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Isolation of a Chinese hamster fibroblast mutant defective in hexose transport and aerobic glycolysis: Its use to dissect the malignant phenotype

(growth control/tumorigenicity/tritium suicide/phosphoglucose isomerase mutant/regulation of glucose transport)

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ABSTRACT **A procedure is described for the selection of glucose uptake mutants based upon radiation suicide of Chinese hamster fibroblasts by 2-deoxy[^{3}H]glucose. In one of these mutants, DS 7, the ability to transport either 2-deoxyglucose or 3-*O*-methylglucose was decreased to one-fifth to one-fourth. Besides this defect, DS 7 produces $^1/_{14}$th the lactic acid produced by the parent when grown on 5 mM glucose. This block in aerobic glycolysis is due to a mutation that affects the expression of the phosphoglucose isomerase gene because no isomerase activity is detected in cell extracts of DS 7. This glycolytic block makes that cell line dependent exclusively on respiration for its energy requirement. Consequently, DS 7 survives well after removal of glucose but dies quickly in the presence of oligomycin. The parental line O 23 (subclone of CCl 39) grows at low serum concentration, is anchorage-independent, and is tumorigenic in *nude* mice. The derived glycolytic mutant DS 7 has retained both the *in vitro* transformed phenotype (low serum dependence and loss of anchorage dependence) and the tumor-forming capability. The tumor cells derived from the injection of DS 7 cells have kept the original glycolytic defect. This finding suggests that the transformed properties (high hexose transport and aerobic glycolysis) that can be uncoupled from abnormal growth control are not necessary for the expression of the malignant phenotype in fibroblasts.**

So far, among the various properties that accompany viral or spontaneous transformation (for review, see refs. 1–4), it has been difficult to assign to any particular transformed property a primary role in the establishment and maintenance of the abnormal growth control. A set of surface and membrane changes—including low adhesion to substratum, rounded shape, increased agglutinability by plant lectins, altered cell locomotion, decreased cell alignment, and loss of microfilament bundles—has been dissociated from the mechanism that alters the control of division (5–9).

Increases in hexose transport and in aerobic glycolysis are two other characteristics that one finds closely linked to transformation and increased proliferation in cells (10–14). It is still unknown whether or not these two properties (derepressed glucose transport and high glycolytic flux) are required for the expression of the transformed state and for tumorigenicity.

To approach this question, we attempted to isolate various mutants defective in glucose uptake. We report here (*i*) a method of selection of such mutants by using tritium suicide and (*ii*) the properties of DS 7, a Chinese hamster fibroblast mutant in which glucose transport and aerobic glycolysis are impaired. In spite of a low glucose transport activity and a block in aerobic glycolysis, DS 7, like the parental cell line, grows in agarose with high frequency and is tumorigenic in *nude* mice. This finding strongly suggests that increase in glucose transport and high aerobic glycolysis are not necessary for the expression of the transformed and malignant phenotype. A preliminary report of this work has been presented (15).

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MATERIALS AND METHODS

3-*O*-Methyl[1-^{3}H]glucose (2–5 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), 2-deoxy[1-^{3}H]glucose (15–25 Ci/mmol), and 2-deoxy[1-^{14}C]glucose (50 mCi/mmol) were obtained from the Radiochemical Centre (Amersham). 3-*O*-Methylglucose and ethyl methanesulfonate (EtMes) were obtained from Sigma.

Cell Lines and Culture Conditions. The Chinese hamster lung fibroblast line O 23 used in this study is an ouabain-resistant subclone of the established line CCl 39 (American Type Culture Collection). These cells were maintained as described (16).

Mutagenesis. Exponentially growing cells ($1–2 \times 10^4$ cells per cm^2) were treated with EtMes at 0.25 μl/ml in regular medium for 16 hr. In these conditions, 50% of the cell population survived. The cells were washed twice with Dulbecco's phosphate-buffered saline, cultured, and passaged for 6 days in Falcon flasks before selection.

2-Deoxyglucose and 3-*O*-Methylglucose Transport Assays. Cells were planted in 35-mm dishes, cultured 2–3 days near confluency, and assayed for hexose uptake 1 day after medium change as described (16). To improve the transport kinetics of 3-*O*-methylglucose, cells were precharged for 1 hr with 50 mM 3-*O*-methylglucose prior to the transport assay.

Assays of Glycolytic Enzymes. Hexokinase, glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase were measured at room temperature in 50 mM phosphate, pH 7.5/2 mM ATP/2 mM $MgCl_2$/1 mM $NADP^+$ containing 5 mM substrate (glucose or glucose 6-phosphate or fructose 6-phosphate). For hexokinase and phosphoglucose isomerase assays, an excess of purified glucose-6-phosphate dehydrogenase was added. Phosphofructokinase was measured as reported by Schneider *et al.* (17).

Measurements of Aerobic Glycolysis. DS 7 and O 23 cell lines were planted at 5×10^5 and 2.5×10^5 cells, respectively, per 60-mm dish. One day later the cells were washed twice with phosphate-buffered saline and the assay was initiated by addition of growth medium containing 5 mM glucose and 10% dialyzed fetal calf serum. Aliquots of the medium were assayed for glucose content by using the Sigma glucose assay kit and for lactate by using lactate dehydrogenase coupled to glutamate/pyruvate transaminase (18).

Analysis of "*in Vivo*" 2-Deoxyglucose Phosphorylation. The $BaSO_4$ precipitation procedure of Somogyi as described by Kletzien and Perdue (19) was used to measure the phosphorylated 2-deoxyglucose.

Growth in Soft Agar. The method used was essentially that described by MacPherson and Montagnier (20) in which agar was replaced by agarose.

Abbreviation: EtMes, ethyl methanesulfonate.

Tumorigenicity Assays. Cells were harvested by trypsinization, and 2–6 × 10^6 washed cells suspended in 0.2 ml of medium were injected at a single subcutaneous site into each *nude* mouse.

RESULTS

Principle of Mutant Selection. Suicide selection by using radioactive labeled substrates has proved to be a potent method for the isolation of bacterial and mammalian cell mutants (21–23). The glucose analog 2-deoxyglucose enters fibroblasts *via* the D-glucose-facilitated transport system and accumulates inside the cell, mainly as 2-deoxyglucose 6-phosphate. We predicted that any mutation that would decrease either hexokinase or glucose transport activity would give the cell resistance against the toxicity of ^{3}H-labeled 2-deoxyglucose by decreasing the intracellular radiotoxic pool. Fig. 1 shows the schema of selection used. The mutagenized cell population was trypsinized and incubated for 1 hr in glucose-free medium containing 2-deoxy[1-^{3}H]glucose. By the end of the incubation period, the intracellular 2-deoxyglucose pool had reached a value corresponding to about 10^4 decays per cell per day. The cells were immediately centrifuged to eliminate the external labeled hexose, frozen in regular medium supplemented with 10% dimethyl sulfoxide, and stored in liquid N_2. Periodically, aliquots were thawed and analyzed for survival of clones (Fig. 1). After 25 days, by which time survival had decreased by a factor of 10^4, clones were isolated and tested for their ability to transport 2-deoxyglucose. One-third of the clones analyzed were found to have less than 50% of the wild-type hexose uptake activity. DS 7, one of the most markedly affected clones, was further investigated.

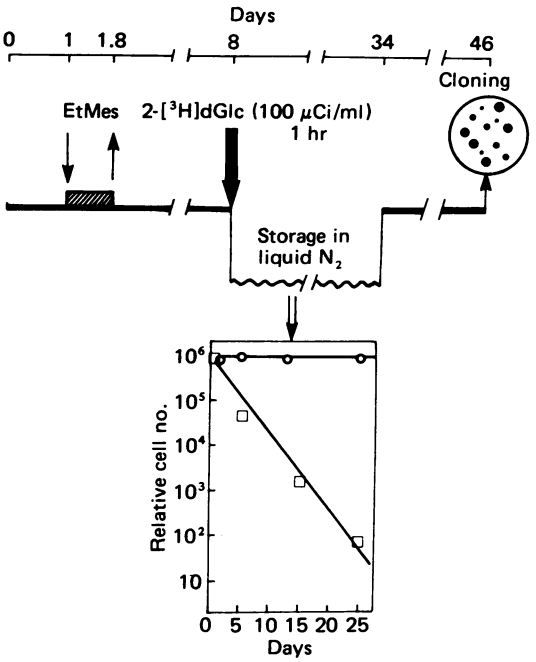

FIG. 1. Schematic representation of the tritium suicide method for the selection of glucose uptake mutants. The cells were mutagenized, grown for 6 days, trypsinized, and resuspended at a density of 8 × 10^6 cells per ml. This cell suspension was incubated for 1 hr at 37°C in glucose-free medium containing 2-deoxy[1-^{3}H]glucose (dGlc) (100 μCi/ml; 17 Ci/mmol). The cells were centrifuged to eliminate the labeled sugar, frozen in 10% dimethyl sulfoxide (10^6 cells per ml), and stored in liquid N_2. Periodically, samples were thawed and the cells were plated to analyze survivors. □, Cells incubated with the radioactive sugar; ○, cells incubated with 10 μM unlabeled 2-deoxyglucose.

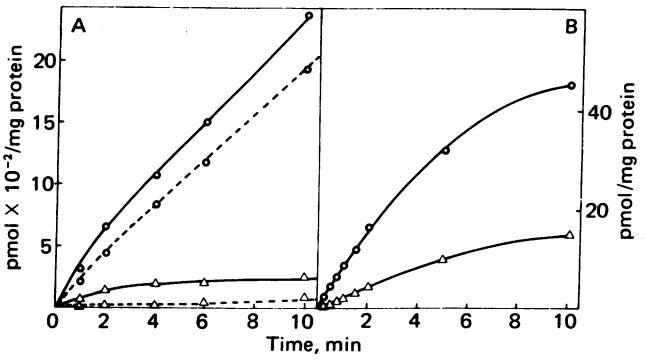

FIG. 2. Uptake and phosphorylation of 2-deoxyglucose (*A*) and transport of 3-*O*-methylglucose (*B*) in O 23 (○) and DS 7 (△) cells lines. Monolayers were incubated with 50 μM 2-deoxy[1-^{3}H]glucose or 1.2 μM 3-*O*-methyl[1-^{3}H]glucose. At indicated times, aliquots of homogenates were taken for protein determination, scintillation counting, and determination of intracellular phosphorylated 2-deoxyglucose (dashed line) by the $BaSO_4$ precipitation method. 2-Deoxyglucose uptakes were measured at 37°C; 3-*O*-methylglucose transport was measured at 20°C with precharged cells.

Characteristics of Hexose Transport. The ability to take up 2-deoxyglucose was very low in mutant DS 7 [10% of the parental value at 10 min uptake (Fig. 2*A*)]. Whereas 80–90% of the deoxysugar taken up was phosphorylated in the parental strain (dashed line), 90% of the pool present in DS 7 was in the free form. Indeed, after a 2-min incubation with 2-deoxy[^{14}C]glucose, chromatographic analysis of the soluble pool showed that the mutant was unable to phosphorylate the deoxysugar (Fig. 3). These results suggested at least two alterations in DS 7 cells: one in hexose transport because the initial rate of 2-deoxyglucose influx was decreased to one-fifth, and one in the phosphorylation step. The defect in hexose transport was confirmed by the kinetics of 3-*O*-methylglucose flux (Fig. 2*B*). The transport of α-aminoisobutyric acid, a nonmetabolizable amino acid, did not show any significant difference between the two cell lines. This result, which points to a specific defect in hexose uptake, validates the specificity of 2-deoxyglucose tritium suicide for selection of glucose uptake mutants.

It has been reported that, for many types of fibroblasts, removal of glucose from the external medium results in increased hexose transport activity (24–27). We recently found that, in

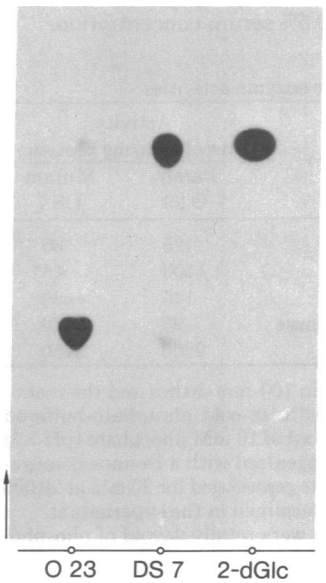

FIG. 3. Autoradiogram of chromatographic analysis of intracellular pool of O 23 and DS 7 cells exposed to 2-deoxy[1-^{14}C]-glucose. Cells were incubated for 2 min at 37°C with 100 μM 2-deoxy[1-^{14}C]glucose. Intracellular pools were extracted in 70% ethanol (pH 3.5) and analyzed by chromatography on thin-layer cellulose plates in 95% ethanol/1 M ammonium acetate, pH 3.8 (7.5:3, vol/vol) with a standard of 2-deoxyglucose (2-dGlc). The plate was exposed 3 days to a Kodak film (Kodirex).

the Chinese hamster fibroblast line O 23, the increase in transport is mainly due to a mechanism of "carrier activation" (16). DS 7 has kept intact this mechanism of carrier activation upon glucose removal. Glucose depletion for 5 hr resulted in a 3-fold stimulation of glucose transport in both cell lines. However, when starvation was extended over 24 hr, the glucose transport activity of DS 7 cells increased to reach the value of the wild-type starved cells (unpublished data). In contrast, 30 min of glucose starvation was enough to restore the ability of DS 7 cells to phosphorylate 2-deoxyglucose. This result suggested that the defect of *in vivo* phosphorylation was due to an inhibition of hexokinase secondary to an accumulation of a glucose metabolite. Accordingly, we found that, when growing on glucose, DS 7 tends to accumulate gradually a large intracellular pool of glucose 6-phosphate. This accumulation was found to result from a specific defect in phosphoglucose isomerase. Indeed, the activity of this enzyme in cell homogenates of DS 7 represents <1% of the parental corresponding activity (Table 1).

Aerobic Glycolysis. The data in Fig. 4 point up that DS 7 has lost the ability to utilize glucose as a glycolytic substrate, the production of lactic acid being barely detectable. We calculated that, for an increment of 1 mg of total protein, O 23 cells utilized 14.5 μmol of glucose and produced 28 μmol of lactic acid. This result indicates that this fibroblast line converts almost 100% of the glucose taken up into lactic acid. In contrast, for an increment of 1 mg of protein, DS 7 utilized only 3 μmol of glucose and produced $^1/_{14}$th the amount of lactic acid. The implication of these data is that DS 7 derives all of its energy from respiration. Removal of glucose from the culture medium does not affect its viability for at least 3 days. However, addition of oligomycin (1 μg/ml) results in a rapid cell death whereas parental cells which derive ATP both from respiration and glycolysis are not affected by the ATPase inhibitor. These observations are in agreement with the existence in DS 7 of a glycolysis block.

Growth and Anchorage Dependence. In regular medium (10% serum and 25 mM glucose), the doubling time was 10–12 hr for O 23 and 15–17 hr for DS 7. This slight difference disappeared when both cell lines were grown on other sugars such as galactose, glucose 6-phosphate, fructose, and xylose. Characteristics expressed in DS 7—low hexose transport and absence of glycolysis—belong to the phenotype observed in nontransformed cells when they stop proliferation, at confluency. We therefore analyzed the ability of these cells to express the transformed phenotype. Like the parental cells, DS 7 showed a low dependence on serum concentration for growth. Both cell lines still were able to grow at 0.5% serum concentration.

Table 1. Glycolytic enzyme activities

Enzymes	Activity, nmol/min/mg protein: Parent O 23	Mutant DS 7
Hexokinase	25	30
Phosphoglucose isomerase	1100	<5*
Phosphofructokinase	145	90
Glucose-6-phosphate dehydrogenase	95	70
Lactate dehydrogenase	2200	1750

Cells were grown to confluency in 100-mm dishes and the monolayers were washed three times with ice-cold phosphate-buffered saline. The cells were removed in 1 ml of 10 mM phosphate (pH 7.5) with a rubber policeman and homogenized with a Dounce homogenizer (20 strokes). Homogenates were centrifuged for 20 min at 20,000 × *g* and enzyme activities were determined in the supernatant.

* Whole homogenates of DS 7 also were totally devoid of phosphoglucose isomerase activity.

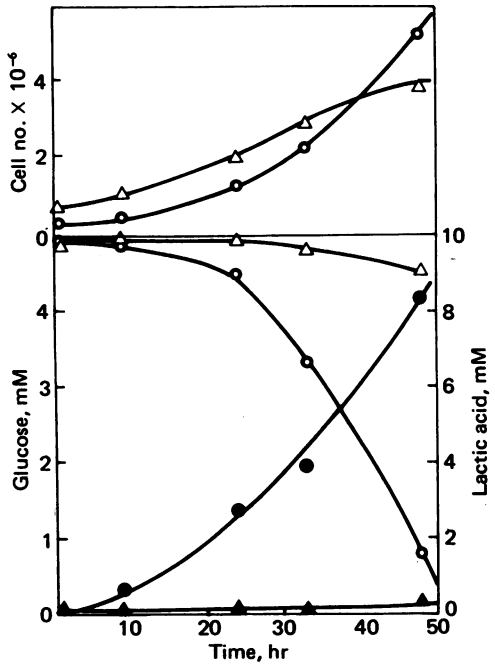

FIG. 4. Glucose consumption and lactic acid production in exponentially growing O 23 and DS 7 cells. Glucose (open symbols) and lactic acid (solid symbols) present in the culture medium were assayed starting at 24 hr after cell plating. Cell numbers were determined in parallel dishes. △, ▲, DS 7; ○, ●, O 23.

In regard to the growth in suspension, O 23 cells grew at high frequency in soft agarose (20–25%, Table 2). Interestingly, the derived cell line DS 7 displayed the same anchorage-independent growth: 16% of the cells grew in agarose, and the size of the colonies was slightly smaller (cells grew more slowly) than that of the parental strain O 23. Eight independent colonies growing in agarose tested have retained the original phenotype of DS 7 (data not shown).

Tumorigenicity in *Nude* Mice. The correlation between loss of anchorage dependence and tumorigenicity (28) can be extended to the two cell lines O 23 and DS 7 because both form tumors in *nude* mice (Table 2). The only differences noted between O 23 and DS 7 cells are that: (*i*) tumors derived from DS 7 appeared with a lag of 4–8 weeks instead of the 2 weeks seen with O 23 cells; and (*ii*) tumors from DS 7 grew more slowly than did the tumors from the parent cells.

Two tumors derived from DS 7 cells were removed from the animals, passaged twice in culture, and analyzed for phenotype. We found that these cells kept the glycolytic defect of the original injected DS 7 cells (low glucose transport and phos-

Table 2. Anchorage dependence and cellular tumorigenicity in *nude* mice

Cell type	Origin	Growth in soft agar,* %	Tumorigenicity: No. of cells injected × 10^{-6}	Tumorigenicity: Tumors†
O 23	CCl 39 (ouabain-resistant)	24	2	5/5
			6	8/9
DS 7 (pgi⁻, this study)	O 23	16	2	5/6
			6	6/10

* Colony numbers are expressed as percentage of cell input (8 × 10^3 cells in 60-mm dishes).

† Number of mice with tumors/number of mice injected. Cells were scored as tumorigenic if a palpable nodule appeared at the site of injection within 10 weeks.

phoglucose isomerase block). Therefore, this result excludes the possibility that tumors derived from DS 7 cells originated from isomerase$^+$ spontaneous revertants of the cell population injected.

DISCUSSION

The unique properties of the mutant line DS 7 demonstrate that tritiated 2-deoxyglucose suicide can be used as a powerful technique to select either glucose transport or glycolytic mutants in mammalian cells. We have shown that this mutant has a specific block in glycolysis due to a single mutation affecting the activity of phosphoglucose isomerase. A result of this defect early in the glycolytic pathway is a detectable increase of the intracellular glucose 6-phosphate pool which in turn elicits a short-term inhibition of hexokinase and a longer term "suppression" of the glucose transport activity (unpublished data). As a consequence of the glycolytic block, DS 7 utilizes glucose at a low rate and only *via* the oxidative pathway of the pentose phosphate shunt. This restricted utilization of glucose in DS 7 prevents the cell from deriving energy from glycolysis and therefore makes it very sensitive to anaerobic conditions or to inhibitors of respiration such as rotenone or oligomycin.

A second objective of this study was to analyze the transformed and malignant phenotypes of the DS 7 line impaired in hexose transport and in aerobic glycolysis. Since the original observation by Warburg (12) that malignant cells have enhanced aerobic glycolysis, several studies have suggested that transport changes and rate of glucose metabolism may be important in the escape of malignant cells from normal growth control (11–14, 29–31). The results we report here indicate that, when hamster fibroblasts have lost their growth control, a mutation leading to a decrease in hexose transport and to a block in the glycolytic pathway does not restore their original growth regulatory properties. This conclusion was based on the fact that DS 7 cells have retained the *in vitro* transformed phenotype of the parent line O 23 (low serum dependence and loss of anchorage dependence for growth).

These data suggest that (*i*) glycolysis *per se* is not absolutely required for growth and (*ii*) the increase in hexose transport activity and aerobic glycolysis associated with malignant transformation is not necessary for the expression of the *in vitro* transformed phenotype. Thrash and Cunningham (32) have previously shown that increased hexose transport and initiation of proliferation can be uncoupled in density-inhibited 3T3 fibroblasts. Our conclusion agrees with this report and extends the uncoupling to aerobic glycolysis. Another example of dissociation was reported for a glutamine-independent variant of polyoma-transformed fibroblast (33).

Concerning the analysis of the malignant phenotype, we have seen that the parental cells O 23, like many other cell lines showing anchorage-independent growth, are tumorigenic in *nude* mice (Table 2; ref. 28). Interestingly, the phosphoglucose isomerase mutation which abolishes aerobic glycolysis and reduces glucose transport influx does not prevent the tumor-forming capability. However, although a quantitative study of tumorigenicity has not yet been performed, tumors from DS 7 appeared after a longer lag period and developed more slowly. This apparent lower tumorigenicity of DS 7 cells, compared to that of O 23 cells might be explained by their high sensitivity to anaerobiosis. Soon after injection, many DS 7 cells may die as a consequence of local anoxia before vascularization has taken place to promote tumor expansion.

A general conclusion of this study is that the two transformed characters—high hexose transport and high aerobic glycolysis—closely associated with malignancy can be uncoupled from abnormal growth control. This report, complementing our work (5, 7) with a cell-surface-carbohydrate-deficient mutant, illustrates the use of somatic cell genetics as a powerful approach to dissect the complex phenotype of malignant fibroblasts.

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