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Expression of GLUT-2 cDNA in human B lymphocytes: analysis of glucose transport using flow cytometry

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The molecular characterization of transport proteins is often limited by transient functional expression or the need for a simple method to select functional cDNA clones. We used a mammalian expression system to obtain long-term expression of GLUT-2, an isoform of glucose permease. Rat GLUT-2 cDNA was ligated into an EBV vector (pLPP) and transfected into B lymphocytes which lack GLUT-2. Northern and Western analyses confirmed expression of GLUT-2 protein in membranes of transfected cells. Two functional assays using flow cytometry were developed to distinguish GLUT-2 transfectants from control/pLPP transfectants. Uptake of NBD-glucosamine, a fluorescent analogue of glucose, was increased in GLUT-2 transfectants. In addition, when exposed to hypertonic glucose medium, GLUT-2 transfectants and control/pLPP transfectants exhibited a difference in forward-angle light scatter (FALS), an index of cell volume, indicating a difference in glucose permeability. Independent measurements of glucose uptake (isotopic) and cell volume (video microscopy) confirmed the flow cytometry observations. This expression system used in combination with flow cytometry is useful for studying the functional properties of glucose and other solute transporters.

Introduction

GLUT-2, one of a family of Na^+-independent glucose transporters, is a low-affinity hexose transporter. It was cloned from liver but is also expressed on the basolateral membrane of renal and intestinal epithelia and in pancreatic β cells [1,2]. In β cells, GLUT-2 is thought to function as part of the glucose sensor for insulin release and down-regulation of this transporter may play a role in the pathogenesis of type I and II diabetes mellitus [3,4]. While the isoforms of glucose permease share significant homology at the nucleotide and amino acid level, kinetic properties, regulation, and inhibitor sensitivity differ [5–7]. For example, the K_m of GLUT-2 (20–40 mM) is greater than that of GLUT-1 (2–20 mM), which is the native transporter expressed in B lymphocytes. The molecular basis for these functional differences is largely unknown. Expression of GLUT-2 cDNA in mammalian cells will be important for studying structure-function relationships of this transporter.

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Several expression systems have been used to characterize cDNAs encoding transport proteins (e.g., *Xenopus* oocytes, COS cells) [8]. However, the molecular characterization of transport proteins using these methods can be limited by transient functional expression or the need for a rapid and efficient method to select functional clones. Margolskee et al. [9] used an EBV expression vector to transfect B lymphocytes and then identify and select functional clones using a fluorescence-activated cell sorter (FACS). In combination with an antibody, this approach was used to clone a TNF receptor [10,11]. This method has the advantages of stable cDNA expression and rapid selection of functional clones using the FACS. In general, however, identification of functional clones using flow cytometry has involved the binding of an antibody or high-affinity ligand to the protein of interest.

The transport of uncharged solutes such as glucose has traditionally been studied by isotopic uptake. However, fluorescent substrate analogues are available and there are several advantages of using these substrates to study transport [12]. Most significantly, measurement of fluorescence requires little biological material for study and can be used to measure solute uptake by individual cells rather than the average uptake rate of

a heterogeneous population of cells as is done with isotopic uptake. There are many fluorescent probes available including NBD-glucosamine (NBD-G), an analogue of glucose which can readily enter cells via the glucose permease pathway [13].

Changes in cell volume can also reflect differences in solute transport rates. Moreover, light scatter has been used to measure permeability of cells to various solutes based on changes in cell volume [14,15]. Light scatter can be routinely measured by FACS and has been used successfully to discriminate different populations of cells [16].

The purpose of this study was to transfect B cells with glucose permease-2 (GLUT-2) cDNA using the EBV vector pLPP and to develop functional assays of glucose transport using the FACS to identify and select cells which expressed this transporter. Two functional assays of glucose transport were developed using the flow cytometer: (1) uptake of a fluorescent glucose analogue, NBD-glucosamine and (2) forward-angle light scatter (FALS) of cells exposed to hypertonic glucose. Using these assays, GLUT-2 transfectants exhibited significantly greater glucose transport capacity than control/pLPP transfectants.

Methods

Cell culture and DNA transfection

BJAB cells, an EBV-negative B lymphocyte cell line (kindly provided by Dr. F. Wang), were grown in RPMI 1640 medium (Whittacker Bioproducts) supplemented with 10% fetal bovine serum (JRH Biosciences) at 37°C/5% CO_2. Transfected cells were maintained in medium supplemented with hygromycin B 400 μg/ml (Calbiochem). Cells were split 1:1 every 5–7 days.

Full length rat GLUT-2 cDNA, 2573 bp, (a gift from B. Thorens) was ligated into the EBV expression vector EBO-pLPP (a detailed description of this vector is provided in Ref. 17). For transfection of these plasmid vectors 50000 cells were resuspended in RPMI medium and transferred to electroporation cuvettes containing 15 μg of pLPP/GLUT-2 (for GLUT-2 transfectants) or plasmid alone (for control/pLPP transfectants). Electroporation was performed at room temperature, 0.2 kV, 960 μF using a Bio-Rad Gene Pulser. After electroporation the cells were resuspended in 20 ml medium pre-warmed to 37°C and incubated for 24 h at 37°C/5% CO_2. Cells were then seeded at 1250–5000 cells per 200 μl well (96 well plates, Costar) in medium containing 400 μg/ml hygromycin B to select for transfectants.

RNA preparation and Northern analysis

Total cellular RNA was extracted from untransfected cells and GLUT-2 and control/pLPP transfectants as previously described [18]. Briefly, cells were pelleted and lysed with 4 M guanidine isothiocyanate (BRL), 22 μM sodium acetate pH 6 and 0.0075% β-mercaptoethanol and subjected to ultracentrifugation through 5.7 M cesium chloride (BRL) and 25 mM sodium acetate pH 6. Total RNA was quantitated spectrophotometrically; 7 μg of each sample was fractionated on a 1% agarose-formaldehyde gel and then transferred overnight onto a Genescreen membrane (New England Nuclear) with 20 × SSC (1 × consists of 150 mM NaCl, 15 mM Na citrate, pH 7.0) and UV crosslinked (UV Stratalinker, Stratagene). Membranes were then pre-washed with 1 M NaCl, 10 mM Tris (pH 8), 1 mM EDTA, 0.1% SDS at 47°C for 2 h and pre-hybridized with 40% (v/v) formamide, 10% dextran sulfate (American Bioanalytical), 4 × SSC, 7 mM Tris (pH 7.6), 0.8 × Denhardt's solution (1 × consists of 0.02% polyvinylpyrrolidine, 0.02% ficoll, 0.02% bovine serum albumin), 0.02 mg/ml salmon sperm DNA (Sigma), and 0.5% SDS at 42°C for 1 h. Membranes were hybridized overnight under the above conditions with 10^6 cpm/ml of ^{32}P-cDNA probe. The GLUT-2 probe was synthesized form the 2573 bp, full length cDNA by random primer extension. Membranes were washed twice (2 × SSC, 0.1% SDS) for 20 min at 25°C and then once in 0.2 × SSC, 0.1% SDS for 30 min at 50°C. Membranes were exposed on Kodak XAR-5 film at −80°C in the presence of an intensifying screen for 48 h.

Preparation of microsomes and western analysis

$5 \cdot 10^6$ cells were washed twice in PBS, resuspended in 2 ml of low ionic strength buffer (10 mM Tris (pH 7.5), 0.5 mM $MgCl_2$) and incubated on ice for 10 min. Proteinase inhibitors (PMSF 0.1 mM and aprotinin 100 U/ml) were added and the cells homogenized with a 7 ml Dounce tight A pestle as follows: 40 strokes, addition of 2 ml sucrose buffer (0.5 M sucrose, 20 mM Tris (pH 7.5), 6 mM β-mercaptoethanol, 2 mM EDTA), 20 strokes. The homogenate was spun at 800 × *g* (SA-600 rotor, Sorvall) at 4°C for 10 min. The supernatant was removed and spun at 8000 × *g* at 4°C for 20 min. The supernatant was then transferred to ultracentrifuge tubes and spun at 36000 rpm at 4°C for 1 h (T1270 rotor Sorvall). The final pellet (crude microsomes) was resuspended in 50 μl of a 1:1 dilution of the sucrose buffer.

In parallel, rat liver microsomes were prepared. Fresh rat liver, 0.5–0.6 g, was finely diced, suspended in 3 ml of a sucrose buffer consisting of 0.25 M sucrose, 10 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 1 mM EDTA in the presence of proteinase inhibitors (PMSF 0.1 mM and aprotinin 100 U/ml) and homogenized in a Dounce homogenizer with 15–20 strokes. Crude microsomes were then prepared exactly as outlined above for BJAB cells. Protein was determined by the Brad-

ford assay (Bio-Rad) using bovine serum albumin as the standard.

Liver microsomes (10 μg) and BJAB microsomes (30 μg) from GLUT-2 and control/pLPP transfectants were suspended in Laemmli buffer and resolved on a 10% SDS-polyacrylamide gel (Bio-Rad Mini-Protean II apparatus) and electrotransferred to nitrocellulose filters (Micron Separations, Inc.) [19]. The filters were washed at room temperature in distilled water for 10 min; for 20 min in TBS (20 mM Tris (pH 7.4), 150 mM NaCl)/0.1% Tween-20; and then blocked for 30 min in TBS/5% non-fat dry milk (SACO, Mix'n'Drink)/0.2% NP-40 at 37°C. The filters were incubated with a rabbit antibody to GLUT-2 diluted 1:200 in blocking solution at 4°C for 12 h. The GLUT-2 antibody (kindly provided by B. Thorens) was raised in rabbits against a peptide consisting of amino acids 477–492 of the carboxy terminal of GLUT-2. The filters were washed twice at room temperature for 20 min in TBS/0.2% NP-40, once in TBS/0.1% Tween-20 and reblocked as above. The blocked filters were then incubated at room temperature for 1 h with anti-IgG alkaline phosphatase conjugate (Promega) diluted 1:7500 in blocking solution. The filters were washed twice as above and developed using a 1:1 molar ratio of the chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BRL) in 10 ml 0.1 M Tris (pH 9), 0.1 M NaCl, 5 mM $MgCl_2$.

3-O-Methyl-D-[^{3}H]glucose (^{3}H-3-OMG) uptake

Cells were resuspended in RPMI medium (which contains 11 mM unlabeled glucose) to achieve a concentration of $(2–5) \cdot 10^7$ cells/ml. At time zero 10 μl of ^{3}H-3-OMG, 1.5 μCi/ml, was added to 400 μl of cell suspension and incubated at 20°C with gentle shaking. Preliminary experiments indicated that under these conditions uptake was linear for 3 min. Therefore, the relative initial velocity of 3-OMG uptake was calculated as the slope of the linear regression line for the plot of time (min) vs. uptake (nmol 3-OMG/mg protein) based on five time points. Each time point was performed in triplicate. The reaction was stopped by layering the cell suspension on 500 μl of oil (1 part silicone: 1 part dioctyl phthalate, Aldrich) in ice-cold microfuge tubes and immediately spun at 1200 *g* in a microcentrifuge for 15 s. The cell pellet was collected by cutting off the bottom of the microfuge tube and agitating it for 12–18 h in 5 ml scintillation vials containing 500 μl 10% SDS to lyse the cells. 4 ml of scintillation fluid (Optifluor, Packard) was added to each tube and they were counted in a liquid scintillation analyzer (Packard).

Video microscopy

500 μl of cells, suspended in RPMI medium to achieve a concentration of $1 \cdot 10^6$ cells/ml, were transferred to 1 ml, six well plates (Costar). At time zero 500 μl of RPMI medium alone (isotonic control) or 500 μl of RPMI medium containing 900 mM glucose, sorbitol or urea was added to the 500 μl cell suspension to achieve a final concentration of 450 mM of the added solute. The cells were incubated at 37°C/5% CO_2 in the presence of hypertonic glucose or sorbitol and at 20°C in the presence of hypertonic urea. At selected time points 300-μl aliquots were placed on a glass slide and viewed with the 25 × objective (Zeiss). Differential interference contrast microscopy images were recorded on videocassettes (3M) as previously described [20]. Cell perimeter was measured by the tracing the outlines of video images using Image 1 software (Universal Imaging Corp.). 50–55 cells were counted for each time point and condition. Cell volume was calculated as the volume of a sphere. Cells appeared round under both control isotonic and hypertonic conditions. Roundness was assessed quantitatively by a shape factor using Image-1 software. The shape factor $= 4\pi a/p^2$, where a = area and p = perimeter. The equation is based on the fact that a circle has the highest area to perimeter ratio of any object. Values above 0.8 represent objects which are very round, with a perfect circle having a value of 1. Under all conditions cells had a shape factor ≥ 0.9, indicating a high degree of roundness.

Flow cytometer analysis

Cells were suspended in medium to a concentration of $1 \cdot 10^6$ cells/ml and transferred to 1 ml, six well plates (Costar) and exposed to hypertonic conditions exactly as outlined above for video microscopy measurements. Flow cytometric analysis was performed on a Coulter Epics 750. Light scattered at angles of 1°–19° was collected for forward-angle light scatter (FALS). The change in FALS in the presence of 450 mM urea was performed at 20°C. FALS was measured before the addition of urea (isotonic) and at selected time points after exposure to 450 mM urea.

To measure NBD-G uptake 1 μl of 30 mM NBD-G (Molecular Probes) was added to 1 ml of cell suspension ($1 \cdot 10^6$ cells/ml) in RPMI medium and incubated at 37°C/5% CO_2. At selected time points a 500-μl aliquot was removed to a tube containing 1 ml of ice-cold stopping solution (RPMI medium + 100 mM phloretin) and spun down in a table top centrifuge at $1200 \times g$ for 15 s. The cell pellet was washed twice in 1 ml ice-cold stopping solution and resuspended in 500 μl ice-cold stopping solution. All optical emissions were derived from an argon laser tuned to 488 nm. NBD-G emission was measured at 530 nm–560 nm. Each time point was done in triplicate. For experiments in which an inhibitor was used, cells were incubated with 100 μM phloretin 20 min prior to and during dye uptake. In all experiments $1 \cdot 10^4$ cells were

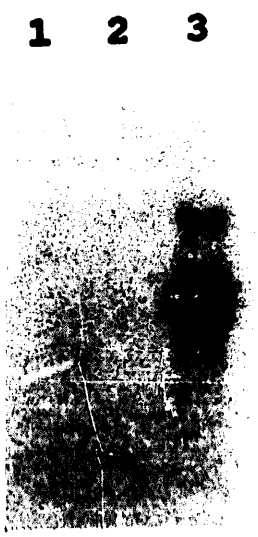

Fig. 1. Northern analysis of GLUT-2 mRNA in untransfected BJAB cells (lane 1) and control/pLPP (lane 2) and GLUT-2 (lane 3) transfected cells. 7 μg of total RNA was loaded per lane.

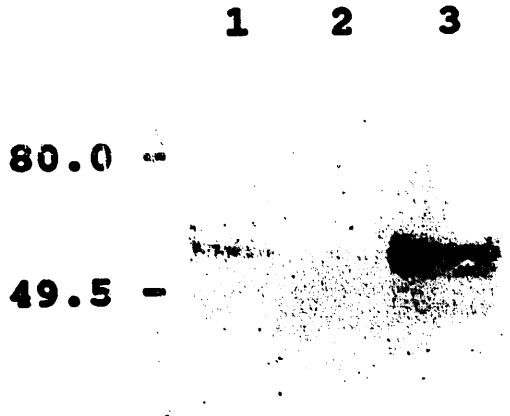

Fig. 2. Western blot detection of GLUT-2 transporter in microsomes prepared from rat liver (lane 1), control/pLPP transfectants (lane 2) and GLUT-2 transfectants (lane 3) using an antibody against a peptide consisting of carboxy-terminal amino acids 477–492. 10 μg of protein was loaded of liver microsomes and 30 μg each of control/pLPP and GLUT-2 microsomes. Molecular mass markers in kDa are on the left.

analyzed per sample. Simultaneous staining with propidium iodide was used to exclude dead cells. For sorting, $(2–5)\cdot 10^4$ cells were collected in 4 ml of medium, spun down and resuspended in 400 μl RPMI medium and seeded in two 200 μl wells.

Reagents

All chemicals were purchased from Sigma unless otherwise indicated.

Results

Expression of GLUT-2 cDNA in transfected cells

Northern and Western analyses. B Lymphocytes (BJAB) were transfected with pLPP-GLUT-2 (GLUT-2 transfectants) or plasmid alone (control/pLPP transfectants). Fig. 1 is a Northern blot demonstrating that GLUT-2 mRNA is not expressed in untransfected cells (lane 1) or control/pLPP transfectants (lane 2). In contrast, there was a significant level of GLUT-2 mRNA in the GLUT-2 transfected cells (lane 3). Fig. 2, a Western blot using an antibody to GLUT-2, confirms the presence of GLUT-2 protein in microsomes isolated from GLUT-2 transfectants (lane 3). Liver microsomes (lane 1) serve as a positive control since GLUT-2 is natively expressed in liver. Microsomes from control/pLPP transfectants did not bind the GLUT-2 antibody (lane 2). The apparent molecular mass (63 kDa) of GLUT-2 protein was identical in liver microsomes and GLUT-2 transfectants and is in agreement with published reports [2]. Thus, GLUT-2 cDNA was transcribed and translated by the transfected BJAB cells and the mature protein was detected in the membrane fraction of these cells.

Glucose uptake

Flow cytometric analysis. Having demonstrated expression of the transfected cDNA at the mRNA and protein level, we next sought to develop functional assays to show that GLUT-2 transfectants exhibited greater glucose transport than control/pLPP transfectants. We reasoned that if GLUT-2 transfectants have increased glucose transport, they should possess an enhanced ability to take up NBD-glucosamine, a fluorescent glucose analogue. Alternatively, this increase in glucose permeability should be evident when the cells are exposed to hypertonic glucose since the GLUT-2 transfectants would shrink less and/or return more quickly towards control volume.

NBD-glucosamine (NBD-G) is a fluorescent analogue of glucose which has been used to study hexose transport [13]. It is able to enter cells via a glucose permease pathway [13]. Fig. 3 compares NBD-G uptake at 20 min in GLUT-2 versus control/pLPP transfectants. The GLUT-2 histogram is shifted to the right, indicating increased dye uptake and hence increased glucose transport. Phloretin, an effective inhibitor of

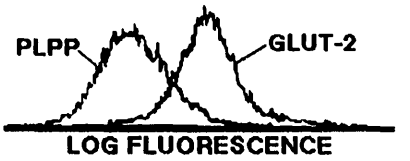

Fig. 3. Comparison of histograms of NBD-glucosamine (NBD-G) uptake in control/pLPP and GLUT-2 transfectants. The ordinate represents cell number. $1\cdot 10^4$ cells were analyzed for each sample. Cells were incubated in 30 μM NBD-G for 20 min at 37°C/5% CO_2 and fluorescence due to NBD-G uptake was measured in control/pLPP and GLUT-2 transfectants. Propidium iodide staining was used to exclude dead cells. Autofluorescence was insignificant in control/pLPP and GLUT-2 transfectants.

Fig. 4. Inhibition of NBD-G uptake by phloretin, an inhibitor of Na^+-independent hexose uptake. Conditions are identical to those in Fig. 3 except cells were incubated with 100 μM phloretin for 15 min prior to and during dye uptake. Inhibition of NBD-G uptake in control/pLPP (A) and GLUT-2 (B) transfectants by phloretin.

sodium independent glucose transport, inhibited NBD-G uptake in both control/pLPP (Fig. 4A) and GLUT-2 (Fig. 4B) transfectants. Thus GLUT-2 transfectants exhibited enhanced uptake of NBD-glucosamine via a phloretin-inhibitable pathway.

3-O-Methyl-D[^{3}H]glucose (^{3}H-3-OMG) uptake. To confirm that the assays developed on the FACS reflected true functional differences between GLUT-2 and control/pLPP transfectants, we performed independent measurements of hexose uptake. The relative initial velocity of ^{3}H-3-OMG uptake was 1.9-fold greater in GLUT-2 transfectants compared with control/pLPP (49.5 ± 5.1 nmol/mg protein vs. 26.6 ± 7.1 nmol/mg protein, $P < 0.05$, $n = 3$). This finding is in agreement with Fig. 3 which shows increased uptake of NBD-G, the fluorescent hexose analogue, by GLUT-2 transfectants compared with control/pLPP.

Cell volume changes in hypertonic media

Forward-angle light scatter (FALS). Light scatter has been used successfully to discriminate cell populations on the basis of different cell properties. Under certain well-defined conditions FALS correlates with cell size. However, other properties of the cell clearly influence FALS [16]. To demonstrate that light scatter was related to cell volume in this experimental system, we compared video microscopy measurements of cell volume with light scatter in the presence of 450 mM urea. Since cells have a relatively high urea permeability, in hypertonic urea the cells should shrink initially but return rapidly to control (isotonic) volume as urea enters the cell. Fig. 5 shows that the time course of change in light scatter parallels changes in cell volume, with a maximum decrease in cell volume occurring by 5 min and the subsequent recovery to near control cell volume in isotonic medium by 30 min. The magnitude of change in FALS and cell volume at 5 min (30% and 35%, respectively) and the degree of recovery (to 86% and 94% of control cell volume in isotonic medium) were also comparable. Thus there is a correlation between cell volume and FALS.

Next, FALS was used to characterize differences in glucose permeability following exposure of transfected cells to either isotonic or hypertonic medium. As shown in Fig. 6, exposure of control/pLPP transfectants to hypertonic glucose medium (i.e., supplemented with 450 mM glucose) caused a significant increase in FALS, indicative of cell shrinkage. GLUT-2 transfectants also exhibited a significant increase in FALS when exposed to hypertonic glucose medium (not shown). Figs. 7A–C are representative histograms comparing the FALS of control/pLPP and GLUT-2 transfectants exposed to various media. In isotonic medium (Fig. 7A) there was no significant difference in FALS of the two transfectants. With exposure to medium supplemented with

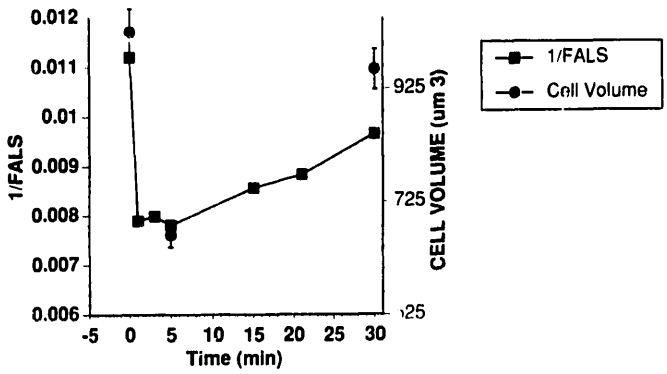

Fig. 5. Time course of 1/linear FALS (closed squares) and cell volume (μm^3) measured by video microscopy (closed circles) in control/pLPP transfectants exposed to 450 mM urea ($n = 3$). Identical results were obtained with GLUT-2 cells (data not shown).

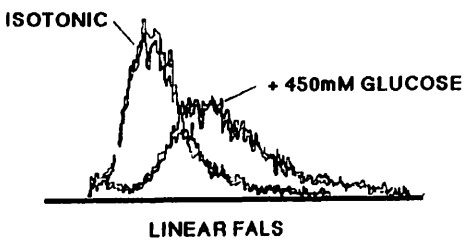

Fig. 6. Comparison of histograms of linear forward angle light scatter (FALS) in control/pLPP transfectants in isotonic vs. hypertonic medium. The ordinate represents cell number. Cells were incubated at 37°C/5% CO_2 for 2 h in isotonic RPMI or RPMI supplemented with 450 mM glucose. For each sample $1 \cdot 10^4$ cells were analyzed for linear FALS. Propidium iodide staining was used to exclude dead cells.

450 mM sorbitol, a relatively impermeant solute, control/pLPP and GLUT-2 transfectants displayed an identical increase in FALS indicative of cell shrinkage (Fig. 7B). However, in the presence of 450 mM glucose for 2 h, these two cell populations could be reliably distinguished on the basis of linear FALS. Fig. 7C shows that the FALS distribution for GLUT-2 transfected cells is shifted to the left compared to the control/pLPP cells. Quantitation of these data was performed by comparing the means of the FALS distribution between control/pLPP and GLUT-2 transfectants for each of the three experimental conditions. In isotonic medium the difference in the mean FALS was 1.5 ± 1.2% (n.s., $n = 5$). When exposed to 450 mM sorbitol the difference in the means FALS was 0.5 ± 2.0% (n.s., $n = 4$). With exposure to 450 mM glucose the difference in mean FALS of 13.1 ± 2.1% was significant ($P < 0.001$, $n = 5$). Thus, the GLUT-2 transfectants appeared less shrunken in 450 mM glucose as would be expected if they possessed a greater permeability to glucose.

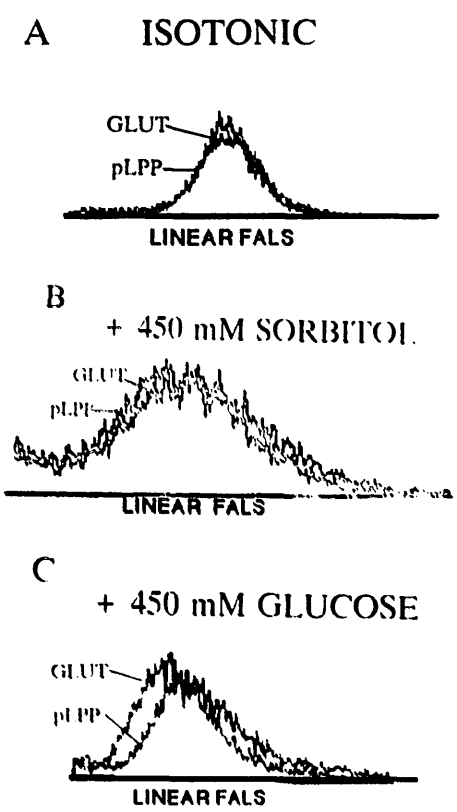

Fig. 7. Comparison of histograms of linear FALS in control/pLPP versus GLUT-2 transfectants. Experimental conditions were exactly as outlined in the legend to Fig. 6. Control/pLPP vs. GLUT-2 transfectants in isotonic medium (A, difference in mean FALS = 0.2%), in medium supplemented with 450 mM sorbitol (B, difference in mean FALS = 2.0%) and in medium supplemented with 450 mM glucose (C, difference in mean FALS = 13.4%). Note that the x-axes in panels A, B and C are not aligned and therefore cannot be compared directly. FALS is shifted to the right under the hypertonic conditions (B and C) compared with the isotonic condition (A) (see Fig. 6.).

TABLE I

Cell volume measured by video microscopy

Condition	Control/ pLPP	GLUT-2
450 mM sorbitol ($n = 4$)	56 ± 1.8%	55 ± 3.9%
450 mM glucose ($n = 4$)	50 ± 4.9%	66 ± 3.8% *

* $P < 0.05$ compared with Control/pLPP.

Cell volume measurement by video microscopy. To confirm that the light scatter measurements demonstrated in Fig. 7 correlated with cell volume changes, we measured cell volume in hypertonic medium supplemented with 450 mM glucose or sorbitol. Control/pLPP and GLUT-2 transfectants were incubated in isotonic or hypertonic medium for 2 h and imaged by video microscopy. The results shown in Table I are in agreement with the FALS data in Fig. 7. In the presence of 450 mM glucose GLUT-2 transfectants were at 66% of their volume in isotonic medium, whereas control/pLPP transfectants were significantly smaller at 50% of their isotonic volume. To confirm that this effect was specific for glucose, cells were incubated in 450 mM sorbitol. Under these conditions the sizes of GLUT-2 and control/pLPP transfectants were not significantly different (Table I).

Viability

After flow cytometric analysis of either NBD-G uptake or FALS the top and bottom 5–10% of each cell population were sorted on the FACS and grown for greater than five passages indicating that these conditions were not toxic to cells and can therefore be used to sort and expand a population of cells.

Discussion

We obtained long-term expression of GLUT-2 cDNA in a human B lymphocyte cell line, BJAB, using an EBV expression vector, oLPP. Expression was confirmed both by demonstration of GLUT-2 mRNA and protein and by functional criteria. The apparent molecular mass (63 kDa) of GLUT-2 in transfected B lymphocytes was identical to that of liver, a tissue which has native GLUT-2 expression. This suggests that GLUT-2 protein expressed in transfected B cells underwent very similar or identical post-translational modifications (e.g., glycosylation) to that in the native tissue of expression (liver). Several assays of glucose transport confirmed that a mature, functional protein was expressed in the transfected B cells. Uptake of NBD-glucosamine or ^{3}H-3-OMG was greater in GLUT-2 transfectants than control/pLPP. Furthermore, video microscopy and FALS indicated that GLUT-2 transfectants shrank less in hypertonic glu-

cose confirming increased permeability of these cells to glucose.

A method was devised to rapidly and efficiently select B cells expressing GLUT-2 using flow cytometry. Two functional assays of glucose transport using flow cytometry were developed: (1) uptake of NBD-glucosamine, a fluorescent analogue of glucose and (2) changes in linear forward-angle light scatter (FALS) in hypertonic glucose. Both of these functional parameters assayed by flow cytometry were confirmed by independent video microscopy and isotopic uptake measurements.

Fluorescence due to NBD-glucosamine (NBD-G) was greater in GLUT-2 transfectants than in control/pLPP cells indicating increased hexose uptake. NBD-G has previously been used to study hexose transport. Speizer et al. [13] showed that NBD-G uptake into RBC ghosts is inhibited by D-glucose and cytochalasin B but not by L-glucose. The influx of NBD-G was found to be slower than that of D-glucose probably due to decreased affinity of NBD-G for the external site of the transporter. We found that NBD-G uptake was inhibited by phloretin. This supports the finding of Speizer et al. [13] that NBD-G enters cells via the glucose permease pathway. The native glucose transporter in B lymphocytes is the phloretin-inhibitable GLUT-1 permease. For this reason, phloretin also reduced NBD-G uptake in the control/pLPP transfectants. However, the phloretin-inhibitable uptake of NBD-G was greater in GLUT-2 transfected cells than in control transfectants. Increased 3-OMG uptake confirmed increased glucose permease activity in GLUT-2 transfectants. Whether GLUT-1 and GLUT-2 have different affinities for NBD-G was not determined in this study. However, these findings suggest that this fluorescent hexose analogue can be valuable for the study of facilitated glucose transport.

In the presence of 450 mM glucose both GLUT-2 and control/pLPP transfectants demonstrated increased linear forward-angle light scatter (FALS). However, the histogram for GLUT-2 cells remained closer to the isotonic histogram than control/pLPP cells. As with the NBD-G uptake data, this suggests increased permeability of GLUT-2 cells to glucose.

FALS is generally believed to be a good measure of cell size [14–16]. In this study we observed an inverse correlation between cell size and FALS. In the presence of 450 mM urea the magnitude and time course of change in linear FALS strongly correlated with independent measurements of cell volume by video microscopy. We found, however, that FALS increased with decreasing cell volume (Fig. 6), whereas FALS is generally believed to increase with increasing cell volume [14,15,21,22]. However, a direct correlation between cell size and FALS is only valid under narrowly defined conditions. Many cell properties can influence FALS, including but not limited to, the internal structure of the cell, its biochemical composition and its surface-to-volume ratio [16]. For example, Grogan et al. [23] showed that neutrophils have much higher FALS than larger monocytic cells probably due to the granular inclusions and segmented nuclei of neutrophils. There are many other instances in which FALS does not vary directly with cell size [16].

In hypertonic glucose, GLUT-2 transfectants showed a FALS distribution closer to that in isotonic conditions than did control/pLPP transfectants (Fig. 7C). This suggests that the enhanced ability of the GLUT-2 cells to take up glucose led to lesser morphological and/or biochemical changes that affect FALS. Since control/pLPP and GLUT-2 transfectants have identical FALS in isotonic medium (Fig. 7A) and hypertonic medium supplemented with sorbitol (Fig. 7B), this difference in FALS in hypertonic glucose is not likely to be due to some other effect of the transfection or an artifact of the assay. Rather, increased glucose permeability seems responsible for the measured difference in FALS in hypertonic glucose.

Under all circumstances studied, we observed an inverse relationship between FALS and volume measured by video microscopy but the exact relationship between FALS and cell volume is not clear. The relationship was observed in hypertonic glucose, urea, and sorbitol. This suggests that, under these conditions, FALS is determined by one or more cell properties that closely correlates with changes in cell size. We hypothesize that changes in cell granularity, ruffling of the cell membrane (which was observed by video imaging in cells exposed to hypertonic medium) and/or changes in surface-to-volume ratio, all of which may be a consequence of cell volume changes, are responsible for the differences in FALS we measured. The rapidity of the changes in FALS and the observed biphasic response to hypertonic urea makes biochemical and/or metabolic effects related to increased glucose uptake less likely.

The changes in light scatter that occur with alterations in cell size depend on the angle at which scattered light is collected [24]. The flow cytometer used in this study collects light scatter at angles of 1–19° and this setup for FALS is typical of many commercially available machines. FALS would be expected to increase directly with increasing cell size only at much narrower angles of light scatter (3–5°) [25]. Thus the direction of change in FALS observed in this study could be due in part to the angle at which scattered light was collected. Since external factors, such as the angle of scatter and composition of the medium, influence FALS, the use of independent measures to confirm the validity of this assay were needed. Though not performed in this study, measurement of cell volume can also be performed with a coulter counter. Previous

studies have shown that a coulter counter, when coupled to a flow cytometer, can be used to analyze and sort cells according to size [26]. Such an approach could potentially provide greater sensitivity than FALS in detecting cell volume changes.

The GLUT family of facilitative transporters promote equilibration of the extracellular and intracellular glucose concentration. Thus, at steady-state, cells with differing rates of glucose permease-mediated glucose transport will contain similar intracellular levels of glucose or its analogues. For this reason, it is critically important to select a pre-steady-state time point for analysis of glucose uptake by the cells. In the present study, NBD-glucosamine uptake was measured at 20 min whereas FALS in the presence of 450 mM glucose was measured at 2 h. Pilot experiments using flow cytometry, isotope fluxes, and video microscopy indicated that these time-points were optimal for distinguishing differences in glucose uptake. Studies of other cells or transporters will thus require similar attention to identifying optimal conditions for distinguishing differences in transport.

The method described here has several advantages for the expression of cDNA encoding transport proteins. Most important among these is the relatively long-term expression of cDNA. This enables the isolation of transfected clonal B cell lines. Repeated sorts on the FACS can be used to isolate clones with high or low expression and/or different inhibitor sensitivity yielding important information about structure-function relationships. These transport assays could also be used to determine the effects of site-directed mutagenesis of GLUT-2 on its function or to isolate functional clones following random mutagenesis. To study regulation, two dyes could be used simultaneously to relate glucose transport to another important cell function, for example intracellular pH or $[Ca^{2+}]$, for which fluorescent probes are available. In addition to the study of regulation and structure-function relationships, Margolskee et al. outlined a cloning strategy using this expression system which takes advantage of flow cytometry to select and amplify clones of interest [9].

Although we have used B cells as the recipient cell line, many other cell lines are permissive for this vector [27]. This will facilitate selection of a cell line with low native expression of the function of interest. Since many of the recipient cell lines are mammalian, processing of the cDNA transcript and post-translational modifications are likely to be accurate, increasing the likelihood of functional expression.

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References

1 Thorens, B., Sarkar, H.K., Kaback, H.R. and Lodish, H.F. (1988) Cell 55, 281–290.
2 Thorens, B., Cheng, Z.-Q., Brown, D. and Lodish, H.F. (1990) Am. J. Physiol. (Cell Physiol.) 259, C279–C285.
3 Johnson, J.H., Ogawa, A., Chen, L., Orci, L., Newgard, C., Alam, T. and Unger, R.H. (1990) Science 250, 546–549.
4 Unger, R.H. (1991) Science 251, 1200–1205.
5 Bell, G.I., Kayano, T., Buse, J.B., Burant, C.F., Takeda, J., Lin, D., Fukumoto, H. and Seino, S. (1990) Diabetes Care 13, 198–208.
6 Thorens, B., Charron, M. and Lodish, H.F. (1990) Diabetes Care 13, 209–218.
7 Kasanicki, M.A. and Pilch, P.F. (1990) Diabetes Care 13, 219–227.
8 Sigel, E. (1990) J. Membr. Biol. 117, 201–221.
9 Margolskee, R.F., Kavathas, P. and Berg, P. (1988) Mol. Cell. Biol. 8, 2837–2847.
10 Heller, R.A., Song, K., Villaret, D., Margolskee, R., Dunne, J., Hayakawa, H. and Ringold, G.M. (1990) J. Biol. Chem. 265, 5708–5717.
11 Heller, R.A., Song, K., Onasch, M.A., Fischer, W.H., Chang, D. and Ringold, G.M. (1990) Proc. Natl. Acad. Sci. USA 87, 6151–6155.
12 Eidelman, O. and Cabantchik, Z.I. (1989) Biochim. Biophys. Acta 988, 319–334.
13 Speizer, L., Haugland, R. and Kutchai, H. (1985) Biochim. Biophys. Acta 815, 75–84.
14 Fischbarg, J., Kuang, K., Hirsch, J., Lecuona, S., Rogozinski, L., Silverstein, S. and Loike, J. (1989) Proc. Natl. Acad. Sci. USA 86, 8397–8401.
15 Fuhrmann, G.F. (1989) Methods Enzymol. 173, 263–280.
16 Grogan, W.M. and Collins, J.M. (1990) Guide to Flow Cytometry Methods, pp. 86–122, Marcel Dekker, New York.
17 Canfield, V., Emanuel, J.R., Spickofsky, N., Levenson, R. and Margolskee, R.F. (1990) Mol. Cell. Biol. 10, 1367–1372.
18 Chirgwin, J.M., Przybyal, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
19 Laemmli, U.K. (1970) Nature 277, 680–685.
20 Hebert, S.C. (1986) Am. J. Physiol. (Cell Physiol.) 250, C907–919.
21 Grinstein, S. Fuyura, W. and Cragoe, E.J., Jr. (1986) J. Cell Physiol. 128, 33–40.
22 Visser, J.W.M., Van den Engh, G.J. and Van Bekkum, D.W. (1980) Blood Cells 6, 391–407.
23 Grogan, W.M., Haar, J.L., Scott, R.B. and Collins, J.M. (1980) Blood CElls 6, 625–644.
24 Latimer, P. and Pyle, B.E. (1972) Biophys. J. 12, 764–773.
25 Mullaney, P.F., Crowell, J.M., Salzman, G.C., Martin, J.C., Hiebert, R.D. and Goad, C.A. (1976) J. Histochem. Cytochem. 24, 298–304.
26 Lauf, P.K. and Valet, G. (1983) J. Cell. Physiol. 116, 35–44.
27 Yates, J.L., Warren, N. and Sudgen, B. (1985) Nature 313, 812–815.