Compartmentalization of transport and phosphorylation of glucose in a hepatoma cell line

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The first steps of glucose metabolism are carried out by members of the families of GLUTs (glucose transporters) and HKs (hexokinases). Previous experiments using the inhibitor of glucose transport, CB (cytochalasin B), revealed that compartmentalization of GLUTs and HKs is a major factor in the control of glucose uptake in L6 myotubes [Whitesell, Ardehali, Printz, Beechem, Knobel, Piston, Granner, Van Der Meer, Perriott and May (2003) *Biochem. J.* **370**, 47–56]. In the present paper, we evaluate compartmentalization of GLUTs and HKs in a hepatoma cell line, H4IIE, which is characterized by excess GLUT activity, HKI in a particulate and a cytosolic fraction, and insignificant G6Pase (glucose-6-phosphatase) activity. The measured activity of glucose transport exceeded the rate of phosphorylation approx. 30-fold. Treatment with 25 μ M CB ($K_i \sim 3 \mu$ M in H4IIE cells) paradoxically increased the excess of GLUTs over phosphoryl-

ation (GLUTs are inhibited 80 %, while phosphorylation is inhibited 98 %). The global relationships of the data could be reconciled most simply by a two-compartment model. In this model, phosphorylation of glucose is carried out by a subset of HK molecules supplied by a subset of GLUTs that are more sensitive to CB than the other GLUTs. The agent, DCC (dicyclohexylcarbodi-imide) caused HKI to translocate from the particulate compartment to the cytosolic compartment and potentially inhibited glucose phosphorylation. The particulate compartment may represent the mitochondria, to which the more CB-sensitive GLUTs may control the transport of glucose.

Key words: dicyclohexylcarbodi-imide, flux control, global analysis, hexokinase, hexose transport, sensitivity analysis.

INTRODUCTION

Glucose phosphorylation results from the action of GLUTs (glucose transporters) and HKs (hexokinases). Certain of the 12 known members of the GLUT family and HKs (isoforms I–IV) are present in tissue-specific combinations, where their unique kinetic properties contribute to specialized functions [1,2]. GLUTs are thought to be rate-limiting in muscle because levels of intracellular glucose are very low [3]. However, increased HK activity in muscle augments glucose disposal, suggesting that control of glucose uptake in the cell is also shared by its HK [4,5]. Glycogen synthase exerts a major control over glucose uptake in muscle [6] by a downstream effect to lower G6P (glucose 6-phosphate), a potent feedback inhibitor of HKI and HKII. Phosphorylation may be affected further by compartmentalization of HK activity within the cell. HKI and HKII are bound to mitochondria in cells through their N-terminal domains. Some of the HKI and HKII in cells have a truncated N-terminal domain, and these proteins are not bound. HKIII is free in the cytoplasm [7,8], while HKIV is predominantly a cytosolic enzyme, with the exception of a small, but important, component bound to mitochondria [9]. HKI attached to mitochondria is thought to be more active than that in the cytosol because of preferred access to ATP [10,11].

Even where there is an excess of transport activity, the glucose concentration of a small cellular compartment can still limit phosphorylation, as we found in previous work with L6 myotubes [12]. In that work, we studied CB (cytochalasin B) inhibition of

glucose, deoxyglucose and methylglucose uptake to measure the intracellular activity of HKs. Our results suggested that transport limited intracellular glucose concentrations, but only to a part of the cell. When the control of glucose usage is dependent on such compartmentalization of HKs and GLUTs, a derangement of this organization might be extremely important in metabolic diseases such as cancer and diabetes.

In an effort to learn more about the physical nature of compartmentalization in a relatively simple system, we studied H4IIE hepatoma cells, since, unlike muscle, intracellular glucose in hepatocytes is in equilibrium with the extracellular milieu [13]. We evaluated the effects of CB on transport and phosphorylation of glucose and deoxyglucose, an analogue of glucose that has high affinity for GLUTs. It has a 10-fold lower affinity for HK, so there is a proportionately greater excess of GLUTs facilitating uptake of this tracer. A set of data combining specificity for glucose compared with deoxyglucose and perturbation of transport by CB was analysed globally using Globals Unlimited software (written by J. M. Beechem, E. Gratton and W. W. Mantulin, University of Illinois, Urbana, IL, U.S.A.) [14]. The data suggest a model in which only a fraction of the cells' HKI and GLUTs participate in transporting and phosphorylating glucose.

We also show that HK binding is a potential factor in control of glucose uptake. The agent DCC (dicyclohexylcarbodi-imide) has been shown to prevent binding of HKI to mitochondria [7]. We found that DCC has the effect of reducing glucose phosphorylation without lowering ATP, while promoting the dissociation of

Abbreviations used: CB, cytochalasin B; DCC, dicyclohexylcarbodi-imide; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; G_i , intracellular glucose; G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase; GLUT, glucose transporter; HBSS, Hanks balanced salt solution; HK I–IV, hexokinase isoform I–IV; K_i , non-competitive inhibition coefficient.

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bound HK, assumed to represent the mitochondrial compartment in intact H4IIE cells. Thus the mitochondria may define a compartment for glucose that is isolated from the rest of the cytoplasm, providing an important link between subcellular structures and metabolic control.

EXPERIMENTAL

Cell culture and preparation

A rat hepatoma cell line, H4IIE (passages 12–15), was grown to confluence in 48-well plates (5×10^5 cells/well) using AMEM (alpha-modified Eagle's minimum essential medium) containing 10% (v/v) foetal bovine serum prepared by the Vanderbilt Diabetes Center Tissue Culture Core Facility. In some experiments, cells were grown to confluence in six or 96-well plates.

Assays of hexose transport, phosphorylation and ATP

Phosphorylation by the coupled activities of GLUTs and HKs in intact cells on 48-well plates was estimated from $^3\text{H}_2\text{O}$ that was produced by detritiation of $[2\text{-}^3\text{H}]\text{glucose}$, as in previous work [12]. The substrates included along with unlabelled glucose in the incubations were $[2\text{-}^3\text{H}]\text{glucose}$ (0.05 μCi) and either 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$ (0.0025 μCi) or 3- $O\text{-}[^{14}\text{C}]\text{methylglucose}$ (0.01 μCi), in combinations for double-label experiments depending on the experimental protocol. H4IIE cells incubated with 3- $O\text{-}[^{14}\text{C}]\text{methylglucose}$ accumulated no significant metabolites (determined from an absence of anionic c.p.m., results not shown). When $[2\text{-}^3\text{H}]\text{glucose}$ is phosphorylated, the exchange reaction of hexose phosphates by phosphoglucose isomerase releases a variable quantity of the ^3H to $^3\text{H}_2\text{O}$ recoverable in the medium [15]. In L6 myotubes, we found this release to be complete [12]. In liver, the method is not practical, because 20–80% of phosphorylated $[2\text{-}^3\text{H}]\text{glucose}$ does not release ^3H to water [16]. To validate the technique in H4IIE cells, the cells and medium were fixed in 70% propan-2-ol and applied to anion-exchange resin. The anionic and propan-2-ol-insoluble metabolites containing ^3H that were found in the incubation medium and the cell residue comprised only 2% of the total uptake in comparison with the $^3\text{H}_2\text{O}$ that was released and assayed as described below. We conclude that total glucose metabolized is adequately represented by the release of $^3\text{H}_2\text{O}$ from $[2\text{-}^3\text{H}]\text{glucose}$ in H4IIE cells. In the case of 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$, metabolism is represented by accumulated phosphorylated label [17]. This was validated by experiments on the fate of phosphorylated deoxyglucose as discussed below.

The phosphorylated and unphosphorylated hexoses in extracts of cells were separated using anion exchange as described previously [12]. The levels of free hexoses in cells were used to estimate rates of transport and intracellular spaces. The anionic ^{14}C radioactivity after subtraction of a blank (a parallel incubation with 100 mM unlabelled glucose to saturate phosphorylation) represented the corrected phosphorylation of deoxyglucose tracer.

G6Pase (glucose-6-phosphatase) in H4IIE cells and dog liver extracts (used for a positive control and provided by Dr Owen McGinnis, Department of Molecular Physiology and Biophysics, Vanderbilt University) was quantified from the rate of phosphate release from G6P measured by a kit (Biomol Green; Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.). The reaction was linear with phosphate standards up to 2 nmol. Cells or frozen tissues were extracted with approx. 10 vol. of buffer [20 mM Hepes, pH 7.4, 40 mM KCl, 2 mM MgCl_2 , 2.7 mM EDTA and 2.7 mM DTT (dithiothreitol)]. A serial dilution of this extract was incubated with a range of G6P concentrations and incubated for 1 h in a total volume of 50 μl in a clear 96-well

plate. To stop the reaction, 100 μl of the reagent (Biomol Green) was added, and the plate was developed for 30 min and absorbance was read at 630 nm. The data were normalized to double-stranded DNA concentration that was measured with a kit (Fluo-Reporter Blue; Molecular Probes, Eugene, OR, U.S.A.), according to the manufacturer's instructions.

Transport turnover was directly measured as efflux of 3- $O\text{-}[^{14}\text{C}]\text{methylglucose}$ corrected for non-specific flux using L- $[^3\text{H}]\text{glucose}$. Wells of a 96-well plate containing cells were rinsed three times with 36 μl of Hepes-buffered HBSS (Hanks balanced salt solution) and incubated for 30 min at 37°C. Then the medium was replaced with 36 μl of HBSS containing 5 μCi of L- $[^3\text{H}]\text{glucose}$ and 0.5 μCi of 3- $O\text{-}[^{14}\text{C}]\text{methylglucose}$, and incubated another 10 min before adding inhibitors in 4 μl aliquots. To assay efflux, the medium from eight wells was transferred to a 96-well scintillation plate using a multi-channel pipette. Efflux was started with a 100 μl aliquot of HBSS added from a second pipette, then this medium was transferred to the second section of the scintillation plate using the first pipette. This cycle was repeated at approx. 10 s intervals for the duration of efflux. The last section of the scintillation plate was reserved for the cell residue, lysed with 50 μl of 0.2 M NaOH, and transferred with two 50 μl rinses of HBSS. This sample was analysed for protein as well as radioactivity. The scintillation plate was placed in a 50°C convection oven to dry overnight, then 20 μl of water was added to each well before adding 80 μl of scintillant (Microscint 40; PerkinElmer Life Sciences, Boston, MA, U.S.A.). The plate was shaken for 5 min (Jitterbug shaker; Bloekel Scientific, Philadelphia, PA, U.S.A.), then 100 μl of scintillant was added, and the plate was shaken again. The plate was sealed with top-sealing tape and counted (Topcount, PerkinElmer) set for double-label discrimination. Counts in preset low- and high-energy windows were used to evaluate c.p.m. from ^3H and ^{14}C by a calculation based on the proportion of c.p.m. in each window from standards of unmixed isotopes. The corrected c.p.m. for each isotope were summed from each sampling time to the end of the assay and divided by the summed c.p.m. over the entire row of samples. This value, giving the total hexose present in the system with time, was corrected for extracellular hexose by subtraction of the value for L-glucose to give transporter-specific intracellular 3- $O\text{-methylglucose}$ efflux as a function of time.

Phosphorylation of 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[2\text{-}^3\text{H}]\text{glucose}$ was measured in other experiments. A 96-well plate with cells was rinsed three times with 100 μl of DMEM (Dulbecco's modified Eagle's medium) followed by the addition of 100 μl of DMEM containing the isotopes plus unlabelled glucose and agents, as indicated. A previously described high-throughput 96-well-plate method [18] was used to measure the production of $^3\text{H}_2\text{O}$.

After removal of medium to measure production of $^3\text{H}_2\text{O}$, the cells were fixed with 50 μl of propan-2-ol and dried for measurement of phosphorylated 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$ by anion exchange. A 100 μl volume of a 2% slurry by dry mass of DEAE or DE52 cellulose (Whatman BioScience) was applied to each well of a 96 well filter (Unifilter GFC; PerkinElmer). Cellular radioactivity was extracted by two 50 μl aliquots of water that were applied to the filters with a gentle vacuum applied after each addition, using the Multifilter (Millipore) system. Then a high vacuum was applied, and the filters were rinsed from the top with water, removed from the vacuum, and rinsed thoroughly underneath. This process was repeated twice, after which the underside of the filter was blotted dry. After the plate was dried by standing overnight at room temperature (22°C) or for 1 h in a convection oven at 50°C, a bottom-sealing tape was applied and the filter wells were wetted with 50 μl of scintillation fluid (Microscint 20; PerkinElmer). Top-sealing tape was applied, the

radioactivity was measured (Topcount), and the c.p.m. from ^3H and ^{14}C were calculated as described above. Control experiments established that phosphorylated 2-deoxy-[1- ^{14}C]glucose was extracted successfully and trapped on the filters. Non-phosphorylated radioactivity was not trapped, except approx. 0.1 % of the total, which was corrected for by using a blank sample containing cells, isotope and 100 mM unlabelled glucose.

ATP was measured in parallel wells using a kit (ATP-lite; PerkinElmer) according to the manufacturer's instructions.

Measurement of HK activity in cell extracts

Cell extracts for assay of HK activity were prepared by removing the incubation medium from cells grown to confluence in six-well plates, rinsing with two aliquots of 2 ml of ice-cold PBS, and adding 0.5 ml of ice-cold extraction buffer. The buffer consisted of 250 mM sucrose, 10 mM Tris/HCl, 5 mM MgSO_4 , 2 mM NaF, 0.4 mM EDTA, 1 mM DTT, 10 mM glucose and one protease inhibitor tablet (Roche, Indianapolis, IN, U.S.A.) per 10 ml of extraction buffer. The contents of the plates were suspended with a cell scraper and transferred to a 1.5 ml centrifuge tube, with another 0.5 ml of extraction buffer used to rinse the residue. This was centrifuged for 30 min at 18 000 g at 4 °C, and the supernatant fraction was removed and used for assay of cytosolic enzymes. It was determined that disruption of cells was complete before the first centrifugation, because the cytosolic enzyme, lactate dehydrogenase, was present only in the first supernatant fraction (results not shown). In another test, 2-deoxy-[1- ^{14}C]glucose that was loaded in the cells before scraping was released completely to the medium and was not concentrated in the pellet. The pellet was suspended in 0.5 ml of the extraction buffer, plus 0.5 % (v/v) Triton X-100, and centrifuged again (particulate HK fraction). For assay of HK activity, 10 μl of each fraction was transferred to a black 96-well plate (Costar, Corning, Acton, MA, U.S.A.) and 200 μl of HK assay buffer was added [50 mM Tris/HCl, pH 7.5, 75 mM KCl, 15 mM MgCl_2 , 0.4 % sodium azide, 2 mM DTT, 2 mM ATP, 0.2 mM NADP (Roche), 0.05 m-unit/ml G6P dehydrogenase (Sigma-Aldrich, St. Louis MO, U.S.A.) and 0.1 mg/ml BSA (Sigma fraction V)], yielding a final glucose concentration of 0.5 mM. In some cases, 10 mM glucose was added to each well to test for saturation. Fluorescence was read with excitation at 340 nm and emission at 460 nm in a plate reader (Polarstar, BMG Labtechnologies, Offenburg, Germany). Fluorescence was calibrated using 10 nmol of NADP that reacted completely with an excess of G6P. A radioisotopic assay was used to compare the ability of HK in extracts to phosphorylate deoxyglucose and glucose, as described previously [19].

The HK activities present in cell and tissue extracts were separated by anion exchange over a 1 ml column (HiTrap Q; Amersham Biosciences) using a two-piston pump column controller (BioLogic; Bio-Rad, Hercules, CA, U.S.A.). Cell or tissue extract containing approx. 300 μg of protein was diluted 4:1 in running buffer (10 mM Tris/HCl, pH 7.5, 10 mM glucose, 5 mM MgSO_4 and 2 mM DTT), and eluted with a gradient up to 400 mM KCl. Samples of 0.2 ml each were collected in black 96-well plates, and tested for activity by adding a 5 $\times$ concentrated reagent for the assay of HK activity by fluorescence as described above.

RT-PCR (reverse transcription PCR) was used to detect the expression of mRNAs for HKI, HKII and cyclophilin. H4IIE cells were extracted for mRNA using a kit (Rneasy; Qiagen Sciences, MD, U.S.A.). cDNA was made against the mRNA using another kit (RevTaq; Applied Biosystems). PCR (30 cycles) was performed on the cDNA using three sets of primers and a blank. Primers to rat HKI were 5'-GCTTTGTCCACGTGATCAGT-AC-3' (reverse) and 5'-GACACCCAGAGAACATCGTGCA-3'

Figure 1 Pre-steady-state measurements of tracer uptakes

H4IIE cells in 48-well plates were tested for hexose transport activity as described in the Experimental section. The assay was conducted at room temperature with 0.1 mM glucose in 100 μl of experimental medium that contained two tracers, 2-deoxy[1- ^{14}C]glucose (□); [2- ^3H]glucose (■). The unphosphorylated tracers were isolated from the cell extracts as described in the Experimental section, and the effects of the transport inhibitor, CB, were measured. Control experiments are shown in (A) and cells treated with 3 μM CB are shown in (B). Bars represent the S.D. from duplicate samples from a typical experiment.

(forward). Presence of rat HKII was tested with primers 5'-GTA-GATCTGGTTCTCCATCTC-3' (reverse) and 5'-ACCTTTGTG-AGGTCAACTCC-3' (forward). Rat Cyclophilin was detected using the primers 5'-AGTTGTCCACAGTCGGAGATGG-3' (reverse) and 5'-AAGGTGAAAGAAGGCATGAGCA-3' (forward).

Protein measurements of cell extracts were performed using a kit (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard.

Calculations

Pre- and steady-state uptake of glucose, deoxyglucose and methylglucose tracers were expressed as the ratio of the intracellular to the extracellular concentration (cell/medium ratio), and kinetic parameters were calculated globally from the data using methods and models outlined previously [12] and a commercial software package, Globals Unlimited [14].

RESULTS

Measurement of tracer uptakes in H4IIE cells

After separation of the phosphorylated hexoses, values for the cell/medium ratios for [2- ^3H]glucose and 2-deoxy-[1- ^{14}C]glucose were measured at various times (Figures 1A and 1B). Similar initial rates of uptake were seen for both substrates. Their cell/medium ratio reached nearly the same value as that reached by methylglucose, which was not phosphorylated (methylglucose

tracer was pre-equilibrated, and is represented by the value of 1.0 in the Figures). The cell/medium ratio of the phosphorylated tracers would be reduced to a value < 1 if GLUTs were rate-determining for glucose phosphorylation. To make it more likely for GLUTs to be rate-limiting, we added $3 \mu\text{M}$ CB (results shown in Figure 1B). With the inhibitor present, and at the low concentration of glucose (0.1 mM) in the assay, we expected that GLUTs would be rate-limiting, and that the cell/medium ratios of glucose and deoxyglucose would be reduced compared with the methylglucose tracer. At this concentration, CB inhibited the shortest uptakes of both tracers by approx. 50%. If GLUTs had appreciable rate control, cell/medium ratios for inhibited tracer uptakes should have been lower after 5 min. The fact that inhibited uptakes were similar to uninhibited uptakes suggests that GLUTs do not limit the concentration of glucose in H4IIE cells.

HK identities and activities in cells

The anion-exchange profile for HK activity in the particulate extract of H4IIE cells shows two peaks (Figure 2A). Reverse transcription of the mRNA produced cDNAs that could be amplified by PCR using primers for rat HKI, but not for rat HKII (Figure 2B). The ability of cell extracts to phosphorylate $[\text{U-}^{14}\text{C}]$ glucose and 2-deoxy- $[\text{3H}]$ glucose was tested as described in the Experimental section (Figure 2C). Fitting the data mathematically (lines) established the K_c (competitive inhibition coefficient) of glucose as a competitive inhibitor of both tracers (0.18 and 0.12 mM respectively). The kinetics of phosphorylation ruled out significant contributions from glucokinase (high K_m activity) or HKIII (substrate-inhibited activity), and shows that HK in the extracts phosphorylated the deoxyglucose tracer at a much lower rate than the glucose tracer.

Steady-state phosphorylation of glucose and deoxyglucose in intact cells

CB and extracellular glucose altered the uptake of glucose and deoxyglucose tracers, allowing us to evaluate the reactions of GLUTs and HK separately. As the glucose concentration is increased, the transport and phosphorylation of the tracers are competitively inhibited (Figure 3A). With increasing concentrations of CB, glucose tracer phosphorylation is reduced. Phosphorylation of deoxyglucose is not significantly reduced even when GLUTs are inhibited by $10 \mu\text{M}$ CB (Figure 3B). In fact, there is a significant increase in its uptake at intermediate levels of transport inhibition. This phenomenon was predicted from the interaction of GLUTs and HK as shown in Figure 3.

G6Pase activity in cell extracts and intact cells

Accumulation of phosphorylated deoxyglucose does not reflect glucose uptake in liver, because of the high activity of G6Pase. This possibility was also evaluated in H4IIE cells. G6Pase is highly expressed in liver, and its mRNA is expressed in H4IIE cells [20]. However, G6Pase activity in extracts of H4IIE cells was found to be at most 2% of the activity found in a liver sample (dog) that was used as a positive control (Figure 4A). The efflux of accumulated radiolabelled deoxyglucose was used to estimate the possible impact of G6Pase on rates of hexose uptake (Figures 4B and 4C). The total of the radiolabelled deoxyglucose of cells declined by 10% per h after an initial rapid phase of efflux that was complete within 5 min (Figure 4B). The starting content of deoxyglucose in the cells was highest when the concentration of glucose in the pre-loading medium was the lowest, but the rate of decline of deoxyglucose was noted at all concentrations. To determine if this decline was the result of G6Pase activity,

Figure 2 HK activity of particulate cell extracts

(A) Particulate cell extracts were prepared from H4IIE cells, separated by anion exchange, and assayed for HK activity as described in the Experimental section. The Figure is representative of three to five similar experiments. (B) Expression of HKs in H4IIE cells was tested using primers as described in the Experimental section. DNA markers were present to demonstrate that the products had the expected molecular mass (sizes given in kDa). Expression of HKII was shown in a positive control performed with the same primers on a rat intestinal epithelial cell line (not shown). (C) Cell extracts were assayed for their ability to phosphorylate the tracers $[\text{U-}^{14}\text{C}]$ glucose (■) and 2-deoxy- $[\text{3H}]$ glucose (□) with unlabelled glucose varied from 0.01 to 3 mM . The data are from an experiment representative of three similar ones. The solid lines through the data were generated from the coefficients of the global fit to the data discussed in the text for a two-compartment model, while the broken line was generated from the HK activity that appears to be kinetically confined in the cell (see the text description of Figure 5).

we separated out the phosphorylated deoxyglucose present in the cells at each time using anion-exchange filters (Figure 4C). In this measurement, no significant trend of radiolabel loss with time was noted. The slow loss of label seen in Figure 4(B) therefore consisted of deoxyglucose that was accumulated, but not phosphorylated. A similar phenomenon of concentration and efflux of non-phosphorylated deoxyglucose has been noted in other cells [21]. We concluded from these experiments that G6Pase activity would not affect measurements of glucose or deoxyglucose phosphorylation in H4IIE cells.

Figure 3 Clearance of hexose tracers as functions of unlabelled glucose and CB

(A) Medium samples from cells incubated for 30 min with $[2\text{-}^3\text{H}]\text{glucose}$ and 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$ were analysed for $^3\text{H}_2\text{O}$ resulting from metabolism of $[2\text{-}^3\text{H}]\text{glucose}$ with the range of unlabelled glucose concentrations shown. Lines through the data represent fits that were globally optimized for the complete data set with the coefficients presented discussed below. Four conditions are represented: $\blacksquare$ and — , no inhibitor; $\blacktriangle$ and $-\text{--}$, $3\text{ }\mu\text{M}$ CB; $\blacktriangledown$ and $\cdots$, $10\text{ }\mu\text{M}$ CB; $\blacklozenge$ and $-\text{--}$, $30\text{ }\mu\text{M}$ CB. (B) Cells were incubated with 10 mM glucose and the series of CB concentrations indicated as in (A), but extracted for 2-deoxy- $[1\text{-}^{14}\text{C}]\text{G6P}$. Open bars represent the observed clearances ($\mu\text{l}/\text{mg}$ of protein per h) $\pm$ S.D. ($n=3$). Hatched bars were generated from the coefficients of Figure 5.

Analysis of glucose uptake with coefficients for a two-compartment model

In theory, the results shown in Figure 1 contain all the information necessary to derive the control coefficients for GLUTs and HK, since the equilibrium space for free hexose should be smaller than the water space of the cell as a function of the degree that GLUTs limit the access of glucose to HK. CB inhibited phosphorylation, with an apparent K_i (non-competitive inhibition constant) of $3\text{ }\mu\text{M}$, at low concentrations of glucose (Figure 3A). The initial rate of glucose transport was inhibited by CB with roughly the same K_i , but without the reduction in the equilibrium space for free hexose (Figure 1) expected to result from limitation of phosphorylation by inhibition of GLUTs. To determine if the experiments shown in Figures 1 and 3 are compatible with this model, we combined the data sets, and tested a model for coupled activities of transport and phosphorylation of glucose using a multidimensional analysis program called Globals Unlimited [12,14]. A two-compartment model gave a good fit to the data. The coupled reactions revealed their kinetic parameters by glucose competition, and inhibition by CB (Figure 3). Comparisons of the rates of phosphorylation of $[2\text{-}^3\text{H}]\text{glucose}$ and 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$ were also part of the analysis, since the substrate specificities of the component reactions are reflected in the coupled reactions. The ratio of activity for deoxyglucose/glucose tracer used in the global model was approx. 1.2 for GLUTs (derived from

Figure 4 G6Pase activity in H4IIE cells and its effect on deoxyglucose phosphate recovery

(A) Extracts prepared from H4IIE cells and a positive control (from frozen dog liver tissue) were tested for G6Pase activity normalized to DNA as described in the Experimental section. (B) A 96-well plate of H4IIE cells was incubated for 30 min in serum-free DMEM, with the glucose concentrations indicated, 0.1 mg/ml BSA and $0.025\text{ }\mu\text{Ci/well}$ of 2-deoxy- $[1\text{-}^{14}\text{C}]\text{glucose}$. The medium was then aspirated from the wells and replaced with non-radioactive medium three times during 5 min. At the times after rinsing shown in the Figure, the medium was aspirated a final time, and the cells were fixed with $50\text{ }\mu\text{l}$ of propan-2-ol. The cell residue was dried and the radioactivity was extracted with $40\text{ }\mu\text{l}$ of water. After removal of an aliquot of $20\text{ }\mu\text{l}$, radioactivity in the plate was assayed as described in the Experimental section and normalized for the volume sampled. Results are expressed in μl (c.p.m. in the sample divided by the c.p.m. in $0.5\text{ }\mu\text{l}$ of medium)/mg of cellular protein. (C) The $20\text{ }\mu\text{l}$ aliquots from (B) were applied to a filter plate prepared for anion exchange that was washed and counted as described in the Experimental section. Results are expressed in μl (c.p.m. in the sample divided by the c.p.m. in $0.5\text{ }\mu\text{l}$ of medium)/mg of cellular protein.

Figure 1), while for HK it was 0.1 (from Figure 2). The kinetic parameters for GLUTs and HK activity were determined from the global fitting of the data of Figure 3. The uncertainties of the parameters based on the original data are shown graphically in Figure 5. Figure 5 shows the global χ^2 , a measure of the quality of the fit of the model to the data set, for each parameter as tested over a range of possible values around the optimum. The critical χ^2 was calculated to be 1.04, above which the values had less than

Figure 5 Confidence analysis of the parameters

The curves give the global χ^2 associated with each of the parameters of a model characterized by reversible transport and irreversible phosphorylation presented previously [12]. The units of the parameters are mM glucose for K_t (K_m for 0-*trans* transport) and B_g (K_m for equilibrium exchange transport of glucose), and nmol of glucose/mg of protein per h for V_t (V_{max} of GLUTs), and K_p and V_p (HK K_m and V_{max} respectively). 'HK space' is expressed as a cell/medium ratio. The value of each parameter that best fits the data has the lowest χ^2 . The error testing provided by the program Globals Unlimited generates a range of hypothetical data to show the value of the parameter $\pm$ S.D. The normalized χ^2 rises as the parameter value deviates further from the fit to the data, and the point where the lines cross the horizontal broken line represents the χ^2 of data with two S.D.

67 % confidence, with the limit shown in Figure 5 by a horizontal broken line. In other words, attempts to fit the data which resulted in global χ^2 values higher than 1.04 were rejected, since they were more than two S.D. away from the global mean. In the model, the fraction 0.85 of the cell volume has GLUTs, but no HK activity, while the fraction comprising the 'HK space', 0.15, has both GLUTs and active HK. The mean value for this parameter was between the probable limits of 0.09 and 0.2, or in equivalent terms, 0.15 ± 0.06 S.D. Values for K_t (0-*trans* K_m) and B_g (equilibrium exchange K_m) of GLUTs were not distinguished from one another, having overlapping values at the critical χ^2 . The K_m for HK, K_p , and the V_{max} of the total HK activity, V_p , and the V_{max} of GLUTs, V_t , were more sharply defined by the analysis of the data, their mean values being indicated on the axis under the lowest point of each curve.

Compartmentalization of HK activity

As the concentration of non-radioactive glucose approaches 0, the clearance of the tracer in the coupled reaction of transport and phosphorylation extrapolates to the value of V_{max}/K_m for the transport reaction, which should be limiting at such low substrate concentrations. Both the compounds DCC and CB, at 25 μ M, suppress this value by over 90 % (Figure 6A). Clearances by GLUTs were measured directly in parallel incubations using the radioactive glucose analogues L-[3 H]glucose and 1 mM 3-*O*-[14 C]methylglucose in an efflux experiment that also defines V_{max}/K_m for the transport reaction (Figure 6B). Clearance by GLUTs was clearly less susceptible to CB than phosphorylation, and not at all to DCC.

Summarized curve-fitting parameters (Table 1) show that CB inhibits the flux by GLUTs by approx. 80 %, while it inhibits the phosphorylation flux by more than 95 %. The K_m of the coupled reaction that leads to phosphorylation of extracellular glucose is approx. 0.4 mM, and approx. 4 mM in the presence of CB. The lower value is intermediate between the K_m values of GLUTs and

HK, reflecting their shared control of phosphorylation, while the higher K_m reflects uptake limited by GLUTs in the presence of CB. Still, clearance by GLUTs, even when inhibited by CB, is > 100 -fold in excess of the clearance by phosphorylation. These data suggest that there is a 'HK space' supplied by an independent set of GLUTs that are more susceptible to CB than the GLUTs that are responsible for entry of tracers into the larger space.

Some evidence suggests that the physical location of the HK space may be defined by the mitochondrial compartment. Treatment of cells by DCC led to a concentration-dependent translocation of HK from a particulate fraction, assumed to represent the mitochondrial compartment, to a cytosolic location (Figure 6C). Treatment with this agent did not inhibit GLUTs activity (Figure 6B) in H4IIE cells, and similar concentrations of DCC added to lysed cells did not inhibit HK activity directly (results not shown), but it did inhibit glucose phosphorylation in intact cells (Figure 6A). Since phosphorylation activity, but not transport activity, was inhibited, the fold excess of GLUTs over HKs rose to more than 100 after treatment with 25 μ M DCC (Table 1). Inhibition of glucose phosphorylation by DCC did not significantly lower ATP levels (Figure 6D). Glucose metabolism is not critical for ATP, possibly because an alternate substrate (glutamine) was present in the incubation medium.

DISCUSSION

In liver and hepatoma cells, there is a large excess of GLUT activity over HK activity, so that control of glucose usage rests with HK [13]. A potential mode of HK control is its binding to the mitochondria [11]. Our data show that, despite the large excess of GLUT activity in H4IIE cells, there is a tight coupling between GLUTs and HKs, because a small subset of GLUTs and HKs are kinetically confined in a compartment, probably defined by the vicinity of the mitochondria. This compartmentalization is an important aspect of the metabolic control potential of GLUTs, as well as HKs, that should be systematically studied.

Flux control coefficients are used in metabolic control analysis to predict the potential sensitivity of metabolic pathways to altered levels of individual effectors or enzymes. The sensitivity of glucose usage to a minute change in GLUT or HK activity for H4IIE cells is shown in the flux control coefficients illustrated in Figure 7. The summation theorem of Kacser and Burns [22] accounts for the fact that, for the pathway defined by GLUTs and HKs, the control coefficient for HK is high when that for GLUTs is low, and vice versa, so that the two always add up to 1. At low glucose concentrations, the control coefficient for the GLUTs is significantly above zero, because GLUTs are partially rate-determining. At slightly higher concentrations of glucose, HK becomes rate-determining, and the control exerted by GLUTs drops to zero. The flux control exerted by GLUTs and HK over deoxyglucose uptake is shown in Figure 8. Because HK and not GLUTs show a preference for glucose tracer over deoxyglucose tracer, inhibiting GLUTs and not HKs is expected to have a greater effect in lowering the concentration of glucose than deoxyglucose. This would lead to decreased levels of unlabelled glucose available to compete against deoxyglucose phosphorylation. Consistent with this hypothesis, the control coefficient for transport of deoxyglucose is negative at higher glucose concentrations, predicting that the transport inhibitor, CB, would increase deoxyglucose phosphorylation slightly, as is seen in Figure 3(B). The observation that deoxyglucose and glucose reach similar cell/medium ratios in CB-inhibited cells (Figure 1B), creates a problem for the hypothesis, however. The problem is resolved by the postulation of a second compartment.

Figure 6 Effects of DCC and CB on glucose phosphorylation, transport, HK translocation and intracellular ATP concentration

(A) Clearance of [2-³H]glucose against glucose concentration. H4IIE cells were incubated in 96-well plates in glucose-free medium for 30 min and treated for another 30 min before the assay with 25 μM DCC (◆) or 25 μM CB (■) [(added from a 2.5 mM concentration in DMSO, with an equivalent concentration of DMSO added to control incubations (▲)]. Then glucose and tracers were added, and phosphorylation of [2-³H]glucose was measured by the high-throughput methods discussed in the Experimental section. (B) Parallel incubations were tested for efflux of 3-*O*-methyl-[¹⁴C]glucose corrected for non-specific efflux using L-[³H]glucose in a double-label washout experiment as discussed in the Experimental section. Symbols as in (A). (C) HK activity was measured in cell extracts after incubating for 60 min with 1 mM glucose and the concentrations of DCC indicated. Extracts were prepared and assayed as described in the

Table 1 Effects of DCC and CB on the parameters of glucose transport and phosphorylation

The fitting parameters of the curves of Figure 6 are shown for the purpose of comparing the activities of glucose transport, which was measured dynamically, and phosphorylation, which was measured at steady-state.

	Agents		
	DMSO vehicle	25 μM DCC	25 μM CB
Phosphorylation parameters*			
V_{max}/K_m ($\mu\text{l}/\text{mg}$ per h)	300 $\pm$ 90	34 $\pm$ 18	6 $\pm$ 0.6
Apparent K_m (mM)	0.16 $\pm$ 0.1	0.41 $\pm$ 0.2	3.81 $\pm$ 1
Transport parameters†			
Turnover (h^{-1})	229 $\pm$ 72	199 $\pm$ 48	50 $\pm$ 6
Intracellular vol. ($\mu\text{l}/\text{mg}$)	44 $\pm$ 18	44 $\pm$ 20	39 $\pm$ 12
Flux ($\mu\text{l}/\text{mg}$ per h)	10000 $\pm$ 3660	8760 $\pm$ 3060	1920 $\pm$ 480
Fold excess transport activity	34	260	336

* The data from three experiments similar to that shown in Figure 6(A) were summarized to show S.E.M. for the parameters K_m and V_{max}/K_m that describe the curves. The units of V_{max}/K_m are $\mu\text{l}/\text{mg}$ per h, which is the activity of the (transport-limited) coupled reaction as glucose approaches zero.

† The data from three transport experiments similar to that shown in Figure 6(B) were summarized to show S.E.M. for the fitting parameters h^{-1} and $\mu\text{l}/\text{mg}$ that describe the curves shown according to the equation $\mu\text{l}/\text{mg}$ per h = $\mu\text{l}/\text{mg}$ ($e^{-(s-1)} \cdot 3600$).

Figure 7 Sensitivity of glucose phosphorylation to activity of GLUTs and HK

The effect of a minuscule change in GLUTs or HK V_{max} to cause a corresponding fractional change in glucose phosphorylation (flux control coefficient) was calculated for a range of glucose concentrations.

The coefficients illustrated in Figure 4 include a factor for 'HK space'. This factor is necessary to account for the cell/medium ratios of glucose shown in Figure 1 that were incompatible with the data presented in Figure 3. Figure 9 represents a replot of the data shown in Figure 1, with the transport predictions that are compatible with the kinetically confined HK compartment shown as a broken line. Predictions from the model having an additional compartment with levels of each hexose up to the extracellular concentration are shown by the solid lines. This model, using the coefficients shown in Figure 4, produced the optimum agreement between the three types of evidence presented: pre-steady state

Experimental section. Sum represents the total of the supernatant and the pellet extracts and is shown to illustrate that the treatments did not result in changes in the HK content of the cells. Results are means $\pm$ S.D. for duplicate determinations. (D) ATP was measured in parallel incubations using the ATP-lite kit (PerkinElmer) according to the manufacturer's instructions.

Figure 8 Sensitivity of deoxyglucose phosphorylation

The same calculations as in Figure 7 were performed for 2-deoxy- ^{14}C glucose phosphorylation.

Figure 9 Two-compartment versus one-compartment fitting of the data of Figure 1

The results are as shown in Figure 1 with (A) representing the control and (B) representing CB-treated cells. The broken lines represent predictions compatible with global fitting only of the data of Figure 3. The solid lines are the predicted accumulations (in the HK space) of each hexose tracer (■, glucose tracer; □, deoxyglucose tracer) from a global fitting that incorporated all the data of Figures 1–3 with a two-compartment model.

hexose uptake (Figure 1), steady-state phosphorylation of glucose (Figure 3) and the various levels of substrate preference observed between glucose and deoxyglucose, shown in Figures 1, 2 and 3.

The low K_m HKs are thought to be more active when bound to mitochondria [7]. The distribution of HK between the cytosol and the mitochondria is a potential control point for glucose phosphorylation and compartmentalization in H4IIE cells. Cells treated with DCC show a concentration-dependent reduction of HK activity in the particulate fraction and increased HK activity in the cytosolic fraction. The bound fraction of HK whose dis-

placement affects glucose phosphorylation is assumed to represent the mitochondrial compartment.

Some cancerous cells may be able to resist apoptosis because a greater than normal proportion of HKII is bound to the mitochondria [23]. The drug clotrimazole has also been used as an agent to displace the enzymes of glucose metabolism [24] in cancerous tissues, in order to study the importance of their subcellular location to apoptosis. However, the cytotoxicity of clotrimazole may be dependent on its effect in lowering ATP [25]. The binding of HKI to mitochondria is looser than that of HKII, but it also prevents apoptosis [26]. A subtype of HKI that is peculiar to transformed cells has been identified by phenyl-Sepharose chromatography [27]. The first peak that we see in the particulate fraction of HK in H4IIE cells after resolution of HK activities by anion-exchange chromatography might be such a subtype, or it might be bound to a molecule lowering its pI, the basis for separation by anion exchange. Like HKII, its binding may be particularly effective in preventing apoptosis, and the agent that is able to displace it, DCC, may therefore be of some therapeutic interest.

DCC and oligomycin inhibit the same ATPase of mitochondria, and can lower ATP in hepatocytes [28], although not in H4IIE cells under the conditions used in the present study, perhaps because of the availability of glutamine. The effect of DCC to prevent HK binding is independent of its ATPase inhibition, and occurs through a specific interaction with porin [29,30]. In the present study, DCC was found to displace two subtypes of bound HKI coincidentally with its inhibition of glucose phosphorylation, suggesting that glucose is phosphorylated only near a particulate fraction. The glucose compartment that is accessible to HK through special GLUTs would reside in the same location, assumed to represent the mitochondrial compartment. A physiological consequence of such compartmentalization of HK and GLUTs is that cells are 'transparent to glucose', so that glucose can diffuse through cells to neighbouring cells without saturating the HK of the first cell. Breakdown of compartmentalization may account for increased intrinsic activity of GLUTs in certain types of metabolic stimulation [31]. GLUTs may be involved in the regulation of glycolysis in the presence of oxygen, so that a breakdown in the compartmentalization of cells may lead to the 'Warburg effect', or excessive glycolysis in spite of the availability of oxygen that occurs in cancers [23] that is part of their 'selfish' phenotype. If such a selfish phenotype is part of cancerous cells' ability to resist apoptosis, DCC or related compounds that affect compartmentalization of HKs may aid cancer therapies.

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