Expression of a Functional Glucose Transporter in *Xenopus* Oocytes†

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ABSTRACT: A cDNA encoding the rat brain glucose transporter was inserted between the 5′ and 3′ untranslated regions from the *Xenopus* globin gene and downstream of an SP6 RNA polymerase start site. RNA synthesized from this vector was microinjected into oocytes from *Xenopus laevis*; this resulted in expression of the glucose transporter, as determined by both immunoblotting and the appearance of transport activity. The properties of the transporter were those expected from previous studies: it was glycosylated, and its activity, measured by 3-*O*-methylglucose transport, was inhibited by D-glucose and cytochalasin B, but not by L-glucose. The low level of endogenous glucose transport activity found in water-injected oocytes makes this a useful system in which to determine the kinetic parameters of transport. The K_m for 3-*O*-methylglucose was found to be 20 mM under equilibrium exchange conditions. Despite the fact that oocytes exhibit insulin-dependent responses, insulin did not stimulate 3-*O*-methylglucose transport by injected oocytes.

Glucose enters most animal cells by means of transporters of the facilitated diffusion type. To data, three such glucose transporters have been described, including the cloning of their cDNAs. One type of transporter is abundant in human erythrocytes, HepG2 cells, and brain, but is also present in many other tissues (hereafter referred to as the brain-type glucose transporter) (Mueckler et al., 1985; Birnbaum et al., 1986; Flier et al., 1987). A second type of transporter is found in tissues which exhibit insulin stimulation of glucose transport, such as muscle, heart, and fat (hereafter referred to as the muscle-type glucose transporter) (James et al., 1988, 1989; Birnbaum, 1989; Charron et al., 1989; Kaestner et al., 1989); a third species is present in liver and a few other tissues (Thorens et al., 1988; Fukumoto et al., 1988). In addition, a cDNA that appears to encode a fourth type of glucose transporter has been cloned from a fetal human muscle library, and the mRNA corresponding to this cDNA has a widespread distribution in adult tissues (Kayano et al., 1988).

The elucidation of the function and the regulation of these transporters requires a system in which they can be individually expressed in a functionally active and assayable form. Such a system would allow the identification by site-directed mutagenesis of amino acid residues critical for both the transport function and the regulation. As an initial step toward this goal, we undertook the expression of the rat brain glucose transporter in *Xenopus laevis* oocytes. *Xenopus laevis* oocytes have been shown to faithfully translate foreign mRNAs introduced into the cell by microinjection. The proteins synthesized are generally correctly modified (e.g., glycosylated, phosphorylated) and targeted to the correct intracellular location (e.g., plasma membrane, secreted) (Berridge & Lane, 1976; Colman & Morser, 1979; Colman et al., 1981; Matthews et al., 1981); thus, oocytes are an ideal system with which to address many complex biochemical questions.

Our results show that the rat brain glucose transporter is functionally expressed with its expected characteristics. Since the level of glucose transport in control injected oocytes is very low, this system proved to be ideal for the study of the kinetics and other properties of the expressed transporter. This study, together with the simultaneous demonstration that the muscle-type transporter can also be expressed in oocytes (Birnbaum, 1989), indicates that this system will be very useful for the detailed investigation of the properties of the various glucose transporters.

Materials and Methods

Materials. Type II collagenase (C 6885) and gentamyacin sulfate were purchased from Sigma. Sodium diguanosine triphosphate was purchased from Pharmacia. SP6 polymerase, RNasin, and nucleotides were purchased from Promega Biotech. All restriction endonucleases were from Bethesda Research Labs. Uridine [α-^{32}P]triphosphate and 3-*O*-methyl-D-[1-^{3}H]glucose ([^{3}H]3-*O*-MG)[1] were from Amersham. All other reagents were as described (Gibbs et al., 1988).

Oocyte Isolation and Injection. Female *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI) and maintained in the laboratory at 18–22 °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 medium [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, and gentamyacin sulfate at 10 mg/L]. All subsequent procedures were performed in Barths medium. Oocytes were incubated with collagenase (2 mg/mL) for 1 h with agitation and washed 6 times with 10 mL of fresh Barths and incubated overnight at 18 °C. Surviving oocytes (generally greater than 80%) were subsequently injected with 50 nL of RNA at various concentrations or 50 nL of water as a control (Melton, 1987). Oocytes were incubated in Barths at 18 °C for 12–96 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 cDNA encoding the rat brain glucose transporter (including 19 bp

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[1] Abbreviations: 3-*O*-MG, 3-*O*-methyl-D-glucose; PBS, phosphate-buffered saline; IGF-I, insulin-like growth factor I.

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of 5′ and 89 bp of 3′ untranslated sequences) was isolated from prGT1 by *Xho*I and *Bgl*II digestion (Birnbaum et al., 1986). Following the addition of *Bgl*II linkers, the cDNA was ligated into the *Bgl*II site of pSP64T (Krieg & Melton, 1984). The plasmid thus constructed (pSPGT1) contains the entire coding sequence of the rat brain glucose transporter, flanked by 89 bp of 5′ and 141 bp of 3′ untranslated sequence from the *Xenopus* β-globin gene; this construct did not generate any new start codons. *Sal*I-linearized plasmid DNA was used as a template for mRNA synthesis. A total of 3–5 μg of DNA was incubated in 50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each of ATP, CTP, and UTP, 0.1 mM GTP, and 0.5 mM diguanosine triphosphate in a volume of 100 μL; 0.1 μCi of [α-^{32}P]UTP was included as a means of quantitating the RNA synthesized. Twenty units of SP6 RNA polymerase were added and incubated at 39 °C for 45 min. After this time, an additional 10 units of SP6 polymerase were added, and the reaction was incubated for a further 45 min. Following this incubation, 2 units of RQ1 DNase were added, and the mixture was incubated at 37 °C for 10 min. It was then extracted once with phenol/$CHCl_3$ (1:1) and once with $CHCl_3$ and the RNA recovered by two rounds of ethanol precipitation with potassium acetate. The yield of RNA was estimated from the amount of trichloroacetic acid insoluble ^{32}P. RNA was injected as an aqueous solution.

Translation of mRNA synthesized from the SP6 polymerase site in reticulocyte lysates in the presence of canine pancreatic microsomes (Promega Biotech) yielded a protein that was immunoprecipitated by antibodies against the C-terminal peptide of the rat brain glucose transporter and which migrated as a broad band of molecular weight approximately 50 000 (data not shown).

Immunoprecipitation of the Glucose Transporter. Groups of 20 oocytes were homogenized by hand in a glass tube with a Teflon pestle in 600 μL of 50 mM Tris-HCl, pH 7.5, containing 2% octaethyleneglycol dodecyl ether, 5 mM *N*-ethylmaleimide, 1 μg/mL pepstatin A, 10 μM L-*trans*-epoxysuccinylleucylamido-3-methylbutane, and 0.2 mM diisopropyl fluorophosphate. The homogenate was centrifuged at 12000*g* in a microfuge for 5 min (Melton, 1987). The fat layer was carefully aspirated and the supernatant recovered from the pellet of yolk with a Pasteur pipet. The supernatant was incubated at 4 °C with 2 μg of affinity-purified rabbit antibodies against the C-terminus of the rat brain glucose transporter (Birnbaum et al., 1986; Davies et al., 1987) or irrelevant rabbit IgG. The immune complex was recovered by incubation with protein A–Sepharose for 2 h at 4 °C (Gibbs et al., 1986). Electrophoresis and immunoblotting of the immunoprecipitate were performed as described in Gibbs et al. (1988).

Hexose Transport in Oocytes. The transport of 3-*O*-methyl-D-glucose was determined under both zero-trans and equilibrium exchange conditions:

Zero-Trans Transport. Groups of five oocytes were incubated at room temperature in 500 μL of Barths medium (pH 7.4) for 15 min prior to addition of 3-*O*-MG to the medium (26 μM; 1.0 μCi of [^{3}H]3-*O*-MG per assay) for the requisite time. The reaction was stopped by quickly aspirating the medium 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, a potent transport inhibitor (Krupka, 1971). The oocytes were washed in this fashion a further 2 times and dispensed to scintillation vials, one oocyte per vial. These three washes were completed within 30 s; 0.5 mL of 1% sodium dodecyl sulfate was added to each scintillation vial, and the vials were incubated at room temperature for 1 h with agitation before addition of Hydrofluor (National Diagnostics) and measurement of radioactivity.

Equilibrium Exchange. Groups of five oocytes were incubated for 6 h at room temperature in 0.5 mL of Barths medium (pH 7.4) containing 3-*O*-MG at various concentrations. To start the reaction, 10 μL of Barths medium 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. The reaction was stopped and the counts per oocyte determined exactly as described for zero-trans conditions.

2-Deoxyglucose Uptake. Groups of five oocytes were incubated in Barths buffer at pH 7.4 for 15 min prior to initiation of uptake by the addition of an aliquot of [1,2-^{3}H]-deoxyglucose, such that the final concentration was 25 μM and 1.0 μCi/mL. After the required time, transport was stopped, and the radioactivity in individual oocytes was determined as described above for 3-*O*-MG.

Oocyte Maturation Assays. Groups of 30–50 oocytes were incubated in Barths (pH 7.6) containing 1 mg/mL bovine serum albumin (type V, Sigma A 7030) with and without 3 μM insulin (porcine, zinc). After 10 h at room temperature, oocytes were examined under the microscope and scored for the appearance of a white spot on the animal pole, an indicator of germinal vesicle breakdown (Maller & Koontz, 1981).

RESULTS

Expression of the Rat Brain Glucose Transporter Polypeptide in Oocytes. Groups of 20 oocytes were injected with 50 nL of rat brain glucose transporter mRNA at various concentrations. Two days following injection, the oocytes were homogenized in detergent and centrifuged to remove the abundant yolk proteins. The supernatant was divided in half and incubated with either antibodies raised against the C-terminal peptide of the brain glucose transporter (Birnbaum et al., 1986; Davies et al., 1987) or irrelevant IgG as described under Materials and Methods. The immunoprecipitate was recovered, electrophoresed, and then immunoblotted with the same anti-C-terminal peptide antibodies. As shown in Figure 1A, oocytes injected with synthetic mRNA encoding the rat brain glucose transporter expressed a protein of molecular weight 45 000–55 000 which was recognized by antibodies against the C-terminal peptide (lane 1); no such protein was detected in water-injected oocytes, even upon prolonged exposure of the autoradiogram (lane 3). The brain-type transporter is known to be a glycoprotein (Lienhard et al., 1984), and observation that the immunoreactive protein migrated as a broad band of molecular weight 45 000–55 000 suggested heterogeneous glycosylation. In this regard, we have found that treatment of the immunoprecipitate with endoglycosidase F resulted in the protein migrating as a sharp band of molecular weight about 40 000 (data not shown). We therefore conclude that the transporter is glycosylated in oocytes.

The amount of transporter polypeptide expressed was proportional to the amount of mRNA injected (Figure 1B); although it is not evident in this figure, at the mRNA dilution of 1:250, a weak signal was detected upon prolonged exposure of the autoradiograph. The amount of transporter expressed per oocyte was found to increase with time over a 2-day period (Figure 1C). In this experiment, the actual nanograms of transporter was estimated by quantitative immunoblotting using the erythrocyte glucose transporter as a standard, since these two transporters have identical C-termini (Mueckler et al., 1985; Birnbaum et al., 1986; Davies et al., 1987).

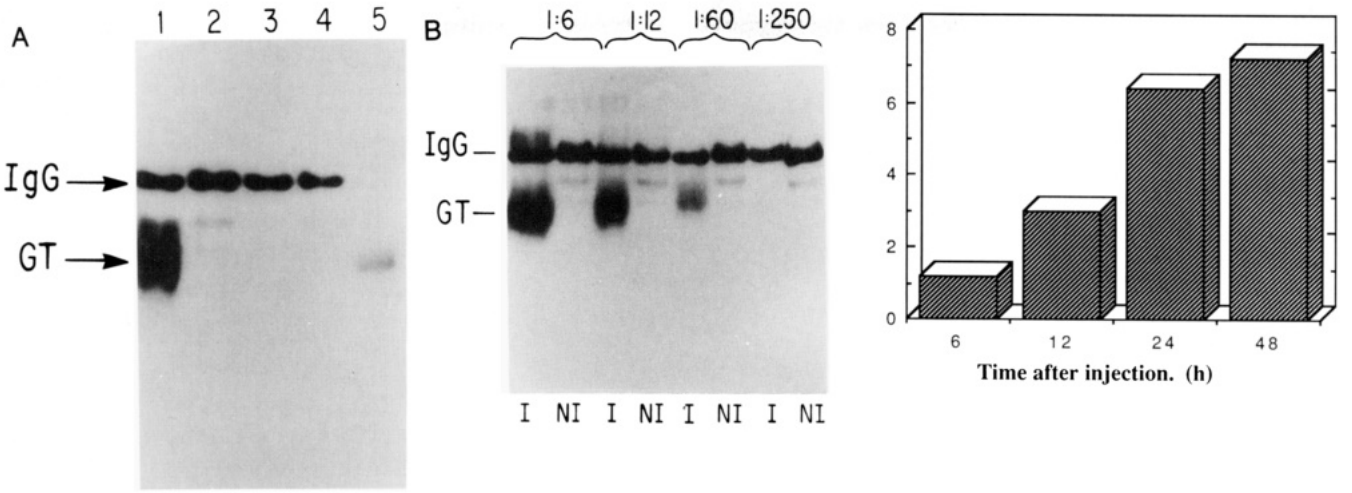

FIGURE 1: Immunological identification of the glucose transporter. (Panel A) Oocytes were injected with 50 nL of 80 μg/mL transporter mRNA (lanes 1, 2) or 50 nL of water (lanes 3, 4), and the transporter was immunoprecipitated with either anti-C-terminal peptide antibodies (lanes 1, 3) or irrelevant IgG (lanes 2, 4) 48 h after injection. Electrophoresis (sample from eight oocytes) and immunoblotting were performed as described under Materials and Methods, and a sample of 3T3-L1 adipocyte membranes containing about 2 ng of brain-type transporter was included as a comparison (lane 5). The upper band on the gel is the heavy–light-chain dimer of the antibody used in the immunoprecipitate (indicated IgG); the glucose transporter (GT) runs as a characteristically broad band of M_r 45 000–55 000. The entire running gel is shown. (Panel B) Groups of 20 oocytes were injected with 50 nL of mRNA (stock concentration 0.5 mg/mL) diluted in water as indicated (1:6, 1:12, etc. mRNA/water, v/v). Oocytes were incubated in Barths medium for 48 h following injection, and the transporter was immunoprecipitated with either antibodies against the C-terminus (I) or irrelevant IgG (NI). Following electrophoresis and immunoblotting with the same anti-C-terminal peptide antibody and ^{125}I-labeled goat anti-rabbit IgG, the autoradiograph was exposed for 24 h. Each lane is the signal obtained from eight oocytes. The entire gel is shown. (Panel C) Oocytes were injected with 50 nL of mRNA (145 μg/mL) and incubated in Barths buffer at 18 °C. The transporter content was determined as a function of time after injection by immunoprecipitation and immunoblotting as described under Materials and Methods. The amounts of GT were estimated by comparison with standards of human erythrocyte transporter run on the same immunoblot. A repetition of this experiment gave similar results.

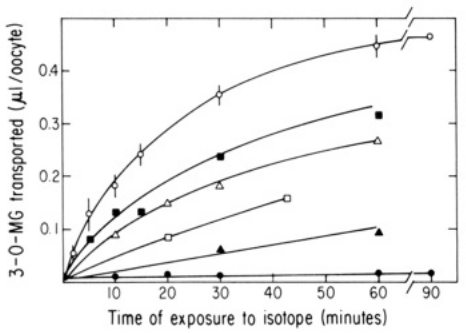

FIGURE 2: 3-*O*-MG transport in oocytes expressing the rat brain glucose transporter. Oocytes were microinjected with 50 nL of mRNA (0.26 mg/mL) diluted with water 1:3 (○), 1:6 (■), 1:12 (△), 1:24 (□), and 1:100 (▲) or 50 nL of water as a control (●) and incubated for 48 h at 18 °C in Barths medium. After 15-min incubation in Barths medium at room temperature, 3-*O*-MG (26 μM; 1.0 μCi) was added for the times indicated, the reaction was stopped, and the radiolabel in individual oocytes was determined as described under Materials and Methods. The data are presented as microliters of 3-*O*-MG transported per oocyte. The vertical bars on the top curve show the typical standard error of the mean (n = five oocytes).

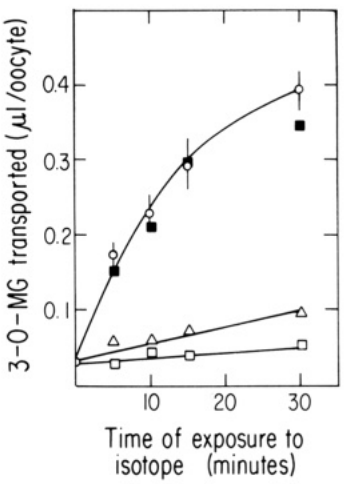

FIGURE 3: Characteristics of transport in oocytes expressing the rat brain glucose transporter. The uptake of 3-*O*-MG (26 μM; 1.0 μCi per assay) under zero-trans conditions was determined as described under Materials and Methods. 3-*O*-MG transport was measured in Barths medium alone (○), with 100 mM D-glucose (△), with 100 mM L-glucose (■), or with 25 μM cytochalasin B (□). Cytochalasin B was added from an ethanol stock solution at 25 mM; addition of 0.1% ethanol alone was without effect.

Expression of Glucose Transport Activity in Oocytes. The data presented above indicate that oocytes injected with mRNA encoding the rat brain glucose transporter express a protein that reacts strongly with antibodies against the carboxy terminus of this transporter. In order to determine whether this glucose transporter is functional in oocytes, the transport of 3-*O*-MG was assayed. Figure 2 shows the results obtained when oocytes were injected with either water (as a control) or increasing amounts of transporter mRNA. In water-injected oocytes, there was no detectable uptake of 3-*O*-MG over a 90-min period (closed circles). By contrast, oocytes injected with mRNA clearly exhibited 3-*O*-MG transport, and the rate of equilibration of the oocyte intracellular water space with 3-*O*-MG was found to increase with increasing amounts of mRNA injected. Thus, the glucose transporter was functionally expressed at the cell membrane. Typically, the water space equilibrated by 3-*O*-MG was 0.45–0.55 μL per oocyte; this value is in satisfactory agreement with a value of 0.5–0.6 μL/oocyte that we have measured by the equilibration with radiolabeled glycerol (data not shown). Figure 3 illustrates that the properties of the transporter were those expected on the basis of studies of transport in erythrocytes (Bloch, 1973; LeFevre, 1961); cytochalasin B and D-glucose inhibited the transport of 3-*O*-MG, whereas L-glucose did not.

The K_m for 3-*O*-MG was determined under equilibrium exchange conditions at concentrations of 3-*O*-MG of 0.1–100 mM. The data from a representative experiment (Figure 4)

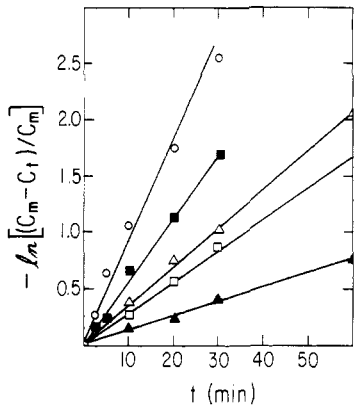

FIGURE 4: Rate of 3-*O*-MG transport as a function of substrate concentration under equilibrium exchange conditions. Groups of five oocytes were incubated for 6 h in 0.5 mL of Barths medium at room temperature containing unlabeled 3-*O*-MG at 100 (▲), 50 (□), 25 (△), 10 (■), and 1 mM (○); this incubation was sufficient to allow full equilibration of the 3-*O*-MG into the oocytes (data not shown). Following this incubation, radiolabeled 3-*O*-MG was added (1 μCi per assay) 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 per oocyte at time t.

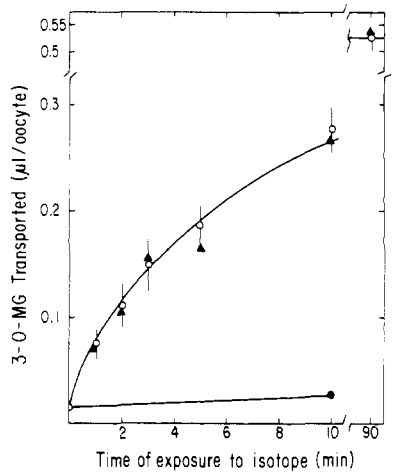

FIGURE 5: Effect of insulin on 3-*O*-MG transport. Oocytes were dissected manually, injected with 50 nL of mRNA at 125 μg/mL, and assayed 30 h following injection. Groups of five oocytes were incubated in Barths buffer (pH 7.6) containing 1 mg/mL BSA, with and without 3 μM insulin for 1 h at room temperature. After this time, 3-*O*-MG was added to a final concentration of 26 μM (1.0 μCi per assay), and the oocytes were incubated for the times indicated. The uptake was stopped and the radioactivity per oocyte determined as described under Materials and Methods. (▲) mRNA-injected, insulin-treated oocytes, (○) mRNA-injected, basal oocytes; (●) water-injected, insulin-treated oocytes [similar rates were recorded with water-injected, basal oocytes (data not shown)]. In a parallel experiment with oocytes from the same animal isolated using collagenase digestion, there was also no stimulation of transport by insulin (data not shown). In addition, experiments were performed on oocytes from the same preparation to determine whether the oocytes contained functional insulin receptors. Groups of 30–50 oocytes were incubated in the same buffer with and without insulin for 10 h. After this time, oocyte maturation was determined as described under Materials and Methods. In the presence of insulin, 71% of the oocytes exhibited a maturation response, compared to 2% in the control group. Furthermore, oocytes isolated from the same animal but using collagenase digestion also exhibited an insulin response, 39% maturation in the presence of insulin compared to 3% in the control group.

show the rate of equilibration of labeled 3-*O*-MG plotted in the form of a first-order process. It demonstrates that, as expected for equilibrium exchange (Eilam & Stein, 1984), the process was first-order. The rate constants (k_{obs}) obtained from such plots were used in Lineweaver–Burk plots to determine the K_m by plotting $1/k_{obs}$[3-*O*-MG] against 1/[3-*O*-MG]. The value of the K_m at 20 °C was 20.1 ± 2.9 mM (mean of three separate determinations).

Effect of Insulin on the Expressed Transport Activity. The effect of insulin on the rate of 3-*O*-MG uptake under zero-trans conditions was examined. Oocytes injected with mRNA (50 nL, 125 μg/mL) 12–48 h earlier were incubated in Barths buffer (pH 7.4) containing 1 mg/mL bovine serum albumin with or without 3 μM insulin for 1 h prior to assay, and the uptake of 3-*O*-MG was measured at various times after the addition of the labeled hexose. Insulin was found to be without effect on the rate of 3-*O*-MG transport (data not shown). This experiment was performed on four different preparations of injected oocytes with the same result.

Since the preparation of oocytes involves collagenase treatment for 1 h to remove the follicular cell layer, the experiment was repeated using oocytes that had been manually dissected, in order to be certain that the collagenase treatment had not inactivated the insulin receptors. Also, insulin-induced maturation was assayed on the same preparation to test for the presence of functional insulin receptors. The results are presented in Figure 5. As shown, insulin did not stimulate the transport of 3-*O*-MG in oocytes expressing the rat brain glucose transporter, even though 71% of oocytes from this preparation exhibited insulin-induced maturation. By comparison, we found 30–60% maturation in preparations of oocytes that had been collagenase treated. In contrast to the data of Janicot and Lane (1989), we recorded no stimulation of the rate of 3-*O*-MG transport in water-injected or noninjected oocytes, isolated with or without collagenase.

DISCUSSION

In this paper, we demonstrate the expression of the rat brain glucose transporter in oocytes after injection of its mRNA. The transporter protein was detected by immunoblotting and shown to be glycosylated. In addition, it was shown to be functional by the development of 3-*O*-methylglucose transport, which was inhibited by both cytochalasin B and D-glucose.

The very low rates of 3-*O*-MG transport recorded in water-injected oocytes under these conditions make this a useful system in which to examine the kinetic characteristics of this transporter. To this end, we have determined the K_m for 3-*O*-MG under equilibrium exchange conditions at 20 °C. Unfortunately, to our knowledge, the K_m for 3-*O*-MG transport by the brain-type transporter has not been determined under these conditions in any other cell type. However, the value obtained (20 mM) is in reasonable agreement with that for D-glucose in human erythrocytes under equilibrium exchange conditions at 20 °C (17 mM) (Lowe & Walmsley, 1986).

The following calculation indicates that the turnover number of the expressed transporter is also in the expected range. For any vesicle, the first-order rate constant for equilibration (k_{obs}) at a substrate concentration much below the K_m is given by

$$k_{obs} = k(T)_t/K_m V_i$$

where k is the turnover number for equilibrium exchange, $(T)_t$ is the moles of transporter per vesicle, K_m is the half-saturation constant for equilibrium exchange, and V_i is the internal volume of the vesicle (Baldwin et al., 1981). The oocytes containing 7 ng of transporter (Figure 1C) showed a rate constant for 26 μM 3-*O*-MG equilibrium of 0.14 min^{-1} at 20 °C. From these data, the K_m value of 20 mM, and the aqueous volume of 0.5 μL per oocyte, we calculate that the value of the turnover number for equilibrium exchange is 10^4 min^{-1}.

This calculation assumes that all the expressed transporter is at the oocyte plasma membrane; since in other cells, such as adipocytes, a significant pool of transporter is located intracellularly, this assumption should be viewed with caution. Nonetheless, this value is only slightly less than that for equilibrium exchange of D-glucose by the transporter in human erythrocytes at 20 °C (3×10^4 min^{-1}) (Lowe & Walmsley, 1986).

While this paper was in preparation, Janicot and Lane reported that uninjected *Xenopus laevis* oocytes exhibit a measurable rate of 2-deoxyglucose uptake, which is stimulated 3-fold by insulin (Janicot & Lane, 1989). As shown in Figures 2 and 5, the endogenous transport activity by our preparations of oocytes was very low. In one experiment, where the uptake of 25 μM 2-deoxyglucose over a 30-min period at 20 °C by uninjected oocytes was measured, the rate was approximately 0.04 pmol min^{-1} oocyte^{-1}, whereas oocytes injected with brain transporter mRNA showed a rate of 1.6 pmol min^{-1} oocyte^{-1}. By comparison, Janicot and Lane report an endogenous basal rate of 2-deoxyglucose uptake equivalent to 0.15 pmol min^{-1} oocyte^{-1} at 25 μM substrate, a value that is 4 times higher. Birnbaum has also found a much lower rate of endogenous 2-deoxyglucose uptake by insulin-treated oocytes than that of Janicot and Lane; when normalized to the same substrate concentration, his value is 10 times lower than theirs (Birnbaum, 1989). We have no explanation for these differences between laboratories but emphasize that the expressed transporter is the overwhelming contributor to transport activity here.

A number of insulin-dependent processes have been documented in oocytes, including maturation (Maller & Koontz, 1981; Deshpande & Kung, 1987), a reduction in cAMP level (Sadler & Maller, 1988), and phosphorylation of the ribosomal protein S6 (Stith & Maller, 1984; Stefanovic et al., 1986). Additionally, as noted above, it has been shown that insulin and IGF-I stimulate endogenous 2-deoxyglucose uptake in oocytes (Janicot & Lane, 1879). These effects are probably due to an endogenous IGF-I receptor, with lower affinity for insulin (Janicot & Lane, 1989). Insulin stimulates the translocation of intracellular transporters to the plasma membrane in adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Blok et al., 1988). Since the brain-type glucose transporter is known to be one type of transporter that undergoes insulin-stimulated translocation in adipocytes (Calderhead & Lienhard, 1988; Blok et al., 1988; Gibbs et al., 1988; James et al., 1988, 1989), we examined the effect of insulin on 3-*O*-MG transport in the injected oocytes. No stimulation of transport occurred, even though the oocytes responded to insulin by maturation. One explanation for this finding may be that oocytes lack proteins required to confer insulin responsiveness on the expressed transporter. If it is possible to obtain responsiveness by the simultaneous microinjection of mRNAs or proteins from adipocytes, this system may provide an assay for the identification of such proteins.

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References

Baldwin, J. M., Gorga, J. C., & Lienhard, G. E. (1981) *J. Biol. Chem. 256*, 3683–3689.

Berridge, M. V., & Lane, C. D. (1976) *Cell 8*, 283–297.

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.

Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W., & Gueze, H. J. (1988) *J. Cell Biol. 106*, 69–76.

Calderhead, D. M., & Lienhard, G. E. (1988) *J. Biol. Chem. 263*, 12171–12174.

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

Colman, A., & Morser, J. (1979) *Cell 17*, 517–526.

Colman, A., Lane, C. D., Craig, R., Boulton, A., Mouhn, T., & Morser, J. (1981) *Eur. J. Biochem. 113*, 339–348.

Cushman, S. W., & Wardzala, L. J. (1980) *J. Biol. Chem. 255*, 4758–5762.

Davies, A., Meeram, K., Cairns, M. T., & Baldwin, S. A. (1987) *J. Biol. Chem. 262*, 9347–9352.

Deshpande, A. K., & Kung, H.-F. (1987) *Mol. Cell. Biol. 7*, 1285–1288.

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

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

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

Gibbs, E. M., Allard, W. J., & Lienhard, G. E. (1986) *J. Biol. Chem. 261*, 16597–16603.

Gibbs, E. M., Lienhard, G. E., & Gould, G. W. (1988) *Biochemistry 27*, 6681–6685.

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

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

Janicot, M., & Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2642–2646.

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.

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.

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

Krupka, R. M. (1971) *Biochemistry 10*, 1143–1153.

LeFevre, P. G. (1961) *Pharm. Res. 13*, 39–70.

Lienhard, G. E., Crabb, J., & Ransome, K. J. (1984) *Biochim. Biophys. Acta 769*, 404–410.

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

Maller, J. L., & Koontz, J. W. (1981) *Dev. Biol. 85*, 309–316.

Marcus-Sekura, C. J., & Hitchcock, M. J. M. (1987) *Methods Enzymol. 152*, 284–287.

Matthews, J. A., Brown, J. W. S., & Hall, T. C. (1981) *Nature (London) 294*, 175–178.

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

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.
Sadler, S. E., & Maller, J. L. (1989) *J. Biol. Chem. 264*, 856–861.
Stefanovic, D., Erikson, E., Pike, L. J., & Maller, J. L. (1986) *EMBO J. 5*, 157–160.
Stith, B. J., & Maller, J. L. (1984) *Dev. Biol. 102*, 79–82.
Suzuki, K., & Kono, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2542–2546.
Thorens, B., Sarkar, H. K., Kaback, H. R., & Lodish, H. F. (1988) *Cell 55*, 281–290.

Mitochondrial NADH:Ubiquinone Reductase: Complementary DNA Sequence of the Import Precursor of the Bovine 75-kDa Subunit†

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ABSTRACT: The 75-kDa subunit of complex I (NADH:ubiquinone oxidoreductase) from bovine heart mitochondria is its largest subunit and is a component of the iron–sulfur (IP) fragment of the enzyme. It is encoded in nuclear DNA and is imported into the organelle. Protein sequences have been determined at the N-terminus of the intact protein and on fragments generated by partial cleavage with cyanogen bromide and with *Staphylococcus aureus* protease V8. Parts of these data have been used to design two mixtures of oligonucleotides 17 bases long, containing 192 and 256 different sequences, which have been synthesized and used as hybridization probes for identification of cognate cDNA clones. Two different but overlapping clones have been isolated, and the sequences of the cloned DNAs have been determined. Together they code for a precursor of the 75-kDa subunit of complex I. The mature protein is 704 amino acids in length, has a calculated molecular mass of 75961 daltons, and contains no segments of sequence that could be folded into hydrophobic α-helixes of sufficient length to span the inner membrane of the mitochondrion. Its precursor has an N-terminal extension of 23 amino acids to specify its import into the mitochondrion from the cytoplasm. Seventeen cysteine residues are dispersed throughout the 75-kDa subunit; some of them are close to each other in the sequence in three separate groups and, by analogy with other iron–sulfur proteins, could be involved in iron–sulfur clusters. The sequence of the 75-kDa subunit of complex I is not related closely to any known protein sequence, and the protein is not, as has been proposed, the major human biliary cirrhosis autoantigen.

Complex I (NADH:ubiquinone oxidoreductase) is the least understood of all the components of the mitochondrial respiratory chain [for a review, see Ragan (1987)]. This is due to the astonishing complexity of the enzyme. It contains one FMN plus at least seven Fe-S clusters, and analyses by SDS–PAGE[1] and two-dimensional PAGE of the bovine heart enzyme resolve 26–30 different subunits, of which seven are encoded in mitochondrial DNA (Chomyn et al., 1985, 1986; Fearnley & Walker, 1987). Complex I is located in the inner mitochondrial membrane where it catalyzes the oxidation of NADH in the matrix and the reduction of ubiquinone-10 in the bilayer. Of particular interest is the fact that the electron transfer reaction catalyzed by complex I is coupled to the generation of a protonmotive force with about two protons translocated per electron (Wikström, 1984). Mechanisms by which this might be accomplished have been discussed recently (Krishnamoorthy & Hinkle, 1988).

With the use of chaotropic agents, bovine complex I has been split up into distinct multisubunit fragments which maintain some structural and/or functional integrity. Two water-soluble fragments can be derived from the detergent-solubilized enzyme; the flavin-containing or FP fragment has three subunits (53, 24, and 10 kDa), contains the FMN plus two Fe-S clusters, and retains some NADH dehydrogenase activity. The FP subunits are probably all located on the matrix side of the inner membrane (Han et al., 1988). The iron-containing fragment, IP, contains six subunits (75, 49, 30, 18, 15, and 13 kDa) and at least three Fe-S clusters that retain their characteristic EPR spectral line shapes. Chemical labeling studies show that the 75-kDa subunit is located predominantly on the matrix side of the mitochondrial inner membrane, whereas the 49-kDa subunit is exposed on the cytoplasmic side. This would suggest that the IP fragment is transmembranous (Patel et al., 1988). However, the IP fragment appears to be water-soluble, though it is not monodisperse (Ragan, 1987). In addition to the three or more iron–sulfur clusters within the IP fragment, the 15-kDa subunit of the IP fragment is reported to bind ubiquinone (Suzuki & Ozawa, 1986). No catalytic activity has been ascribed to isolated IP. The remaining subunits in complex I, following removal of those comprising IP and FP, constitute the HP fraction, the main hydrophobic component of the enzyme. This fraction contains at least two Fe-S clusters and a subunit which binds to rotenone, a potent inhibitor of the enzyme (Earley

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[1] Abbreviations: CAPS, 3-(cyclohexylamino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride).

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