Evidence for a Functional Change in the Plasma Membrane of Murine Sarcoma Virus-infected Mouse Embryo Cells

TRANSPORT AND TRANSPORT-ASSOCIATED PHOSPHORYLATION OF $^{14}$C-2-DEOXY-D-GLUCOSE*

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SUMMARY

Enhancement of sugar uptake occurs in murine sarcoma virus-infected mouse cells at the time of transformation. Direct analysis of the sugar transport and transport-associated phosphorylation system of mouse cells infected with the Harvey strain of MSV was performed with 2-deoxy-D-glucose-$^{14}$C. It was shown that 2-deoxy-D-glucose-$^{14}$C was taken up at various concentrations ($10^{-5}$ to $10^{-6}$ M) at a greater rate and was transported with a lower $K_m$ (21.0 to 23.3 $\times 10^{-4}$ M for uninfected and 3.5 to 5.0 $\times 10^{-4}$ M for infected cells) and higher $V_{max}$ (18.2 to 19.2 nmoles per mg of protein per min at 37° for uninfected and 52.3 to 83.5 nmoles per mg of protein per min for infected cells) in the infected cells. The transported substrate was found mainly as a phosphorylated product, 2-deoxy-D-glucose 6-phosphate. The increased phosphorylation of 2-deoxy-D-glucose-$^{14}$C by the infected cells was obtained only with intact cells, but not with cell homogenates, indicating that this phosphorylation is not due to increased intracellular hexokinase activity but rather based on a membrane-bound transport system. These data support the hypothesis that alterations in a plasma membrane sugar transport (penetration plus phosphorylation) system occurred during cellular transformation by murine sarcoma virus.

EXPERIMENTAL PROCEDURE

The Harvey strain of mouse sarcoma virus obtained from Dr. R. Ting, formerly of the National Cancer Institute, was used throughout. The virus was propagated on secondary cultures of NIH Swiss mouse embryo fibroblasts. Exponentially growing cell cultures in 50-mm diameter plastic Petri dishes were infected with MSV (Harvey) ($4 \times 10^4$ focus-forming units per $4 \times 10^6$ cells). The cells were grown in Eagle’s minimal essential medium containing 10% fetal bovine serum for the first 2 days after infection; then minimal essential medium plus 2% fetal bovine serum was used and the medium was changed every 2 to 3 days. The cell cultures were previously washed five times before assay with 10 ml of Hanks’ solution minus glucose which was previously warmed to the assay temperature. The cultures were then incubated with 2.0 ml of previously warmed 2-deoxy-glucose-$^{14}$C (0.2 $\mu$Ci per ml in Hanks’ solution minus glucose with various concentrations of unlabeled 2-deoxyglucose) at 37° for various periods of time. After incubation, the cultures were washed five times with 10 ml of cold Hanks’ solution minus glucose. The remaining solution was removed under vacuum and the cells were scraped into 0.5 ml of water, transferred to a small tube, and homogenized by vibration with a Vortex mixer for 5 sec. An aliquot (0.2 ml, 200 to 600 $\mu$g of protein per ml) was mixed with 5 ml of Bray’s solution and counted in a Beckman LS250 liquid scintillation system. Protein was assayed by the method of Lowry et al. (12), 2-Deoxy-D-glucose-$^{14}$C (8.2 mCi per mmole) and inulin carboxyl-$^{14}$C (0.05 mCi/32.1 mg/10 ml) were purchased from New England Nuclear. 2-Deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate were obtained from Sigma and purified alkaline phosphatase was from Worthington.
RESULTS

Sugar uptake was linear with time in both control and infected cells over periods ranging from less than 1 min to 20 min at 37°C (Fig. 1), thus permitting comparisons of uptake rates and $K_m$ calculations based on determinations at a given time within this period. The difference shown in Fig. 1 between infected and control cells was obtained with $\mu$M 2-deoxyglucose; however, the rate difference was clearly maintained over a very wide concentration range (Table I). This uptake was not simply due to diffusion of 2-deoxyglucose since conditions could be found in which intracellular concentrations of isotope greatly exceeded extracellular concentrations (Table II). In addition, intracellular isotope was mainly found as 2-deoxyglucose phosphate (see below), indicating a clear difference between infected and control cells in the sum of the related processes of transport and transport-associated phosphorylation. Inulin-$^{14}$C was used as a control for simple diffusion and, as shown in Table II, no differences in rate of penetration were observed with this compound. The $K_m$ of 2-deoxyglucose uptake was determined in the concentration range $10^{-3}$ to $10^{-4}$ M. Lineweaver-Burk plots ($1/V$ against $1/X$) were linear in this concentration range, permitting graphic estimation of $K_m$ and $V_{max}$. Table III shows the marked reduction in the $K_m$ of MSV (Harvey)-infected cells. The values are markedly similar to those reported previously for glucose transport (3).

![Figure 1](http://www.jbc.org/)

**Identification of Product of 2-Deoxyglucose Transport**—Cells were incubated for 15 min at 37°C with $10^{-4}$ M 2-deoxyglucose-$^{14}$C and processed as described above. Cell homogenate, 0.5 ml, and 0.5 ml of cold 10% perchloric acid with 1 drop of phenol red added were mixed and incubated in an ice bath for 15 min. The supernatant solution, after centrifugation at 3000 rpm (IEC, 10 min), together with three supernatant washes was adjusted to $pH$ 8.0 with 50% KOH and the solution was chilled for 30 min. The potassium perchlorate precipitate was then removed by low speed centrifugation in the cold. The final volume was adjusted to 2 ml with 0.01 M Tris buffer, $pH$ 8.0. A 0.2-ml aliquot was incubated with 0.02 ml of alkaline phosphatase or bovine serum albumin (each 1 mg per ml in 0.01 M Tris, $pH$ 8.0) for

**Table I**

| Sugar | Uninfected | Infected |
|-------|------------|----------|
| 2-Deoxyglucose $^{14}$C | 15.6 | 53.2 |
| Inulin-carboxyl | 6.4 | 52.2 |

**Table II**

| Sugar                  | Uninfected | Infected |
|------------------------|------------|----------|
| 2-Deoxyglucose-$^{14}$C | 2.7       | 15.9     |
| Inulin-carboxyl-$^{14}$C | 0.3      | 0.3      |

* Ratio of isotope in packed cells to isotope in equal volume of supernatant solution, after 10-min incubation at 37°C with 0.2 ml of $\mu$M $^{14}$C sugar. Cells were separated from supernatant fluid at 1500 rpm for 10 min in an IEC centrifuge with tubes graduated to 0.001 ml. Then 0.05 ml of cells and supernatant fluid was taken for assay. Cells were mixed with 0.05 ml of Hank's salt solution and the supernatant was mixed with 0.05 ml of nonlabeled packed cells before radioactivity determinations were carried out.

**Table III**

| Cells             | $K_m$ (M) $\times 10^4$ | $V_{max}$ mmol/mg protein/min |
|-------------------|-------------------------|-------------------------------|
| A                 | B                        | A                             | B                             |
| Uninfected        | 21.0                     | 23.3                          | 18.2                          | 19.2                          |
| MSV-infected      | 3.5                      | 5.0                           | 52.3                          | 83.5                          |

* The two values shown in each case were obtained over the concentration range 10 mM to max (A) and max to 0.1 mM (B). Values were obtained from graphic analysis of double reciprocal plots (Lineweaver-Burk) of velocity against 2-deoxyglucose concentration.

**Fig. 1.** Initial entry rates of 2-deoxyglucose-$^{14}$C into control (– – –) and MSV-infected (O—O) cells. Duplicate cultures at 5 days postinfection or postplating were previously washed and incubated with 2 ml of $\mu$M 2-deoxyglucose-$^{14}$C. Samples were harvested for analysis at intervals from 0.25 to 20 min. The protein values of sequential samples did not vary more than 10% and averaged 300 $\mu$g per ml for the control and 630 $\mu$g per ml for MSV (Harvey) infected cells. Aliquots, 0.2 ml, were analyzed and results presented are the average of two plates uncorrected for the slight variations in protein content.
deproteinized, mixed with carrier 2-deoxyglucose and 2-deoxyglucose-6-phosphate, and subjected to paper electrophoresis. The two sugars were separated by 5 cm under the conditions described and 0.5-cm lengths were taken for radioactivity determination. The sum of the activity corresponding to each sugar is recorded in this table and no significant activity was located other than these fractions. An aliquot of each sample was incubated with alkaline phosphatase for 1 hour at 37° before electrophoresis.

### Table IV

**Transport-associated phosphorylation of 2-deoxyglucose**

|                | 2-Deoxyglucose | 2-Deoxyglucose 6-phosphate |
|----------------|----------------|----------------------------|
|                | A | B | A | B |
| **Uninfected** | Background | 240 | 175 | 180 |
| **Uninfected +** | 125 | 262 | 1140 | Background |
| **MSV-infected** | 230 | 330 | Background | 1030 |
| **MSV-infected +** | 1430 | 1070 | Background | 82 |

### Table V

**Phosphorylation of 2-deoxyglucose-14C by intact and disrupted cells**

|                | Disrupted cells | Intact cells |
|----------------|-----------------|--------------|
| **Experiment 1** |                 |              |
| Uninfected      | 0.820/0.142 (5.8) | 0.024/0.397 (0.06) |
| MSV-infected    | 0.142/0.170 (7.9) | 0.295/0.555 (0.54) |
| **Experiment 2** |                 |              |
| Uninfected      | 0.150/0.062 (2.4) | 0.003/0.410 (0.0008) |
| MSV-infected    | 0.300/0.130 (2.3) | 0.036/0.522 (0.068) |

For the homogenate assay, 0.1 ml of disrupted cells was incubated with 0.4 ml of assay mixture (see legend to Fig. 2) for 10 min at 37° and processed as described (Fig. 2). The concentration of 2-deoxyglucose was 10-fold lower than used for the experiments of Fig. 2 (0.1 mM instead of 1.0 mM). Thus, over a wide range of substrate concentration no differences in hexokinase activity was detected between control and MSV-infected cells. For the intact cell, 40 (a) or 6 (b) nmoles of 2-deoxyglucose-14C were added to cultures which were then incubated at 37° for 10 min. After incubation, cells were washed and homogenized and 0.5-ml aliquots were incubated with either bovine serum albumin or alkaline phosphatase (0.1 ml, 1 mg per ml) for 10 min at 37°. Carrier 2-deoxyglucose was then added and phosphorylated sugars were precipitated with the barium-zinc reagent of Somogyi. The supernatant after low speed centrifugation was taken for radioactivity determinations. The difference between the alkaline phosphatase and bovine serum albumin supernatants was taken as a measure of phosphorylated 2-deoxyglucose.

1 hour at 37°, and then 0.1 ml of 2-deoxyglucose and 2-deoxyglucose-6-phosphate was added to the samples. Then 0.02 ml of each sample was spotted on Whatman No. 3MM paper strips and subjected to electrophoresis at 360 volts, 5 to 7 ma for 14 hours with Veronal buffer, pH 8.5 (ionic strength 0.05). Under these conditions the phenol red in the samples moved 10 cm toward the anode from the origin. The paper was dried after electrophoresis and sprayed with 0.5% benzidine in ethanol-acetic acid (80:20) and heated for 10 min in a 10° oven. The dark brown spots with fine gray dots of 2-deoxyglucose and 2-deoxyglucose 6-phosphate (separated by about 5 cm) were marked, and then the paper was cut into 0.5-cm segments from the origin for a 20-cm length. The paper fragments were put into 5 ml of toluene scintillation solution and the radioactivity in each fraction was determined. The results are shown in Table IV. Most of the radioactivity is located in the spot of 2-deoxyglucose 6-phosphate, indicating that both uninfected and infected cells produce 2-deoxyglucose 6-phosphate in transporting 2-deoxyglucose. Alkaline phosphatase treatment reforms 2-deoxyglucose, confirming that the product of the transport is indeed 2-deoxyglucose 6-phosphate. However, frequently 5 to 20% of the transported sugar was observed as 2-deoxyglucose itself. Areas of radioactivity other than those containing 2-deoxyglucose and 2-deoxyglucose 6-phosphate were quite few (less than a few per cent of the total recovered radioactivity). Paper chromatography with butanol-1-acetic acid-water (2:1:1) showed the same results (not presented).

**Requirement for Intact Cells**—The increased uptake of 2-deoxyglucose in infected cells was assumed to be based on alterations...
in a membrane transport and associated phosphorylation system; however, other possibilities such as greatly increased levels of hexokinase were also considered. To test the latter possibility specifically, hexokinase determinations were carried out on cell homogenates. The assay used 2-deoxyglucose-14C as substrate in the presence of ATP and MgCl2. The phosphorylated product was quantitatively precipitated by the barium-zinc reagent of Somogyi as described by Van Steveninck (13). This assay has the advantage over the conventional assay with glucose as substrate in that end product inhibition by glucose 6-phosphate is not a factor. The kinetic measurements obtained with 2-deoxyglucose showed linearity with both time and enzyme concentration (Fig. 2, A and B) thus permitting accurate estimations of hexokinase levels in control and infected cells. The results depicted in Fig. 2 show little difference in the levels of hexokinase activity between control and MSV-infected cells. Additional experiments were carried out with substrate concentrations 10-fold lower than for the assays of Fig. 2. The results of two such experiments are shown in Table V. The differences in specific activity between control and infected cells again appear trivial of hexokinase levels in control and infected cells. The results depicted in Fig. 2 show little difference in the levels of hexokinase activity between control and MSV-infected cells. Additional experiments were carried out with substrate concentrations 10-fold lower than for the assays of Fig. 2. The results of two such experiments are shown in Table V. The differences in specific activity between control and infected cells again appear trivial and could not explain the differences in uptake obtained with intact cells. This tends to eliminate a nonstructural enzyme, such as hexokinase, as a determinant factor and provides compelling evidence in favor of specific alteration of a membrane-bound system.

**DISCUSSION**

The results presented here show that the significant increase of 2-deoxyglucose-14C uptake in MSV-infected cells is based on change in a sugar transport and phosphorylation system, irrespective of other possible changes in sugar metabolism. This change occurs at the same time as cell transformation (3), leading to the conclusion that functional modification of plasma membrane is a key event in the process of MSV transformation. Insofar as we are aware, this is the first demonstration of functional alteration of the plasma membrane during viral transformation, although virus-specific antigenic changes at the cell surface are well known (14-19).

After these experiments were completed, an interesting report concerning amino acid transport in 3T3 and poloma virus-transformed 3T3 cells (20) drew our attention. It was shown that poloma-transformed cells had lost the ability to limit amino acid transport which is characteristic of normal cells in the confluent state. The authors concluded, however, that this change was not a general alteration in membrane transport caused by transformation. In contrast, the sugar transport and phosphorylation system induced by MSV infection or transformation has a lower Km than the normal system (Table III, Reference 3), regardless of growth phase. SV40-transformed or leukemia virus-infected cells did not show this change. These original results have now been confirmed with the use of an extremely well controlled system; i.e., cells transformed by different tumor viruses all derived from the same single clone of Balb/3T3 (21). In these experiments only MSV transformants showed the characteristic low Km (22). These data collectively support the hypothesis that specific alteration in sugar transport of the plasma membrane is an integral event in MSV transformation.

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