Mammalian Facilitative Glucose Transporters: Evidence for Similar Substrate Recognition Sites in Functionally Monomeric Proteins†

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ABSTRACT: Four facilitative glucose transporters isoforms, GLUT1/erythrocyte, GLUT2/liver, GLUT3/brain, and GLUT4/muscle-fat, as well as chimeric transporter proteins were expressed in *Xenopus* oocytes, and their properties were studied. The relative K_m's of the transporters for 2-deoxyglucose were GLUT3 (K_m = 1.8 mM) > GLUT4 (K_m = 4.6 mM) > GLUT1 (K_m = 6.9 mM) > GLUT2 (K_m = 17.1 mM). In a similar fashion, the uptake of 2-deoxyglucose by GLUT1-, GLUT2-, and GLUT3-expressing oocytes was inhibited by a series of unlabeled hexoses and pentoses and by cytochalasin B in a similar hierarchal order. To determine if the functional unit of the glucose transporter was a monomer or higher-order multimer, the high-affinity transporter GLUT3 was coexpressed with either the low-affinity GLUT2 or a GLUT3 mutant which contained a transport inactivating $Trp^{410} \rightarrow$ Leu substitution. In oocytes expressing both GLUT2 and GLUT3, the transport activity associated with each transporter isoform could be distinguished kinetically. Similarly, there was no alteration in the kinetic parameters of GLUT3, or the ability of glucose or cytochalasin B to inhibit 2-deoxyglucose uptake, when coexpressed with up to a 3-fold greater amount of functionally inactive mutant of GLUT3. These studies suggest that the family of glucose transporters have similar binding sites which may be in the form of a functional monomeric unit when expressed in *Xenopus* oocytes.

In most cells, the utilization of glucose is initiated by the translocation of the sugar across the plasma membrane by an energy-independent process (Burant et al., 1991; Mueckler, 1990). Facilitative glucose transport is mediated by a family of structurally-related intregal membrane proteins, designated GLUT1/erythrocyte,[1] GLUT2/liver, GLUT3/brain, GLUT4/muscle-fat, and GLUT5/small intestine. Of these, GLUT1 (Thorens et al., 1990a; Burant et al., 1991), GLUT2 (Thorens et al., 1990b), GLUT3 (Maher et al., 1991), and GLUT5 (Davidson et al., 1992) appear to be localized primarily to the plasma membrane. By contrast, in the basal state, GLUT4 is associated primarily with a population of intracellular vesicles that translocate to the plasma membrane in the presence of insulin (Simpson & Cushman, 1986; Zorzano et al., 1989). Although glucose is the primary cellular carbohydrate energy source, other sugars are also taken up and utilized. Studies suggest that most hexoses are transported into the cell by passive diffusion through the same proteins which transport glucose (LeFerve, 1961; Wheeler et al., 1985). Although the structural requirements for the transport of sugars by the erythrocyte glucose transporter, GLUT1, have been well described (Walmsley, 1988; Carruthers, 1990), the substrate selectivities of other glucose transporters and the role of higher-order multimers in determining the properties of the facilitative glucose transporters is only beginning to be studied (Hebert & Carruthers, 1991; Pessino et al., 1991).

In this report, we have examined the interactions of a series of hexose and pentose sugars with the glucose transporter isoforms when expressed in *Xenopus laevis* oocytes. The results show that the rank-order affinities of GLUT1, GLUT2, and GLUT3 for glucose, other hexoses, and pentoses parallel one another, indicating that the sugar recognition site is similar for each of the these transporter isoforms. Although efficiently expressed as determined by Western blot analysis, the transport activity of GLUT4 was too low to allow detailed examination of its functional properties. We have also studied the transport properties of oocytes coexpressing two different glucose transporter isoforms and show that they reflect the kinetic characteristics of the parental transporters rather than a protein with intermediate properties implying that heteromultimers having different kinetic properties are not formed. Similar studies of oocytes expressing different amounts of normal and functionally inactive forms of the same isoform showed no inhibition of transport activity, which is also consistent with the monomer being the functional unit of transport activity.

MATERIALS AND METHODS

Isolation of Oocytes. Female *X. laevis* (Nasco, Ft. 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 ice immersion, and oocytes were removed by a lateral ventral incision, rinsed in OR-2 medium minus $CaCl_2$ (Maller, 1983), and incubated in two changes of OR-2 minus $CaCl_2$ containing 2 mg/mL of collagenase (Sigma, St. Louis, MO) for 1 h each at room temperature. The oocytes were then extensively washed in OR-2 containing 2 mg/mL gentamicin (GIBCO, Grand Island, NY). Individual oocytes were dissected free of connective tissue and associated vasculature and then kept

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[1] Abbreviations: GLUT, glucose transporter; dGlc, 2-deoxy-D-glucose; 3-*O*-MeGlc, 3-*O*-methylglucose; sRNA, synthetic messenger RNA; PBS, phosphate-buffered saline.

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in OR-2 with 2 mg/mL gentamicin for 18–24 h at 19 °C before use. Only oocytes in stage 5 or 6 were used in these studies.

Synthetic mRNA Synthesis and Oocyte Injection. sRNA synthesis and oocyte injection was performed as previously described (Kayano et al., 1990). Briefly, cDNAs encoding rat GLUT1 (Gould & Lienhard, 1989) or human GLUT2, GLUT3, and GLUT4 were cloned into pSP64T at a unique *Bgl*II site which is flanked by 89 bp of 5′- and 141 bp of 3′-untranslated region of the *Xenopus* β-globin mRNA (Kreig & Melton, 1984). The plasmids were linearized, and SP6-directed sRNA synthesis was performed using a Riboprobe kit (Promega Biotec, Madison, WI) and capped with 2.5 mM m^7(5′)GpppG(5′) (Pharmacia/LKB, Piscataway, NJ). Constructs encoding human GLUT3Leu410 or a chimeric transporter designated $GLUT3^{1-458}/GLUT4^{477-509}$ (described below) were cloned into the *Sal*I/*Bam*HI site of pGEM-4Z (Promega Biotec). sRNA synthesis was quantitated by the inclusion of 0.1 μCi of [α-^{32}P]UTP (Amersham, Arlington Heights, IL) in the reaction mixture. DNA was eliminated by incubation of the sRNA mixture with RNase-free RQ1 DNase. Quantitation of nucleotide incorporation was determined by trichloroacetic acid precipitation of ^{32}P-labeled RNA and Cherenkov counting. The RNA was dissolved in sterile water at concentrations of 0.2–0.5 mg/mL and used for injection. The integrity of the sRNA was assessed by formaldehyde–agarose gel electrophoresis.

Sterile water (100 nL) or an equal volume of sRNA solution was injected into oocytes near the vegetal hemisphere utilizing a micromanipulator. Oocytes were incubated at 19 °C in OR-2 supplemented with 2 mg/mL gentamicin for 48 h with media change every 12 h.

Measurement of Hexose Uptake in Oocytes. Hexose uptake was determined by incubation of oocytes in 250 μL of OR-2 media containing 2-[1,2-^{3}H]deoxyglucose, [1-^{14}C]galacotse, [*glucose*-^{14}C(U)]methyl-α-D-glucopyranoside, 3-*O*-[*methyl*-^{3}H]methyl-D-glucose, D-[^{14}C(U)]fructose (all from Dupont-NEN Products, Boston, MA), or L-[1-^{14}C]glucose (Amersham, Arlington Heights, IL) as described in the figure legends. Uptake was terminated by addition of 750 μL of ice-cold PBS solution containing 100 mM of D-glucose and D-galactose, 0.1 mM phloretin (Sigma, St. Louis, MO), and 50 μM cytochalasin B (Sigma). The oocytes are washed quickly three times with ice-cold PBS, individual eggs were solubilized in 0.5% sodium dodecyl sulfate, and radioactivity was determined by liquid scintillation counting. For GLUT3, the accumulation of dGlc, galactose, and 3-*O*-MeGlc was linear for 30, 5, and 2 min, respectively (data not shown), and no lag in uptake was noted.

In studies in which dGlc uptake was competed by other sugars, 50-nL of [γ-S]ATP (Sigma) was injected into oocytes 5–15 min prior to assay; the calculated final intracellular concentration was 50 mM assuming a 0.5-μL water space in an average oocyte (Gould & Leinhard, 1989). To initiate the transport, 25 μL of a 10-fold concentrated solution of the competitor was added immediately before that of 25 μL of a 500 μM solution of 2-[1,2-^{3}H]deoxyglucose, and uptake was terminated after 30 s as described above. The concentration of the competitor sugar that inhibited 50% of the dGlc uptake (IC_{50}) was calculated from the inhibition curves. In other experiments, cytochalasin B was added to the incubation media 5 min prior to the addition of 2-[1,2-^{3}H]deoxyglucose.

Measurement of Deoxyglucose Phosphorylation. The fraction of phosphorylated intracellular dGlc was measured as described by Olefsky (1978) at [^{14}C]dGlc concentrations of 50 μM and 20 mM in the presence of 0.1 μM [^{3}H]inulin as an extracellular marker. After 1 min, groups of 5 oocytes were transferred immediately to 1 mL of boiling water for 10 min. The oocytes were removed, the aqueous solution was then passed through Dowex 1X-8, and phosphorylated and nonphosphorylated dGlc was separated as described (Olefsky, 1978). The amount of [^{14}C]dGlc present in media that transferred with the oocytes to the boiling water solution was determined from the amount of [^{3}H]inulin present in the sample, and the results were corrected accordingly.

Mutagenesis of GLUT3. Mutagenesis was performed using a PCR-based protocol (Vallette et al., 1989). Nucleotide primers containing a A → G substitution in base 1056 of the cDNA (Kayano et al., 1988) were used to generate a GLUT3 protein in which TRP^{410} was replaced by Leu. A chimera of GLUT3 and GLUT4 was constructed by PCR in a similar manner to effect the replacement of the last 34 amino acids of GLUT3 with the corresponding region of GLUT4. The sequences of the mutated and chimeric cDNA constructs were confirmed by sequencing (Sambrook et al., 1989).

Western Blot Analysis. Groups of 10 oocytes injected 48 h previously with 50 ng of glucose transporter sRNA were homogenized in 500 μL of a solution of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10% sucrose, and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Coleman, 1984). The homogenate was layered on a step gradient of 1 mL of 20% sucrose over 1 mL of 50% sucrose, both in 10 mM Tris-HCl, pH 7.6, and 10mM $MgCl_2$ and centrifuged at 15000*g* for 30 min. The interface between the 20% and 50% layers was collected, diluted 10-fold with the homogenization buffer, and recentrifuged at 15000*g* for 60 min. The pellet was solubilized for 1 h in 100 μL of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 0.5% Na-deoxycholate, and 1 mM PMSF after which the insoluble material was removed by centrifugation at 14000*g* for 10 min. Supernatant protein (100 μg) was resolved on a 10% SDS–PAGE and electroblotted to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with a solution of 5% nonfat powedered milk (Carnation) in 0.05% Tween-20, 10 mM Tris-HCl, pH 7.4, and 500 mM NaCl and then incubated with 1:1000 dilution of a GLUT3 antisera directed against amino acids 477–492 (Graeme I. Bell, unpublished results), anti-GLUT4 serum (8105, kindly provided by S. Cushman, NIH) directed against the C-terminal 15 amino acids of GLUT4, or with normal rabbit serum washed with three changes of a solution of 0.05% Tween-20, 10 mM Tris-HCl, pH 7.4, and 500 mM NaCl. Antibody binding was detected after incubation with ^{125}I-labeled protein A (Amersham) by autoradiography using Kodak XAR-5 film at –80 °C for 12–36 h.

Kinetic and Statistical Analyses. K_m values were determined by best fit of Eadie–Hofstee transformation of the data and were determined with the help of ENZ-FIT (Elsevier-Biosoft, Cambridge, U.K.). Statistical significance was determined using Student's *t*-test. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

Kinetic Properties of Glucose Transporters. As shown previously (Birnbaum, 1989; Gould & Leinhard, 1989; Keller et al., 1989; Vera & Rosen, 1989; Kayano et al., 1990; Gould et al., 1990), *Xenopus* oocytes are an efficient system for studying the heterologous expression of mammalian facilitative glucose transporters. Oocytes injected with GLUT1, GLUT2, GLUT3, or GLUT4 sRNA showed an increase in accumulation of the glucose analogues dGlc, 3-*O*-MeGlc, and galactose over those injected with water (Figure 1). No transport of α-methylglucopyranoside or L-glucose was seen in uninjected

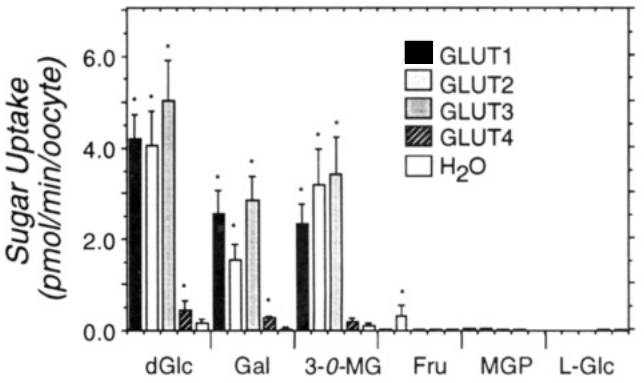

FIGURE 1: Transport of 2-deoxyglucose (dGlc), galactose (Gal), 3-*O*-methylglucose (3-O-MeGlu), fructose (Fru), α-methylglucopyranoside (MGP), and L-glucose (L-Glu). sRNA (25 ng) encoding to GLUT1–4 or water was injected into oocytes 48 h prior to the addition of 50 μM of the indicated ^{3}H- or ^{14}C-labeled sugar. After 30 s, uptake was terminated and the radioactivity accumulated was determined by scintillation counting of individual oocytes. Shown is a representative experiment with 5–7 oocytes present in each group. Bars represent mean ± SEM. * represent significantly ($p < 0.05$) increased transport over water-injected controls.

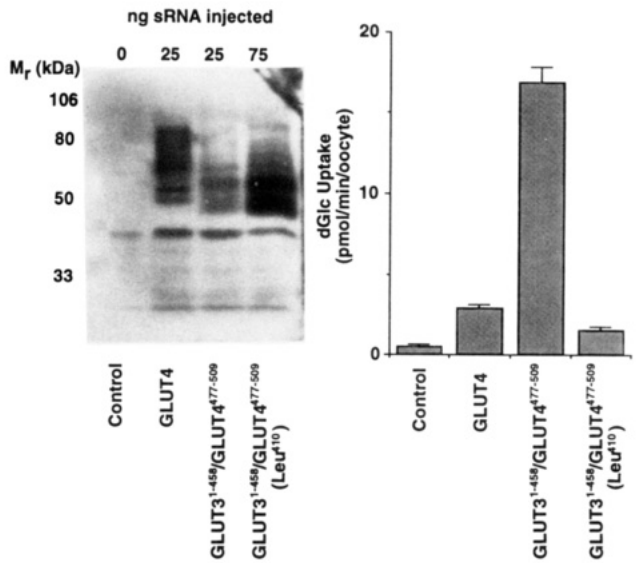

FIGURE 2: Western blot and dGlc uptake by oocytes expressing GLUT4, GLUT3$^{1-458}$/GLUT4$^{477-509}$ or GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing the Trp^{410} → Leu mutation. (Panel A, left) Membranes were prepared from groups of 10 oocytes injected 24 h previously with water, 25 ng of GLUT4, 25 ng of GLUT3$^{1-458}$/GLUT4$^{477-509}$, or 75 ng of GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing Trp^{410} → Leu sRNA and subjected to Western blotting as described under Materials and Methods. (Panel B, right) An identical group of oocytes was incubated with 500 μM [^{3}H]dGlc for 1 min at 25 °C, and the radioactivity accumulated was determined by scintillation counting of individual oocytes. Bars represent mean ± SEM for 5–8 oocytes from each group. * represent significantly ($p < 0.05$) increased transport over uninjected controls.

oocytes or in oocytes expressing any of the facilitative glucose transporters. Only GLUT2 sRNA injected oocytes showed any significant uptake of fructose, which was much less than the uptake of dGlc, 3-*O*-MeGlc, or galactose.

In repeated experiments, oocytes injected with 25 ng of GLUT3 sRNA demonstrated the highest rate of accumulation of each of the sugars tested. By contrast, oocytes injected with equivalent amounts of GLUT4 sRNA consistently showed only a small stimulation of glucose transport (Figure 1), even when injected with sRNA transcribed from vectors containing identical 5′ and 3′ noncoding regions as present in the efficiently expressed GLUT3 isoform (data not shown). The relatively small increase in dGlc transport by GLUT4-expressing oocytes was not due to the inability of the oocyte to synthesize protein

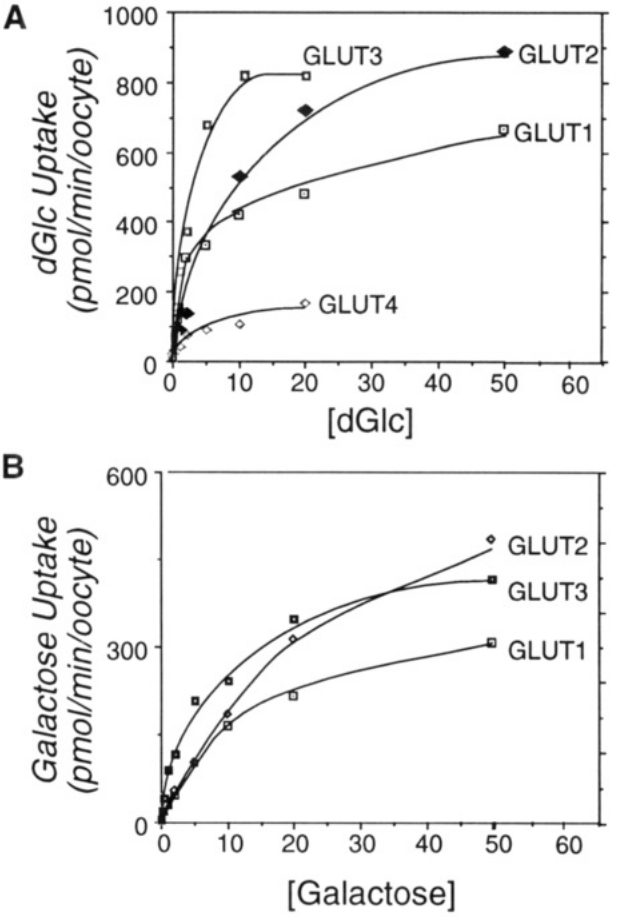

FIGURE 3: Uptake of dGlc and galactose by oocytes expressing GLUT1, GLUT2, GLUT3, and GLUT4. Transport was carried out, and the data were analyzed as described in the Materials and Methods section. Shown is a representative experiment in which increasing concentrations of dGlc (A) or galactose (B) was added to the incubation media of oocytes expressing the indicated glucose transport isoform. Each data point on the graphs represents the mean from 4–6 oocytes. The calculated K_m values for multiple experiments is presented in Table I.

since Western blot analysis of oocyte membranes showed that GLUT4 was synthesized (Figure 2A). In fact, the levels of protein expressed in these oocytes were comparable to or greater than oocytes expressing a chimeric glucose transporter in which the 34 C-terminal amino acids of GLUT3 were replaced with the corresponding region from GLUT4 (chimera-GLUT3$^{1-458}$/GLUT4$^{477-509}$). This chimeric glucose transporter is recognized by the GLUT4 antiserum (Figure 2A), however it mediates the uptake of dGlc as efficiently as oocytes expressing GLUT3 (Figures 2B and 3B).

To assess the kinetic characteristics of the facilitative glucose transporters, the uptake of dGlc and galactose was performed at increasing concentrations of substrate (Figure 3). The apparent K_m's of rat GLUT1 and human GLUT2, GLUT3, and GLUT4 for dGlc and galactose (GLUT1, 2, and 3 only) are summarized in Table I. The rank order of apparent affinity for dGlc and galactose were identical for GLUT1, GLUT2, and GLUT3 (the uptake of galactose by oocytes injected with GLUT4 sRNA was too low to accurately assess kinetic constants). The apparent affinity of each transporter for galactose was 2.5–3-fold lower than for dGlc (Table IA), and the estimated V_{max} was 60–75% of that noted for dGlc transport when assayed at the same time (data not shown).

Inhibition of dGlc Transport by Hexose and Pentose Sugars and Cytochalasin B. To estimate the relative affinities of other sugars for the glucose transporters, the ability of a number of sugars to inhibit the uptake of a 50 μM solution

Table I: Kinetic Parameters of Glucose Transporter Isoforms

	Section A[a] transporter isoform			
	GLUT1	GLUT2	GLUT3	GLUT4
dGlc	6.9 ± 1.5	16.2 ± 2.4	1.8 ± 0.6	4.6 ± 0.2
Gal	17.1 ± 1.6	36 ± 1.8	6.0 ± 1.0	ND[b]
	Section B[c]			
glucose	5.0	12	1.0	ND
dGlc	7.5	15	1.5	ND
mannose	5	15	2.0	ND
3-*O*-MeGlc	20	60	7.5	ND
galactose	15	50	10	ND
xylose	35	75	25	ND
talose	35	75	25	ND
arabinose	>100	>100	100	ND
	Section C[d]			
cytochalasin B	0.1	7.5	0.075	ND

[a] K_m (mM) calculated for the uptake of dGlc or galactose. Transport was carried out, and the data were analyzed as described under Materials and Methods. Shown is the mean ± SEM of 3–6 individual transport experiments with each individual experiment having 8–13 data points with 4–8 oocytes in each data point. [b] ND = not determined because of low transport activity. [c] Approximate IC_{50} (mM) for inhibition of 50 μM dGlc transport by oocytes. Transport was determined as described under Materials and Methods. Inspection of three inhibition curves with 5–8 data points with 4–7 oocytes in each data point on each curve, yielded an apparent IC_{50}, and the mean of these determinations is presented. [d] Approximate IC_{50} (μM) for cytochalasin B inhibition of 50 μM dGlc transport by oocytes. Note that these values are presented for comparison of the transporters and are not meant to represent actual affinities of the unlabeled sugars. Methods are described in Section B.

of [^{3}H]dGlc was determined. Sucrose was added at a concentration to equalize the osmolality of each uptake solution. The addition of sucrose did not affect transport of dGlc (data not shown). To ensure that the inhibition of transport was being measured rather than the inhibition of hexokinase or other posttransport metabolism of dGlc, oocytes were injected with [γ-S]ATP 5–15 min prior to assessing transport of [^{3}H]dGlc. Injection of [γ-S]ATP inhibits phosphorylation of the transported dGlc (Figure 4A) without affecting the ability of the oocyte to accumulate dGlc or the ability of D-glucose or cytochalasin B to inhibit uptake (Figure 4B,C). As shown in Table IB, D-glucose, dGlc, and D-mannose were about 10-fold more effective at inhibiting dGlc transport than the other sugars tested. In parallel to their K_m for dGlc, GLUT3 had the highest affinity and GLUT2 the lowest affinity for the other hexoses and pentoses as estimated by their IC_{50} on dGlc transport. The low level of dGlc transport by oocytes expressing GLUT4 precluded an accurate evaluation of the relative ability of different sugars to inhibit its transport by this protein.

As determined by the ability of cytochalasin B to inhibit dGlc uptake, GLUT3 had the highest affinity for the cytochalasin B, but in contrast to the sugars studied, the IC_{50} was only slightly lower than GLUT1 (Table IC). Similar to the experience with hepatocytes (Craik & Elliot, 1979), the liver-type glucose transporter GLUT2 had a 7.5-fold lower affinity for cytochalasin B as compared to the erythrocyte-type transporter GLUT1.

Coexpression of Normal and Mutant Glucose Transporters. Several studies have suggested that glucose transporters may function as dimers or tetramers (Sogin et al., 1980; Rampal et al., 1986; Hebert & Carruthers, 1991). The expression of two or more glucose transporter isoforms having different kinetic properties in the same cell raises the possibility that glucose transporter heteromultimers may be formed which could possess unique kinetic properties. To test this possibility, sRNA encoding GLUT2 and GLUT3 was mixed and then injected into oocytes. GLUT3 showed a high affinity for dGlc uptake (K_m = 2.5 mM, Figure 5A) whereas GLUT2 had a lower affinity (K_m = 15.7 mM, Figure 5B). The Eadie–Hofstee plot of the transport data from oocytes injected simultaneously with both GLUT2 and GLUT3 sRNA clearly shows two components with K_m's of ≈2.3 and 20 mM (Figure 5C) that are similar to the values determined for each alone, indicating that the monomer is likely the functional unit of transport activity in this system.

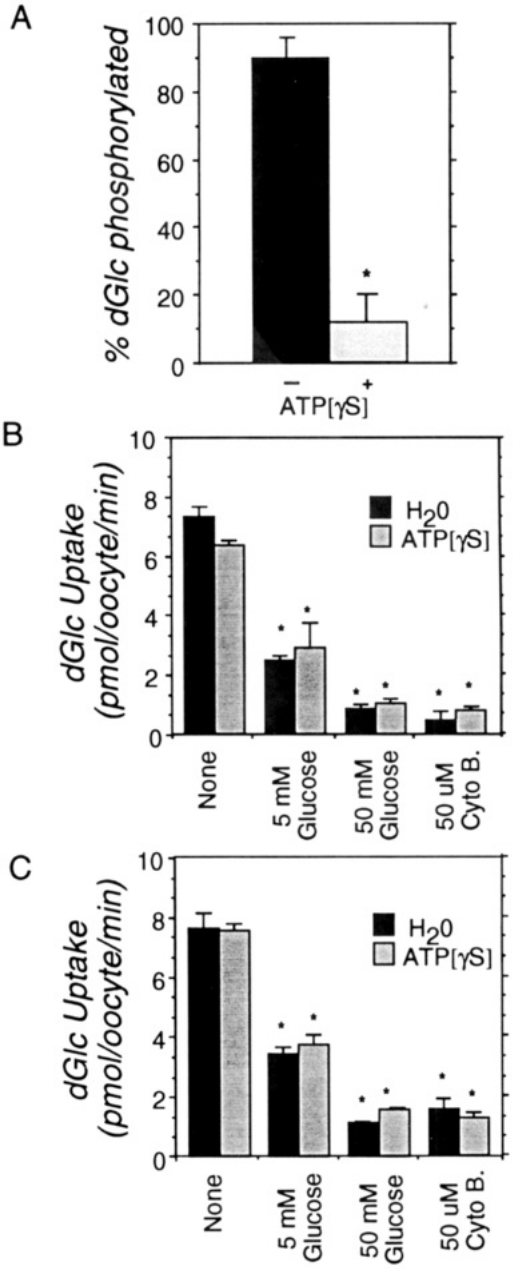

FIGURE 4: Effect of injection of [γ-S]ATP on dGlc transport and phosphorylation. Oocytes expressing GLUT3 (A) or GLUT1 (B) or GLUT2 (C) were injected with 100 nL of sterile water or a 250 mM solution of [γ-S]ATP in sterile water. Later, 5–15 min, oocytes were placed in a solution of 50 μM [3H]dGlc for 1 min at 25 °C with or without the indicated addition of unlabeled glucose or cytochalasin B. (Panel A) Oocytes were placed in boiling water, and phosphorylated and nonphosphorylated dGlc was separated as described under Materials and Methods. The percent of dGlc in the phosphorylated form is presented. Bars represent mean ± SEM for 8–10 oocytes assayed in triplicate. * represents significantly ($p < 0.05$) decreased phosphorylation over water-injected controls. (Panels B and C) After uptake, individual oocytes were solubilized and radioactivity was determined as described under Materials and Methods. Bars represent mean ± SEM for 4–5 oocytes from each group. * represents significantly ($p < 0.05$) decreased uptake as compared to no competitor.

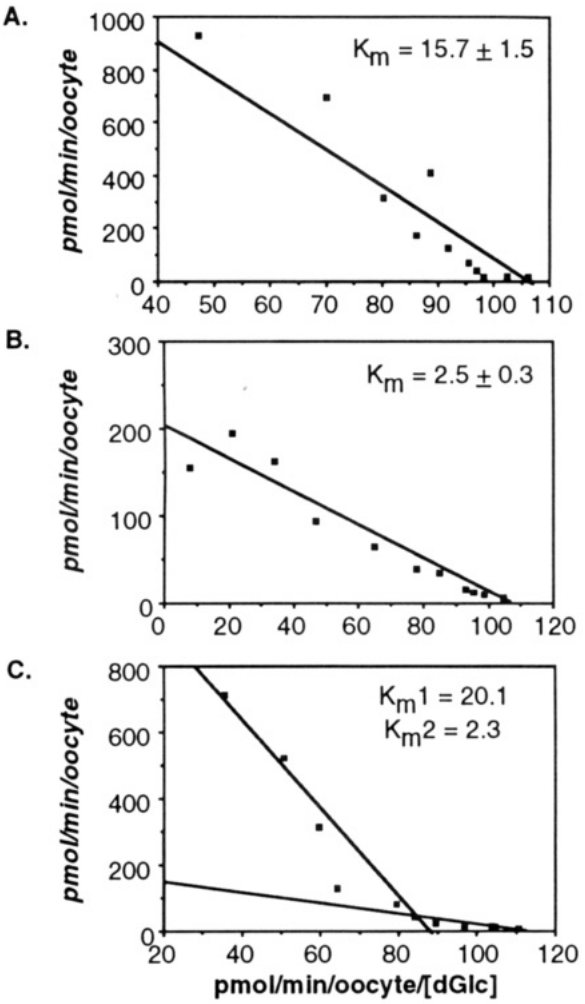

FIGURE 5: Kinetics of transport in oocytes expressing GLUT2 (A) and GLUT3 (B) or coexpressing GLUT2 and GLUT3 (C). Shown are Eadie–Hofstee plots of transport data from oocytes injected with (panel A) 25 ng of GLUT2 sRNA, (panel B) 10 ng of GLUT3 sRNA, and (panel C) 25 ng of GLUT2 and 10 ng of GLUT3 sRNA. Each data point represents the mean from 5–6 oocytes. Inserts show the K_m for the transport from the best-fit lines of the data.

It is possible that glucose transporters are unable to form heterodimers or tetramers whereas they are able to form homodimers or tetramers. To test this possibility, GLUT3 and a functionally inactive protein, GLUT3Leu410 were coexpressed in *Xenopus* oocytes. When expressed in both *Xenopus* oocytes (Garcia et al., 1992) and mammalian cells (Katagiri et al., 1991), the corresponding Trp → Leu mutation in GLUT1 has been shown to inhibit glucose transport activity without affecting expression on the cell surface. In *Xenopus* oocytes, GLUT3Leu410 was expressed at levels comparable to those of GLUT3 as determined by Western blotting (Figure 6A) but was unable to mediate increased dGlc uptake (Figure 6B). Injection of 10 ng of wild-type GLUT3 and 30 ng of GLUT3Leu410 resulted in a 3.5-fold increase in protein expression as compared to that expressed by injection of 10 ng of GLUT3 alone (not shown) while injection of 2- and 3-fold more GLUT3 sRNA resulted in a 1.8- and 2.7-fold increase (not shown) respectively, in dGlc uptake (Figure 7A). In contrast, the uptake of dGlc by wild-type GLUT3 was not affected by injection of up to 3-fold more GLUT3Leu410 sRNA (Figure 7A). In addition, the inhibition of uptake of dGlc by D-glucose or cytochalasin B was unaffected (Figure 7A,B) and, the apparent V_{max} and K_m of dGlc uptake by oocytes expressing GLUT3 and a 3-fold greater GLUT3Leu410 (Figure 7, and data not shown) or a $GLUT3^{1-458}$/$GLUT4^{477-509}$ chimera containing the transport inactivating

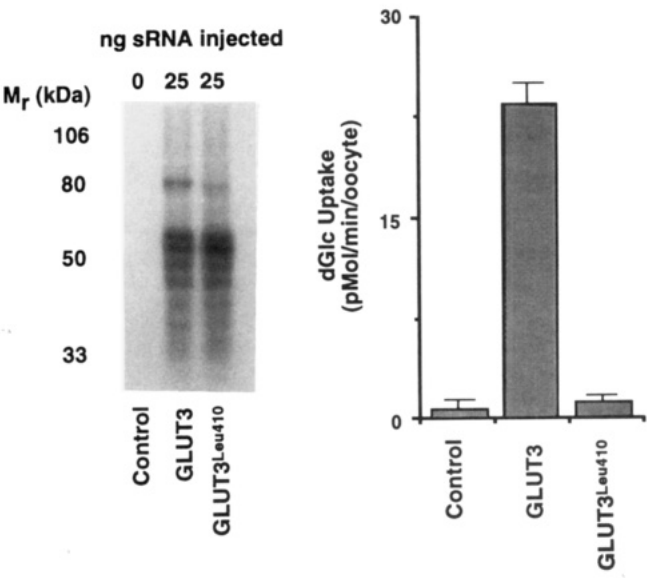

FIGURE 6: Western blot and dGlc uptake by oocytes expressing GLUT3 or GLUT3Leu410. (Panel A, left) Whole oocyte membranes were prepared and subjected to Western blotting as described in the Materials and Methods section from groups of 10 oocytes injected 24 h previously with 25 ng of GLUT3 or 25 ng of GLUT3Leu410 sRNA. (Panel B, right) An identical group of oocytes was incubated with 500 μM [^{3}H]dGlc for 1 min at 25 °C, and the radioactivity accumulated was determined by scintillation counting of individual oocytes. Bars represent mean ± SEM for 6–8 oocytes from each group. * represents significantly ($p < 0.05$) increased transport over uninjected controls.

Leu^{410} mutation (Figure 2A) were similar to oocytes expressing GLUT3 alone (Figure 8).

DISCUSSION

The expression of a family of glucose transporters with different tissue distributions and functional properties allows the body to precisely maintain glucose homeostasis under changing metabolic conditions. Studies of the biochemical properties of the individual glucose transporter isoforms when expressed in *Xenopus* oocytes injected with synthetic mRNA have shown that GLUT1, GLUT2, GLUT3, and GLUT4 are stereoselective glucose carriers (Birnbaum, 1989; Gould & Leinhard, 1989; Keller et al., 1989; Vera & Rosen, 1989; Kayano et al., 1990; Gould et al., 1991). In addition to glucose, they are also able to transport galactose; however, only GLUT2 appears to be able to mediate fructose uptake, although less efficiently than dGlc, 3-*O*-MeGlc, or galactose. Neither GLUT1, GLUT2, GLUT3, or GLUT4 were able to transport α-methylglucopyranoside, confirming that this sugar is specific for the Na^+-dependent glucose cotransporter system.

2-Deoxy-D-glucose was chosen for studying the kinetics of sugar transport for several reasons: (1) zero-trans kinetics can be determined more easily with dGlc than with analogues such as 3-*O*-MeGlc since it is trapped in the cell after transport; (2) 3-*O*-MeGlc appears to have a lower affinity for the glucose transporter than does dGlc whose affinity is similar to that of D-gluocse (LeFerve, 1960); and (3) the affinity of the outward-facing conformation of the glucose transporter system can be examined since little free dGlc exists in the cell after transport because it is rapidly phosphorylated, whereas nonphosphorylatable analogues such as 3-*O*-MeGlc accumulate in the cell and are able to interact with the inward-facing binding site of the transporter. Although there are potential pitfalls in using dGlc for such studies and we cannot completely rule out the possibility that the measured kinetics are influence by hexokinase activity, control experiments indicate the

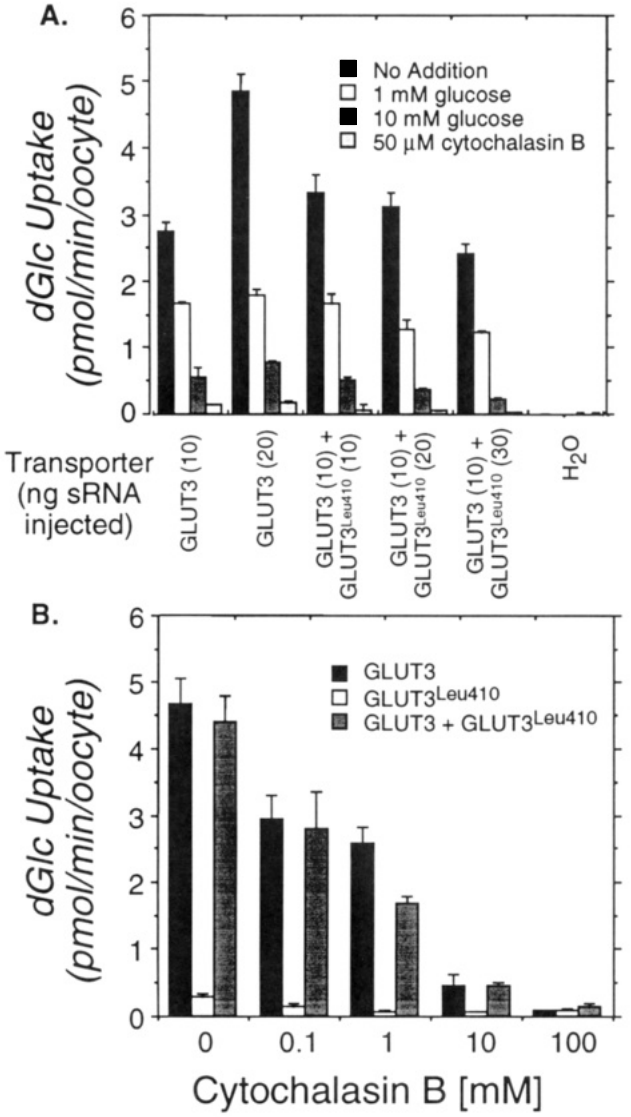

FIGURE 7: Uptake of [^{3}H]dGlc by oocytes expressing GLUT3 and GLUT3Leu410. 24 h after injection with the indicated quantity (parentheses) of GLUT3 and GLUT3Leu410 sRNA or both, groups of 5–7 oocytes were assayed for [^{3}H]dGlc uptake (50 μM) in the absence or presence of the indicated concentration of glucose (A) or cytochalasin B (B). Uptake was quantitated by scintillation counting. Bars represent mean ± SEM for 6–8 oocytes from each group. * represents significantly ($p < 0.05$) different from the uptake seen in oocytes injected with 10 ng of GLUT3 sRNA.

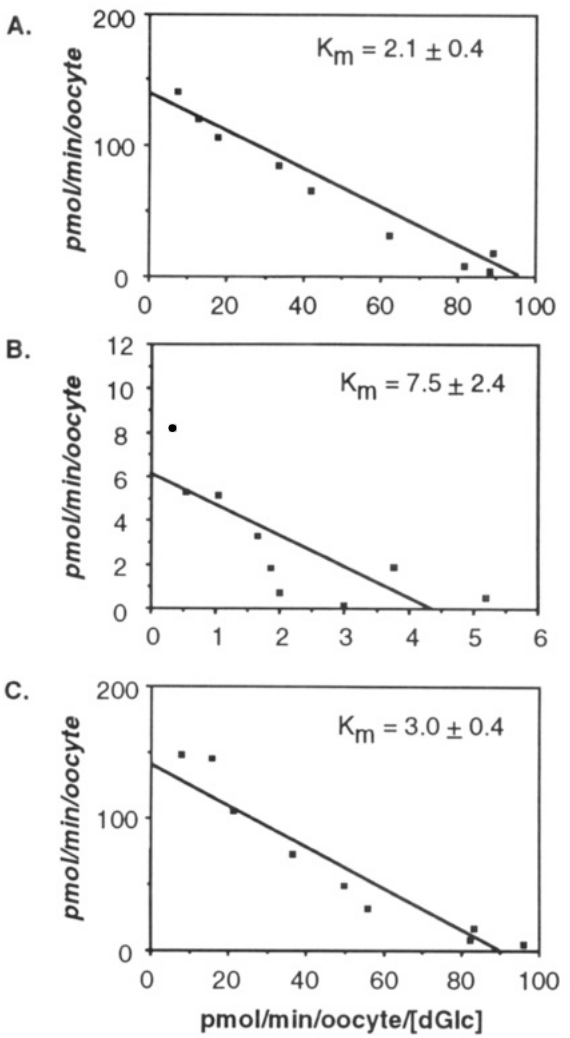

FIGURE 8: Kinetics of transport in oocytes expressing GLUT3 or GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing Trp^{410} → Leu or both GLUT3 and GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing Trp^{410} → Leu. Eadie–Hofstee plots of transport data from oocytes injected with (A) 25 ng of GLUT3^{20} sRNA, (B) 75 ng of GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing Trp^{410} → Leu, or (C) 25 ng of GLUT3 and 75 ng of GLUT3$^{1-458}$/GLUT4$^{477-509}$ containing Trp^{410} → Leu sRNA. Each data point represents the mean from 5–8 oocytes. Inserts show the K_m for transport from the best-fit lines of the data.

kinetics of dGlc uptake were not confounded by hexokinase activity. Thus, in the time intervals in which uptake was measured, >90% of the dGlc was phosphorylated, indicating that hexokinase activity was not limiting (not shown) and free intracellular dGlc was not greater than 10% of equilibrium values (Figure 3). Moreover, the rank-order affinity for dGlc transport as determined by zero-trans uptake was identical to that determined by equilibrium exchange using 3-*O*-MeGlc (Gould et al., 1991): The K_m's of GLUT1, GLUT2, and GLUT3 for dGlc are 6.9, 13.2, and 1.8 mM, respectively, and for 3-*O*-MeGlc they are 20.1, 42.3, and 10.6 mM, respectively.

The relatively low levels of dGlc uptake by oocytes expressing GLUT4 noted in these studies and as reported by others (Birnbaum, 1989; Keller et al., 1989; Gould et al., 1991) are not due to an inability of the oocytes to synthesize the protein since GLUT4 could be readily detected by Western blotting at levels comparable to that of GLUT3 (Figure 2). Rather they may be due to the fact that the most of the GLUT4 protein is in an intracellular location where it cannot participate in glucose transport. This interpretation is consistent with studies of cultured mammalian cells transfected with GLUT4-expressing constructs which have shown that GLUT4 is excluded from the plasma membrane (Haney et al., 1991). This observation may not be unreasonable since in adipocytes and muscle cells, cells in which it is normally expressed, GLUT4 is not normally present on the plasma membrane except when they have been stimulated with insulin but is rather associated with an intracellular compartment (Zorzano et al., 1988). Alternatively, in heterologous expression systems such as *Xenopus* oocytes, additional protein(s) or activation of GLUT4 may be required to obtain higher levels of transport activity with GLUT4.

The finding that dGlc transport in oocytes expressing GLUT1, GLUT2, and GLUT3 is inhibited by a series of test sugars and analogues in a similar hierarchal order suggests that the sugar binding site(s) in these transporter isoforms are similar. Since the highest regions of identity in the amino acid sequences of these transporter isoforms are in the transmembrane domains (Kayano et al., 1990; Burant et al., 1991), the recognition site for glucose may be located in these domains. In addition, the observation that mutation of the Trp^{410} residue in GLUT3 inactivates glucose transport in a manner similar to the homologous mutation in GLUT1

(Katagiri et al., 1991; Garcia et al., 1992) is consistent with the glucose binding site being similar in these two isoforms. The observation that cytochalasin B was a more effective inhibitor of dGlc uptake by GLUT3-expressing oocytes and less so of uptake by GLUT2 is also consistent with the idea that cytochalasin B is recognized by the glucose transporter binding site in a similar manner as glucose (Walmsley, 1988).

Pessino et al. (1991) have recently reported that GLUT1 may form functional homo-oligomers in vivo but could not find evidence for heterooligomeric association of GLUT1 with GLUT4. Although their results are consistent with physical association between GLUT1 monomers, their data do not indicate whether oligomerization is a prerequisite for functional activity. The studies of Nebert and Carruthers (1991) with purified erythrocyte glucose transporters (GLUT1) suggest that this protein may exist as a dimer as well as a tetramers, and they present a model of the glucose transporter in which the individual subunits of the dimer mediate transport of glucose independently but show cooperative effects when in the tetrameric form. In the present study, the distinct kinetic parameters of GLUT2 and GLUT3 could be demonstrated when coexpressed in *Xenopus* oocytes, suggesting that these isoforms do not form heteromultimers with altered kinetic properties. In addition, there was no alterations in the kinetic properties of GLUT3 even when coexpressed with excess amounts of the glucose transport incompetent GLUT3Leu410 protein (Figures 7 and 8). While it is possible that the wild-type transporters are targeted to the surface of the cell in preference to that of the mutated transporters, we observed that the GLUT3Leu410 protein is expressed at the similar levels with the same electrophoresis mobility and banding pattern as wild-type GLUT3 (Figure 6). These results and the studies by Garcia et al. (1992) in oocytes and Katagiri et al. (1991) in mammalian cells using the homologous mutation in GLUT1 suggested that there is no gross disruption of the translational or posttranslational processing or targeting of the mutated protein. These data imply that while the transporter protein may be able to physically associate, at least GLUT3 functions as a monomer in the plasma membrane. It should be noted that the Trp → Leu mutation may affect the association between mutated and wild-type transporters, and this is the reason for the failure to find alterations in the kinetic parameters in the coexpression studies.

In summary, the data presented here suggest that the glucose transporter isoforms recognize substrates in a similar manner and differ primarily in their affinities for sugars. Thus, differential tissue expression of the members of the facilitative glucose transporter family would allow precise distribution of glucose to cells under various metabolic conditions such as such as in the fed and fasting state. The data also suggest that when expressed in the *Xenopus* oocyte, the glucose transporter monomer is sufficient to support translocation of substrate across the plasma membrane. The block in heterooligomer formation would prevent the cell from forming transporters with unintended kinetic properties.

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