed the stop codon for GLUT 1. Using overlap PCR with this C-peptide DNA fragment and the full-length GLUT 1 cDNA, a GLUT 1 cDNA was created that encoded the full-length GLUT protein followed by 31 amino acids of C-peptide protein. The DNA sequence of the GLUT 1−C-peptide cDNA was confirmed by dideoxynucleotide sequencing.

RNA Synthesis from cDNA Templates. The cDNAs encoding the human GLUT1 and GLUT4 glucose transporters were subcloned into the oocyte expression vector pGOV-B (Morita et al., 1994). All wild-type GLUT cDNAs were prepared by PCR so that they included only the coding region of the cDNA and suitable restriction sites on each end. For *in vitro* mRNA synthesis, the corresponding cDNAs were linearized downstream of the 3′ globin untranslated region and transcribed *in vitro* into capped RNA by T7 RNA Polymerase (Stratagene mRNA capping kit). The yield of the *in vitro* transcription was assessed by spectrophotometry at 260 nM. Verification of full-length transcription of the cDNA was confirmed by electrophoresis on a formaldehyde−agarose gel. For microinjection, mRNA was dissolved in diethylpyrocarbonate-treated water at a concentration of 2 μg/μL and stored at −70 °C.

Oocyte Preparation. Female *Xenopus laevis* (Xenopus I, Ann Arbor, MI, or from NASCO, Fort Atkinson, WI) were maintained in a 12-h light cycle at 20 °C and fed a diet of minced bovine liver. Frogs were anesthetized by immersion in 0.15% 3-aminobenzoic acid ethyl ester (Sigma), and ovarian fragments were removed through a lateral ventral incision. The tissue fragments were rinsed in oocyte Ringer's (OR) buffer #2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) and then digested in this buffer containing 4 mg/mL collagenase (Type II, Worthington) for 2 h at room temperature on a circular rotator. Healthy oocytes were selected under a dissection microscope and maintained at 18 °C in OR buffer (82.5 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5), supplemented with penicillin-G/streptomycin (1%, Gibco). Oocytes were microinjected with capped RNA within a day of isolation. Microinjection of oocytes was carried out using a Picospritzer II (General Valve Corp., Fairfield, NJ) and a micromanipulator from Singer Instruments Co. (Somerset, England). The diameters of circular drops of RNA solution on the injection needle were measured using an ocular micrometer, from which the injection volume was calculated. Following microinjection of RNA, oocytes were incubated at 18 °C for 2−3 days, during which time the OR media was changed daily and any unhealthy oocytes were removed.

Measurement of 3-O-Methylglucose Influx. Zero-*trans* influx of 3-*O*-methylglucose was initiated by incubating 5−10 oocytes in 200 μL of OR medium containing 1.2 μCi of 3-*O*-[^{3}H]methyl-D-glucose and unlabeled 3-*O*-methylglucose at concentrations of 1−50 mM. After a specified time at room temperature, the transport assay was stopped by rapidly washing the oocytes four times in 2 mL of ice-cold OR containing 0.2 mM phloretin. After the final wash, the oocytes in 0.5 mL of stop solution were transferred to scintillation vials, lysed by mixing in 5 mL of EcoLume scintillation fluid (ICN), and counted for radioactivity in a Packard 2000CA liquid scintillation spectrometer. The uptake time was chosen such that less than 10% of equilibrium 3-*O*-methylglucose was occupied during the transport period, based on preliminary transport measurements in each batch of oocytes. The transport times were usually 1−5 min for GLUT1 and up to 60 min for GLUT4 and other constructs with low rates of transport.

For equilibrium exchange influx measurements involving GLUT1-injected cells, oocytes were incubated in the indicated concentration of 3-*O*-methylglucose for 60 min at room temperature, followed by the transport assay as described above, also at room temperature. Overnight incubation of GLUT1-expressing oocytes with radiolabeled 3-*O*-methylglucose resulted in intracellular 3-*O*-methylglucose spaces similar to those in oocytes incubated for 1 h, so the latter time point was used in subsequent studies.

In zero-*trans* and exchange entry measurements, uptake was also measured in noninjected oocytes at the same time point and the resulting space subtracted from that observed for the injected oocytes. It was established that water-injected oocytes had uptakes no different from noninjected cells, and that the rate of uptake by noninjected cells was independent of the extracellular 3-*O*-methylglucose concentration (not shown). Following correction for nonspecific uptake and trapping, duplicate or triplicate clearance measurements at each hexose concentration were converted to units of pmol·min^{-1}·oocyte^{-1} before analysis. Since influx (Nishimura et al., 1993) and efflux (Whitesell et al., 1993) of 3-*O*-methylglucose are known to follow biexponential kinetics, we measured transport at early times, when rates of influx or efflux were clearly linear (i.e., <10% of equilibrium 3-*O*-methylglucose space).

Efflux of 3-O-Methylglucose. Immediately before starting the efflux assay, single oocytes were injected with radiolabeled and unlabeled 3-*O*-methylglucose calculated to result in the desired intracellular 3-*O*-methylglucose concentration. Following injection, the oocyte was removed from the injection well, washed briefly in 1.5 mL of OR, and transferred to a scintillation vial containing 0.5 mL of OR at time zero. After 1 min the oocyte was transferred to another scintillation vial. The efflux rate was calculated as the radioactivity in the efflux vial divided by the total radioactivity originally in the oocyte. The latter was taken as the sum of the radioactivity in the efflux vial and that left in the oocyte at the end of the incubation. Fractional efflux calculated in this manner was less than 10% of equilibrium space. These initial rates of efflux at various hexose concentrations were used in the curve-fitting analysis to derive the Michaelis−Menten parameters.

Curve-Fitting and Data Analysis. The hyperbolic transport data from kinetic experiments were fit using global analysis as described previously for measurements of glucose transport coupled to phosphorylation (Whitesell et al., 1993). In the present analysis the steady-state uptake model was simplified to include only two compartments, the extracellular and intracellular spaces, connected by the transport step. To increase the degrees of freedom and enhance discrimination between parameters in global analysis, repetitive experi-

ments and experiments from different models were linked where possible by their common parameters (Beechem et al., 1991). Data were fit using a nonlinear least-squares approach based on the Marquardt–Levenberg algorithm to determine the best-fit parameter estimate corresponding to the minimum χ^2. In the error analysis routine, the error surface for a specified range of each parameter (e.g., K_m, V_{max}, or nonsaturable uptake) is determined by allowing all of the other unknown parameters to vary independently. In this manner a multidimensional error surface can be established for two or more parameters. All of the higher order correlations that may exist between a given set of fitting parameters are considered. The statistical significance is determined from the χ^2 distribution by means of an F-test (Box, 1960). A difference between two parameters is considered significant when there is no overlap in their χ^2 values below a specified confidence level (usually 0.67).

Oocyte Plasma Membrane Preparation. Purified oocyte plasma membrane ghosts were prepared by an adaptation of the method described by Chaney and Jacobson (1983). From 10 to 50 oocytes were rinsed three times in 2 mL of attachment buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid, 140 mM sorbitol, pH 6.5]. The cells were resuspended in 0.3 mL of attachment buffer, and 0.3 mL of a 6–8% suspension of cationic silica was added to the cells with gentle mixing. The cells were immediately diluted with 7–10 mL of wash buffer [20 mM 2-(*N*-morpholino)-ethanesulfonic acid, 70 mM NaCl, pH 6.5]. The cells were mixed and allowed to settle, and the supernatant was discarded. The cells were resuspended in 0.3 mL of wash buffer and treated with 0.3 mL of 2 mg/mL polyacrylic acid [M_r = 90 000 (Sigma)], prepared in wash buffer (pH 6.5). Cells were washed a total of four times with 7–10 mL of the wash buffer. The bead attachment procedure was repeated once or twice with similar results. Following the last wash, the cells were resuspended in 2 mL of ice-cold oocyte homogenization buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, pH 7.6). All subsequent steps were performed on ice or at 4 °C. The cells were lysed manually with watchmaker's forceps, and yolk and cytoplasmic contents were separated from the ghost membranes by gentle pipetting up and down 8–10 times with a Pasteur pipette. The membrane suspension was centrifuged for 20–30 s at 200*g*, the supernatant was removed and saved, and the ghosts were resuspended in 1–2 mL of ice-cold homogenization buffer. The low-speed centrifugation wash was repeated twice. After the last wash, any yolk remaining attached to the ghosts was removed by pipetting up and down under a dissecting microscope. The membranes were washed twice in 1 mL of homogenization buffer by centrifugation for 30 s at 200*g*. Following the last wash, the membranes were resuspended in 0.2 mL of homogenization buffer for assay of enzyme activity or for immunoblotting later. The wash supernatants (6–8 mL) were combined and saved for the same assays.

Cell Surface Labeling with [^{125}I]Concanavalin A. Surface labeling of oocytes was carried out by incubating 30 oocytes in 0.1 mL of OR buffer with 0.01 μCi of [^{125}I]concanavalin A for 10 min at 23 °C, followed by three rinses in 5 mL of OR buffer to remove the unbound lectin. After purified plasma membranes were prepared from cells coated with the silica pellicle as described above, the total amount of radioactivity in the plasma membrane and homogenate fractions was determined by γ counting. The fractional yield of plasma membrane was calculated by dividing the radioactivity in the plasma membrane fraction by the sum of the radioactivity in the plasma membrane and total cytoplasmic fractions (i.e., all radioactivity bound initially).

Sodium Dodecyl Sulfate Electrophoresis and Immunoblotting. The oocyte plasma membrane or homogenates were dissolved in an equal volume of sample buffer containing 125 mM Tris-HCl, 20% glycerol (v/v), 4% sodium laurel sulfate, 10% β-mercaptoethanol (v/v), and 0.0025% bromphenol blue (w/v), pH 6.8. Samples were incubated at 37 °C for 5 min and microfuged for 10 s, and the solubilized material was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Electrophoresis was performed using a 4% acrylamide stacking gel and a 10 × 6 × 0.075 cm running gel of 8% acrylamide. Prestained molecular weight markers ("Rainbow" standards, Amersham) were run in lanes next to the samples. Following electrophoresis, the gel was soaked for 10 min in transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, and 20% methanol (v/v). A sandwich was formed to the size of the gel with four pieces of transfer buffer-soaked Whatman chromatography paper (0.18 mm thickness) on the bottom, followed by the gel, then by a section of methanol-prewetted poly(vinylidene difluoride) microporous membrane (Immobilon, Millipore), and finally by four more pieces of buffer-soaked chromatography paper on top. Electrotransfer was performed for 18 min at 24 V using a Bio-Rad Semi-Dry apparatus (Bio-Rad Inc.). Transport proteins were detected on the membrane by immunoblotting using a chemiluminescence detection method. In this method, the membrane is rewetted in methanol, and nonspecific sites are blocked by vigorous shaking for 1 h in 5% nonfat dry milk in 50 mL of a phosphate-buffered saline-Tween (PBS-T) solution consisting of 10 mM phosphate, 150 mM NaCl, and 0.05% Tween 20 (v/v), pH 7.4. The membrane is washed twice by gentle shaking in 30 mL of PBS-T for 5 min and then incubated for 1 h at room temperature with the primary antibody added in the same buffer containing 10 mg/mL bovine serum albumin. The antibodies used were as follows: GLUT1 monoclonal antibody 7F7.5 at a 1:5 dilution of tissue culture supernatant (Tai & Carter-Su, 1988), a gift from Dr. Christin Carter-Su, and affinity-purified GLUT4 polyclonal antibody at a 1:5000 dilution (Goto et al., 1992). Following incubation with the primary antibody, the membrane was washed once with 30 mL of PBS-T for 15 min and then four additional times for 5 min each in the same volume of PBS-T. Antibody 7F7.5 was detected with horseradish peroxidase conjugated to goat anti-mouse antibody (1:1000 dilution in PBS-T with 10 mg/mL of bovine serum albumin). The polyclonal antibody was detected with horseradish peroxidase coupled to protein A (1:10 000 dilution in PBS-T with 10 mg/mL of bovine serum albumin, Boehringer Mannheim). The detection reagents were incubated with the membrane for 1 h with shaking and washed as described for the primary antibody. The chemiluminescence reagent (Renaissance, Dupont, New England Nuclear) was prepared and used according to the manufacturer's instructions. For detection, the moist membrane was covered in clear acrylic plastic and exposed to Kodak XRP-5 film for 10–40 s before developing. The intensity of individual bands on the X-ray film was quantified by laser-scanning densitometry, using an LKB

Table 1: Recovery and Purity of Oocyte Plasma Membrane[a]

marker for	% in plasma membrane	*N*
plasma membrane	87 ± 3	4
lysosomes	0.4 ± 0.32	5
endoplasmic reticulum	0.66 ± 0.54	5
mitochondria	0.82 ± 0.64	4

[a] Oocytes were labeled with [^{125}I]concanavalin A and cytoplasmic and plasma membranes were prepared as described under Experimental Procedures. Marker enzymes were as follows: lysosomes, *N*-acetylglucosaminidase; endoplasmic reticulum, NADH diaphorase; and mitochondria, succinate−cytochrome *c* reductase. All amounts are shown as a percent of the total in the cell.

Ultroscan XL densitometer with data analysis by Gelscan XL software (version 2.1) on a 486 computer. The absorption of the immunoblotted transporter band (or doublet) for both oocyte plasma membrane and homogenate was determined and corrected for background absorption present in a blank area of the film. The absorption of the plasma membrane band was expressed as a percent of the total absorption of plasma membrane and homogenate bands. The resulting percentage was corrected for dilutions and the amount of protein loaded on the gel.

Other Assays and Data Analysis. The following marker enzymes were assayed in the plasma membranes and in the combined supernatants remaining after plasma membrane removal: NADH diaphorase (endoplasmic reticulum) (Wallach & Kamat, 1966), succinate−cytochrome *c* reductase (mitochondria) (Vaes & Jacques, 1965), and *N*-acetylglucosaminidase (lysosomes) (King, 1967). Protein was measured by the BCA method (Pierce Chemical Co.). Except where noted, data are shown as mean ± standard error. Statistical comparisons were made using the Student's nonpaired "*t*"-test.

RESULTS

Expression and Function of Transporters in the Oocyte Plasma Membrane. To correlate the expression of glucose transporters with their function, it was necessary to develop a method for estimating the relative amounts of transporter in the plasma and internal membranes of oocytes. Our attempts to prepare oocyte plasma membranes by published methods (Sadler & Maller, 1981; Keller et al., 1989) were unsuccessful because of fragmentation of the delicate oocyte plasma membrane. We therefore adapted a method of coating cells with a pellicle of cationic silica before disruption (Chaney & Jacobson, 1983). This treatment was found to markedly strengthen the plasma membrane of oocytes so that large translucent ghost membranes could be prepared, essentially free of visible yolk platelets or dark cortical granules. When first allowed to bind fluorescein-tagged concanavalin A, the resulting ghosts were brightly fluorescent (not shown). Recovery of plasma membrane was quantified by allowing the oocytes to bind [^{125}I]concanavalin A before ghost preparation. Contamination of plasma membranes with internal membranes and organelles was assessed by measuring the activities of several marker enzymes in the ghost membranes. The results of such measurements on several oocyte preparations are shown in Table 1, expressed as a percentage of the total marker enzyme initially present in the oocyte. Recovery of plasma membrane was consistently high and allowed membrane preparation from as few as 10 oocytes for gel electrophoresis. The lack of complete recovery is attributed to some fragmentation of the silica-treated membranes and failure to pellet these in low-speed centrifugations. The results of the marker enzyme measurements show that even a concentrated pellet of plasma membranes contained a low and at times unmeasurable contamination of intracellular membranes. This method was used for plasma membrane preparation in subsequent experiments in which glucose transporters were quantified in the plasma membrane and in internal membranes.

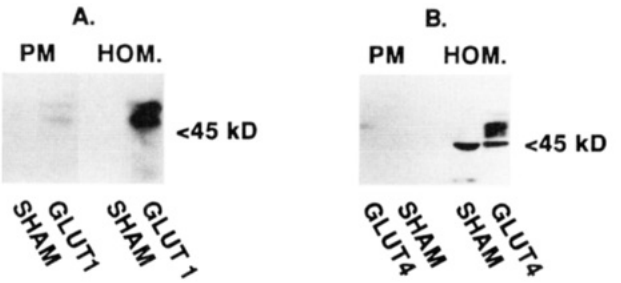

FIGURE 2: Expression of GLUT1 and GLUT4 in the plasma membrane of oocytes. Plasma membrane was prepared from thirty oocytes expressing GLUT1 (A) or GLUT4 (B) as described under Experimental Procedures. Aliquots of the plasma membrane (PM) and combined homogenate (HOM) for control oocytes (SHAM) or oocytes expressing the indicated glucose transporter (GLUT1, GLUT4) were subjected to gel electrophoresis and immunoblotting as described under Experimental Procedures. For GLUT1, gel lanes were loaded with 2.0 μg of plasma membrane from a total of 87 μg recovered, and 50 μg of homogenate from a total of 2.4 mg. For GLUT4, 30 μg of plasma membrane from a total of 96 μg recovered, and 10 μg of homogenate from 3.2 mg were loaded in each lane. The location of the 45 000 kDa marker is indicated.

Figure 2 shows representative immunoblots of GLUT1 and GLUT4 expressed in oocyte cytoplasm and plasma membrane. The amounts of both transporter isoforms were adjusted by dilution of cytoplasmic transporters to give measurable signals in the immunoblots. Both transporter types typically migrated as a singlet or doublet at 45−50 kDa. GLUT1 immunoreactivity was prominent in the plasma membrane fraction, whereas GLUT4 was difficult to detect in plasma membranes (Figure 2). The densely stained band at 45 kDa in the cytoplasmic fraction of GLUT4 was present in noninjected oocytes and was considered to be nonspecific. When corrected for dilutions, in five oocyte preparations 4.3 ± 0.5% of GLUT1 was found in the plasma membrane, whereas in the same number of preparations only 0.6 ± 0.2% of GLUT4 was found in the plasma membrane fraction ($p < 0.001$). This difference in membrane expression did not account for all the decrease in transport rates found in GLUT4 (Table 2). It likely reflects residual contamination of the plasma membranes with intracellular GLUT4, since the amount of GLUT4 expressed in the plasma membrane was similar to the amount of intracellular marker enzyme contamination.

To confirm that expressed transporters are functional, oocytes were injected with increasing amounts of GLUT1 mRNA and both their function and plasma membrane expression were measured. In Figure 3 is shown the zero-*trans* influx of 1 mM 3-*O*-methylglucose into oocytes as a function of the mRNA injected. Transport rates showed a nonlinear relationship with the amount of mRNA injected. In other experiments a distinct plateau was evident above 15 ng of injected mRNA (not shown). This decrease in transport rate with injected mRNA was associated with a comparable decrease in expression of GLUT1 in the plasma

Table 2: Kinetic Parameters of GLUT1, Truncation Mutants, and Chimeras[a]

construct	assay	K_m (mM)	V_{max} (pmol/oocyte·min)	N
GLUT1	eq. exch.	37.9 (19.5−90)[b]	1627	3
GLUT1	Z-T influx	9.24 (5.1−17.1)[b]	396	4
GLUT1	Z-T efflux	23.2 (7.5−>100)[b]	997	5
GLUT1-HIS	eq. exch.	39.9 (18.8−>100)[b]	940	3
GLUT1-HIS	Z-T influx	5.5 (2.5−12.3)[b]	130	3
G1-GLY(470)	Z-T influx	7.7 (5.0−12.3)[c]	702 (593−894)[c]	3
G1-PHE(450)	Z-T influx	11.0 (7.5−91)[c]	23 (9.5−30)[c]	3
GLUT4	Z-T influx	2.93 (2.6−6.2)[c]	2.6 (1.8−4.2)[c]	3
G1/4-B	Z-T influx	5.4 (4.2−8.1)[c]	5.4 (4.5−6.2)[c]	2
G4/1-B	Z-T influx	13.8 (7.1−28)[c]	93.1 (70−140)[c]	3
G4/1-N	Z-T influx	15.4 (7.3−22.1)[c]	200 (149−343)[c]	3

[a] Kinetic parameters of oocytes expressing native GLUT1 and the various constructs were measured as described under Experimental Procedures. Values in parentheses are confidence intervals for linked[b] and unlinked[c] fits in global analysis. "N" indicates the number of experiments performed.

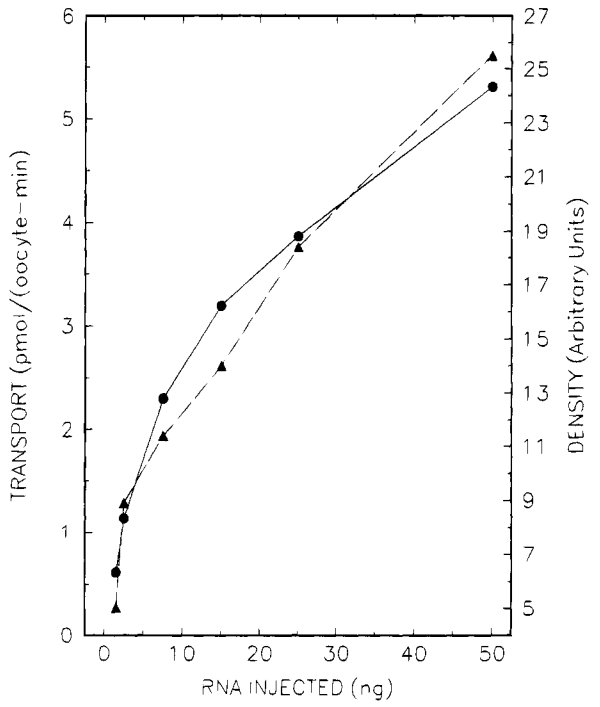

FIGURE 3: Expression of increasing amounts of GLUT1 mRNA in *Xenopus* oocytes. Oocytes were injected with the indicated amount of GLUT1 mRNA in the same volume. After 48 h the oocytes were divided into groups for measurement of 3-*O*-methylglucose transport or were used to prepare purified plasma membrane, both as described under Experimental Procedures. The concentration of 3-*O*-methylglucose was 1 mM, and transport times were varied (5−15 min) to ensure that no more than 10% of the 3-*O*-methylglucose equilibrium distribution space was occupied by the transported hexose. Transport data are means of 3−5 determinations at each mRNA concentration. The uptake of 3-*O*-methylglucose is shown as solid circles, and the relative densitometric readings are shown as solid triangles.

membrane (Figure 3). The latter was measured as the relative density of bands on an immunoblot of transporter protein. Although not shown, intracellular accumulation of GLUT1 followed the same relationship as in the plasma membrane. Similar results were obtained in another experiment. These data suggest that whereas transporter expression in the plasma membrane is saturable, the transporters that reach the plasma membrane are functional.

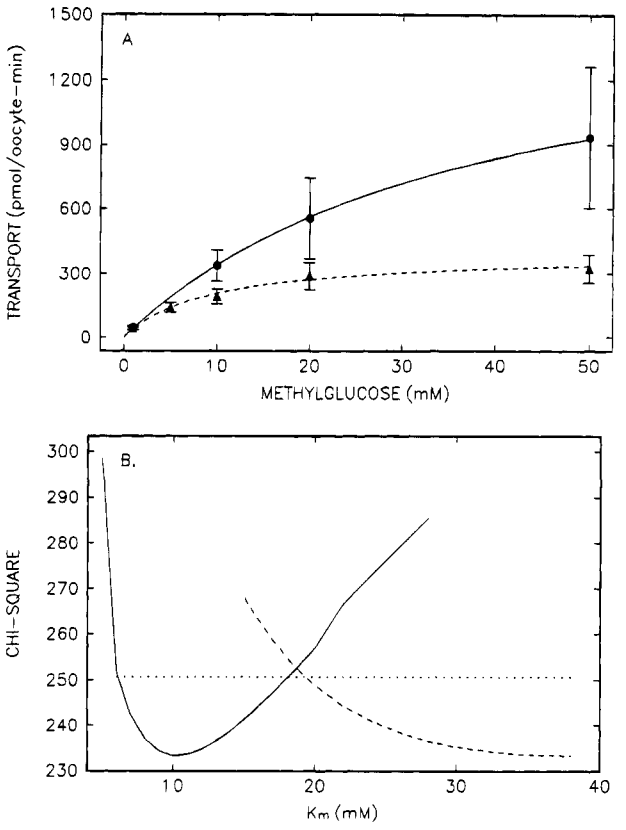

FIGURE 4: Zero-*trans* and equilibrium exchange kinetics and error analysis of GLUT1 expressed in oocytes. (Panel A) Transport of the indicated concentration of 3-*O*-methylglucose was measured under zero-*trans* (solid circles, $N = 4 \pm$ SD) or equilibrium exchange entry conditions (solid triangles, $N = 3 \pm$ SD) as described under Experimental Procedures. Nonlinear fits of initial transport rates at each substrate concentration were carried out in global analysis as also described under Experimental Procedures and are shown by the curved lines through the respective data points. (Panel B) Error analysis of the fits depicted in Panel A was performed in global analysis and is shown as the χ^2 derived from zero-*trans* entry (solid line) or from equilibrium exchange entry (dashed line) as a function of the 3-*O*-methylglucose concentrations in the medium. The horizontal dotted line indicates the 0.67 level of significance.

Transport Kinetics in Oocytes Expressing GLUT1. Since we wish to correlate transport kinetics of native with modified transporters, it is important to first show that GLUT1 retains its kinetic transport characteristics when expressed in *Xenopus* oocytes. Thus GLUT1 should have a similar substrate affinity and an asymmetrical transport mechanism in oocytes. In other words, we should find a higher affinity for substrate on entry than on exit (Lowe & Walmsley, 1986). This hypothesis was assessed by measuring the kinetics of zero-*trans* entry, zero-*trans* efflux, and equilibrium exchange entry of 3-*O*-methylglucose in oocytes. These three experiments have been shown to define the essential features of the one-site model of GLUT1 transport in human erythrocytes (Lowe & Walmsley, 1986; Devés & Krupka, 1979). In Panel A of Figure 4 are shown the initial rates of 3-*O*-methylglucose transport as a function of the extracellular 3-*O*-methylglucose concentration for equilibrium exchange and zero-*trans* influx. The zero-*trans* efflux data are not shown for the sake of clarity. Visual inspection suggests that both the K_m and V_{max} of exchange entry are greater than the corresponding parameters for zero-*trans* entry. We used an approach termed global analysis to fit initial transport rates to the Michaelis−Menten equation. This

analysis derives the kinetic parameters from the three transport experiments. Since global analysis allows common parameters in different but related experiments to be linked, we took advantage of the fact that the Haldane relationship requires that the ratio V_{max}/K_m be equal for the three different transport methods (Lowe & Walmsley, 1986). In the fitting analysis, all the data from 12 experiments (150 data points) were linked by a common V_{max}/K_m, with inverse weighting to compensate for changes in the specific activity of 3-*O*-methylglucose over the range of concentrations used. This analysis yielded a V_{max}/K_m of 42.9 μL·oocyte^{-1}·min^{-1} for the three experimental types. The curved lines in Figure 4A represent the global fits to the hyperbolic transport data. Two-dimensional error surfaces for the zero-*trans* and exchange entry K_m values are shown in Figure 4B. The χ^2 values do not overlap below the 0.67 confidence level (dotted horizontal line), indicating a significant difference between these parameters. The K_m and V_{max} values and their corresponding asymmetric global confidence limits for the three types of experiments are listed in Table 2. Efflux kinetic parameters did not differ from influx or exchange measurements in these experiments, mainly because the results of efflux measurements were more variable than those of influx or influx exchange. The lower K_m for zero-*trans* than exchange uptake (Table 2) suggests that the expressed human GLUT1 has kinetic asymmetry. The higher equilibrium exchange V_{max} (42.9 μL·oocyte^{-1}·min^{-1} × 37.9 pmol/μL = 1626 pmol·oocyte^{-1}·min^{-1}) indicates that the slowest step during influx is return of the unloaded carrier to an outward-facing conformation (May & Mikulecky, 1982). Both features resemble those of erythrocyte GLUT1 (Lowe & Walmsley, 1986). Finally, the apparent K_i values for transport inhibition by cytochalasin B and EGlc (Table 3) were also in the range expected for the erythrocyte GLUT1 under these conditions (Jung & Rampal, 1977). These two agents were selected for study since they are known from kinetic studies in erythrocytes to bind to opposite faces of the transporter: cytochalasin B binds to the inward-facing conformation (Devés & Krupka, 1978) and EGlc binds to the outward-facing form (Baker et al., 1978).

Constructs with Additions to and Deletions from the C-Terminal Tail of GLUT1. To determine whether additional amino acids on the C-terminal tail of GLUT1 affect its transport kinetics, we prepared a construct containing six additional histidine residues and a construct containing part of the connecting peptide of the proinsulin molecule with 31 additional amino acids. The kinetics of zero-*trans* and equilibrium exchange entry were evaluated for the GLUT1-His construct and are given in Table 2. When fits in global analysis were linked by a common Haldane parameter, this construct showed asymmetry of transport comparable to that of native GLUT1. The K_m for equilibrium exchange entry was almost 8-fold higher than for zero-*trans* entry in the GLUT1-His construct, with a comparable inverse relationship in the V_{max} values (Table 2). This result suggests that addition of histidines did not affect the transport mechanism of the parent GLUT1. The lower calculated V_{max} values for the GLUT1−His construct than for native GLUT1 given in Table 2 are attributed to seasonal variation of oocyte transport, since the experimental series for each were performed several months apart. Direct comparisons between GLUT1 transport rates and those of the GLUT1−His construct are given in Figure 5A. The construct containing

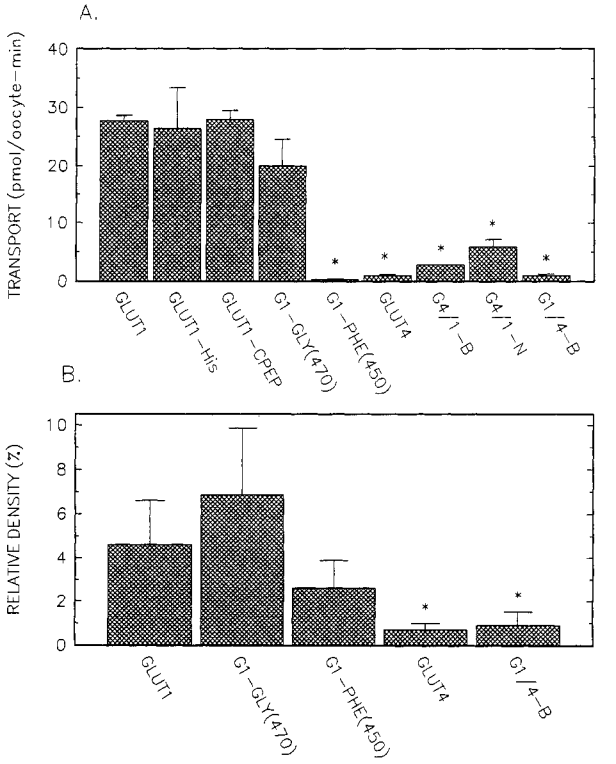

FIGURE 5: Plasma membrane expression and influx of 3-*O*-methylglucose in native and modified transporters. (Panel A) Transport of 3-*O*-methylglucose was measured at 1 mM 3-*O*-methylglucose and rates are shown for 3−6 experiments. (Panel B) Relative expression of each transporter construct was measured by densitometry of immunoblots as described under Experimental Procedures for 3−5 experiments, ±SD. An asterisk "*" indicates $p < 0.01$ compared to GLUT1.

part of the C-peptide sequence also showed similar transport to GLUT1 (Figure 5A). Thus adding as many as 31 amino acid residues to the C-terminal tail of GLUT1 had no significant effect on function of the protein.

To evaluate the effects of removing part of the C-terminal cytoplasmic tail on GLUT1 expression and function, two truncation mutants were engineered and expressed in oocytes. It was possible to assess the plasma membrane expression of the truncation mutants using immunoblotting with antibody 7F7.5, which recognizes epitopes located elsewhere on the protein than the C-terminal tail (Tai & Carter-Su, 1988). The G1-GLY(470) truncation mutant had plasma membrane expression (Figure 5B), zero-*trans* influx kinetics (Figure 5A, Table 2), and inhibitor binding affinities (Table 3) that were similar to those observed for GLUT1. The G1-PHE-(450) truncation mutant had very low rates of transport (Figure 5A and Table 2). Because transport rates were so low and variable from preparation to preparation, the transport K_m was difficult to measure in the G1-PHE(450) construct. Expression of the G1-PHE(450) truncation in the plasma membrane was decreased to about 50% that of GLUT1 (Figure 5B), whereas transport was decreased to less than 5% the rates observed for GLUT1 (Figure 5A). Thus, a decrease in plasma membrane expression alone does not explain the marked decrease in transport rates observed for the G1-PHE(450) truncation mutant. The affinity of the G1-PHE(450) construct for cytochalasin B was normal, but the affinity of this construct for EGlc was decreased several-fold compared to GLUT1 or to the G1-GLY(470) construct (Table 3). This indicates that GLUT1 lacking the entire

Table 3: Effect of Side-Selective Inhibitors on Oocyte Transport[a]

construct	cytochalasin B K_i (μM)	EGlc K_i (mM)
GLUT1	0.9 ± 0.3	14.5 ± 1.6
G1-GLY(470)	0.5 ± 0.1	8.0 ± 1.0
G1-PHE(450)	1.0 ± 0.3	42.9 ± 3.4[b]
GLUT4	1.0 ± 0.2	11.2 ± 5.3
G1/4-B	0.6 ± 0.01	11.9 ± 3.9
G4/1-B	1.9 ± 1.1	11.2 ± 1.4
G4/1-N	1.2 ± 0.8	12.8 ± 4.7

[a] Oocytes expressing the indicated transporter construct were incubated with a range of inhibitor concentrations and transport measured as described under Experimental Procedures at 1.0 or 0.1 mM 3-*O*-methylglucose. Apparent inhibitory constants (K_i) were determined from Dixon plots as previously described (May et al., 1993). Data are shown as means ± SE for two or three separate experiments. [b] $p <$ 0.01 versus GLUT1.

C-terminal tail cannot assume the outward-facing conformation required to bind EGlc.

Chimeras of GLUT1 and GLUT4. When expressed in *Xenopus* oocytes, the two chimeras composed mostly of GLUT4 had zero-*trans* transport rates about one-fourth to one-half that of GLUT1. However, these were markedly higher than observed for native GLUT4 (Figure 5A). The K_m values for both the GLUT4/1-B and the GLUT4/1-N chimeras were significantly higher than for GLUT4, but no different from the GLUT1 K_m (Table 2). On the other hand, the chimera composed mostly of GLUT1 (GLUT1/4-B) had very low rates of transport (Figure 5A), with a K_m resembling that of GLUT4 (Table 2). It was not possible to carry out immunoblotting studies on the GLUT4/1 chimeras due to lack of a C-terminal antibody with adequate affinity and low background. However, only 0.4% of the GLUT1/4-B chimera was found in immunoblots of plasma membrane compared to homogenate, a distribution similar to that noted previously for GLUT4. This suggests that decreased plasma membrane expression largely accounts for decreased transport in the GLUT1/4-B construct. Despite very low transport rates in GLUT4 and in the GLUT1/4-B chimera, inhibitor affinities were comparable and were similar to those observed for the constructs containing more GLUT1 (Table 3).

DISCUSSION

Xenopus laevis oocytes have proved to be a useful expression system for study of the structure–function relationships of the "GLUT" family of glucose transporters (Keller et al., 1989; Nishimura et al., 1993). At the stage of development used, these oocytes lack appreciable endogenous glucose transport and express exogenous transporter isoforms with cellular targeting sites and simple transport kinetics resembling those of other cells (Keller et al., 1989; Nishimura et al., 1993). Transporter protein expression in the plasma membrane varies with different batches of cells, with the time after introduction of mRNA, and with the type of transporter introduced. Therefore, it is necessary to have a method for estimating the amount of transporter in the plasma membrane. This is complicated by the fact that the oocyte plasma membrane contains a small fraction of total transporters synthesized from injected mRNA (Garcia et al., 1992; Nishimura et al., 1993). Any contamination of the plasma membrane fraction with intracellular membranes will cause an overestimation of the transporter content in the plasma membrane. Essentially pure oocyte plasma membrane "ghosts" can be prepared from manually dissected cells by the method of Wall and Patel (1989). Using this method, Nishimura et al. (1993) calculated that about 8% of rat GLUT1 and 2% of rat GLUT4 transporters/cell are expressed in the plasma membrane of oocytes. These estimates correlated well with those made by surface labeling cells using a photosensitive bis(mannose) derivative (Nishimura et al., 1993). However, manual dissection of oocytes is time-consuming, and the residual vitelline membrane makes it difficult to inject mRNA into the cells and to measure transport. Collagenase digestion has been used to prepare oocyte "ghost" membranes that contained about 12% of human GLUT1 in the plasma membrane (Keller et al., 1989; Garcia et al., 1992). Collagenase treatment removes the vitelline membrane and attached follicular cells, but it is difficult to avoid fragmentation of the thin plasma membrane during separation from intracellular yolk and membrane (Wall & Patel, 1989). Because of this problem, we adapted a method for strengthening the plasma membrane developed by Chaney and Jacobson (1983). In this method the cell is coated with one or more layers of positively charged colloidal silica entrapped in a meshwork of polyacrylic acid. The method is rapid, reproducible, and results in plasma membrane ghosts that are suitable for gel electrophoresis and immunoblotting. Our results show that despite injection of saturating amounts of mRNA, only about 4% of GLUT1, and less than 1% of GLUT4 are present in the plasma membrane. These values are lower for each transporter than previously reported (Keller et al., 1989; Garcia et al., 1992; Marshall et al., 1993; Thomas et al., 1993). Since maximal transport rates observed in this study are similar to values obtained by most other investigators (Keller et al., 1989; Marshall et al., 1993; Thomas et al., 1993; Nishimura et al., 1993), comparable numbers of transporters appear to have reached the plasma membrane in the present studies. Additionally, plasma membrane recoveries in our experiments were measured directly and were usually 80–90%. Decreased contamination of plasma membranes with intracellular membranes probably accounts for the lower estimates of transporter content in the plasma membrane obtained in this study. The relative distributions of GLUT1 and GLUT4 between cytosol and plasma membrane in oocytes generally reflect those of the two transporters in insulin-sensitive tissues, with GLUT4 located primarily in internal membranes and more GLUT1 in the plasma membrane (Bell et al., 1990). However, oocytes retain a much larger fraction of GLUT1 molecules in intracellular locations than do insulin-sensitive cells, possibly due to incomplete processing.

In a previous study, Garcia et al. (1992) reported that rates of 3-*O*-methylglucose transport decreased more than protein expression in the plasma membrane with increasing amounts of mRNA injected. This suggests that transporters have decreased functional capacity at high levels of expression in the plasma membrane. Although we injected similar amounts of mRNA as did Garcia et al., we found that transport and plasma membrane expression of GLUT1 declined in a coordinate manner at high levels of injected mRNA (Figure 3). A possible explanation for the difference between the two studies is the following. Garcia et al. (1992) measured transport over a 30 min period at all levels of GLUT1 expression. Assuming an intracellular 3-*O*-methylglucose space of 0.25 μL/oocyte, intracellular 3-*O*-methylglucose had reached about 40% of equilibrium space at

the higher levels of expression in the experiment of Garcia et al. (1992). Nishimura et al. (1993) reported that oocytes show biexponential kinetics of 3-*O*-methylglucose transport, with saturation of a rapidly filling compartment at intracellular 3-*O*-methylglucose concentrations well below those measured following longer periods of equilibration. It is possible that the apparent decrease in function of transporters at high levels of expression in the study of Garcia et al. (1992) was due to filling of such a compartment. To reduce this problem, we shortened transport times to result in filling of less than 10% of the equilibrium space of oocytes. With this modification, our results suggest that the intrinsic activity of GLUT1 at high levels of expression in oocyte plasma membrane is not compromised.

To simplify comparison of transport kinetics between native and mutated glucose transporters expressed in oocytes, we have adapted an approach termed global analysis for fitting and error analysis of hyperbolic transport data (Whitesell et al., 1993). Besides the nonlinear fitting, global analysis provides an estimate of the goodness-of-fit of the data based on the χ^2 distribution. This is the most rigorous analysis of the error surface possible (Beechem, 1992). The confidence intervals obtained from this analysis are derived from *all* the weighted data points of the curves and can be directly compared between analyses. An additional advantage of global analysis is that common parameters can be linked directly in the fitting procedure, allowing comparison of the data from multiple experiments within the same model. We used linked analyses with a common V_{max}/K_m to compare the results of transport experiments defining the kinetic model for GLUT1: equilibrium exchange entry, zero-*trans* influx, and zero-*trans* efflux (Figure 4 and Table 2). We found asymmetry of substrate binding affinities on opposite faces of the transporters, a faster rate of movement of the substrate-loaded than the unloaded transporter, and comparable inward- and outward-binding inhibitor affinities. Thus GLUT1 expressed in oocytes has many kinetic similarities to erythrocyte GLUT1 (Lowe & Walmsley, 1986). This suggests that these kinetic transport features are determined by the intrinsic sequence and not due to interaction of the transporter with nearby membrane lipids or proteins.

We made three types of changes in the C-terminal tail of GLUT1: additions, truncations, and substitutions. If the C-terminal tail is necessary for the conformational change of the protein, we reasoned that adding extra amino acids to the tail might impair the ability of the transporter to shift from one form to another. However, addition of up to 31 amino acids to the C-terminal tail of native GLUT1 had no effect on 3-*O*-methylglucose transport in oocytes (Table 2 and Figure 5A). Thus the added length of the GLUT2 and GLUT4 C-terminal tails does not account for their different substrate affinities compared to GLUT1. Removal of up to 22 amino acids from the tail of GLUT1 was also without effect on transporter expression and transport kinetics (Table 2). However, removal of the entire C-terminal tail in the G1-PHE(450) almost abolished transport (Table 2 and Figure 5). The insignificant decrease in plasma membrane expression of this construct did not account for the observed decrease in transport rates (Figure 5B). The decrease in transporter function was due to a fall in the V_{max} and was not associated with a change in the transport K_m (Table 2). The G1-PHE(450) truncation lacks Lys-451. Loss of this positively charged residue may cause a major distortion of the transmembrane stability of GLUT1. Lys-451 is predicted by hydropathy analysis to be the first amino acid outside the lipid bilayer (Mueckler et al., 1985) and thus may be important for proper orientation of the last transmembrane segment. On the other hand, Oka et al. (1990) removed only the terminal 37 amino acids of the rabbit GLUT1. This construct had normal plasma membrane expression but a similar decrease in the ability of the transporter to bind and transport extracellular substrate. Our work has confirmed that of Oka et al. (1990) and further narrowed the range of amino acids required for adequate GLUT1 function to those between residues 457 and 471. Recent observations by Muraoka et al. (1994) using a series of truncation mutants of GLUT1 suggest that Phe-467 and Arg-468 are the crucial amino acids required for proper function of GLUT1.

As expected, the GLUT4 protein targeted mostly to intracellular sites (Figures 2 and 5). However, when a region of GLUT1 only slightly shorter than that removed in the G1-PHE(450) truncation mutant was swapped onto the C-terminal tail of GLUT4, transport increased to about 30–50% that of native GLUT1. Others have shown that the increase in transport of such chimeras compared to that of GLUT4 is due to increased expression in the plasma membrane (Czech et al., 1993; Verhey et al., 1993; Marshall et al., 1993). We also found that adding the C-terminal tail of GLUT1 to GLUT4 lowered substrate affinity (Table 2). A similar increase in the K_m was observed for the G4/1-N chimera, which contained most of the last two transmembrane segments of GLUT1 and its C-terminal tail. Conversely, swapping the C-terminal tail of GLUT4 onto GLUT1 had the opposite result. Expression of this transporter in the plasma membrane was very low, comparable to that found for native GLUT4. Despite its low V_{max}, the K_m of the G1/4-B construct was in the range observed for GLUT4, although over 90% of its sequence was derived from GLUT1. Katagiri et al. (1992) found that replacement of the last 35 amino acids of GLUT1 with the corresponding (larger) region of GLUT2 resulted in a protein with a fourfold lower affinity for substrate than that of native GLUT1. Taken with our results, it appears that the last 29 amino acids of the C-terminal tail are important in determining the affinity of the protein for substrate. The affinity change may be due to an impaired ability to shift conformations, since substrate binding during uptake in the kinetic studies is to the outward-facing substrate binding site.

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