Differential targeting of glucose transporter isoforms heterologously expressed in *Xenopus* oocytes

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We have examined the subcellular distribution of three members of the human glucose transporter family expressed in oocytes from *Xenopus laevis*. Following injection of *in vitro*-transcribed mRNA encoding the transporter isoform to be studied, we have determined the subcellular localization of the expressed protein by immunofluorescence and by subcellular fractionation coupled with immunoblotting using specific anti-peptide antibodies. We have shown that both the liver-type (GLUT 2) and brain-type (GLUT 3) glucose transporters are expressed predominantly in the plasma membranes of oocytes, and in both cases high levels of glucose transport activity are exhibited. In contrast, the insulin-regulatable glucose transporter (GLUT 4) is localized predominantly to an intracellular membrane pool, and the levels

of transport activity recorded in oocytes expressing GLUT 4 are correspondingly lower. The localization of the different transporter isoforms to distinct subcellular fractions mirrors the situation observed in their native cell type and thus demonstrates that oocytes may prove to be a useful system with which to study the targeting signals for this important class of membrane proteins. In addition, the determination of the amounts of the transporters expressed per oocyte together with a knowledge of their K_m values has allowed us to estimate the turnover numbers of these transporters. Insulin was without effect on glucose transport in oocytes expressing any of these transporter isoforms. Microinjection of guanosine 5'-[γ -thio]triphosphate into oocytes expressing GLUT 4 was also without effect on the transport rate.

INTRODUCTION

It has been demonstrated that the transport of glucose across the plasma membranes of mammalian cells is mediated by a family of homologous proteins (for reviews see Gould and Bell, 1990; Mueckler, 1990; Bell et al., 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, four human glucose transporters have been identified: the erythrocyte-type transporter (GLUT 1), which is expressed at highest levels in fetal tissues and is also found in 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); and the muscle/fat-type, or insulin-responsive, transporter (GLUT 4), expressed only in tissues which exhibit insulin-sensitive glucose transport (fat, skeletal muscle and heart) (James et al., 1988, 1989; Birnbaum, 1989; Charron et al., 1989; Kaestner et al., 1989; Fukumoto et al., 1989). An additional member of this family, GLUT 5, has also been identified and found to be expressed predominantly in the small intestine and at low levels in many other tissues (Kayano et al., 1990; Shepherd et al., 1992a). However, recent studies have demonstrated that this protein is a high-affinity fructose transporter and its role in glucose transport is at present uncertain (Burant et al., 1992). These proteins exhibit 39–65% amino acid identity and 50–76%

sequence similarity, and are predicted to possess identical overall structures and orientations in the plasma membrane.

All of these proteins function as glucose transporters when expressed in *Xenopus* oocytes, bacteria or cultured cells (Gould and Lienhard, 1989; Keller et al., 1989; Permutt et al., 1989; Kayano et al., 1990; Gould et al., 1991; Burant et al., 1992), although with apparently different efficiencies. The *Xenopus* oocyte system is well suited to the study of the functional properties of heterologously expressed proteins. We and others have shown that this system is particularly well suited for the study of facilitative glucose transporters, because of the low levels of endogenous glucose transport activity present in un-injected oocytes. Using this procedure, K_m values for 3-*O*-methyl-D-glucose (3-*O*-MG) transport have been determined under equilibrium exchange conditions for GLUTs 1–4 (Gould and Lienhard, 1989; Keller et al., 1989; Gould et al., 1991).

The stimulation of glucose transport by insulin in adipocytes has been the subject of intense study, mainly as a consequence of the important role of insulin in regulating glucose disposal in peripheral tissues. It is now well established that, upon insulin exposure, adipocytes exhibit up to 30-fold increases in the glucose transport rate within minutes, a stimulation which is independent of new protein synthesis. Studies have shown that the majority of this increase in the glucose transport rate is accounted for by the movement of a pool of intracellular glucose transporters to the plasma membrane, a process termed translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Holman et al., 1990). It is now apparent that this translocation event is chiefly accounted for by the movement of one specific transporter isoform, GLUT 4 (James et al., 1987; Holman et al., 1990; Calderhead et al., 1990; Yang et al., 1992). Immuno-electron microscopy studies have demonstrated that in resting adipocytes

Abbreviations used: 3-*O*-MG, 3-*O*-methyl-D-glucose; deGlc, deoxyglucose; IGF-1, insulin-like growth factor-1; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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and cardiac myocytes the majority of GLUT 4 is localized in the *trans* Golgi region of the cells (James et al., 1987; Slot et al., 1991a,b).

In contrast, other glucose transporter isoforms, such as GLUT 2 and GLUT 3, are found at the plasma membrane of cells, with relatively minor amounts of these isoforms being expressed in an intracellular location (Orci et al., 1989; Thorens et al., 1990a,b; Hughes et al., 1992; Harris et al., 1992). The elucidation of the signals within the transporter molecule that regulate this distinct subcellular localization requires a system in which mutated transporters may be readily expressed and their localization examined. We have evaluated the use of the oocyte expression system for this purpose, and here we demonstrate that, when expressed in oocytes, GLUT 4 is predominantly intracellularly localized, but in contrast GLUTs 2 and 3 reside mainly at the plasma membrane. In addition, we have determined the turnover numbers of GLUT 3 and GLUT 4 in this expression system.

Despite the apparently correct intracellular localization of the transporter protein and the presence of functional insulin receptors on the oocyte surface, insulin was found to be without effect on heterologously expressed GLUT 4.

MATERIALS AND METHODS

Materials

Sodium diguanosine triphosphate was purchased from Pharmacia. SP6 polymerase, RNasin and nucleotides were purchased from Promega Biotech. 3-*O*-Methyl-D-[1-³H]glucose, 2-deoxy-D-[2,6-³H]glucose and ¹²⁵I- labelled goat anti-rabbit IgG were from DuPont/NEN. Optiphas HiSafe 3 was from LKB-Wallac (Milton Keynes, Bucks., U.K.). All other reagents were as described previously (Gould et al., 1991, 1992).

Oocyte isolation and injection

Female *Xenopus laevis* were purchased from the South African Xenopus Facility (Noordhoek, Republic of South Africa) and maintained at 18 °C on a 12 h light/12 h dark cycle. Animals were anaesthetized by ice immersion and oocytes removed by the procedure described by Marcus-Sekura and Hitchcock (1987). Individual oocytes were dissected and stored in Barth's medium [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 5 mM Hepes/NaOH, pH 7.6, plus penicillin at 10 µg/ml and streptomycin at 10 i.u./ml]. All subsequent procedures were performed in Barth's medium. Following isolation, oocytes 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 Barth's medium at 18 °C for 48–72 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 (Gould et al., 1991).

Plasmid construction and synthesis of mRNA

The glucose transporter constructs used for the preparation of synthetic mRNA have been described previously (Kayano et al., 1990). 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). Linearized plasmid DNA was used as a template for mRNA synthesis. RNA synthesis was performed as described (Gould et al., 1991).

Deoxyglucose (deGlc) transport in oocytes

DeGlc transport was determined exactly as described previously (Gould et al., 1991). Groups of 5–10 oocytes were exposed to [³H]deGlc (100 µM; 0.5 µCi per assay) for the times (generally 15–30 min) indicated in the figures. We have previously shown that, under these conditions, the transport and not the phosphorylation of deGlc is rate limiting (Gould et al., 1991; Colville et al., 1993). The reaction was stopped by quickly aspirating the medium and washing the oocytes three times 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). Note that the transport activities reported here are specific transport rates, determined by subtracting the counts associated with each oocyte in the presence of 10 µM cytochalasin B, a specific inhibitor of facilitative glucose transporters (Bloch, 1973). Typically, this value was < 5% of the counts recorded in the absence of cytochalasin B. Individual oocytes were solubilized by incubation in 0.5 ml of 1% SDS and the radioactivity associated with each oocyte was determined by liquid scintillation counting in OptiPhase HiSafe 3.

Zero-trans 3-*O*-MG transport assays

Groups of five oocytes were incubated at room temperature in 450 µl of Barth's medium (pH 7.4) for 15 min prior to the addition of 50 µl of 3-*O*-MG to the medium (100 µM final concentration; 1.0 µCi of [³H]3-*O*-MG per assay) for the requisite time. The reaction was stopped and the radioactivity associated with each oocyte was determined as described above for deoxyglucose.

Equilibrium-exchange 3-*O*-MG transport assays

Groups of five oocytes were incubated at room temperature in 490 µl of Barth's medium (pH 7.4) containing 0.1 mM 3-*O*-MG for 5 h with occasional agitation. After this time, 10 µl of 0.1 mM 3-*O*-MG (1.0 µCi/10 µl) was added and the contents of the tube were mixed. After the requisite period of exposure to the isotope, the transport was stopped and the radioactivity associated with each oocyte determined as described above for deGlc. The maximum level of radiolabel associated with each oocyte was determined by exposure of oocytes to radiolabel for 4 h.

Subcellular fractionation of oocytes

Preparation of total oocyte membranes

The procedure used was that described by Geering et al. (1989), with minor modifications. Oocytes were suspended in homogenization buffer (83 mM NaCl, 1 mM MgCl₂, 10 mM Hepes, pH 7.9, 0.5 mM phenylmethanesulphonyl fluoride, 1 µg/ml pepstatin A, 0.2 mM di-isopropyl fluorophosphate and 10 µM *L*-trans-epoxysuccinyl-leucylamido-3-methylbutane) in a volume of 50 µl per oocyte and homogenized with 20 hand-driven strokes in a Teflon/glass homogenizer. Typically, 50 oocytes were homogenized for each experiment. Homogenates were centrifuged at 1000 *g*_{av} for 5 min at 4 °C to pellet the yolk granules and melanosomes, and the supernatant was removed. The yolk granules and melanosomes were subsequently rehomogenized in 500 µl of homogenization buffer, recentrifuged at 1000 *g*_{av} for 5 min and the supernatants combined. The pooled supernatants were centrifuged again at 1000 *g*_{av} for 5 min to remove residual yolk protein, and the resulting supernatant was centrifuged at 100 000 *g*_{av} for 90 min at 4 °C to obtain a total membrane fraction. The pellet obtained was resuspended in

either homogenization buffer or SDS/PAGE sample buffer as the experiment dictated.

Isolation of subcellular fractions

The procedure used was adapted from that described previously (Geering et al., 1989; Keller et al., 1989; Garcia et al., 1992). Oocytes were placed in a small Petri dish containing 1 ml of ice-cold homogenization buffer plus protease inhibitors (see above). Using a watchmaker's forceps, oocytes were gently squeezed and the plasma membrane 'ghosts' were separated from the yolk/cellular contents, which were expelled into the buffer. The resulting oocyte ghosts were placed in 1 ml of ice-cold homogenization buffer with the final NaCl concentration raised to 0.5 M and gently stirred for 10 min on ice. The buffer containing the yolk protein and intracellular debris was placed on ice throughout this time.

After stirring, the buffer was centrifuged at 1000 g_{av} for 10 min at 4 °C. The resulting pellet (designated P1) was resuspended in SDS/PAGE sample buffer. The supernatant containing plasma membrane sheets was further centrifuged at 10000 g_{av} for 20 min at 4 °C and the resulting pellet (designated P2) was resuspended in SDS/PAGE sample buffer.

The supernatant from this step was combined with the yolk protein/intracellular debris from the initial oocyte disruption and centrifuged repeatedly at 1000 g_{av} to pellet the yolk protein. The supernatants were then centrifuged at 100000 g_{av} for 60 min at 4 °C to pellet the intracellular membranes. The pellet was resuspended directly in SDS/PAGE sample buffer and is designated P3.

The original subcellular fractionation procedure elucidated by Geering et al. (1989) utilized several enzyme activities as markers of the subcellular fractions. We have assayed adenylate cyclase activities in the membrane fractions and our results were comparable to those of Geering et al. (1989), with the exception that activities of adenylate cyclase measured in our hands were 2–4-fold lower (results not shown). In addition, we have examined these fractions by electron microscopy. The P1 fraction contains most of the dense secretory granules, and little or no contamination of the P3 fraction with these granules was observed. P3 was found to contain the majority of the rough endoplasmic reticulum as well as other membranous structures.

Immunofluorescence studies

Paraffin-embedded oocyte sections (15 μ m) were fixed by incubation in acetone at 4 °C and air-dried. Non-specific binding was blocked by incubation with 2% goat serum, followed by staining with the specific antiserum, generally at a 1:50 dilution. After washing, bound antibody was detected using fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG.

Immunoblotting of expressed transporters

SDS/PAGE and immunoblotting were performed exactly as described (Gibbs et al., 1991; Gould et al., 1991). The anti-GLUT 2, anti-GLUT 3 and anti-GLUT 4 antibodies were raised against peptides corresponding to the C-terminal 14 amino acids of the human homologues of GLUTs 2, 3 and 4 and have been previously described (Brant et al., 1992; Shepherd et al., 1992a,b). The standards used in the quantitative immunoblotting were purified GLUT 1 (a gift from Dr. S. A. Baldwin, Royal Free Hospital, London, U.K.) and membranes isolated from cells engineered to stably express GLUT 3 (Maher et al., 1992) or GLUT 4 (a gift from Dr. Graeme Bell, University of Chicago, U.S.A.). For all quantitative blotting experiments, samples

containing known amounts of the standards (typically between 1 and 20 ng) were loaded on a gel along with at least two dilutions of the oocyte membranes to be analysed.

5'-Nucleotidase assay

5'-Nucleotidase activities were determined exactly as described (Brown et al., 1988). Briefly, membrane fractions were resuspended in 150 mM KCl, 20 mM Hepes and 2 mM $MgCl_2$, pH 7.2, then incubated at 37 °C with 0.3% Triton X-100, 5 mM 2',3'-AMP and 0.2 mM [3H]5'-AMP (1 μ Ci/ml). At various times (10–90 min) duplicate samples were removed and added to 50 μ l of 0.25 M $ZnSO_4$, followed by the addition of 100 μ l of $BaCl_2$. The radioactivity in the supernatants from these samples after centrifugation was determined by liquid scintillation spectrometry. The protein concentration was adjusted such that the rate of adenosine release was linear with time.

RESULTS

We have observed some variability in the rate of deGlc transport in control (non-injected) oocytes isolated from different

Table 1 DeGlc transport rates in oocytes expressing GLUTs 1–4

Oocytes were microinjected with 25 ng of mRNA encoding the different transporters and assayed for deGlc transport 48 h after injection as described. Shown are the transport rates at 100 μ M deGlc (means $\pm$ S.D.; $n = 5$) from a representative experiment measured in the absence or presence of 10 μ M cytochalasin B. The transport rates in non-injected oocytes (controls) are shown. Note that we have observed some variability (between 0.1 and 0.5 pmol/min per oocyte) in the deGlc transport rates measured in non-injected oocytes isolated from different animals (for example, compare control rates below with those of Table 5). However, the incremental increases in transport rates observed following injection of mRNA such as shown below from a representative experiment have been determined to be consistent between preparations. We have found no differences in transport rates between water-injected and non-injected oocytes in any preparation (results not shown).

Isoform	DeGlc transport rate (pmol/min per oocyte)	
	No cytochalasin B	+ 10 μ M Cytochalasin B
GLUT 1	2.6 $\pm$ 0.14	0.22 $\pm$ 0.03
GLUT 2	3.1 $\pm$ 0.11	0.72 $\pm$ 0.02
GLUT 3	2.9 $\pm$ 0.2	0.30 $\pm$ 0.05
GLUT 4	0.4 $\pm$ 0.01	0.08 $\pm$ 0.01
Control	0.12 $\pm$ 0.01	0.03 $\pm$ 0.02

Table 2 Circulation of the transport activity as a function of the amount of expressed transporter

Rates of deGlc (100 μ M) transport were determined for a series of oocyte preparations expressing the different transporter isoforms. Total membranes were prepared from the same preparations and the absolute levels of GLUTs 1, 3 and 4 were determined by quantitative immunoblotting against known standards of each isoform (note that the lack of a standard for GLUT 2 has precluded this analysis for this isoform). The measured transport rates were then expressed as pmol/min per ng of GLUT. The data presented are the means $\pm$ S.D. of at least four independent determinations of each isoform. Within these experiments, the levels of GLUTs 1, 3 and 4 were between 1 and 3 ng per oocyte. n.d., not determined.

Isoform	Transport rate (pmol/min per ng of GLUT)
GLUT 1	3.14 $\pm$ 0.18
GLUT 2	n.d.
GLUT 3	2.68 $\pm$ 0.32
GLUT 4	0.24 $\pm$ 0.05

Figure 1 Immunofluorescence analysis of GLUTs 2, 3 and 4 in oocytes

Oocytes expressing GLUTs 2, 3 or 4 were sectioned and stained as described in the Materials and methods section. Expression was verified by the measurement of deGlc transport in a parallel group of oocytes. (a) GLUT-2-expressing oocytes stained with anti-GLUT 2; (b) GLUT-3-expressing oocytes stained with anti-GLUT 3; (c) GLUT-3-injected oocytes stained with anti-GLUT 2 antibodies; (d) GLUT-4-expressing oocytes stained with anti-GLUT 4 antibodies. The presence of the transporter protein in oocytes in this experiment was also demonstrated by immunoblot analysis (not shown).

Table 3 5'-Nucleotidase activities in oocyte membrane fractions

Oocyte membrane fractions were prepared from 20 oocytes and assayed for 5'-nucleotidase activities as described. Shown below are results from a representative experiment. Activities are means ($\pm$ S.D.) of three determinations for each fraction. ND, not detected.

Fraction	5'-Nucleotidase activity (nmol/h per 20 oocytes)	
Total membranes	2.6 $\pm$ 0.3	(100%)
P1	0.4 $\pm$ 0.1	(15%)
P2	1.95 $\pm$ 0.20	(75%)
P3	ND	

animals. The range of transport rates varied between ~ 0.1 and ~ 0.5 pmol/min per oocyte (at 0.1 mM deGlc), depending on the animal used for the oocyte isolation. In addition, we have observed no difference between the deGlc transport rates in water-injected and non-injected oocytes. Consequently, the majority of the experiments in this paper used non-injected oocytes as controls unless otherwise stated.

Microinjection of *in vitro*-transcribed mRNAs encoding members of the human glucose transporter family into oocytes resulted in an increased rate of deGlc transport into the oocyte (Table 1). In a series of experiments, however, we have noted that injection of comparable amounts of mRNA encoding the different isoforms did not lead to comparable increases in the rate of deGlc transport; in particular, low rates of deGlc transport were consistently observed in oocytes injected with GLUT 4 mRNA (see, for example, Table 1). (Note that the low levels of transport activity for GLUT4 compared with GLUTs 1, 2 or 3 were observed in oocyte preparations which exhibited different non-injected transport rates; compare Table 1 and Table 5.) Similar results have been obtained from over 20 separate experiments. In an attempt to address this, we have quantified the amounts of heterologously expressed glucose transporters in

oocytes by quantitative immunoblotting against known GLUT 1, 3 and 4 standards.

The data in Table 2 compare the amount of glucose transporter expressed (determined by quantitative immunoblotting; see the Materials and methods section) and the transport rate into the oocyte population in question. The amount of GLUT 4 protein expressed per oocyte was similar to that for GLUTs 1 and 3 (typically between 1 and 3 ng/oocyte; results not shown), but the transport rate into oocytes expressing GLUT 4 was substantially less than that recorded for GLUTs 1 and 3 (note that we have been unable to accurately quantify the levels of GLUT 2 expressed in oocytes) (Table 1). When these data are expressed as a rate per ng of expressed transporter (Table 2), it is apparent that the activity of GLUT 4 is lower than that of both GLUT 1 and GLUT 3, despite this transporter having the highest affinity for deGlc (Keller et al., 1989; Gould et al., 1991; Colville et al., 1993). This result may be explained in two ways. (i) It is possible that there are substantial differences in the turnover numbers of the different isoforms. If GLUT 4 had a lower activity than GLUTs 1, 2 and 3 then, in the event that broadly similar levels of each isoform were expressed at the surface of the oocyte, the transport rate in GLUT 4-expressing oocytes would be expected to be less than with the other isoforms. (ii) A second alternative is that GLUT 4 may not be targeted to the plasma membrane of oocytes. It is well established that, in adipocytes and muscle, which are major sites of expression of GLUT 4, more than 90% of the GLUT 4 is present in an intracellular compartment in resting cells (see, for example, James et al., 1989; Holman et al., 1990; Calderhead et al., 1990; Slot et al., 1991a,b). If this were the case in oocytes it would presumably be reflected by a low glucose transport rate into the cell. In their native cell types, GLUTs 2 and 3 are predominantly localized to the plasma membrane (Orci et al., 1989; Thorens et al., 1990a,b; Gerhart et al., 1992). In addition, GLUT 1 resides in the plasma membrane of most cells; however, an intracellular pool of GLUT 1 has been identified (Yang et al., 1992). Given that oocytes have been shown to correctly target heterologously expressed proteins (e.g. Colman and Morser, 1979; Colman et al., 1981; Matthews et al.,

Figure 2 Subcellular distribution of GLUTs 2 (a), 3 (b) and 4 (c) expressed in oocytes

Oocytes were injected with mRNA encoding the appropriate isoform and incubated for 48 h. After this time, oocytes were fractionated as described in the Materials and methods section. Samples were electrophoresed and immunoblotted with antibodies specific to each isoform. Shown are representative immunoblots from oocytes injected with GLUT 2, 3 or 4. P1 contains the majority of the dense vesicles and some plasma membranes, P2 is enriched with plasma membranes, and P3 contains the total remaining membranes (mainly intracellular). In all blots, each lane contains membranes from an equal number of oocytes. Data obtained from three immunoblot analyses from three independent oocyte fractionations are presented in Table 3.

Table 4 Subcellular distribution of GLUT isoforms determined by subcellular fractionation and quantitative immunoblotting

Oocytes expressing the different glucose transporter isoforms were fractionated as described in the Materials and methods section. The relative levels of immunoreactive glucose transporter in each fraction is expressed as a percentage of those in a total membrane fraction. P1 and P2 represent plasma-membrane-containing fractions, and P3 contains predominantly intracellular membranes. Shown are the means $\pm$ S.D. of three separate determinations for each isoform.

Fraction	Transporter level (%)		
	GLUT 2	GLUT 3	GLUT 4
P1	19 $\pm$ 10	15 $\pm$ 2	9 $\pm$ 3
P2	40 $\pm$ 14	72 $\pm$ 5	14 $\pm$ 7
P3	38 $\pm$ 7	15 $\pm$ 7	75 $\pm$ 8

1981), we set out to determine the subcellular location of GLUTs 2, 3 and 4 expressed in oocytes.

We have used two approaches to determine the subcellular distribution of heterologously expressed glucose transporters in oocytes: immunofluorescence of oocyte sections, and subcellular fractionation with immunoblotting using antibodies specific for each of the transporter isoforms. The results of the immunofluorescence analysis are presented in Figure 1. In oocytes expressing GLUT 2 or GLUT 3, a bright ring of immunofluorescence was observed at the perimeter of the oocyte, consistent with the expression of the transporter at or close to the cell surface. In the case of GLUT-4-expressing oocytes, no such staining was observed. In these oocytes, quantitative immunoblotting of total oocyte membranes indicated that GLUT 3 and GLUT 4 were present at comparable levels (results not shown). Intracellular localization of GLUT 4 cannot be accurately determined using immunofluorescence, because non-specific antibody binding to the yolk protein resulted in background immunofluorescence in all sections with all antibodies. Nonetheless, the data in Figure 1 do indicate that GLUT 2 and GLUT 3 are located at the surface of the oocyte, with no immunodetectable GLUT 4 recorded in a comparable location in GLUT 4-injected oocytes. Previous studies have demonstrated that GLUT 1-injected oocytes stained with antibodies specific to GLUT 1 also exhibit this characteristic bright ring of immunofluorescence at the cell surface (Garcia et al., 1992).

We have therefore undertaken a simple subcellular fractionation of oocyte membranes in an attempt to demonstrate the localization of GLUT 4 to a distinct compartment from GLUTs 1, 2 or 3. The subcellular fractionation procedure utilized the ability of oocyte ghosts to be isolated separately from the majority of the yolk protein by manual dissection (Keller et al., 1989; Garcia et al., 1992). Oocytes were fractionated using this procedure into three membrane fractions: P1, which contained the majority of the dense secretory vesicles (as assessed by electron microscopy), and probably some elements of the plasma membrane; P2, the plasma-membrane-ghost-enriched fraction; and P3, which represents an intracellular membrane pool. Assay of the plasma membrane marker enzyme 5'-nucleotidase in these fractions (Table 3) showed that P2 contained about 75% of the 5'-nucleotidase activity (enriched 2–3-fold over the total membrane activity) and P1 contained ~15% of the activity, suggesting that the P1 fraction contained some elements of the plasma membrane. The activity of 5'-nucleotidase was below the limit of detection in the P3 fraction, suggesting that most of the plasma membranes had been efficiently removed from this fraction.

Immunoblots of these membrane fractions from oocytes

Table 5 Effect of insulin on the transport activity of heterologously expressed transporters

Oocytes expressing the indicated isoforms were incubated in Barth's medium in the presence or absence of 2 μ M insulin for 60 min prior to the determination of deGlc transport rates as described. Values presented are the means $\pm$ S.D. ($n = 5$) from a representative experiment. The values presented below have been corrected for the transport rate in non-injected oocytes. In this experiment this rate was 0.5 pmol/min per oocyte (see the legend to Table 1).

Isoform	DeGlc transport rate (pmol/min per oocyte)	
	No insulin	2 μ M insulin
GLUT 1	5.9 $\pm$ 0.8	6.5 $\pm$ 0.2
GLUT 2	8.8 $\pm$ 0.6	7.5 $\pm$ 0.6
GLUT 3	3.5 $\pm$ 0.2	3.7 $\pm$ 0.4
GLUT 4	0.7 $\pm$ 0.1	1.0 $\pm$ 0.1

Figure 3 Effect of GTP[S] on 3-O-MG transport into oocytes expressing GLUT 4

Oocytes were injected with 20 nl of GLUT 4 mRNA (0.5 mg/ml) and incubated for 48 h in Barth's buffer. After this time, 50 nl of 10 mM GTP[S] (●) or 50 nl of water (○) was microinjected into the oocyte. Groups of five oocytes were then incubated for a further 1 h at room temperature in 450 μ l of Barth's buffer. Subsequently, 50 μ l of 3-O-MG (0.1 mM, 1.0 μ Ci per assay) was added to the tube and incubation was continued for the times indicated. Transport was stopped and the radiolabel associated with each oocyte was measured as described in the Materials and methods section. The data are expressed as a first-order plot, and each point is the mean of five determinations ($\pm$ S.D.). Data from a representative experiment are shown. C_m and C_i are the counts associated with the oocyte after 5 h and time t respectively.

expressing GLUTs 2, 3 or 4 is presented in Figure 2. In the representative blot shown, GLUT 4 is predominantly (> 70%) located in P3. In contrast, GLUT 2 and GLUT 3 are chiefly resident in fractions P1 and P2. The result of three quantifications of this type (Table 4) show that GLUT 4 is predominantly localized to the P3 (intracellular) fraction, whereas GLUT 2 and GLUT 3 are localized mainly to fractions P1 and P2.

It is well established that oocytes contain functional insulin/insulin-like growth factor-1 (IGF-1) receptors (Janicot and Lane, 1989). Hence we have evaluated the effect of insulin on deGlc and 3-O-MG transport into oocytes expressing any of the transporter isoforms. Using a preparation of permeabilized adipocytes, Lodish and colleagues demonstrated that the addition of guanosine 5'-[γ -thio]triphosphate (GTP[S]) resulted in the

translocation of GLUT 4 to the plasma membrane (Baldini et al., 1991). Consequently we evaluated the effect of GTP[S] on the transport of the non-metabolizable glucose analogue 3-*O*-MG in oocytes expressing GLUT 4 (Figure 3). Microinjection of this non-hydrolysable GTP analogue was without effect on the GLUT-4 mediated 3-*O*-MG transport rate.

DISCUSSION

It is well established that the insulin-responsive glucose transporter, GLUT 4, is unique amongst the transporter family by virtue of its sequestration into an intracellular pool in resting adipocytes and muscle. Upon insulin-stimulation there is a substantial movement of GLUT 4 to the plasma membrane, resulting in an approximate 20-fold increase in GLUT 4 at the surface of rat adipocytes (Holman et al., 1990; Calderhead et al., 1990; Slot et al., 1991a,b). Several laboratories have heterologously expressed GLUT 4 in a variety of cells, such as CHO cells, several fibroblastic cell lines and the neuronal-like cell line PC12 (Hudson et al., 1992; Piper et al., 1992; Shibasaki et al., 1992). In all of these studies GLUT 4 has been shown to be predominantly sequestered inside the cells, present in the perinuclear region and sequestered in vesicles throughout the cytoplasm. In addition, most of these studies have demonstrated that insulin did not result in the movement (translocation) of heterologously expressed GLUT 4 to the surface, even in cell lines expressing high levels of the insulin receptor (Hudson et al., 1992). To date, however, no data have been presented regarding the nature of the localization of heterologously expressed GLUT 2 and GLUT 3. It is established that insulin does not stimulate glucose transport into isolated hepatocytes, the major site of expression of GLUT 2, and immunofluorescence has provided no evidence for a substantial intracellular pool of GLUT 2 in either liver or pancreatic β -cells (Orsi et al., 1989; Thorens et al., 1990a,b). In the present work, we have examined the subcellular location and insulin-responsiveness of GLUTs 2, 3 and 4 expressed in *Xenopus* oocytes. This system was chosen for study because of the ease with which heterologous expression may be achieved. Also, these cells possess functional insulin receptors, are known to be insulin-sensitive (see, for example, Sadler and Maller, 1988) and thus may provide a somewhat more appropriate system for study than fibroblasts over-expressing the insulin receptor.

In a series of experiments, we have consistently observed that microinjection of comparable amounts of mRNA encoding GLUTs 1–4 into oocytes resulted in a lower level of transport activity in GLUT 4-injected oocytes than in any of the other populations (see, for example, Table 1 and Gould et al., 1991). In an attempt to investigate the nature of this effect, we quantified the relative levels of expression of GLUTs 1, 3 and 4 expressed in oocytes using quantitative immunoblotting against purified GLUT 1 or against membranes containing a known amount of GLUT 3 or 4 (the lack of a suitable standard of GLUT 2 has precluded a similar analysis for this isoform). The expression of the transport rates as pmol/min per ng of transporter (Table 2) indicated that GLUT 4 did not exhibit as high levels of transport activity as the other transporters. This result may be explained either by a substantial difference in the affinity and/or turnover numbers of the different transporters, or else by the localization of GLUT 4 to an intracellular pool resulting in only a small fraction of the GLUT 4 residing at the cell surface (such as occurs in adipocytes and muscle). Each of the transporter affinities has been determined in the oocyte system (Gould and Lienhard, 1989; Keller et al., 1989; Gould et al., 1991), and the K_m values indicate that GLUT 4 has the highest affinity for

glucose amongst the isoforms studied here. Hence, under the conditions of the assays used in Table 2 (0.1 mM deGlc), it would be predicted that if all transporters had similar turnover numbers and were expressed at the plasma membrane at comparable levels, GLUT 4 should exhibit the highest transport rate.

Given that oocytes have been well-documented to correctly target heterologously expressed proteins to the appropriate cellular compartment (Colman et al., 1981; Matthews et al., 1981), we felt it most likely that the data in Table 1 could be explained mainly by differences in subcellular distributions between the transporter isoforms. Immunofluorescence analysis (Figure 1) demonstrated that significant staining at the periphery of the oocyte was exhibited by GLUT-2- and GLUT-3-expressing oocytes. In contrast, immunofluorescence analysis of GLUT-4-expressing oocytes did not show any evidence of staining at or close to the cell surface. This result may be cautiously interpreted to imply only low levels of expression of GLUT 4 at the oocyte surface, but the high level of non-specific binding to the yolk protein in the oocyte interior precluded the identification of GLUT 4 inside the cell. We therefore used a simple subcellular fractionation procedure to isolate a plasma membrane-enriched fraction from oocytes. The results of this analysis (Figure 2, Table 4) demonstrated that GLUTs 2 and 3 were predominantly located in the plasma membrane-enriched fractions (P1 and P2), whereas the majority (~75%) of the GLUT 4 immunoreactivity was present in the intracellular membranes (P3). Taken together, these data demonstrate that, when expressed in oocytes, GLUT 2 and GLUT 3 are predominantly targeted to the plasma membrane of the cell, whereas GLUT 4 resides in an intracellular compartment, the nature of which is not currently understood. It is unlikely, however, that the intracellular compartment to which the GLUT 4 is localized is the endoplasmic reticulum, since the GLUT 4 protein has been glycosylated in oocytes. In experiments not presented here, we have shown that GLUT 4 expressed in oocytes can be effectively deglycosylated with endoglycosidase F (A. M. Brant, and G. W. Gould, unpublished work). Similar results have also been published for GLUT 1 expressed in oocytes (Gould and Lienhard, 1989).

Given that oocytes express abundant functional insulin (or more likely IGF-I) receptors, we examined the ability of insulin to stimulate glucose transport activity in oocytes expressing the different transporter isoforms (Table 5). As shown, we have been unable to detect any significant increase in glucose transport in response to insulin in oocytes expressing any of the transporter isoforms. In all experiments there was a trend towards a slight (less than 2-fold) increase in response to insulin in oocytes expressing GLUT 4, but this effect seldom approached statistical significance. It should be noted that we have examined the ability of insulin to stimulate GLUT 4 transport rates in oocytes expressing different levels of GLUT 4 in an attempt to determine whether the expression of high levels of GLUT 4 may have 'swamped' the insulin-responsive machinery in the cell. At no point did we record any significant increase in transport rate in response to insulin (results not shown). We have also examined the effect of intracellularly injected GTP[S] on the transport of 3-*O*-MG into oocytes expressing GLUT 4 (Figure 3). This non-hydrolysable GTP analogue has been shown to induce the translocation of GLUT 4 to the surface of permeabilized 3T3-L1 adipocytes (Baldini et al., 1991). However, no effect of GTP[S] was observed in oocytes, as judged by a lack of stimulation of the transport of 3-*O*-MG.

The lack of insulin-responsiveness of expressed GLUT 4 may be due to several factors. Firstly, it has not been demonstrated in any heterologous expression analysis of GLUT 4 (including this paper) that the transporter is targeted to the same intracellular

compartment as that to which it is targeted in adipocytes or muscle cells. At present this analysis is difficult, since the precise biochemical nature of this compartment (possibly the *trans* Golgi reticulum) is unknown. It is possible that adipocytes and muscle contain an intracellular compartment unique to these tissues and that it is only from this compartment that GLUT 4 can be translocated. Alternatively, while oocytes clearly respond to insulin, it is probable that this effect is mediated via IGF-1 receptors (Janicot and Lane, 1989), which may be functionally distinct from the insulin receptor. More likely, oocytes may lack a component of the insulin-signalling pathway necessary for insulin-stimulated glucose transport. This expression system may prove useful in the screening of candidate proteins in the response to insulin.

It is interesting to note that the level of expression of the transporters achieved in this analysis (typically between 1 and 3 ng of transporter/oocyte) is less than reported in other studies (Garcia et al., 1992). In their study, Garcia et al. (1992) reported that only 12% of the expressed GLUT 1 was present in the oocyte plasma membrane; however, these authors expressed about 12 ng of GLUT 1 per oocyte. Hence, the apparent discrepancy between the results reported here, where typically 75% of the expressed GLUTs 2 and 3 was localized to a plasma membrane fraction, may be due to the lower level of expression. This would potentially result in a decreased likelihood of overloading the oocyte intracellular membranes with heterologously expressed protein. Alternatively, the results may be a reflection of different intrinsic localizations of GLUTs 2 and 3 compared with GLUT 1. It is established that both 3T3-L1 adipocytes (Calderhead et al., 1990) and 3T3-L1 fibroblasts (Yang et al., 1992) contain a significant pool of intracellularly localized GLUT 1. In both fibroblasts and adipocytes insulin induces a translocation of GLUT 1 to the surface of the cell which, although small when compared with the translocation of GLUT 4 in adipocytes, is nonetheless significant (5-fold in 3T3-L1 adipocytes; 2-fold in 3T3-L1 fibroblasts; Calderhead et al., 1990; Yang et al., 1992).

Quantification of the total amounts of GLUTs 1, 3 and 4 in oocytes has allowed the calculation of the turnover numbers of these isoforms. In these experiments we have used the non-metabolizable glucose analogue 3-*O*-MG. Previously we reported the 3-*O*-MG equilibrium exchange K_m values of GLUTs 1, 2 and 3 expressed in oocytes as 17, 42 and 10.6 mM respectively (Gould et al., 1991), and a value of 1.8 mM has been reported for the rat homologue of GLUT 4 (Keller et al., 1989). We have determined the K_m for the human GLUT 4 homologue to be 2.0 mM, in close agreement with the K_m for rat GLUT 4 (results not shown). For any vesicle, the first-order rate constant for equilibration (k_{obs}) at a substrate concentration much below the K_m is given by:

$$k_{\text{obs}} = k(T_i)/K_m V_i$$

where k is the turnover number for equilibrium exchange, (T_i) is the amount (mol) of transporter per vesicle and V_i is the internal volume of the vesicle (oocyte) (Baldwin et al., 1981). We therefore undertook a series of experiments to measure the k_{obs} at 0.1 mM 3-*O*-MG, to determine the amount of the transporter isoform expressed in the same oocyte population, and hence calculate the turnover number for equilibrium exchange. The values obtained from three independent experiments were $2000 \pm 170 \text{ min}^{-1}$ for GLUT 3 and $150 \pm 60 \text{ min}^{-1}$ for GLUT 4 at 20 °C. These calculations, however, assume that all of the expressed transporter is at the oocyte plasma membrane, and it is clear from the data presented herein that this is not the case, particularly for GLUT 4. Taking these calculations at face value, the turnover number for equilibrium exchange for GLUT 3 is broadly

comparable with that previously reported for GLUT 1 expressed in oocytes (10^4 min^{-1} at 26 μM 3-*O*-MG; Gould and Lienhard, 1989). The value obtained for GLUT 4 is clearly an underestimate, by perhaps as much as an order of magnitude, as a consequence of the predominant intracellular localization of this isoform when it is expressed in oocytes (see above). Thus, if only 10% of the total GLUT 4 expressed in oocytes were present at the cell surface, then the turnover number for this isoform would be comparable with those of GLUTs 1 and 3.

In summary, we have shown that oocytes expressing GLUTs 2 and 3 exhibit high rates of glucose transport, consistent with the predominant localization of these two isoforms in the plasma membrane. In contrast the transport rates exhibited by oocytes expressing GLUT 4 were consistently lower and were correlated with the localization of GLUT 4 in an intracellular compartment. Insulin was without effect on the glucose transport rates in oocytes expressing any of the transporter isoforms, and microinjection of GTP[S] did not stimulate the translocation of GLUT 4 in oocytes. We have determined the levels of transporter expressed in oocytes by quantitative immunoblotting and estimated values of the turnover numbers for these transporters have been presented.

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