Transport and accumulation of 2-deoxy-D-glucose in wild-type and hexokinase-deficient cultured Chinese-hamster ovary (CHO) cells

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Hexokinase-deficient mutants and wild-type Chinese-hamster ovary cells have been used to investigate the role of hexokinase in uptake and accumulation of 2-D-deoxyglucose (2-dGlc). The evidence for a specific sugar transport system in both types of cells is that there is similar saturable phloretin-sensitive uptake of 2-dGlc and 3-O-methyl-D-glucose (3-OMG) in both types of cell. In wild-type cells, 2-dGlc is accumulated to a tissue:medium ratio of 10- and in the mutant only 3-fold; 3-OMG is not accumulated by either mutant or wild-type cells. The evidence that hexokinase affects the membrane transport process is that the rate of exit of free 2-dGlc from wild-type cells is 5-fold less than from mutant cells, whereas there is no difference in the rate of loss of 3-OMG between mutant and wild-type cells.

INTRODUCTION

Glycolysis-deficient mutants from Chinese hamster cells have been used to investigate the role of glycolytic enzymes in the uptake and metabolism of sugars (Morgan & Faik, 1981; Pouyssegur *et al.*, 1980; Faik & Morgan, 1984; Ullrey *et al.*, 1982) and the role of insulin receptors and insulin-dependent kinase reactions in control of metabolism (Chou *et al.*, 1987). One such mutant, designated 'R1.1.7', is deficient in two enzymes of glycolysis: glucose phosphate isomerase (GPI) and phosphoglycerate kinase (PGK) (Morgan & Faik, 1981).

As a consequence of these defects, R1.1.7 cells, unlike the parental (CHO-K1) cells, do not grow in media containing mannose as sole carbon source (Morgan & Faik, 1977) and mannose is, in fact, toxic (Faik & Morgan, 1977b).

Revertants of R1.1.7 now able to grow in mannose media and resistant to mannose toxicity have been isolated. Such revertants are now also deficient in hexokinase.

Here we have used a hexokinase-deficient mutant of Chinese-hamster ovary (CHO) cells, M + R 42 (which is also deficient in GPI and PGK), to investigate the role of hexokinase in the control of 2-D-deoxyglucose (2-dGlc) transport and accumulation within Chinese-hamster ovary cells.

In the preceding paper (Naftalin & Rist, 1989), it was shown that: (a) 2-dGlc transport and accumulation are coupled to hexokinase activity in rat thymocytes; (b) stimulation of thymocyte activation and accumulation of 2-dGlc transport by phorbol 12-myristate 13-acetate is accompanied by tighter coupling of transport to hexokinase activity. The main evidence in favour of this view is that: (i) there is a phorbol-stimulated steady-state accumulation of free 2-dGlc within the cytosol of thymocytes to a concentration 25–40-fold above the external concentration of 0.1 mM; (ii) net exit of 2-dGlc from phorbol-activated cells after a period of accumulation of 2-dGlc is slower than from control cells;

(iii) the high-affinity inhibitor of hexokinase, mannoheptulose, inhibits uptake of 2-dGlc in both control and activated cells; (iv) phorbol has no effect on exit of 3-O-methyl D-glucoside (3-OMG), despite the fact that this non-metabolized sugar interacts with the 2-dGlc transporter.

These data are consistent with a model for active transport of sugar where hexokinase activity at the cytosolic surface of the transporter transforms the transported 2-dGlc to impermeant hexose phosphate, thereby reducing sugar reflux and creating an energy-dependent rectifier for sugar movement across the membrane (Naftalin & Smith, 1987).

The availability of a hexose-deficient CHO mutant is a useful and unambiguous way of demonstrating linkage between hexokinase activity and transport. It is a prerequisite that the hexokinase-deficient mutant cells must have a fully competent sugar transporter. In the present study we used wild-type and mutant CHO cells to demonstrate that: (a) there is a phloretin-sensitive transport system for 2-dGlc and 3-OMG in both wild-type and mutant cells of approximately the same activity; (b) hexokinase activity is deficient in the mutant cells; (c) free 2-dGlc is accumulated to a tissue:medium ratio of approx. 10 in wild-type cells, but only by 1–2 in mutants; (d) exit of 2-dGlc is much slower from wild-type cells than from mutants; and (e) no difference in the rate of 3-OMG exit is seen between mutant and wild-type cells.

The use of 2-dGlc, which is not a substrate for Na⁺-dependent co-transport (Bihler *et al.*, 1962), precludes any possibility that this latter system might obscure the interpretation of 2-dGlc accumulation. Furthermore the absence of any accumulation of 3-OMG by CHO cells indicates that Na⁺ co-transport is absent from the system.

METHODS

Cell culture

CHO-K1 (wild-type) cells were grown in Ham's F12

Abbreviations used: GPI, glucose phosphate isomerase; PGK, phosphoglycerate kinase; 2-dGlc, 2-D-deoxyglucose; 3-OMG, 3-O-methyl-D-glucose; CHO, Chinese-hamster ovary.

medium containing 5 mM-glucose and supplemented with 8% (v/v) foetal-bovine serum as described previously (Faik & Morgan, 1977a). The hexokinase-deficient cells (M+R 42), which are also deficient in GPI and PGK activity, were grown in the same medium, except that pyruvate and glutamine concentrations were increased to 2 mM.

Transport studies

Cells were plated out at approx. 2×10^4 cells per 35 mm-diameter dish containing 1.5 ml of glucose-containing medium. After 24 h the medium was changed to fresh medium. After a further 24 h, duplicate plates were trypsin-treated and the cell numbers determined by using a Coulter counter as described previously (Faik & Morgan, 1977a). The cell numbers were usually between 5×10^5 and 7×10^5 cells/dish. The variation between plates in cell numbers was low, usually $< 5\%$. Before uptake measurements, the cell monolayers were washed twice with warmed (37 °C) saline G (Kao & Puck, 1974) and 1.0 ml of warm (37 °C) modified Ham's F12 (Ham's without glucose) supplemented with the macromolecular components of foetal-bovine serum at 10% (FCM10) (Faik & Morgan, 1977a). The plates were incubated at 37 °C for approx. 15 min before modified F12 medium containing the radioactive sugar was added.

Time courses of uptake were measured over periods varying from 2 min to 3 h. Uptake of sugars into the cells was measured by using ^3H -labelled 2-deoxy-D-[1- ^3H]-glucose (Amersham International; initial sp. radioactivity 11.5 Ci/mmol at a final isotope concentration of 0.2 $\mu\text{Ci/ml}$) or ^{14}C -labelled 3-O-[U- ^{14}C]methylglucose (also from Amersham International; initial sp. radioactivity 74.2 mCi/mmol at a final isotope concentration of 0.05 $\mu\text{Ci/ml}$).

After incubation, sugar uptakes were halted by removing the labelled incubation solution from the plates and replacing with ice-cold stopping solution. The plates were washed twice more in 2 ml of stopping solution to remove adherent isotope. The composition of stopping solution was iso-osmotic Hepes-buffered saline, pH 7.3, containing phloretin (100 μM) and HgCl_2 (1 μM) ('S.soln.'). The cells were then lysed in ice-cold distilled water containing 0.02% Triton X-100. This lysate was used to determine the total uptake, free sugar and hexose phosphate concentrations within the cells. The efficacy of the stopping solution was tested by observing the effect of stopping solution on the initial rate of 2-dGlc uptake. This background was $< 5\%$ of sugar uptake when the cells were incubated for 2 min with 1.0 mM-2-dGlc. Similar effects were observed with 3-OMG uptake (Table 1d).

The efficacy of the ion-exchange-filtration procedure and the extent of degradation of hexose phosphate in the cell lysates from CHO cells was tested, as previously described for thymocytes [the preceding paper (Naftalin & Rist, 1989)]. There was no significant extracellular degradation of hexose phosphates at 0 °C. The proportions of label present in the hexose phosphate spot and 2-dGlc spot on t.l.c. of the freeze-dried extracts from CHO cells were the same as those obtained using ion-exchange filtration.

In the present paper three kinds of transport study are reported, namely: (a) initial rates of uptake, where the initial rate of 2-dGlc uptake and 3-OMG uptake are measured during the initial 2 min exposure; (b) steady-

state uptake, where isotope uptake after exposure for 3–4 h is monitored; (c) the loss of cell-free sugar and hexose phosphate pools after preincubation with radioisotope for 3 h and exposure to an external medium nominally free of radioactivity; additionally, it is demonstrated that (d) the mutant cells have a low hexokinase activity.

Determination of cell total, free sugar and hexose phosphate pool was done by using Whatman DE81 anion-exchange filters as described in the preceding paper (Naftalin & Rist, 1989). No difference in the efficiency of the method was detected with CHO cells compared with rat thymocytes.

Hexokinase assay

The activity of hexokinase in cell supernatants was measured spectrophotometrically, as described previously (Morgan & Faik, 1981). Protein was de-

Table 1. (a) Initial rate of uptake of 2-dGlc, (b) steady-state uptake of 2-dGlc, (3 h uptake; 1 mM-2-dGlc), (c) uptake of 1 mM-3-OMG and (d) effect of stopping solution (S.soln; see the text) on the uptake of 2-dGlc (1 mM) during 2 min incubation

Significance: *** $P < 0.001$; ** $P < 0.01$; ns, not significant.

(a)	Initial rate of uptake of 2-dGlc ($\mu\text{mol}/\text{min}$ per 5×10^8 cells)			
[2-dGlc] (mm)	Wild-type		Mutant	
0.1	0.160 ± 0.01		0.11 ± 0.01 (5) **	
1.0	1.40 ± 0.23		0.92 ± 0.12 (5) ns	
10.0	9.09 ± 1.87		6.38 ± 0.75 (5) ns	
(b)	Steady-state uptake of 2-dGlc ($\mu\text{mol}/5 \times 10^8$ cells)			
	Wild-type		Mutant	
Total uptake	24.44 ± 1.33 (12)		5.34 ± 0.62 (9) ***	
Free sugar	11.72 ± 0.61 (9)		3.31 ± 0.52 (8) ***	
Hexose phosphate	8.2 ± 0.66 (7)		2.16 ± 0.32 (8) ***	
(c)	Uptake of 1 mM 3-OMG ($\mu\text{mol}/5 \times 10^8$ cells)			
Time	Wild-type		Mutant	
2 min	0.84 ± 0.10 (5)		0.83 ± 0.12 (4) ns	
2 h	0.60 ± 0.02 (4)		0.81 ± 0.09 (4) ns	
(d)	Effect of stopping solution on uptake of 2dGlc (1 mM) during 2 min incubation ($\mu\text{mol}/5 \times 10^8$ cells)			
	Wild-type		Mutant	
	Control	S.soln.	Control	S.soln.
	2.00	0.06	1.45	0.06

terminated by the Coomassie Blue method (Bradford, 1976).

RESULTS

Hexokinase activity

The hexokinase activity ($\text{nmole} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) was 28.5 in CHO-K1 cells, 25.8 in R1.1.7 cells and 1.1 in M+R 42 cells, indicating a severe deficiency in hexokinase activity in the mutant cells.

Transport studies

Initial and steady-state uptakes. To determine if there is any difference in specific sugar transport activity between wild-type and mutant cells, the initial rates of sugar uptake were measured over a range of external concentrations. The phloretin-sensitive initial uptakes of 2-dGlc and 3-OMG in wild-type and mutant cells are shown in Table 1. There is no significant difference in 2-dGlc uptake or 3-OMG uptake during the initial 2 min between wild-type and mutant cells (Table 1a). This indicates that the membrane transporter for sugars is functioning normally in both wild-type and mutant cells. However, after 3 h incubation there is a considerable

difference between sugar accumulation in wild-type and mutant cells. Free 2-dGlc is accumulated 10-fold in wild-type cells and only 2–3-fold in mutant cells. There is a corresponding decrease in the amount of hexose phosphate present in the mutant cells (Table 1b). These studies confirm: that (a) in wild-type cells hexokinase and transport work independently as well as in the coupled mode and also indicate that free-sugar accumulation is dependent on the functioning of hexokinase and (b) there is no accumulation of 3-OMG within either the mutant or wild-type cells (Table 1c). The steady-state 3-OMG space is a useful marker of cell water volume.

Efflux. Exit of 2-dGlc from wild-type and mutant is shown in Fig. 1(a). Exit of free 2-dGlc from wild-type cells is approx. 5 times slower than from mutant cells (Fig. 1b). The time course of sugar exit from cells preloaded with 50 mM-3-OMG for 2 h, the cytosol concentration being approx. $50 \mu\text{mol}/5 \times 10^8$ cells, is identical for both mutant and wild-type cells. These results are very similar to those observed for 2-dGlc and 3-OMG exit from rat thymocytes [the preceding paper (Naftalin & Rist, 1989)] and indicate that functioning hexokinase reduces the rate of free sugar exit from the cytosol, as previously predicted by the hypothesis that coupling between hexokinase activity and 2-dGlc transport accelerates net influx of sugar and promotes accumulation of free sugar within the cells (Naftalin & Smith, 1987).

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Fig. 1. Time course (a) of exit of labelled 2-dGlc from wild-type CHO cells (●) and hexokinase-deficient mutants (○) and (b) of percentage loss of exchangeable radioactivity of labelled 2-dGlc from wild-type (●) and mutants (○)

(a) Each point represents the mean $\pm$ S.E.M. for four independent estimates of total sugar within the cells. This is one of three similar experiments. The free sugar is 10.5 ± 0.2 (4) $\mu\text{mol}/5 \times 10^8$ cells at zero time, and at 60 min free sugar is 7.0 ± 0.4 (4) in wild-type cells; in mutant cells at zero time the free sugar is 2.12 ± 0.19 (4) and at 60 min falls to 0.95 ± 0.06 (4) $\mu\text{mol}/5 \times 10^8$ cells. (b) Each symbol is the mean of four independent determinations, the $t_{1/2}$ of 2dGlc loss from mutant cells is 5.0 ± 0.7 min, whereas that from wild-type cells is 25 ± 3.8 min ($P < 0.01$). The $t_{1/2}$ of 3-OMG exit preloaded to 50 mM from wild-type cells (■) is 12.8 ± 1.4 min, and the $t_{1/2}$ from mutant cells is 11.4 ± 1.8 min (□). The non-exchangeable radioactivity is taken to be that remaining after 60 min.

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