Modulation of Glucose Uptake in Animal Cells

STUDIES USING PLASMA MEMBRANE VESICLES ISOLATED FROM NONTRANSFORMED AND SIMIAN VIRUS 40-TRANSFORMED MOUSE FIBROBLAST CULTURES*

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Plasma membrane vesicles isolated from nontransformed and Simian virus 40-transformed mouse fibroblast cultures catalyze carrier-mediated D-glucose transport without detectable metabolic conversion to glucose 6-phosphate. Glucose transport activity was stereospecific, temperature-dependent, sensitive to inactivation by p-chloromercuriphenylsulfonate, and accompanied plasma membrane material during subcellular fractionation. D-Glucose efflux from vesicles was inhibited by phloretin, an inhibitor of glucose uptake in intact cells. Cytochalasin B, a potent inhibitor of glucose uptake when tested with the intact cells used for vesicle isolation did not inhibit glucose transport in vesicles despite the presence of high affinity cytochalasin binding sites in isolated membranes. The enhanced glucose uptake observed in intact cells after viral transformation was not expressed in vesicles: no significant differences in glucose transport specific activity could be detected in vesicle preparations from nontransformed and transformed mouse fibroblast cultures. These findings indicate that cellular components distinct from glucose carriers can mediate changes in glucose uptake in mouse fibroblast cultures in at least two cases: sensitivity to inhibition by cytochalasin B and the enhanced cellular sugar uptake observed after viral transformation.

The capacity for utilization of glucose from the culture medium of animal cells is coordinated and integrated with diverse cellular functions. Considerable increases in rates of glucose uptake are usually apparent in tumor cells (1) and in animal cells after transformation by tumor virus (2-9), compared with their normal counterparts. Increased uptake rates also accompany mitogen stimulation of proliferation of resting nontransformed rodent and avian fibroblast cultures (5, 10-12) and lymphocytes (13). Induction of hexose transport sites in glucose-deprived cultures of chicken fibroblasts has been reported (14).

It has proven difficult to establish either the point of control or molecular basis of increased hexose uptake using intact cells. Specific increases in either the number or activity of functional glucose transport sites in the plasma membrane, rather than an enhanced intracellular sugar phosphorylation (15, 16), have been implicated in the uptake stimulation.

This work was supported by National Cancer Institute Grant CA 05174 and United States Public Health Service Grant GM 25006. This is the 5th paper in the series "Transport Mechanisms in Membrane Vesicles from Mouse Fibroblasts." The previous paper in this series is Ref. 50. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

(Received for publication, August 21, 1978)

RESULTS

Glucose Transport in Membrane Vesicles—Standard criteria indicated that retention of labeled glucose on filters in the presence of membranes resulted from carrier-mediated transport into vesicles rather than binding to fixed sites or nonspecific trapping. When vesicle preparations from Simian virus 40-transformed 3T3 cells were preloaded with high concentrations of unlabeled D-glucose, competitive exchange diffusion (22) could be demonstrated as a stimulation of D-[1-3H]glucose accumulation compared with controls (Fig. 1A). Furthermore, n-glucose uptake measured at low concentrations was time-dependent, stereospecific (Fig. 1B), and showed a temperature dependence such that both influx (Fig. 1C) and efflux (not shown) were stimulated as temperature was increased in the range 2 to 37°C. D-Glucose accumulated at equilibrium in vesicles was released by addition of sucrose to the external medium (Fig. 1D). This can be attributed to osmotic shrinkage rather than alteration of carriers or competitive exchange since sucrose did not affect initial rates of glucose uptake.

Chromatography of accumulated D-[U-14C]glucose extracted from filters revealed no detectable conversion to glucose 6-phosphate under incubation conditions used for transport. Retention of D-glucose by binding to membranes was negligible (<15% of apparent internal volume) as estimated after osmotic lysis in hypotonic solutions.

Glucose transport activity was primarily associated with subcellular fractions enriched in plasma membrane vesicles.

* Portions of this paper (including Figs. 1, 3, and 5, and Tables I, II, and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 72M-1430, cite author(s), and include a check or money order for $1.50 per set of photocopies.
(Table I), providing evidence that glucose transport activity in vesicles is mediated by components of surface membrane.

Labeled D-glucose, 2-deoxyglucose, and 3-O-methyl-D-glucose were retained at equilibrium (15 min incubation) to a value corresponding to the same intravesicular volume of 0.8 to 1.2 µl/mg of protein. Equilibrium retention of 3-O-methyl-glucose was proportional to the amount of vesicle protein.

Glucose transport activity was not affected by addition of 50 mM NaCl or KCl as a gradient across the membrane, nor was uptake stimulated when an interior-negative membrane potential was generated across the membrane as described previously (20), using valinomycin and K⁺ gradients. CaCl₂ at a 1 mM concentration, did not affect glucose transport but uptake was abolished after omission of MgCl₂ or addition of 1 mM EDTA (not shown).

Effect of Phloretin—The identity of glucose carriers in vesicles as components of a cellular glucose uptake system was established using phloretin (22-24), an inhibitor of glucose uptake in intact cells (Fig. 2A). Addition of phloretin to membrane vesicles markedly decreased glucose efflux compared with controls (Fig. 2C). Phloretin had a similar inhibitory effect when tested at either 1 or 0.1 mM. Phloretin caused an apparent stimulation of labeled glucose influx in vesicles (Fig. 2D), probably a reflection of its inhibition of glucose efflux. This effect of phloretin can be interpreted in terms of evidence by Benes et al. (23) that phloretin is nonpenetrating to the erythrocyte membrane and inhibits sugar flux from the face of the membrane trans to the side exposed to this inhibitor. Phlorizin, an inhibitor of glucose transport in intestine and kidney (22), was without effect on sugar uptake in these fibroblast cultures at concentrations up to 50 µM (Fig. 2B); neither phlorizin, 0.5 mM, nor polyphloretin phosphate, 1 mg/ml, affected glucose uptake in membrane vesicles (not shown).

Kinetics of Glucose Transport in Vesicles—D-Glucose transport into vesicles at room temperature showed an apparent Kₘ = 1.4 ± 0.2 mM with Vₘₐₓ = 1.4 nmol of glucose/min/mg (Fig. 3) when glucose concentration was varied from 0.15 to 1 mM. Higher glucose concentrations resulted in appreciable nonspecific leakage when estimated using 1-[³²P]glucose. Uptake of 3-O-methyl-D-glucose (not shown) into vesicles showed an apparent Kₘ = 5 mM, a value also observed for cellular uptake of this nonmetabolizable analog (6). Time courses of glucose uptake shown in Fig. 1 indicate that the filtration assay may permit only an approximation of true initial rates due to the rapid equilibration of glucose in vesicles, even at 21°C.

Inactivation of Glucose Carrier Activity—Glucose transport activity was decreased after treatment of vesicles with µ-chloromercuriphenylsulfonate as similarly observed for deoxyglucose uptake in cells (8) (Table II).

Effect of Cytochalasin B on Glucose Transport Activity—The sensitivity to cytochalasin B of deoxyglucose transport by nontransformed and Simian virus 40-transformed cells differed strikingly from glucose transport in their isolated membrane vesicles. Fig. 4B shows that uptake of deoxyglucose from growth medium by nontransformed and SV40-transformed mouse fibroblast cultures was inhibited by low concentrations of cytochalasin B, as reported previously by several laboratories (25-29). When membrane vesicles derived from these cells were tested using the same batches of cytochalasin B, no inhibition of glucose transport by cytochalasin B concentrations up to 0.1 mM could be detected (Fig. 4A). Similarly, glucose efflux from vesicles was insensitive to these concentrations of cytochalasin B (not shown).

Binding of Labeled Cytochalasin B to Membrane Vesicles—Cytochalasin B bound to high affinity receptors retained on isolated membranes does not block glucose transport in vesicles isolated from either nontransformed or SV40-transformed cells. Retention of 10.4 pmol of cytochalasin B/mg of vesicle protein (after subtraction of blank values and intravesicular trapped label) was observed after incubation of vesicles with 0.13 µM [³²P]cytochalasin B. This retention of labeled cytochalasin B was abolished in the presence of 1 mM unlabeled cytochalasin B. An apparent dissociation constant (Kd) of 0.5 µM could be estimated for binding to membranes (Fig. 4C).

These observations suggest that the inhibitory effect of cytochalasin B on glucose uptake in mouse fibroblasts is mediated by interactions which are uncoupled after vesicle isolation.

Comparative Glucose Transport Activity of Vesicles from Nontransformed and from SV40-transformed Mouse Fibroblasts—Glucose transport activities of vesicles isolated from nontransformed Balb/c and Swiss mouse fibroblasts and those transformed by SV40 are compared in Fig. 5, A and C, and Table III. No significant variation in glucose carrier specific activity was detectable among these vesicle preparations within the limits of the filtration assay.

As an internal control, cell density and transformation-dependent changes in uridine (21) (Table III) and amino acid (20) uptake were expressed in the same preparations when assayed simultaneously. As another control, the rates of aden-
and poorly understood regulatory mechanisms (8). The initial step, the interaction of glucose with its plasma membrane transport carrier, can be assayed using transport-competent plasma membrane vesicles without complications from intracellular metabolism or the use of nonmetabolizable analogs.

By contrast with the brush border membranes of kidney and intestinal cells, which maintain glucose concentration gradients by a Na⁺ symport mechanism, glucose transport in most other cell types is a nonconcentrative, Na⁺-independent facilitated diffusion process (22). The molecular basis of glucose translocation across cell membranes is unknown. Several types of glucose carrier models have been proposed (22, 24) based on studies using erythrocytes.

Cell-free glucose transport activity fulfilled standard criteria for carrier-mediated transport such as countertransport, osmotic sensitivity, and stereospecificity. Furthermore, a specific property of the cellular sugar uptake system, sensitivity to inhibition by phloretin (22), was also expressed in vesiculated surface membranes.

By contrast, it was observed that cytochalasin B, a potent inhibitor of glucose uptake (25–29), cell division, and locomotion (32) in the intact cells used for vesicle isolation could bind with high affinity to membrane vesicles but did not affect their glucose transport properties. These results are consistent with previous indications that cytochalasins do not bind to the sugar transport system of mouse fibroblasts but exert their effects via sites distinct from the transport carrier. Atlas and Lin (33) found high affinity cytochalasin binding sites \( K_D = 10^{-7} \text{ M} \) on normal and transformed Balb/c 3T3 cells which were not susceptible to competitive inhibition by glucose. However, the relationship of fibroblast plasma membrane cytochalasin binding sites to either the transport-related or contractile-related biological activities of cytochalasin B is unclear.

The apparent uncoupling between cytochalasin B binding and glucose transport observed in these studies using fibroblast membrane vesicles can be interpreted in terms of alternate mechanisms proposed to explain the concurrent inhibitory effects of cytochalasins on sugar transport, locomotion, and cell division. The view that cytochalasin B interacts with separate classes of binding sites to mediate independent effects on sugar transport and microfilaments is supported by several observations. Spudich and Lin (34) demonstrated that cytochalasin B interacts with actin and actomyosin. Subsequently,
Lin and Spudich (35) detected a class of high affinity cytochalasin B binding sites in the red blood cell membrane, in which cytochalasin binding was competitively inhibited by sugar substrates of the erythrocyte transport system. These workers proposed that the majority of these high affinity cytochalasin binding sites were associated with a glucose transport component. This assumption was reinforced when Kasahara and Hinkle (36) demonstrated that glucose transport in reconstituted liposomes which had incorporated Band 3 protein of the erythrocyte membrane was inhibited by cytochalasin B. However, high affinity cytochalasin B binding sites unrelated to sugar transport were found in red cells (37) and multiple classes of high affinity cytochalasin B binding sites have been reported on the plasma membrane of fat cells (38). These sites differ strikingly from those of red cells in that cytochalasin binding is not competitively inhibited by glucose (38), suggesting that glucose transport inhibition by cytochalasin in fat cells occurs at a site distinct from the glucose carrier. Cytochalasin B did not inhibit thymidine, α-aminobutyric acid, uridine (27), or phosphate (29) uptake in cells, indicating a rather specific interaction with glucose transport.

An alternate hypothesis to explain the effects of cytochalasin B on membrane transport proposes cytoskeletal control of surface membrane properties. Evidence for cytoplasmic influences on the cell surface has been reviewed by Nicolson (39). Cytoskeleton would exert its effect on membrane transport by disrupting a microfilament system associated with the surface membrane. This model does not require direct interaction of cytochalasin B with the transport carrier. Rather, insertion of microfilaments into the plasma membrane would exert conformational effects on mobility and topography affecting transport secondarily.

Evidence for association of actin with the plasma membrane has been obtained (40, 41). Actin constitutes 2 to 3% of membrane protein in 3T3 cells and this association is not reduced after cytochalasin B treatment (33). Cytochalasin B was shown to randomize intramembraneous plasma membrane particle topography in 3T3 cells (42) and induced microfilament and cell surface redistribution in a variety of cell lines (43). Ukena and Berlin (44) observed effects of cytoskeletal disruption by colchicine and vinblastine on topographical separation of membrane transport sites.

The failure to observe the expected large differences in glucose transport in membrane vesicles between transformed and nontransformed cells was unexpected. Despite an estimated 120% recovery of glucose transport sites calculated using data obtained with confluent nontransformed cells, the increased transport activity associated with transformed cells (6, 8) did not persist in vesicles. By contrast, similar comparisons had demonstrated that cellular differences in amino acid (20) and uridine (21) transport were expressed in isolated membranes and were confirmed in internal controls accompanying these experiments.

The present study indicates that the accelerated cellular uptake of sugars which accompanies viral transformation of mouse fibroblasts (6, 8) was not expressed in vesiculated plasma membranes which do not phosphorylate glucose. This suggests that the primary target of this oncogenic viral regulatory change is not solely associated with glucose carriers in the surface membrane but leaves unanswered the question of how uptake regulation is accomplished.

It may be possible to reconcile these observations using membrane vesicles with apparently contradictory results obtained using other approaches (15, 16). First, the possibility of uptake enhancement at the level of glucose phosphorylation has not been eliminated. Sugar transport may be regulated by rates of glycolysis as suggested by Romano and Colby (19) and in a more recent study of this question (45). In addition, evidence that a significant component of the uptake of 3-O-methylglucose occurs either by simple diffusion or by utilizing a separate carrier system from glucose (17, 18) would suggest that the use of this nonmetabolizable analog is not an appropriate measure of the activity of glucose carriers. Alternatively, the relatively constant glucose transport activity of these vesicle preparations may result from removal, during membrane isolation, of proteins, lipids, or hormones such as insulin, which may be necessary to maintain the activated carrier state by direct interaction with the membrane. Lee and Lipmann (46) have isolated a glucose-binding protein loosely bound to normal and Rous sarcoma virus-transformed chicken fibroblasts which markedly stimulates cellular glucose uptake. Such a dissociable modulator protein may explain transport differences in intact cells after viral transformation.

Other possible mechanisms of glucose transport activity modulation which have been proposed are sulfhydryl oxidation (47) and protein kinase/phosphoprotein phosphatase-mediated modification (48). Observations of effects of ATP on specific permeability properties of vesicles from these fibroblast cell lines (49) and demonstration of a plasma membrane-associated protein kinase (49) are consistent with a role for membrane protein phosphorylation in mediating transport changes in fibroblasts.

Membrane vesicles from mouse fibroblasts have provided a useful system for elucidation of transport mechanisms and their regulation (20, 21, 31, 49, 50). Whereas glucose transport in fibroblast membrane vesicles displays certain specific properties of cellular glucose uptake, important differences occur after cell disruption. Sensitivity of glucose transport in fibroblasts to cytochalasin B and the increased transport activity observed after viral transformation may require cellular organization. Demonstration of these properties in isolated membranes will require further understanding of cytoplasmic influences on the surface membrane.

Acknowledgments—Most of this work was carried out at the Imperial Cancer Research Fund Laboratories, London, England. I thank Mrs. Patricia Pettican for excellent technical assistance and the Cell Production Department for providing cell cultures.

REFERENCES

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Additional references will be found on p. 2966.
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EXPERIMENTAL PROCEDURES

Preparation of cells. Spleen cells from C3H/HeN mice were prepared as described previously (2). Cells were resuspended at 107 cells per ml in Eagle's medium containing 5% fetal calf serum and nonessential amino acids. The cell suspension was incubated for 2 hours at 37°C.

Neutron activation analysis. Spleen cells were incubated with 125I-labeled glucose for 2 hours at 37°C. The total radioactivity was measured in a y-counter. The specific radioactivity was calculated from the counts per minute in the samples and the initial specific activity of the glucose.

Preparation of plasma membranes. Plasma membranes were purified by a modification of the method of Weisiger et al. (3). The cells were disrupted by sonication in 0.01 M Tris-Cl, pH 7.4, containing 1 mM PMSF and 2 mM EDTA. The suspension was centrifuged at 10,000 g for 15 minutes, and the supernatant was centrifuged at 100,000 g for 1 hour. The resulting membrane pellet was resuspended in 0.01 M Tris-Cl, pH 7.4, and the specific activity of glucose uptake was measured.

RESULTS

Fig. 1. Effect of 125I-labeled glucose uptake in spleen cells from untreated animals and from animals treated with 0.05 M sodium fluoride for 30 minutes. The specific activity of uptake is expressed as counts per minute per milligram of protein.

DISCUSSION

The data presented in this study suggest that fluoride may inhibit glucose uptake in spleen cells. The inhibitory effect of fluoride was reversible, and the specific activity of uptake returned to normal after removal of fluoride from the incubation medium. These results are consistent with previous reports indicating that fluoride inhibits glucose uptake in other tissues, including liver and muscle (4).

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. M. Weisiger for his help in the preparation of the manuscript. This work was supported by grants from the National Institutes of Health and the National Science Foundation.

REFERENCES


Table 1. Subcellular Distribution of Glucose Transport Activity

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<th>Fraction</th>
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<tr>
<td>PMN/Angio</td>
<td>9000</td>
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<tr>
<td>Lymphocytes</td>
<td>1000</td>
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<tr>
<td>Brain cortex</td>
<td>200</td>
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<tr>
<td>Kidney cortex</td>
<td>150</td>
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<tr>
<td>Liver</td>
<td>750</td>
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Fig. 2. Effect of 125I-labeled glucose uptake in spleen cells from untreated animals and from animals treated with 0.05 M sodium fluoride for 30 minutes. The specific activity of uptake is expressed as counts per minute per milligram of protein.

Table 2. Comparison of Glucose Transport Activities in Various Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
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<tr>
<td>Spleen</td>
<td>1000</td>
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<tr>
<td>Liver</td>
<td>500</td>
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<td>Brain cortex</td>
<td>250</td>
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<td>Kidney cortex</td>
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Fig. 3. Effect of 125I-labeled glucose uptake in spleen cells from untreated animals and from animals treated with 0.05 M sodium fluoride for 30 minutes. The specific activity of uptake is expressed as counts per minute per milligram of protein.

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Fig. 4. Effect of 125I-labeled glucose uptake in spleen cells from untreated animals and from animals treated with 0.05 M sodium fluoride for 30 minutes. The specific activity of uptake is expressed as counts per minute per milligram of protein.
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Fig. 3. A. In some experiments, inhibition was constant (50 mM) and the effect of varying the concentration of glucose was studied. B. In other experiments, the effect of varying the concentration of glucose on the initial rate of glucose uptake was studied. The results are presented as a graph showing the relationship between glucose concentration and initial rate of glucose uptake for each cell type.

Fig. 4. A. The inhibition of glucose uptake by 50 mM glucose in the presence of increasing concentrations of another substrate, sucrose. B. The effect of varying the concentration of sucrose on the initial rate of glucose uptake in the presence of constant glucose concentration (50 mM).

Fig. 5. Comparison of glucose uptake in cells and in cell-free extracts of various tissues. A. Cells from different tissues were incubated with glucose at various concentrations. B. Cells from the same tissue were incubated with glucose at various concentrations and then assayed for glucose uptake. C. Cells from different tissues were incubated with glucose at various concentrations and then assayed for glucose uptake in cell-free extracts. The results are presented as a graph showing the relationship between glucose concentration and initial rate of glucose uptake for each tissue or extract.
Modulation of glucose uptake in animal cells. Studies using plasma membrane vesicles isolated from nontransformed and simian virus 40-transformed mouse fibroblast cultures.

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