Expression of Human Glucose Transporters in *Xenopus* Oocytes: Kinetic Characterization and Substrate Specificities of the Erythrocyte, Liver, and Brain Isoforms†

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Received September 21, 1990; Revised Manuscript Received December 18, 1990

ABSTRACT: We describe the functional expression of three members of the family of human facilitative glucose transporters, the erythrocyte-type transporter (GLUT 1), the liver-type transporter (GLUT 2), and the brain-type transporter (GLUT 3), by microinjection of their corresponding mRNAs into *Xenopus* oocytes. Expression was determined by the appearance of transport activity, as measured by the transport of 3-*O*-methyl-D-glucose or 2-deoxy-D-glucose. We have measured the K_m for 3-*O*-methyl-D-glucose of GLUTs 1, 2, and 3, and the results are discussed in light of the possible roles for these different transporters in the regulation of blood glucose. The substrate specificity of these transporter isoforms has also been examined. We show that, for all transporters, the transport of 2-deoxy-D-glucose is inhibited by D- but not by L-glucose. In addition, both D-galactose and D-mannose are transported by GLUTs 1–3 at significant rates; furthermore, GLUT 2 is capable of transporting D-fructose. The nature of the glucose binding sites of GLUTs 1–3 was investigated by using hexose inhibition of 2-deoxy-D-glucose uptake. We show that the characteristics of this inhibition are different for each transporter isoform.

Recent evidence has demonstrated that the transport of glucose across the plasma membrane of mammalian cells is mediated by a family of homologous proteins [for review, see Gould and Bell (1990) and Mueckler (1990)]. These proteins have distinct, tissue-specific patterns of expression and exhibit different modes of regulation. This diversity of glucose transporter species presumably allows precise control of blood glucose to be maintained in mammals over a range of physiological conditions. To date, five human glucose transporters have been identified: the erythrocyte-type transporter (GLUT 1), which is expressed at highest levels in fetal tissues, including placenta, erythrocytes, brain microvessels, kidney, and colon (Mueckler et al., 1985; Birnbaum et al., 1986; Flier et al., 1987; Fukumoto et al., 1988a); the liver-type transporter (GLUT 2) expressed in the liver, pancreatic β-cells, kidney, and small intestine (Thorens et al., 1988; Fukumoto et al., 1988b; Orci et al., 1989); the brain-type transporter (GLUT 3) which, in humans, is expressed in many tissues including brain, placenta, and kidney (Kayano et al., 1988); the muscle/fat-type, or insulin-responsive, transporter (GLUT 4), expressed only in tissues that exhibit insulin-sensitive glucose transport (i.e., fat, skeletal muscle, and heart) (James et al., 1988, 1989; Birnbaum, 1989; Charron et al., 1989; Kaestner et al., 1989; Fukumoto et al., 1989); and GLUT 5, expressed predominantly in the small intestine, but also at low levels in many tissues (Kayano et al., 1990). These proteins exhibit between 39% and 65% amino acid identity and 50–76% similarity. They are predicted to possess similar overall structures and orientations in the plasma membrane, including 12 membrane-spanning domains, and intracellularly located N- and C-termini. All have been demonstrated to transport glucose by functional expression in *Xenopus* oocytes, bacteria, or cultured cells (Gould & Lienhard, 1989; Keller et al., 1989; Birnbaum, 1989; Vera & Rosen, 1989; Permutt et al., 1989; Kayano et al., 1990) although with apparently different efficiencies. To evaluate the contribution of each isoform to the maintenance of glucose homeostasis, it is necessary to determine the kinetic characteristics of each of these species and their abundance in each tissue and cell. The *Xenopus* oocyte system is well suited to studying the functional properties of heterologous proteins. We and others have recently shown that this system is particularly well suited for the study of facilitative glucose transporters due to the low levels of endogenous glucose transport activity in uninjected oocytes. Using this procedure, K_m values for the human and rat GLUT 1 and rat GLUT 4 for 3-*O*-methyl-D-glucose (3-*O*-MG)[1] have been determined under equilibrium exchange conditions (Gould & Lienhard, 1989; Keller et al., 1989). The affinities of rat GLUTs 1, 2, and 4 for 2-deoxy-D-glucose (deGlc) have also been described (Vera & Rosen, 1989). In this study, we have expressed the human forms of GLUT 1, GLUT 2, and GLUT 3 in oocytes, determined the K_m for 3-*O*-MG, and examined the substrate selectivity of these species.

Materials and Methods

Materials. Collagenase was obtained from Worthington Scientific Ltd. Sodium diguanosine triphosphate was purchased from Pharmacia. SP6 polymerase, RNasin, and nucleotides were purchased from Promega Biotech. Penicillin/streptomycin solution and all restriction endonucleases were from Gibco/BRL (Paisley, Glasgow). 3-*O*-Methyl-D-[1-

† This work was supported by grants from the Medical Research Council (G.W.G.) and the Juvenile Diabetes Foundation International (G.I.B. and G.W.G.) and by the Howard Hughes Medical Institute (G.I.B.). H.M.T. is an MRC postdoctoral fellow.

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[1] Abbreviations: 3-*O*-MG, 3-*O*-methyl-D-glucose; deGlc, 2-deoxy-D-glucose; PBS, phosphate-buffered saline.

0006-2960/91/0430-5139$02.50/0

[^{3}H]glucose, D-[6-^{3}H]galactose, D-[U-^{14}C]mannose, D-[U-^{14}C]fructose, and 2-deoxy-D-[2,6-^{3}H]glucose were from Amersham International. All other reagents were as described (Gould & Lienhard, 1989).

Oocyte Isolation and Injection. Female *Xenopus laevis* were purchased from Xenopus Ltd. (Redhill, Surrey) and maintained at 18 °C on a 12-h light/dark cycle. Animals were anesthetized by ice immersion and oocytes removed by the procedure described (Marcus-Sekura & Hitchcock, 1987). Individual oocytes were dissected and stored in Barths media [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 5 mM Hepes–NaOH, pH 7.6, penicillin at 10 μg/mL and streptomycin at 10 IU/mL]. All subsequent procedures were performed in Barths media. Oocytes were incubated with collagenase (2 mg/mL) for 30 min–1 h with agitation, washed 6 times with 10 mL of fresh Barths, and incubated overnight at 18 °C. Surviving oocytes (generally greater than 90%) were subsequently injected with 25–50 nL of RNA at various concentrations or with water as a control (Melton, 1987). Oocytes were then incubated in Barths medium at 18 °C for 24–96 h prior to assay; the medium was replaced every 12 h. Immediately prior to assay, any debris associated with the oocytes was removed by manual dissection.

Plasmid Construction and Synthesis of mRNA. The human glucose transporter constructs used for the preparation of synthetic mRNA have been described previously (Kayano et al., 1990). Human GLUTs 1–4 were in pSP64T (Krieg & Melton, 1984). These constructs contain the protein coding region of the cDNA (and various amounts of 5′- and 3′-untranslated region) flanked by 89 bp of 5′- and 141 bp of 3′-untranslated sequence from the *Xenopus* β-globin gene (Kayano et al., 1990). In the case of the rat GLUT 1, the plasmid was a gift of Dr. M. J. Birnbaum (Harvard Medical School, Boston, MA) (Gould & Lienhard, 1989). The human HepG2 cDNA was supplied by Mike Mueckler (Washington University School of Medicine, St. Louis, MO). cDNAs encoding hGLUTs 4 and 5 in pGEM-4Z and pGEM-3Z, respectively, were also used to prepare mRNA in vitro (Fukumoto et al., 1989; Kayano et al., 1990). Linearized plasmid DNA was used as a template for mRNA synthesis. RNA synthesis was performed as described (Gould & Lienhard, 1989).

Hexose Transport in Oocytes. (A) 3-O-MG Equilibrium Exchange. Groups of 5 oocytes were incubated for 8–10 h at room temperature in 0.5 mL of Barths media (pH 7.4) containing 3-*O*-MG at various concentrations. To start the reaction, 10 μL of Barths media containing 1.0 μCi of [^{3}H]-3-*O*-MG was added and mixed. Oocytes were exposed to isotope for the times indicated in the figures (5–90 min). The reaction was stopped by quickly aspirating the media and washing the oocytes with 3 mL of ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin, a potent transport inhibitor (Krupka, 1971). The oocytes were washed in this fashion a further two times and dispensed to scintillation vials, at 1 oocyte per vial. These three washes were completed within 30 s. Sodium dodecyl sulfate (0.5 mL of a 1% solution) was added to each scintillation vial, and the vials were incubated at room temperature for 1 h with agitation before addition of Ecoscint (National Diagnostics) and measurement of radioactivity.

(B) 3-O-MG Zero-trans Transport. Groups of 5 oocytes were incubated at room temperature in 500 μL of Barths media (pH 7.4) for 15 min prior to addition of 3-*O*-MG to the media (50 μM; 1.0 μCi of [^{3}H]-3-*O*-MG per assay) for the requisite time. The reaction was stopped by quickly aspirating the media and washing the oocytes with 3 mL of ice-cold PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin. The oocytes were washed and the counts per oocyte determined as described for equilibrium exchange.

(C) Transport of deGlc and Other Hexoses. Groups of 5 oocytes were incubated in Barths buffer at pH 7.4 plus the sugars indicated in the figure legends for 15 min prior to initiation of uptake by the addition of an aliquot of [2,6-^{3}H]deGlc, such that the final concentration was 100 μM and 1.0 μCi/mL. After the indicated time, transport was stopped and the radioactivity in individual oocytes determined as described above for 3-*O*-MG. Under these conditions, the transport of deGlc is linear over a 1-h period (data not shown). The accumulation of D-mannose, D-fructose, and D-galactose was determined exactly as for deGlc. Groups of oocytes (typically 5) were incubated in Barths buffer at pH 7.4 for 15 min prior to the addition of the appropriate sugar to the media, such that the final concentration was 0.1 mM (0.5 μCi per assay). Uptake was stopped by three washes in ice-cold PBS containing 0.1 mM phloretin and the radiolabel in each oocyte determined as for 3-*O*-MG transport.

Determination of Nonphosphorylated Sugar Levels. The relative levels of nonphosphorylated deGlc were determined by the method of Klienzeller and McAvoy (1976). Briefly, groups of 5 oocytes were incubated in Barths buffer at pH 7.4 plus the sugars indicated in the figure legends for 15 min prior to initiation of uptake by the addition of an aliquot of [2,6-^{3}H]deGlc, such that the final concentration was 100 μM and 1.0 μCi/mL. After the indicated time, transport was stopped by three washes in ice-cold PBS containing 0.1 mM phloretin and 1.5 mL of 90 °C distilled water added to the tube. Oocytes were disrupted by trituration in a Pasteur pipet and incubated at 90 °C for 3 min. Zinc sulfate (1.5 mL of a 0.1 M solution) was added, and the tubes were vortexed. Subsequently, 1.0 mL of 0.15 M barium hydroxide was added, with mixing. Tubes were centrifuged at 500 rpm in a bench top centrifuge and aliquots of the supernatant counted. The supernatant contains only nonphosphorylated sugar species (Klienzeller & McAvoy, 1976). The relative contribution of nonphosphorylated deGlc to the total deGlc (i.e., deGlc and deGlc-6-phosphate) was calculated by measuring total deGlc associated per oocyte exactly as for transport (above).

Results

Expression of the Human Glucose Transporter Family in Oocytes. (A) Stereoselectivity. Each of the five glucose transporter isoforms has been expressed in *Xenopus* oocytes and demonstrated to be a cytochalasin B inhibited glucose carrier (Birnbaum, 1989; Gould & Lienhard, 1989; Keller et al., 1989; Kayano et al., 1990). The data in Figure 1 extend these observations to show that all the glucose transporters exhibit the expected D and L stereoselectivity with respect to glucose transport.

As suggested by the data presented in Figure 1, the levels of expression of the five human isoforms are quite different. In our hands, injection of GLUT 1, 2, and 3 RNA always stimulates glucose transport to a greater degree than an approximately equivalent amount of GLUT 4 or GLUT 5 RNA. Moreover, the fold stimulation of glucose transport on injection of GLUT 4 RNA was similar for transcripts lacking the *Xenopus* β-globin untranslated regions (data not shown). The data presented by Keller et al. (1989) also suggest a similar difference in the ability of GLUT 1 and GLUT 4 RNA to

FIGURE 1: The human glucose transporter family exhibit D- versus L-stereospecificity. Groups of 5 oocytes were incubated in 0.5 mL of Barths containing 50 mM D-glucose (open boxes), 50 mM L-glucose (hatched boxes), or no glucose (filled boxes) for 10 min prior to the addition of deGlc (0.1 mM final concentration, 0.5 μCi per assay). Following a 30-min incubation, the reaction was stopped and the radiolabel associated with individual oocytes was determined as described under Materials and Methods. In the experiment shown, oocytes were injected with 10 ng of GLUT 1 mRNA, 25 ng of GLUT 2 mRNA, 15 ng of GLUT 3 mRNA, 35 ng of GLUT 4 mRNA, or 30 ng of GLUT 5 mRNA and assayed 48 h following injection. The mean values are presented (n = 5 oocytes). The experiment was repeated with similar results. The rate of deGlc transport in water-injected oocytes was 0.18 pmol/(min·oocyte) and was inhibited by D- but not by L-glucose (data not shown).

Table I: K_m Values of Mammalian Glucose Transporters[a]

transporter isoform	K_m (mM) 3-O-MG	K_m (mM) D-glucose[b]
human GLUT 1	17.6, 16.9	17.0
human GLUT 2	42.3 ● 4.1	66.0
human GLUT 3	10.6 ● 1.3	
human GLUT 4	nd[c]	4–7
rat GLUT 1	20.1 ● 2.9[d]	

[a] K_m values for 3-O-methyl-D-glucose were determined as described under Materials and Methods. Mean and standard deviations from 3 (GLUT 2) or 4 (GLUT 3) independent experiments are reported. The results from two experiments with the human GLUT 1 isoform are presented. nd = not determined. [b] Values from Lowe and Walmsley (1986) for GLUT 1 Eliott and Craik (1982) for GLUT 2, and Whitesell et al. (1989) and Suzuki (1988) for GLUT 4. [c] Keller et al. (1989) report a value of 1.8 mM for rat GLUT 4 when expressed in *Xenopus* oocytes. [d] Gould and Lienhard (1989).

stimulate glucose transport in oocytes. Thus, at least part of this difference may be intrinsic to the transporter rather than the expression construct. The lower activity of GLUT 4 in this system may be a consequence of the predominant localization of GLUT 4 to an intracellular compartment within the oocyte, concomitant with reduced amounts present at the cell surface. This would not be unexpected since, in adipocytes and muscle, cells that normally express GLUT 4, the protein is present in an intracellular pool and moves to the plasma membrane following insulin challenge (James et al., 1988, 1989; Zorzano et al., 1989); however, this awaits definitive proof in oocytes. A similar explanation may underlie the low level of expression recorded with GLUT 5.

(B) Kinetic Characterization of Human GLUTs 1–3. We have undertaken a kinetic characterization of the glucose transporter family. Figure 2 shows the results of a typical experiment designed to determine the equilibrium exchange K_m values for 3-O-MG for expressed transporters in oocytes. Oocytes were incubated with 3-O-MG at a range of concentrations for 8–10 h, and the equilibration of the oocyte water space by carrier-free 3-O-MG was subsequently followed with time. The results are presented in the form of a first-order plot; it demonstrates that for each species the process was first order, as expected for equilibrium exchange (Eliam & Stein, 1984). The rate constants (k_{obs}) obtained from such plots were used in Lineweaver–Burk plots to determine the value of K_m

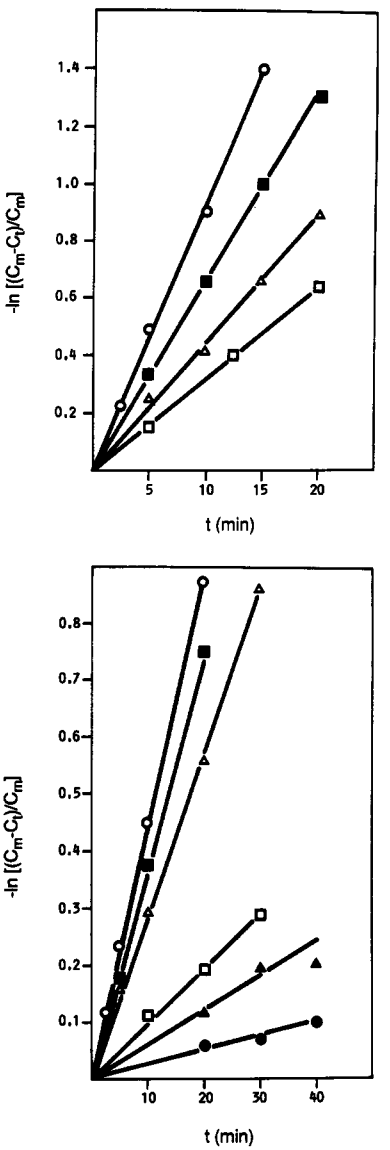

FIGURE 2: Rate of 3-O-methyl-D-glucose transport as a function of substrate concentration under equilibrium exchange conditions for GLUT 2 and GLUT 3. Groups of 5 oocytes were incubated for 8 h in 0.5 mL of Barths buffer at room temperature containing unlabeled 3-O-MG at the concentrations indicated below. This incubation was sufficient to allow full equilibration of the 3-O-MG into the oocytes (data not shown). Following this incubation, radiolabeled 3-O-MG (1.0 μCi per assay) was added and the rate of equilibration of the isotope determined as described under Materials and Methods. The equilibration is expected to be a first-order process, and thus the results are plotted as $-\ln [(C_m - C_t)/C_m]$ versus time for each concentration of substrate, where C_m is the radioactivity per oocyte after full equilibration of the oocyte water space and C_t is the radioactivity at time t. Each point is the mean of 5 oocytes; the variation at each time point was typically less than 10%. Panel A: GLUT 2. (○) 1 mM, (■) 30 mM, (△) 50 mM, (□) 100 mM. Panel B: GLUT 3. (○) 0.1 mM, (■) 1.0 mM, (△) 5.0 mM, (□) 20 mM, (▲) 30 mM, (●) 60 mM.

by plotting $1/k_{obs}$[3-O-MG] against 1/[3-O-MG] (Gould & Lienhard, 1989). The values of K_m obtained by this procedure are presented in Table I. Note that the rate of 3-O-MG transport mediated by both GLUT 4 and GLUT 5 was considered too low to enable an accurate determination of the kinetic constants of these species.

(C) Substrate Selectivity of GLUTs 1–3. We have undertaken an analysis of the effects of other sugars on the rate of deGlc transport mediated by these isoforms in an effort to examine the nature of the glucose binding sites of each isoform. The results from a representative experiment are presented

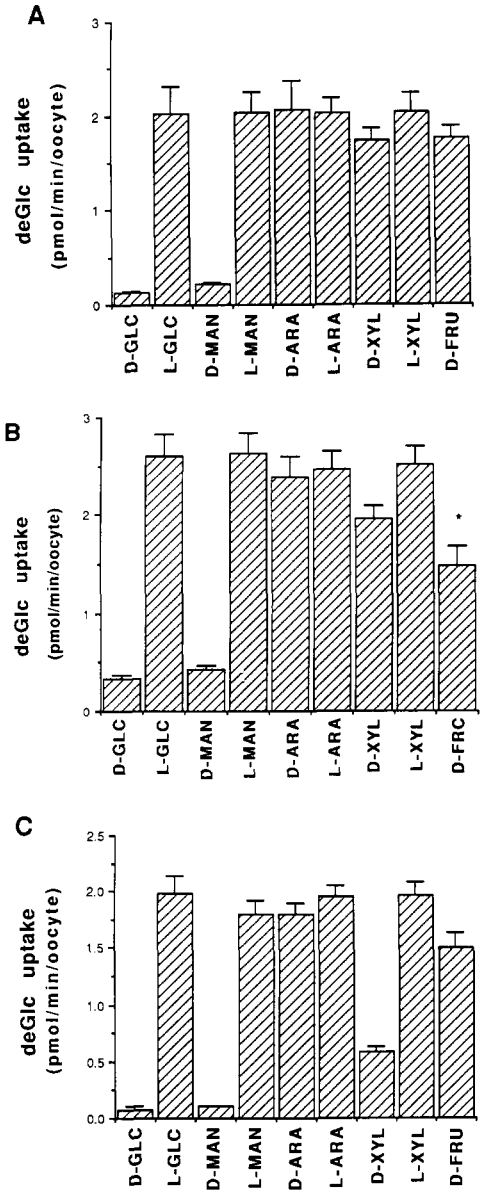

FIGURE 3: Substrate selectivities of the human glucose transporter family. Oocytes were injected with 10 ng of GLUT 1 mRNA (panel A), 25 ng of GLUT 2 mRNA (panel B), or 35 ng of GLUT 3 mRNA (panel C) and incubated for 48 h prior to assay. Groups of 10 oocytes were incubated for 30 min in 0.5 mL of Barths buffer containing 50 mM of the following sugars: D- and L-glucose (D-GLC, L-GLC), D- and L-mannose (D-MAN, L-MAN), D- and L-arabinose (D-ARA, L-ARA), D- and L-xylose (D-XYL, L-XYL), or D-fructose (D-FRU) prior to the addition of deGlc for 1 h (0.1 mM final concentration, 1.0 μCi per assay). Transport was terminated and the radioactivity in each oocyte determined as described under Materials and Methods; the mean and standard deviation (n = 5–10 oocytes) from a representative experiment are presented. These experiments were repeated three times for each transporter isoform. [(*) signifies $p < 0.01$.]

in Figure 3. The data illustrate the effects of 50 mM D- and L-glucose, D- and L-xylose, D- and L-mannose, D- and L-arabinose, and D-fructose upon the transport of deGlc (0.1 mM). As shown, the transport of deGlc mediated by GLUT 1 was inhibited by D-glucose and D-mannose, but the other sugars tested did not significantly inhibit the rate of deGlc uptake (Figure 3A). Similar results were obtained with GLUT 2, with the exception that a small but reproducible (25–35%) inhibition of deGlc uptake was recorded in the presence of D-fructose (Figure 3B). In contrast, GLUT 3 mediated deGlc uptake was significantly inhibited by D-xylose, as well as by D-mannose and D-glucose (Figure 3C). Note that the L-isomers of glucose, arabinose, xylose, and mannose had no effect on the transport of deGlc mediated by any of the transporter isoforms tested.

Table II: Comparison of the Rate of Accumulation of Deoxy-D-glucose, D-Galactose, D-Mannose, and D-Fructose Mediated by GLUT 1, GLUT 2, and GLUT 3[a]

transporter isoform	accumulation rate [pmol/(min·oocyte)]			
	deoxy-glucose	galactose	mannose	fructose
control	0.20 ± 0.02	0.26 ± 0.01	0.56 ± 0.10	0.11 ± 0.01
rat GLUT 1	4.14 ± 0.45	0.90 ± 0.05	11.4 ± 1.43	0.12 ± 0.01
human GLUT 2	4.33 ± 0.15	1.68 ± 0.09	10.6 ± 1.84	0.78 ± 0.09
human GLUT 3	3.39 ± 0.03	0.81 ± 0.02	9.3 ± 1.08	0.11 ± 0.01

[a] Oocytes were injected with water (control), 15 ng of GLUT 1 mRNA, 25 ng of GLUT 2 mRNA, or 35 ng of GLUT 3 mRNA and incubated in Barths buffer for 55 h prior to assay. Groups of 6 oocytes were subsequently assayed. Uptakes were initiated by the addition of labeled sugar solution (0.1 mM, 0.5 μCi per assay in all cases) for 15, 30, 60, and 90 min. Assays were stopped by three washes with ice-cold PBS containing 0.1 mM phloretin, and the radiolabel associated with individual oocytes was determined as described under Materials and Methods. The rates of transport presented are the mean and standard deviation (n = 6 oocytes) recorded 15 min after the addition of sugar; the uptake of mannose and fructose was linear over 90 min and that of deGlc and galactose linear over 60 min. Note that these values reflect the rate of accumulation of the sugar, and not its absolute transport rate.

The possibility that these patterns of inhibition were the result of inhibitory effects of the competing sugars on hexokinase has been ruled out, since similar patterns of inhibition were observed with the nonmetabolizable glucose analogue 3-*O*-MG. In a series of control experiments, we have examined the ability of other hexoses to inhibit the accumulation of 3-*O*-MG by GLUTs 2 and 3. In these assays, the uptake of 50 μM 3-*O*-MG was measured under zero-trans conditions in the presence of 50 mM of the competing hexose. The results were quantitatively similar to those shown for deGlc: for GLUT 2 the rate constant for 3-*O*-MG transport was decreased by 45% in the presence of 50 mM D-fructose and by 80% in the presence of D-mannose but was unaffected by the other sugars. Simiarly, for GLUT 3, both D-mannose and D-xylose inhibited 3-*O*-MG transport by 80–90% (data not shown). Note that this analogue was not used throughout this analysis, since the ability of 3-*O*-MG to be transported in both directions by the transporters results in an inability to study the outward-facing binding site "in isolation", as the competition may be at either the inward-facing or outward-facing glucose site. In order to demonstrate that there was no buildup of nonphosphorylated deGlc in oocytes under the conditions used in this study, we measured the relative contribution of nonphosphorylated deGlc to the total counts associated with each oocyte. In all cases, nonphosphorylated deGlc contributed less than 10% of the radioactivity in each oocyte, and no significant differences were observed between oocytes exposed to sugars with a pronounced inhibitory effect and those with no inhibitory effect. These observations further suggest that the patterns of inhibition may be considered to be due to competition at the level of transport, and not phosphorylation.

Direct measurement of the accumulation of D-fructose, D-mannose, and D-galactose by these isoforms was examined, and the results are shown in Table II. It should be pointed out that these values do not necessarily reflect true transport rates, since these sugars are likely to have distinct kinetics of metabolism by hexokinase. However, these data conclusively show that all three isoforms were capable of mediating the transport of galactose and mannose and, moreover, show that

GLUT 2 is capable of transport fructose, whereas GLUTs 1 and 3 were not.

Discussion

Most, if not all, mammalian cells have integral membrane proteins that translocate glucose down its concentration gradient by a process of facilitative diffusion. These proteins are responsible for equilibrating glucose across the plasma membrane and mediate both the cellular influx as well as efflux of this important nutrient. cDNAs encoding five functional facilitative glucose transporters, as well as an expressed pseudogene, have been identified in mammalian tissues (Gould & Bell, 1990; Bell et al., 1990; Kayano et al., 1990). The elucidation of the functional characteristics of these different glucose transporters requires a system in which they may each be expressed in isolation and their kinetic properties studied. We and others have recently described the use of *Xenopus* oocytes as an expression system with which to examine the kinetics of heterologously expressed glucose transporters (Gould & Leinhard, 1989; Keller et al., 1989; Vera & Rosen, 1989). In a previous report (Kayano et al., 1990), we demonstrated that each of the human glucose transporters was capable of mediating the specific uptake of deGlc following microinjection of the corresponding mRNA into *Xenopus* oocytes and that this transport of deGlc could be inhibited by the potent transport inhibitor cytochalasin B. This report shows that transport by each member of the facilitative glucose transporter family is stereoselective, the transport of deGlc being inhibited by D-glucose, but not by L-glucose (Figure 1).

We have determined the values of K_m for 3-*O*-MG for GLUTs 1–3, and the data are summarized in Table I. The values obtained for the human isoform of GLUT 1 are in good agreement with published results (Keller et al., 1989) and are essentially identical with the value reported for the rat equivalent of this transporter isoform (Gould & Lienhard, 1989). Under equilibrium exchange conditions, the value of the K_m for 3-*O*-MG for GLUT 2 was found to be 42 mM, a value significantly higher than that reported for GLUT 1. The localization of GLUT 2 to tissues involved in the net release of glucose during fasting (liver), glucose sensing (β-cells), and transepithelial transport of glucose (kidney and small intestine) would seem to provide a rationale for this high K_m value, since glucose flux through this transporter at physiological glucose concentrations would be predicted to change in a virtually linear fashion with extracellular/intracellular glucose concentration. This would result in the highly favorable condition that transport of glucose would not be rate limiting. This high K_m value for GLUT 2 is in agreement with data published from intact hepatocytes, where a K_m for glucose of approximately 66 mM has been reported (Eliott & Craik, 1982). As noted by Mueckler (1990), the supraphysiological K_m value for GLUT 2 may also be of considerable importance in the control of insulin secretion from the pancreatic β-cells.

GLUT 3 exhibits a K_m for 3-*O*-MG of about 10 mM (Table I). This relatively low K_m value and ubiquitous tissue distribution, at least in human tissues, may reflect a general "housekeeping" role for this isoform, i.e., constitutive glucose uptake.

In our hands, the level of expression of GLUT 4 (and also GLUT 5) is too low to allow accurate measurement of the K_m for 3-*O*-MG (see Figure 1). However, a value of 1.8 mM for the rat isoform of this transporter has been reported elsewhere (Keller et al., 1989). Recent evidence suggests that the majority of the insulin-stimulated increase in glucose transport is mediated by the appearance of GLUT 4 at the plasma membrane (James et al., 1988, 1989; Birnbaum, 1989; Zorzano et al., 1989; Calderhead et al., 1990). The relatively low K_m value of this transporter would ensure that it operates close to V_{max} under conditions of high blood glucose, ensuring the rapid removal of blood glucose into the body's energy stores.

Table I also gives the estimated K_m values for each transporter isoform for D-glucose, determined from analysis of glucose transport in intact cells. It is notable that the general trends in K_m values for 3-*O*-MG are mirrored by the values obtained for D-glucose; thus although the values of the K_m determined for each sugar are different, the relative affinities of the transporters are similar.

In addition to kinetic differences, the glucose transporters also exhibit differences in substrate selectivity. The effect of other sugars on the uptake of deGlc are shown in Figure 3. DeGlc uptake (0.1 mM) mediated by GLUT 1 was not affected by the presence of 50 mM D-fructose, D- or L-arabinose, L-mannose, or L-xylose. In contrast, both D-glucose and D-mannose significantly inhibited deGlc uptake (Figure 3A). In these experiments, the lack of significant inhibition of deGlc transport by 50 mM D-xylose was surprising since the affinity of GLUT 1 for D-xylose (50 mM) in intact erythrocytes is only slightly lower than that for D-mannose (25 mM) (LeFevre, 1961). One potential explanation for this apparent anomaly could be related to difficulties in accurately determining these affinity values. This interpretation is consistent with preliminary results which show pronounced inhibition of GLUT 1 mediated deGlc transport by D-xylose at higher concentrations (G. W. Gould, unpublished results).

When the same experiment was performed with GLUT 2 (Figure 3B), a small but highly reproducible inhibition of deGlc uptake was observed in the presence of 50 mM D-fructose (between 25% and 35% inhibition). Fructose concentrations of 100 mM and 150 mM inhibited deGlc uptake by 60% and 75%, respectively (data not shown). The small decrease in deGlc transport rate recorded in the presence of 50 mM D-xylose was not statistically significant.

GLUT 3 showed a pattern of inhibition that is different from those of GLUTs 1 and 2. Transport of deGlc by GLUT 3 was inhibited by D-xylose, as well as by D-mannose and D-glucose (Figure 3C), suggesting that GLUT 3 may be a xylose transporter.

One explanation for the different patterns of inhibition of deGlc transport observed with the different transporter isoforms is that they reflect differences in the outward-facing glucose binding site of the proteins. Since the rate of deGlc efflux via the transporter will be essentially zero (due to its phosphorylation; see Results), and the rate of conformational change that results in the glucose binding site reorientating to the intracellular space is fast compared to the rate of substrate association (Appleman & Lienhard, 1985; Lowe & Walmsley, 1989), the measured rate may be taken to reflect the effect of the hexose competing with deGlc for the external binding site. Thus, the results presented in Figure 3 strongly suggest that there are differences in substrate binding site among the different isoforms. However, it is not possible to rule out differential effects of the competing sugars on the distribution of inward/outward-facing conformers of the transporter resulting in the observed patterns of inhibition. Nonetheless, the results do indicate that GLUTs 1–3 are kinetically distinct and may differ in the nature of the outward-facing glucose binding site. This interpretation is also reflected in the affinities of GLUTs 1–3 for 3-*O*-MG (Table I) and their ability to transport other sugars (Table II).

The possibility that these patterns of inhibition were the result of inhibitory effects of the competing sugars on hexo-

kinase has been ruled out, since similar patterns of inhibition were observed with the nonmetabolizable glucose analogue 3-*O*-MG. Note that this analogue was not used throughout this analysis, since the ability of 3-*O*-MG to be transported in both directions by the transporters results in an inability to study the outward-facing binding site "in isolation", as the competition may be at either the inward-facing or outward-facing glucose site.

To determine whether the effects of other sugars on deGlc transport (Figure 3) were a reflection of the ability of GLUTs 1–3 to transport these sugars, we measured the rates of accumulation of D-fructose, D-galactose, and D-mannose (Table II). All three transporter isoforms mediated high rates of D-mannose accumulation. A large proportion of ingested mannose is converted to glucose for metabolism (Baily & Roe, 1944); this observation strongly suggests that both hexoses use the same transport system. This result may not be unexpected since the conformation of mannose exactly resembles that of glucose at the C1, C3, and C4 positions, the sites proposed to be important for the recognition of glucose at the outward-facing binding site of GLUT 1 (Barnett et al., 1973, 1975; Kahlenberg & Dolansky, 1972). Oocytes expressing GLUTs 1–3 were also capable of accumulating galactose at significant rates (Table II). The rates of galactose accumulation, when expressed as a ratio of the rate of deGlc accumulation, appear highest for GLUT 2. The GLUT 2 isoform was also capable of transporting fructose; GLUT 1 and GLUT 3 did not transport fructose under the same conditions. The rate of fructose accumulation recorded was found to be typically about 25% of the rate of deoxyglucose accumulation measured at the same concentration of sugar in the same oocyte population. Since GLUT 2 is localized to the liver, kidney, and gut, it is conceivable that this species is responsible for fructose transport in these tissues. In addition, pancreatic β-cells, the other major site of expression of GLUT 2, have been shown to be readily permeable to D-fructose, indicative of the presence of a fructose transport system in these cells (Sener et al., 1984). However, adipocytes and muscle, tissues that do not express GLUT 2, are also able to transport fructose, indicating that there are likely to be other fructose transporters in addition to GLUT 2.

In summary, the results described in this report demonstrate that GLUTs 1–3 may be distinguished on the basis of their kinetic properties and substrate selectivities. We are attempting to improve the expression of GLUTs 4 and 5 so that similar studies may be performed with these isoforms. These results provide a rational explanation for the presence of tissue-specific glucose transporters each with unique kinetic parameters and substrate specificities. Together with data on the specific numbers of each isoform present in cells, these results will facilitate a better understanding of the regulation of glucose homeostasis under changing physiological conditions.

Acknowledgments

We are grateful to Drs. Bill Cushley and Mark Bushfield for advice and encouragement throughout this study and to Drs. Gus Lienhard, David Calderhead, and Charles Burrant for communicating data prior to publication.

Registry No. 3-*O*-Methyl-D-glucose, 146-72-5; 2-deoxy-D-glucose, 154-17-6; D-glucose, 50-99-7; L-glucose, 921-60-8; D-galactose, 59-23-4; D-mannose, 3458-28-4.

References

Appleman, J. R., & Lienhard, G. E. (1985) *J. Biol. Chem. 260*, 4575–4578.

Bailey, W. H., & Roe, J. H. (1944) *J. Biol. Chem. 152*, 135–139.

Barnett, J. E. G., Holman, G. D., & Munday, K. A. (1973) *Biochem. J. 131*, 211–221.

Barnett, J. E. G., Holman, G. D., Chalkey, R. A., & Munday, K. A. (1975) *Biochem. J. 145*, 417–429.

Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukumoto, H., & Seino, S. (1990) *Diabetes Care 13*, 198–208.

Birnbaum, M. J. (1989) *Cell 57*, 305–315.

Birnbaum, M. J., Haspel, H. C., & Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5784–5788.

Bloch, R. (1973) *Biochemistry 12*, 4799–4801.

Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D. & Lienhard, G. E. (1990) *J. Biol. Chem. 265*, 13800–13808.

Charron, M. J., Brosius, F. C., Alper, S. L., & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2535–2539.

Eilam, Y., & Stein, W. D. (1974) *Methods Membr. Biol. 2*, 283–351.

Eliott, K. R. F., & Craik, J. D. (1982) *Biochem. Soc. Trans. 10*, 12–13.

Flier, J., Mueckler, M., McCall, A., & Lodish, H. F. (1987) *J. Clin. Invest. 79*, 657–661.

Fukumoto, H., Seino, S., Imura, I., Seino, Y., & Bell, G. I. (1988a) *Diabetes 37*, 657–661.

Fukumoto, H., Seino, S., Imura, H., Seino, Y., Eddy, R. L., Fukushima, Y., Byers, M. G., Shows, T. B., & Bell, G. I. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5434–5438.

Gould, G. W., & Lienhard, G. E. (1989) *Biochemistry 28*, 9447–9452.

Gould, G. W., & Bell, G. I. (1990) *Trends Biochem. Sci. 15*, 18–23.

James, D.E., Brown, R., Navarro, J., & Pilch, P. F. (1988) *Nature 333*, 183–185.

James, D. E., Strube, M., & Mueckler, M. (1989) *Nature 338*, 83–87.

Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. H., & Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3150–3154.

Kahlenberg, A., & Dolansky, D. (1972) *Can. J. Biochem. 50*, 638–643.

Kayano, T., Fukumoto, H., Eddy, R. L., Fan, Y.-S., Byers, M. G., Shows, T. B., & Bell, G. I. (1988) *J. Biol. Chem. 263*, 15245–15248.

Kayano, T., Burant, C. F., Fukumoto, H., Gould, G. W., Fan, Y.-S., Eddy, R. L., Byers, M. G., Shows, T. B., Seino, S., & Bell, G. I. (1990) *J. Biol. Chem. 265*, 13276–13282.

Keller, K., Strube, M., & Mueckler, M. (1989) *J. Biol. Chem. 264*, 18884–18889.

Kleinzeller, A., & McAvoy, E. M. (1976) *Biochim. Biophys. Acta 255*, 126–143.

Kreig, P. A., & Melton, D. A. (1984) *Nucleic Acids Res. 12*, 7057–7070.

LeFerve, P. G. (1961) *Pharmacol. Rev. 13*, 39–70.

Lowe, A. G., & Walmsley, A. R. (1986) *Biochim. Biophys. Acta 857*, 146–154.

Lowe, A. G., & Walmsley, A. R. (1989) in *Red Blood Cell Membranes: Structure, Function and Clinical Implications* (Agre, P., & Parker, J. C., Eds.) pp 597–633, Marcel Dekker, New York.

Marcus-Sekura, C. J., & Hitchcock, M. J. M. (1987) in *Guide to Molecular Cloning* (Berger, S., Ed.) Methods in Enzymology 152, pp 284–287, Academic.

Melton, D. A. (1987) *Methods Enzymol. 152*, 288–296.

Mueckler, M. (1990) *Diabetes 39*, 6–11.

Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E., & Lodish, H. F. (1985) *Science 229*, 941-945.
Orci, L., Thorens, B., Ravazzola, M., & Lodish, H. F. (1989) *Science 245*, 295-297.
Permutt, M. A., Koranyi, L., Keller, K., Lacy, P. E., Scharp, D. W., & Mueckler, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8688-8692.
Sener, A., Giroix, M.-H., Malaisse, W. J. (1984) *Eur. J. Biochem. 144*, 223-226.
Suzuki, K. (1988) *J. Biol. Chem. 263*, 12247-12252.
Thorens, B., Sarkar, H. K., Kaback, H. R., & Lodish, H. F. (1988) *Cell 55*, 281-290.
Vera, J. C., & Rosen, O. M. (1989) *Mol. Cell. Biol. 9*, 4187-4195.
Whitesell, R. R., Regen, D. M., & Abumrad, N. A. (1989) *Biochemistry 28*, 6937-6943.
Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinski, B., Ruoho, A. E., & Pilch, P. F. (1989) *J. Biol. Chem. 264*, 12358-12363.

Effects of Phosphorothioate and 2-Amino Groups in Hammerhead Ribozymes on Cleavage Rates and Mg^{2+} Binding†

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Received October 31, 1990; Revised Manuscript Received February 8, 1991

ABSTRACT: Mg^{2+} is important for the RNase activity of the hammerhead ribozyme. To investigate the binding properties of Mg^{2+} to the hammerhead ribozyme, cleavage rates and CD spectra for substrates containing inosine or guanosine at the cleavage site were measured. The 2-amino group of this guanosine interfered with the rate of the cleavage reaction and did not affect the amount of Mg^{2+} bound to the hammerhead RNA. The kinetics and CD spectra for chemically synthesized oligoribonucleotides with a S_p or R_p phosphorothioate diester bond at the cleavage site indicated that 1 mol of Mg^{2+} binds to the *pro-R* oxygen of phosphate. The binding constant for Mg^{2+} was about 10^4 M^{-1}, which represents outer-sphere complexation. The hammerhead ribozyme catalyzes the cleavage reaction via an *in-line* pathway. This mechanism has been proved for RNA cleavage by RNase A by using a modified oligonucleotide that has an S_p phosphorothioate bond at the cleavage site. From these results, we present the reaction pathway and a model for Mg^{2+} binding to the hammerhead ribozyme.

Hammerhead ribozymes, which contain 3 stems and 13 conserved nucleotides, have been found in avocado sunblotch viroid (Hutchins et al., 1986), the plus strand of tobacco ringspot virus (Buzayan et al., 1986; Prody et al., 1986) the virusoid of lucerne transient streak virus (vLTSV; Forster & Symons, 1987a,b) and transcripts of satellite DNA of the newt (Epstein & Gall, 1987). During a rolling circle replication of these RNAs, the hammerhead ribozymes seem to serve as the catalyst that cleaves multimeric replicated RNA into monomeric RNA in the presence of Mg^{2+}. It has been shown that a hammerhead domain was essential for this reaction in short transcribed RNA (Uhlenbeck, 1987). We have also found some crucial base pairs by synthesizing several mutants (Koizumi et al., 1988a). From the results of these mutagenesis experiments, we were able to design riboyzmes that cleavaged targeted RNA by recognizing a sequence of 9-15 nucleotides and hydrolyzed a mutant c-Ha-*ras* mRNA without cleaving the wild-type mRNA (Koizumi et al., 1988b, 1989). Haseloff and Gerlach (1988) reported that RNA enzymes could cleave transcripts of the chloramphenicol acetyltransferase gene. Sarver et al. (1990) showed that a designed hammerhead ribozyme reacted as an anti-HIV-1 therapeutic agent.

Mg^{2+} is necessary for reactions of the other ribozymes that were found in self-splicing RNAs (Grosshans & Cech, 1989) and an RNA component of RNase P (Guerrier-Takada et al., 1986). Sugimoto et al. (1988, 1989) showed that the intervening sequence (IVS) of *Tetrahymena* rRNA bound to 2 mol of Mg^{2+} to act as a catalyst for linearization of IVS with cytidylyluridine. Uhlenbeck (1987) showed that cleavage rates of the hammerhead ribozyme depend on the Mg^{2+} concentration. However, the Mg^{2+} binding properties and mechanisms of participation of Mg^{2+} in the hammerhead ribozyme reaction are not clear.

For this paper, we investigated a mode of Mg^{2+} binding in a hammerhead ribozyme by measuring cleavage rates and CD spectra for substrates containing inosine or guanosine at the cleavage site and concluded that the 2-amino group of guanosine interferes with the rate of cleavage reaction and did not affect the amount of Mg^{2+} bound to the hammerhead RNA. The kinetics and CD spectra for chemically synthesized oligoribonucleotides with an S_p or R_p phosphorothioate diester bond at the cleavage site indicated that 1 mol of Mg^{2+} bound to the *pro-R* oxygen of phosphate at this site. Using a modified oligonucleotide that has an S_p phosphorothionate bond at the cleavage site, we showed that the hammerhead ribozyme catalyzed the cleavage reaction via an *in-line* pathway, which has been shown to be a mechanism of RNA cleavage by RNase A (Usher et al., 1972; Eckstein, 1985).

Materials and Methods

Oligonucleotides. Oligoribonucleotides were synthesized by the phosphoramidite method using 2′-*O*-(tetrahydropyranyl) and 5′-*O*-(dimethoxytrityl) protecting groups as described previously (Koizumi et al., 1989). Oligoribonucleotides con-

†This research was supported by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan.
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0006-2960/91/0430-5145$02.50/0