Biochemical Pharmacology, Vol. 57, pp. 387–396, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00267-6

Distinct Regulation of Glucose Transport by Interleukin-3 and Oncogenes in a Murine Bone Marrow-Derived Cell Line

Nuzhat Ahmed and Michael V. Berridge*

MALAGHAN INSTITUTE OF MEDICAL RESEARCH, WELLINGTON SCHOOL OF MEDICINE, WELLINGTON SOUTH, NEW ZEALAND

ABSTRACT. Growth factors and oncogenes promote glucose uptake, but the extent to which increased uptake is regulated at the level of glucose transporter function has not been clearly established. In this paper, we show that interleukin-3 (IL-3), a cytokine growth factor, and the transforming oncogenes *ras* and *abl* alter the activation state of glucose transporters by distinct mechanisms. Using bone marrow-derived IL-3-dependent 32Dcl3 (32D clone 3) cells and 32D cells transformed with *ras* and *abl* oncogenes, we demonstrated that IL-3 enhanced [^{3}H]-2-deoxyglucose (2-DOG) uptake in parental 32Dcl3 cells by 40–50% at 0.2 mM 2-DOG, and this was associated with a 2.5-fold increase in transporter affinity for glucose (reduced K_m). In comparison, *ras* and *abl* oncogenes enhanced 2-DOG uptake by 72–112%, associated with a 2-fold greater transporter affinity for glucose. The tyrosine kinase inhibitor genistein reversed the effects of both IL-3 and oncogenes on glucose uptake and reduced transporter affinity for glucose. Likewise, with exponentially growing 32D cells in the presence of IL-3, a protein kinase C inhibitor, staurosporine, and a phosphatidylinositol 3-kinase (PI-3) kinase inhibitor, wortmannin, inhibited 2-DOG uptake and decreased transporter affinity for glucose. In contrast, in oncogene-transformed cells, staurosporine inhibited 2-DOG uptake but failed to decrease transporter affinity for glucose, whereas wortmannin did not affect 2-DOG uptake. Inhibition of protein tyrosine phosphatases with vanadate enhanced 2-DOG uptake and transporter affinity for glucose in parental cells and in *ras*-transformed cells but had little effect on *abl*-transformed cells. Consistently, the serine/threonine phosphatase type 2A inhibitor okadaic acid enhanced 2-DOG uptake and transporter affinity for glucose in parental cells but had little effect on *ras*- or *abl*-transformed cells. These results demonstrate differences in the regulation of glucose transport in parental and oncogene-transformed 32D cells. Thus, IL-3 responses are dependent upon tyrosine, serine/threonine, and PI-3 kinases, whereas *ras* and *abl* effects on glucose transport depend upon tyrosine phosphorylation but are compromised in their dependence upon serine/threonine and PI-3 kinases. BIOCHEM PHARMACOL **57**;4:387–396, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. glucose transport; IL-3; oncogenes; *ras*, *abl*; affinity

Glucose is required for survival, growth, proliferation, and function of most mammalian cells. Transport of glucose across the mammalian plasma membrane occurs by a family of stereospecific structurally related "facilitative" glucose transporter (Gluts†) that shift glucose down its concentration gradient without expenditure of energy [1, 2]. These Gluts are expressed in a tissue-specific manner, and their expression and transporter function appear to be regulated intracellularly and also by extracellular signals such as growth factors. Glucose entry into cells has been shown to be regulated during malignant transformation [3, 4], by growth factors [5, 6], following treatment with death-inducing agents [7], and in response to extracellular stress [8, 9]. In insulin-responsive cells, insulin acutely stimulates glucose transport by translocating Glut-4 from an intracellular pool to the plasma membrane [10, 11]. Glut-1, which is less abundant, may also be recruited to the plasma membrane in response to insulin, but this occurs to a much lesser extent [12]. In addition to recruitment, there is also evidence that insulin modulates the intrinsic activity of both Glut-1 and Glut-4 [13, 14]. Apart from insulin-stimulated responses, regulation of glucose transport in other systems has not been widely studied. Increased glucose transport in response to growth factors has been shown to occur in leukemic cells [15], retinal epithelial cells [16], developing myocytes [17], lymphoid cells [6], and other hemopoietic cells [18, 19]. In some cells, increased transport has been shown to occur through increased Glut

* Corresponding author (and present address): Dr. Nuzhat Ahmed, Discipline of Surgical Sciences, Level 4, David Maddison Bldg., Royal Newcastle Hospital, Newcastle, NSW 2300, Australia. Tel. 61-249-23-6168; FAX 61-249-23-6984; E-mail: nahmed@newcastle.mail.edu.au

† *Abbreviations:* Gluts, glucose transporters; Glut-1, glucose transporter subtype 1; Glut-3, glucose transporter subtype 3; Glut-4, glucose transporter subtype 4; IL-3, interleukin-3; 2-DOG, [^{3}H]-2-deoxy-D-glucose; PI-3 kinase, phosphatidylinositol 3-kinase; TBS/T, Tris-buffered saline/0.05% Tween 20; PKC, protein kinase C; GF, glucose-free; and ECL, enhanced chemiluminescence.

Received 31 March 1998; accepted 2 July 1998.

expression or translocation of stored transporters to the plasma membrane [11, 17]. However, in other studies intrinsic activation of transporters at the cell surface was involved in the stimulation of glucose transport [6, 18], suggesting that growth factors other than insulin can also regulate the activation state of Gluts.

Oncogenes circumvent the growth factor requirement of cells by mechanisms including constitutive activation of the signalling molecules used by growth factors. A major difference between tumour cells and cells in surrounding tissue is the nutritional and metabolic environment in which they survive. The functional vasculature of tumours is often inadequate to supply the nutritional needs of the expanding tumour. As a result, hypoxia and nutrient deficiency contribute to cell death within a tumour. Many cells, however, survive stressful conditions by modifying nutrient transport. Hence, changes in cell surface proteins associated with nutrients allow transformed cells to survive in an altered and hostile micro-environment, and this contributes to malignant behavior. Enhanced glucose uptake is a characteristic biochemical feature of tumour cells [20], and this has been associated with increased expression of transporter mRNA and protein [3] and decreased transporter turnover [21]. Acceleration of glucose uptake in some transformed cells correlates with over-expression of Glut-1 and Glut-3 transporters [22]. Increased affinity of Gluts for glucose has been demonstrated in tumour cell lines [23] and in tumourigenic revertants of suppressed hybrid cells compared with the non-malignant phenotype [24], but in these studies cells of widely different backgrounds were used, and the transforming event was not clearly defined.

Growth factors and oncogenes alter the phosphorylation of tyrosine, serine, and threonine residues of many proteins. Inhibitors of tyrosine kinases, serine/threonine kinases, and PI-3 kinases have been shown to inhibit insulin-stimulated glucose transport in adipocytes and muscle cells [25–27]. On the other hand, tyrosine and serine/threonine phosphatase inhibitors have been demonstrated to stimulate basal and insulin-stimulated glucose transport in muscle cells [28, 29]. Kinase inhibitors reduce glucose transport by suppressing the translocation of Glut-4 and Glut-1 to the plasma membrane, but effects on the intrinsic properties of transporters are not known. Furthermore, little is known about the role of phosphorylation in the regulation of glucose transport in systems other than those regulated by insulin.

Using a bone marrow-derived cell line, we and others have shown that growth factors enhance glucose transport by increasing the affinity of Gluts for glucose (K_m) [6, 18, 30–32], and inhibitor studies have indicated the involvement of protein phosphorylation in the process [18, 30]. These results, when considered alongside those of others using transformed cells [23, 24], suggest that oncogenes may act like growth factors to maintain the activation state of Gluts by a mechanism involving phosphorylation.

In the present study, we compared the regulation of glucose transport by IL-3 and specific oncogenes like *ras* and *abl*. We demonstrated that both IL-3 and oncogenes enhance glucose transport by activating existing transporter molecules. Both IL-3 and oncogene-stimulated glucose uptake were abolished by the tyrosine kinase inhibitor genistein, and this was associated with loss of transporter affinity for glucose. Staurosporine (a protein kinase C inhibitor) and wortmannin (a PI-3 kinase inhibitor) also inhibited IL-3-stimulated glucose uptake and the activation state of transporters, but effects on oncogene-stimulated glucose transport were compromised. The protein phosphatase inhibitors vanadate and okadaic acid enhanced both basal and IL-3-stimulated glucose uptake and increased transporter affinity for glucose, but had a differential effect on oncogene-transformed cells. These results suggest that IL-3 and the oncogenes *ras* and *abl* regulate glucose transport by increasing the activation state of transporter molecules, but the role of phosphorylation in the functional activity of the transporters differs between parental and oncogene-transformed cells.

MATERIALS AND METHODS

Cells and Cell Culture

32D clone 3 (32Dcl3) cells, originally derived from long-term bone marrow cultures of C3H/HeJ mice [33], and temperature-sensitive (ts) *ras* and *abl* transfected 32D cells were gifts from Dr. S. T. Anderson, Department of Pathology, University of Colorado Health Science Center. Ts-*ras* and ts-*abl* are clones of v-*ras* and v-*abl* retrovirus-infected 32Dcl3 cells and have been described by Stein *et al.* [34] and Kipreos *et al.* [35]. Cells were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 25 μg/mL of penicillin, 25 μg/mL of streptomycin, 10% (v/v) fetal bovine serum, and 10% WEHI-3-conditioned medium as a source of IL-3. Recombinant murine IL-3 was obtained from Professor J. D. Watson, Genesis Research and Development Corp., and was used where indicated. Cells were maintained at the permissive temperature of 32° in a humidified incubator maintained at 5% CO_2. Even though temperature-sensitive mutants were used in the present study, their temperature-sensitive property was not used. Cell viability was determined by trypan blue exclusion, using a hemocytometer.

[^{3}H]Thymidine Incorporation Assay

Proliferative responses were measured by incubating 5×10^4 cells in 0.1 mL of culture medium in 96-well microtiter plates for periods of 16–20 hr before adding 0.5 μCi [^{3}H]thymidine (Amersham, U.K.) for 3 hr. Incorporation of radioactivity into DNA was determined using an automated cell harvester and liquid-scintillation counting.

[^{3}H]-2-Deoxyglucose Uptake

2-DOG uptake was measured by the zero-trans method using 0.5 μCi [^{3}H]-2-deoxy-D-glucose (2-DOG, 200 μM,

Amersham, U.K.) as described previously [18]. Briefly, cells were washed in RPMI-1640, resuspended at 5×10^5 cells/mL, and treated with or without growth factors and inhibitors where appropriate at 32° for the respective length of time. Then cells were washed twice and preincubated at 37° for 5 min in GF-RPMI 1640. [^{3}H]-2-Deoxy-D-glucose (2-DOG, 200 μM, 0.5 μCi) was added, and glucose uptake was determined for 5 min under conditions where the uptake was linear for 8 min. Uptake was stopped by adding 500 μL of ice-cold GF-RPMI 1640 containing 0.3 mM phloretin and chilling on ice for a further 5 min. Cells were solubilized in 100 μL of 1% Triton X-100, and radioactivity was determined by liquid scintillation counting. All kinetic analysis of 2-DOG uptake used 0.1 to 2.5 mM 2-DOG in extracellular medium and was measured over 5 min. Initial experiments were performed using 0.1 to 10 mM 2-DOG. The effects of growth factors and inhibitors were determined by adding them to exponentially growing cells at 32° for 2 hr. Cells were washed free of the added factors, and glucose uptake was measured and kinetic analysis was performed as described above.

Tyrosine Kinase Assay

Cells were washed twice with PBS (137 mM NaCl, 0.3 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2), resuspended at 1×10^7 cells/mL in PBS, and sonicated at a setting of 7, using an MSE 100 W ultrasonic disintegrator for a total of 90 sec in three 30-sec pulses with an interval of 30 sec between each pulse. The assay was performed using the Protein Tyrosine Kinase Assay System (Gibco-BRL). The ability of cells to transfer phosphate from [γ-^{32}P]ATP to a synthetic peptide (RR-SRC) was measured in the supernatant of crude cell lysates prepared after spinning the sonicate at 900 *g* for 5 min. ^{32}P-Labelled peptide was spotted on PEI-cellulose paper and counted as described by the manufacturer.

Preparation of Crude Membranes

Cells were collected, washed in PBS, and resuspended in Tris buffer [10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Resuspended cells were sonicated, and cellular debris was removed by centrifugation at 900 *g* for 10 min at 4°. The supernatant was centrifuged at 110,000 *g* for 75 min, and the crude membrane was solubilized in 10 mM Tris–HCl, pH 7.4, containing 0.5% Triton X-100 and 1 mM PMSF for 1 hr at 4°. Insoluble material was removed by centrifugation for 5 min in a microfuge, and the solubilized membranes were stored at −70°.

SDS–PAGE and Western Blot Analysis

Solubilized cell membrane preparations (15 μg) were added to Laemmli sample buffer and incubated at 37° for 10 min. Proteins were separated on 8% SDS-polyacrylamide gels using a Mini-Protean II apparatus (Bio-Rad Laboratories). Proteins were transferred electrophoretically to supported nitrocellulose membranes (Hybond-C Super, Amersham International, U.K.) at 15 V for 30 min. Non-specific binding to membranes was blocked by incubating in TBS/T containing 5% non-fat dried milk and 5% BSA for 1 hr at room temperature. Then membranes were incubated with primary antisera diluted in TBS/T containing 1% BSA for 2 hr at room temperature. Rabbit polyclonal antiserum against the C-terminus of rat Glut-1 (1/500 dilution, EastAcres Biologicals) and affinity-purified antibodies against the C-terminus of mouse Glut-3 (1/50 dilution, gift from G. W. Gould, University of Glasgow) were employed. Normal rabbit serum was used as a control in each case. Membranes were washed with TBS/T, and then incubated for 1 hr with affinity-purified swine Ig anti-rabbit IgG-conjugated to horseradish peroxidase (DAKO Corp.) diluted 1/4000 in TBS/T. Membranes were washed and developed using ECL (Amersham International, U.K.) and high performance luminescence detection film (Amersham).

Densitometric analysis used a Macintosh LC 630 computer and the public domain NIH Image program. The density of the immunoblot lanes was linear over the range used.

Protein Determination

Protein was determined using a microplate adaptation of the method of Bradford [36], and absorbance was measured at 570 nm.

Statistical Analysis and Presentation of Results

Results are given as means ± SEM for the number of experiments specified. Significance of differences between control, oncogene-transformed, and treated cells was determined by Student's *t*-test.

RESULTS

Effects of IL-3 and ras *and* abl *Oncogenes on Proliferative Responses and Tyrosine Kinase Activity of 32D Cells*

A saturating concentration of IL-3 (20 ng/mL) stimulated [^{3}H]thymidine incorporation into parental 32Dcl3 cells by 8-fold (Fig. 1A). 32D cells transformed with *ras* and *abl* oncogenes showed a further 2- and 5-fold increase in proliferative response compared with parental cells optimally stimulated with IL-3. Increased proliferative responses in 32D cells in response to IL-3 and transforming oncogenes were associated with increased tyrosine kinase activity. The tyrosine kinase activity of the parental cells was increased by 10-fold in the presence of a saturating concentration of IL-3 (20 ng/mL) (Fig. 1B). A similar level of tyrosine kinase activity was observed in *ras*-transformed cells as in IL-3-stimulated parental cells, while *abl* oncogene

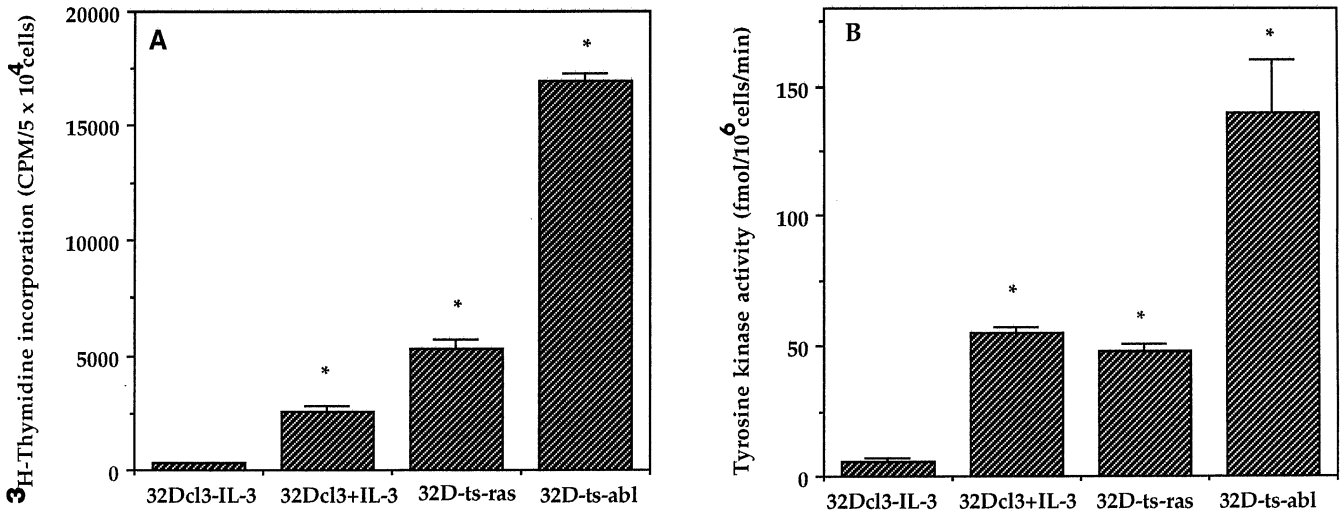

FIG. 1. Effects of ts-*ras* and ts-*abl* on the proliferative responses and tyrosine kinase activity in exponentially growing 32D cells. Parental 32Dcl3 cells and cells transformed with *ras* and *abl* oncogenes were grown exponentially in RPMI 1640 culture medium in the presence of 10% fetal bovine serum. With 32Dcl3 cells, IL-3 (20 ng/mL) was used where appropriate. (A) [^{3}H]Thymidine incorporation and (B) tyrosine kinase activity were determined as described in Materials and Methods. Values are the means ± SEM of at least three independent experiments, each involving triplicate determinations. Key: (*) significantly different from parental cells in the absence of IL-3, $P < 0.01$.

increased tyrosine activity a further 3-fold. Elevated proliferative responses and enhanced tyrosine kinase activity are characteristic features of the malignant phenotype. With *abl*-transformed cells, increased tyrosine kinase activity explains constitutively activated ABL tyrosine kinase.

Effects of IL-3 and ras *and* abl *Oncogenes on [^{3}H]-2-DOG Uptake by 32D Cells*

IL-3 significantly stimulated 2-DOG uptake into parental 32Dcl3 cells, with the greatest increase of 45% being observed between 1 and 4 hr (Fig. 2A). In contrast, IL-3 withdrawal caused the rate of 2-DOG uptake to decline rapidly, 40% loss being observed by 4 hr. Loss of 2-DOG uptake in 32Dcl3 cells under these conditions had no effect on cell viability, which was maintained at 90–95%. 32D cells transformed by *ras* and *abl* oncogenes showed 72–112% increased 2-DOG uptake compared with parental IL-3-dependent cells optimally stimulated with IL-3 (Fig. 2B).

Increased glucose uptake in response to IL-3 in parental cells was associated with changes in the affinity of Gluts for glucose. IL-3 increased the affinity of Gluts for glucose (K_m) by 2-fold (Table 1), resulting in an increase in glucose transport. Enhanced glucose uptake in 32D cells transformed with *ras* and *abl* oncogenes was also observed and was associated with a further 1.75- to 2-fold increase in affinity of Gluts for glucose (Table 1). These kinetic data were derived using 2-DOG in the concentration range of 0.1 to 2.5 mM. At higher glucose concentrations (>2.5 mM), K_m and V_{max} values for parental, *ras*- and *abl*-transformed cells showed similar trends (data not shown). Transport data were analyzed by a Lineweaver–Burk plot using linear regression analysis. Similar trends were obtained using Eadie–Hofstee plots. The R^2 value for each plot was between 0.99 and 1.00.

Glut Expression in Parental and Oncogene-Transformed 32D Cells

IL-3-dependent 32D cells and 32D cells transfected with transforming oncogenes *ras* and *abl* expressed Glut-1 and Glut-3 subtypes (Fig. 3, A and B). The average molecular mass of both Glut-1 and Glut-3 was higher in oncogene-transformed cells than in parental cells. The relative Glut-1/Glut-3 expression in *ras*- and *abl*-transformed cells was 1.3- and 1.5-fold higher (the Glut-1/Glut-3 ratio was arbitrarily set at 1 for parental 32Dcl3 cells), indicating that enhanced 2-DOG uptake in transformed cells was not due to a switch to the high-affinity Glut-3 isoform of the transporter.

Effects of Protein Kinase and Phosphatase Inhibitors on IL-3-Stimulated [^{3}H]-2-DOG Uptake by 32Dcl3 Cells

To determine whether the activation state of Gluts on parental 32Dcl3 cells is dependent on tyrosine, serine/threonine, and PI-3 kinases, inhibitors of these enzymes were used. Involvement of phosphatases was also evaluated using vanadate and okadaic acid, which are potent inhibitors for tyrosine and serine/threonine phosphatase type 2A.

Genistein, staurosporine, and wortmannin inhibited 2-DOG uptake in parental cells in a concentration-dependent manner. Complete inhibition of the "IL-3-stimulated" effect was observed within 2 hr at 0.5 mM, 2 μM, and 100 nM, respectively (Fig. 4). Under these conditions, inhibi-

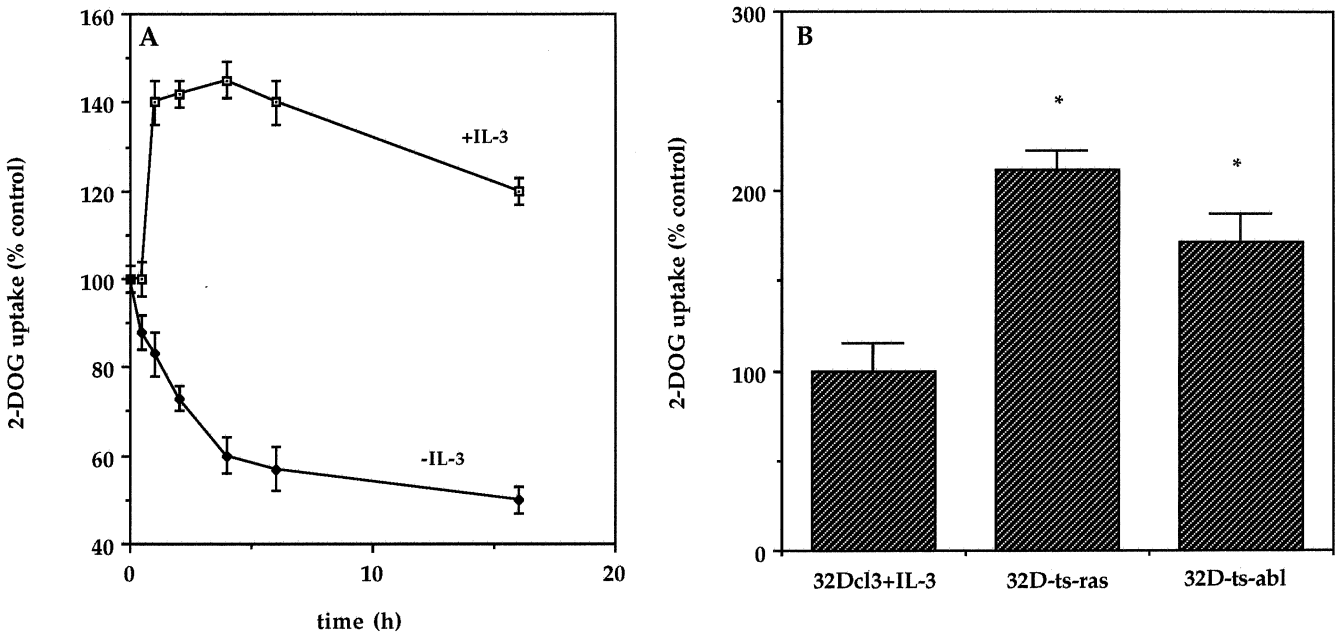

FIG. 2. (A) Effect of IL-3 on 2-DOG uptake by parental 32Dcl3 cells. 32Dcl3 cells were serum-starved in the presence of 2 ng/mL of IL-3 for 16 hr prior to adding 20 ng/mL of IL-3 for the times indicated. 2-DOG uptake was measured using a concentration of 0.2 mM. Control cells are the cells without any IL-3 (2-DOG uptake = 0.16 ± 0.01 nmol/10^6 cells/min). (B) 2-DOG uptake in parental 32Dcl3 cells in the presence of IL-3, and oncogene-transformed cells. 2-DOG uptake in exponentially growing *ras*- and *abl*-transformed cells was measured in the presence of 0.2 mM 2-DOG. Parental 32Dcl3 cells were serum-starved for 2 hr before the addition of 20 ng/mL of IL-3 for an additional 2 hr. Values are the means ± SEM of at least three independent experiments, each involving triplicate determinations. Control cells are 32Dcl3 cells treated with IL-3 (2-DOG uptake = 0.25 ± 0.04 nmol/10^6 cells/min). Key: (*) significantly different from parental cells, $P < 0.02$.

tors had little effect on basal 2-DOG uptake, and cell viability remained unchanged. At a higher inhibitor concentration, basal 2-DOG uptake (0.16 ± 0.01 nmol/10^6 cells/min) in the absence of IL-3 was inhibited with genistein and staurosporine (Fig. 4). Inhibition of 2-DOG uptake by these inhibitors was associated with a 2-fold decrease in transporter affinity for glucose (Table 2). Under similar conditions, vanadate and okadaic acid stimulated both basal and IL-3-dependent responses, increasing transporter affinity for glucose several fold (Table 2). These results suggest that protein kinases and phosphatases are involved in the regulation of 2-DOG uptake in IL-3-stimulated 32D cells. The fact that 2-DOG uptake in the presence and absence of IL-3 can be stimulated by vanadate and okadaic acid suggests effects additional to those of IL-3.

Effects of Inhibitors of Protein Kinase and Phosphatase on ras- *and* abl-*Stimulated [^{3}H]-2-DOG Uptake by Transformed 32D Cells*

Genistein and staurosporine both inhibited 2-DOG uptake by a maximum 40–50% in *ras*- and *abl*-transformed 32D cells (Fig. 4, A and B). With genistein, this was associated with decreased transporter affinity for glucose in *ras*- and *abl*-transformed cells, but with staurosporine, even at a 5-fold higher concentration than that used for IL-3-dependent cells, little effect on K_m was observed, while there was a 2- to 3-fold decrease in V_{max} (Table 3). Similar effects were observed with calphostin C, another potent PKC inhibitor (data not shown). Little effect of okadaic acid on transporter K_m of oncogene-transformed cells was observed

TABLE 1. Effects of IL-3 and oncogenes on glucose uptake by 32D cells

Cell type	IL-3 (20 ng/mL)	K_m (mM)	V_{max} (nmol/10^6 cells/min)
32Dcl3	−	8.33 ± 0.57	7.00 ± 0.21
32Dcl3	+	3.03 ± 0.05*	3.92 ± 0.51
32D-ts-*ras*	−	1.73 ± 0.11†	5.12 ± 0.33
32D-ts-*abl*	−	1.74 ± 0.37†	3.92 ± 0.38

Exponentially growing 32Dcl3 cells were starved of serum for 2 hr before being treated with or without 20 ng/mL of IL-3 for 2 hr. 2-DOG uptake were measured, and kinetic data were derived using Lineweaver–Burk plots and regression analysis. Values are the means ± SEM of a minimum of four separate experiments, each performed in triplicate.

* Significantly different from IL-3-starved cells, $P < 0.01$.

† Significantly different from parental IL-3-treated cells, $P < 0.01$.

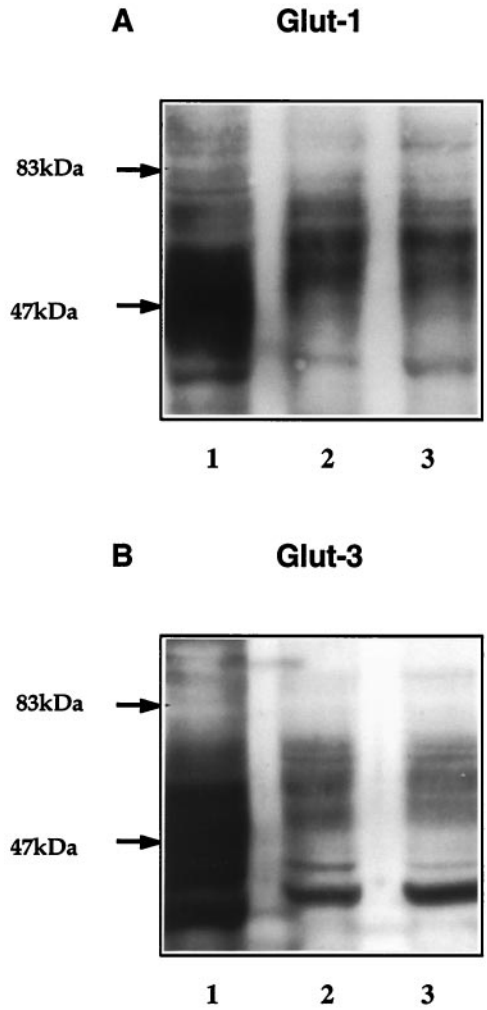

FIG. 3. Expression of Glut-1 and Glut-3 in 32Dcl3 and oncogene-transformed 32D cells. Crude membranes were prepared from exponentially growing cells, and the transporter subtypes (A) Glut-1 and (B) Glut-3 were analyzed by western blotting and ECL. Lane 1, 32Dcl3; lane 2, 32D-*ras*; and lane 3, 32D-*abl*. Each lane contained 15 μg protein.

(Table 3). The protein tyrosine phosphatase inhibitor vanadate enhanced 2-DOG uptake in *ras*-transformed cells and increased transporter affinity for glucose, but had little effect in *abl*-transformed cells. Both tyrosine and serine/threonine phosphatase inhibitors had little or no effect on V_{max}. The PI-3 kinase inhibitor wortmannin at a 5-fold higher concentration than that used with parental 32D cells failed to affect 2-DOG uptake by transformed cells (Fig. 4C and Table 3), suggesting that glucose transport in transformed cells does not require PI-3 kinase activation for function.

DISCUSSION

In this study, we have compared the effects of IL-3 and the transforming oncogenes *ras* and *abl* on glucose uptake in a bone marrow-derived 32D cell line. The results presented support the view that glucose uptake into cells is regulated by growth factors and oncogenes, and that regulation involves a change in the activation state of the plasma membrane transporters. With parental 32D cells, changes in glucose transport were observed between 0.5 and 1 hr of IL-3 addition and withdrawal, and these results are similar to those described previously for Ba/F3 cells [6] and for a different clone of 32D cells [18, 30]. With oncogene-transformed cells, uptake was elevated above that of parental 32D cells optimally stimulated with IL-3. In both cases, increased uptake was associated with increased transporter affinity for glucose (reduced K_m). Both parental and oncogene-transformed 32D cells express universal Glut-1 and high-affinity Glut-3 transporter isoforms. The apparent molecular mass of Gluts in oncogene-transformed cells was greater than in parental cells, indicating that structural changes in the transporter are a consequence of transformation. These results are in general agreement with those of White *et al.* [24], who showed that Gluts in transformed human, mouse, and rat cell lines exhibited a lower K_m for glucose than was found in non-transformed cell lines, and that tumourigenic revertants of suppressed hybrid cells also showed reduced K_m values. In that study, comparisons were of a general nature, cells of diverse origins were used, and the effects of clearly defined transforming events on glucose transport in a single cell line were not explored.

Regulation of the intrinsic properties of Glut isotypes is not well understood. Differences in K_m values in response to IL-3 and oncogene transformation may arise from structural modification to the transporter, which may involve phosphorylation and/or glycosylation [37, 38]. Alternatively, transporter-associated protein may change the intrinsic property of the transporter and enable cells to survive in an environment deficient in glucose and oxygen. In insulin-responsive tissues, insulin stimulates glucose transport several-fold, and in some cases increased glucose transport correlates with translocation of transporters to the plasma membrane [11]. In cases where enhanced glucose transport exceeds transporter translocation, transporter dephosphorylation has been inversely associated with intrinsic activation [13]. Hemopoietic systems are distinct from insulin-responsive systems, and enhanced glucose transport in response to growth factors is small compared with insulin-stimulated responses [6, 22, 30].

Involvement of regulatory proteins that associate with transporters has been postulated to contribute in intrinsic activation in the absence of increased transporter expression. Activation of glucose transport in clone 9 fibroblasts by azide may involve association of cytosolic proteins with the cytoplasmic domain of Glut-1 [39], while other cytosolic proteins have been shown to be associated with the C-terminal domain of Glut-4 [40]. Moreover, different isoforms of hexokinases have been shown to be associated with different transporter subtypes [2], suggesting that protein–protein interactions may play a crucial role in regulating transporter activity. Protein–protein interaction has also been shown to be involved in the regulation of insulin-stimulated glucose transport in adipocytes and fi-

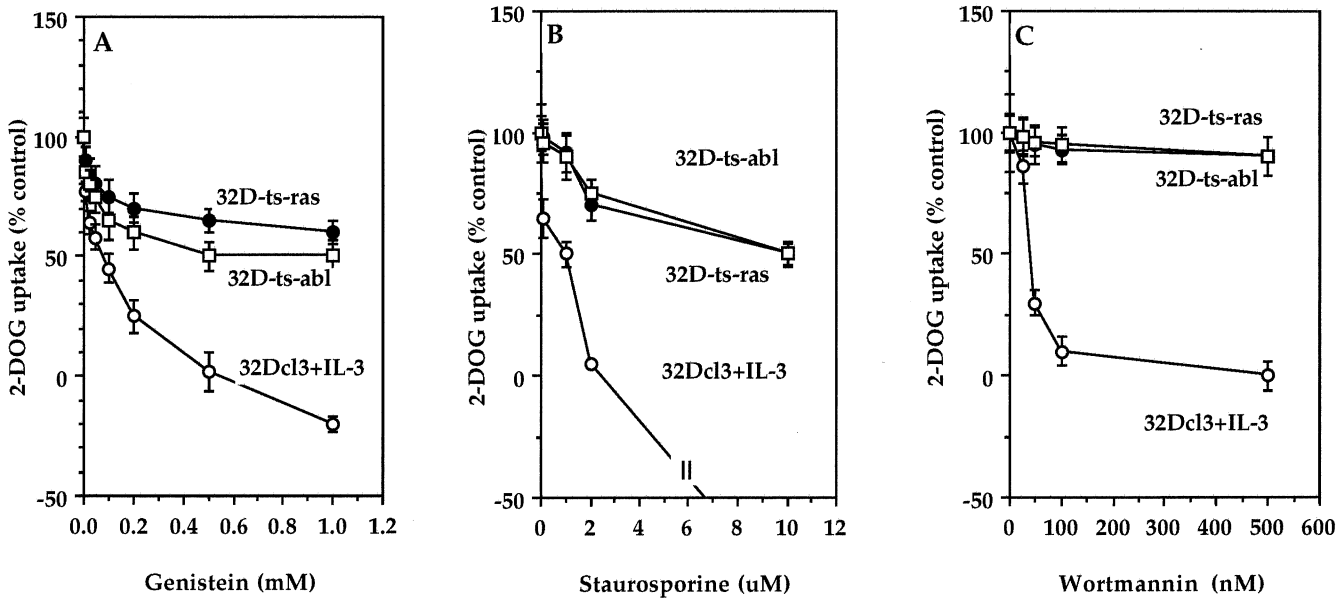

FIG. 4. Effect of inhibitors on 2-DOG uptake in parental and oncogene-transformed 32D cells. 2-DOG uptake in parental and oncogene-transformed 32D cells was measured at a concentration of 0.2 mM. Cells were treated with inhibitors for 2 hr at 32° in RPMI-1640 + 10% fetal bovine serum. For IL-3 effects, cells were starved of serum for 2 hr, and then equilibrated with IL-3 for 10 min before adding inhibitors for a further 2 hr. 32D cells were treated with increasing concentrations of (A) genistein, (B) staurosporine, and (C) wortmannin. Values are the means ± SEM of at least three independent experiments, each involving triplicate determinations. Control responses (100%) are the responses without any inhibitor treatment (2-DOG uptake by 32Dcl3 + IL-3 = 0.25 ± 0.04 nmol/10^6 cells/min; 32D-*ras* = 0.53 ± 0.06 nmol/10^6 cells/min; 32D-*abl* = 0.43 ± 0.07 nmol/10^6 cells/min). With parental cells, 0% is the response in the absence of IL-3 (2-DOG uptake = 0.16 ± 0.01 nmol/10^6 cells/min).

broblasts. The association of VAMP-2 with Syntaxin-4 plays an essential role in the translocation of Glut-4 to plasma membranes [41]. Hence, association of regulating proteins with Gluts can modulate glucose transport into cells by directly activating the transporter or by translocation. This process may involve direct or indirect phosphorylation, which may regulate the intrinsic activity of the transporter protein.

To determine whether phosphorylation plays a role in regulating transporter activation in response to IL-3 and oncogenes, inhibitors of tyrosine kinases (genistein), PKC (staurosporine), and PI-3 kinases (wortmannin) were used. Genistein extensively inhibited 2-DOG uptake promoted by IL-3 and reduced transporter affinity for glucose. The cytoplasmic domains of Glut-1 and Glut-3 contain consensus sites for PKC but lack tyrosine phosphorylation sites,

TABLE 2. Effect of inhibitors of protein kinases and phosphatases on IL-3-stimulated [^{3}H]-2-DOG uptake by 32Dcl3 cells

Inhibitor treatment	IL-3 treatment	2-DOG uptake (nmol/10^6 cells/min)	K_m (mM)	V_{max} (nmol/10^6 cells/min)
(A) Protein kinase inhibitors				
None	−*	0.16 ± 0.01	8.33 ± 0.57	7.00 ± 0.21
None	+*	0.25 ± 0.04	3.03 ± 0.05†	3.92 ± 0.51
Genistein (500 μM)	+	0.18 ± 0.02	6.32 ± 1.50‡	6.10 ± 0.50
Staurosporine (2×10^{-6} M)	+	0.12 ± 0.01	6.50 ± 0.13‡	4.24 ± 0.61
Wortmannin (100 nM)	+	0.16 ± 0.02	5.73 ± 0.80	5.64 ± 1.58
(B) Protein phosphatase inhibitors				
Vanadate (50 μM)	−	0.55 ± 0.27†	1.70 ± 0.50†	4.45 ± 0.97
Vanadate (50 μM)	+	0.43 ± 0.04‡	1.35 ± 0.27‡	3.31 ± 0.84
Okadaic acid (1 μM)	−	0.46§	0.90§	2.36§
Okadaic acid (1 μM)	+	0.58§	1.20§	4.00§

Exponentially growing cells were starved of serum for 2 hr and then treated with or without 20 ng/mL of IL-3 and inhibitors for a further 2 hr before measuring 2-DOG uptake. Kinetic data were derived using Lineweaver–Burk plots and regression analysis. Values are the means ± SEM of a minimum of three separate experiments, each performed in triplicate.

* Data from Table 1 repeated for comparison.

† Significantly different from that in the absence of IL-3, $P < 0.005$.

‡ Significantly different from that in the presence of IL-3 alone, $P < 0.01$.

§ Mean value from two separate experiments performed in triplicate with a variation of 10–15%.

TABLE 3. Effect of inhibitors of protein kinases and phosphatases on [^{3}H]-2-DOG uptake by transformed 32D cells

Inhibitor treatment	Cell type	2-DOG uptake (nmol/10^6 cells/min)	K_m (mM)	V_{max} (nmol/10^6 cells/min)
(A) Protein kinase inhibitors				
None	32D-ts-*ras*	0.53 ± 0.06	1.73 ± 0.11*	5.12 ± 0.33*
Genistein (500 μM)		0.26 ± 0.01†	4.18 ± 0.05†	3.42 ± 0.26
Staurosporine (10^{-5} M)		0.28 ± 0.02†	1.28 ± 0.11	2.14 ± 0.36†
Wortmannin (500 nM)		0.44 ± 0.02	1.77 ± 0.10	4.40 ± 0.80
None	32D-ts-*abl*	0.43 ± 0.07	1.74 ± 0.37*	3.92 ± 0.38*
Genistein (500 μM)		0.22 ± 0.01†	3.88 ± 0.70†	3.62 ± 0.48
Staurosporine (10^{-5} M)		0.20 ± 0.02†	2.50 ± 0.03	1.25 ± 0.04†
Wortmannin (500 nM)		0.49 ± 0.05	1.68 ± 0.02	3.82 ± 0.60
(B) Protein phosphatase inhibitors				
Vanadate (50 μM)	32D-ts-*ras*	0.95 ± 0.18†	0.93 ± 0.11†	4.46 ± 0.50
Okadaic acid (500 nM)		0.42 ± 0.03	1.56 ± 0.10	4.60 ± 0.70
Vanadate (50 μM)	32D-ts-*abl*	0.42 ± 0.01	1.73 ± 0.02	3.90 ± 0.15
Okadaic acid (500 nM)		0.36 ± 0.03	1.78 ± 0.02	3.26 ± 0.04

Exponentially growing cells were treated with inhibitors for 2 hr before measuring 2-DOG uptake. Kinetic data were derived using Lineweaver–Burk plots and regression analysis. Values are the means ± SEM of a minimum of three separate experiments, each performed in triplicate.

* Data from Table 1 repeated for comparison.

† Significantly different from control untreated cells, $P < 0.01$.

indicating indirect effects of genistein on glucose transport in 32D cells. Staurosporine and wortmannin also inhibited IL-3-stimulated 2-DOG uptake in parental cells and inhibited Glut activation, suggesting a role for PKC and PI-3 kinase in the regulation of IL-3-mediated Glut function. These results are similar to those reported for insulin-stimulated glucose transport in adipocytes and muscle cells [27, 28], but in these cases translocation of Glut-4 and Glut-1 was inhibited. Moreover, evidence that tyrosine phosphorylation may be involved in regulating the intrinsic activity of Gluts in adipocytes has been presented previously using genistein [26]. Wortmannin has also been shown to inhibit insulin-, platelet-derived growth factor (PDGF)- and epidermal growth factor (EGF)-stimulated glucose transport in 3T3-adipocytes, but again inhibition of transporter translocation was involved [27, 42]. On the other hand, the tyrosine phosphatase inhibitor vanadate and the serine/threonine phosphatase type 2A inhibitor okadaic acid enhanced both basal and IL-3-stimulated glucose transport and increased transporter affinity for glucose, suggesting that the level of protein phosphorylation on both tyrosine and serine/threonine influences the activation state of transporters. The possibility that the inhibitors of protein kinases and phosphatases may decrease transporter expression at the plasma membrane can be excluded, as 2 hr is insufficient time for substantial transcription, translation, and processing to occur. However, the possibility that these inhibitors might inhibit/stimulate glucose uptake in 32D cells by translocation has not been investigated in this study.

In transformed 32D cells, genistein inhibited glucose transport and reduced transporter affinity for glucose, indicating that inhibition of tyrosine phosphorylation of key proteins might play a central role in the regulation process. Genistein has been shown to inhibit basal 2-DOG uptake in human erythrocytes and in leukemic HL-60 and CHO cells by interacting with Glut-1 directly [43], but that study differed from the present one as 2-DOG uptake in this study was measured in the absence of genistein. Although staurosporine inhibited 2-DOG uptake in oncogene-transformed cells, it did not greatly affect the activation state (K_m) but reduced V_{max}, consistent with the reduced 2-DOG uptake observed. With wortmannin, no effect on either 2-DOG uptake, transporter activation, or V_{max} was observed. Similar effects of wortmannin were seen in transformed RAW 267.4 cells, where wortmannin failed to inhibit *N*-formyl-methionine-leucine-phenylalanine (fMLP) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-stimulated 2-DOG uptake [31]. The lack of effect of staurosporine and wortmannin on the activation state of transformed cells indicates a distinct mechanism of regulation compared with IL-3-stimulated responses. Activation of *ras* and *abl* oncogenes in cells involves tyrosine kinases. On the other hand, different isoforms of PKC are induced in oncogene-transformed cells, which might be resistant to staurosporine and calphostin C. Glucose transport in transformed cells may also bypass the requirement for PI-3 kinases. Vanadate, however, showed differential effects on glucose transport in *ras*- and *abl*-transformed cells. Constitutive activation of tyrosine kinases might override the effect of vanadate on glucose transport in *abl*-transformed cells, while IL-3-like effects were observed in *ras*-transformed cells.

The inhibitor studies provide little information about the specific signalling pathways linking IL-3 and oncogenes with activation of Gluts, but do show that PI-3 kinase involvement in transporter activation is overidden following cell transformation with *ras* and *abl* oncogenes. Preliminary experiments with genistein in 32Dcl3 and *ras*- and *abl*-transformed 32D cells have shown inhibition of ty-

rosine-phosphorylated proteins with a molecular mass of 60–80 and 160–170 kDa. Similar proteins were inhibited when 32Dcl3 was deprived of IL-3 for 24 hr, indicating that growth factor withdrawal and genistein treatment causes an inhibition of the same set of tyrosine-phosphorylated proteins in 32D cells. The involvement of such proteins in the regulation of glucose transport in 32D cells is currently under investigation. Most growth factor signalling pathways that have been studied, e.g. the Ras/Raf/MAP kinase pathway and Jak/Stat pathways, involve transcriptional activation via tyrosine kinases. However, much less is known about membrane proximal signalling events that lead to acute regulation of integral plasma membrane proteins. Both tyrosine kinases and serine/threonine kinases appear to be involved in acute regulation of glucose transport, but the nature of these kinases and whether they are components of other well-studied pathways or uniquely adapted to the regulation of glucose transport remain to be determined.

This study provides evidence that intrinsic activation is an underlying mechanism regulating glucose transport in cells in response to growth factors and oncogenes. Involvement of tyrosine phosphorylation is crucial for both IL-3- and oncogene-induced responses; serine/threonine and PI-3 kinases appear to be less important for oncogene-induced responses but essential for IL-3-stimulated responses. Future studies designed for further understanding the regulation of glucose transport and elucidating the regulatory proteins involved are under investigation.

The authors wish to thank Dr. Kenneth Watson, Department of Molecular and Cellular Biology, University of New England, Armidale, Australia, for his useful comments during the preparation of the manuscript. This work was supported by the Health Research Council and the Cancer Society of New Zealand.

References

1. Carruthers A, Facilitated diffusion of glucose. *Physiol Rev* **70:** 1135–1176, 1990.
2. Gould GW and Holman GD, The glucose transporter family: Structure, function and tissue-specific expression. *Biochem J* **295:** 329–341, 1993.
3. Birnbaum MJ, Haspel HC and Rosen OM, Transformation of rat fibroblasts by FSV rapidly increases glucose transporter gene transcription. *Science* **235:** 1495–1498, 1987.
4. Flier JS, Mueckler MM, Usher P and Lodish HF, Elevated levels of glucose transport and transporter messenger RNA are induced by *ras* or *src* oncogenes. *Science* **235:** 1492–1495, 1987.
5. Dexter TM, Whetton AD and Bazill GW, Haemopoietic cell growth factor and glucose transport. Its role in cell survival and the relevance of this in normal haemopoiesis and leukaemia. *Differentiation* **27:** 163–167, 1984.
6. Nefesh I, Bauskin AR, Alkalay I, Golembo M and Ben-Neriah Y, IL-3 facilitates lymphocyte hexose transport by enhancing the intrinsic activity of the transport system. *Int Immunol* **3:** 827–831, 1991.
7. Berridge MV, Tan AS, McCoy KD, Kansara M and Rudert F, CD95 (Fas/Apo-1)-induced apoptosis results in loss of glucose transporter function. *J Immunol* **156:** 4092–4099, 1996.
8. Haspel HC, Wilk EW, Birnbaum MJ, Cushman SW and Rosen OM, Glucose deprivation and hexose transporter polypeptides of murine fibroblasts. *J Biol Chem* **261:** 6778–6789, 1986.
9. Azevedo JJ, Carey JO, Pories WJ, Morris PG and Dohm GL, Hypoxia stimulates glucose transport in insulin-resistant skeletal muscle. *Diabetes* **44:** 695–698, 1995.
10. Suzuki K and Kono T, Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc Natl Acad Sci USA* **77:** 2542–2545, 1980.
11. Cushman SW and Wardzala LJ, Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J Biol Chem* **255:** 4758–4762, 1980.
12. Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA and Cushman SW, Cell surface labelling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J Biol Chem* **265:** 18172–18179, 1990.
13. Whitesell RR and Abumrad NA, Increased affinity predominates in insulin stimulation of glucose transport in the adipocytes. *J Biol Chem* **260:** 2894–2899, 1985.
14. Toyoda N, Flanagan JE and Kono T, Reassessment of insulin effects on the V_{max} and K_m values of hexose transport in isolated rat epididymal adipocytes. *J Biol Chem* **262:** 2737–2745, 1987.
15. Whetton AD, Bazill GW and Dexter TM, Stimulation of hexose uptake by haemopoietic cell growth factor occurs in WEHI-3B myelomonocytic leukaemia cells: A possible mechanism for the loss of growth control. *J Cell Physiol* **123:** 73–78, 1985.
16. Takagi H, Tanihara H, Seino Y and Yoshimura N, Characterization of glucose transporter in cultured human retinal pigment epithelial cells: Gene expression and effects of growth factors. *Invest Ophthalmol Vis Sci* **35:** 170–177, 1994.
17. Bilan PJ, Mitsumoto Y, Ramlal T and Klip A, Acute and long-term effects of insulin-like growth factor I on glucose transporters in muscle cells. Translocation and biosynthesis. *FEBS Lett* **298:** 285–290, 1992.
18. Berridge MV and Tan AS, IL-3 facilitates glucose transport in a myeloid cell line by regulating the affinity of the glucose transporter for glucose: Involvement of protein phosphorylation in transporter activation. *Biochem J* **305:** 843–851, 1995.
19. Hamilton JA, Vairo G and Lingelbach SR, Activation and proliferation signals in murine macrophages: Stimulation of glucose uptake by hemopoietic growth factors and other agents. *J Cell Physiol* **134:** 405–412, 1988.
20. Hiraki Y, Garcia de Herreros A and Birnbaum MJ, Transformation stimulates glucose transporter gene expression in the absence of protein kinase C. *Proc Natl Acad Sci USA* **86:** 8252–8256, 1989.
21. Shawver LK, Olson SA, White MK and Weber MJ, Degradation and biosynthesis of the glucose transporter protein in chicken embryo fibroblasts transformed by the *src* oncogene. *Mol Cell Biol* **7:** 2112–2118, 1987.
22. Yamamoto T, Seino Y, Fukumoto H, Koh G, Yano H, Inagaki N, Yamada Y, Inoue K, Manabe T and Imura H, Over-expression of facilitative glucose transporter gene in human cancer. *Biochem Biophys Res Commun* **170:** 223–230, 1990.
23. Onetti R, Baulida J and Bassols A, Increased glucose transport in *ras*-transformed fibroblasts: A possible role for *N*-glycosylation of GLUT1. *FEBS Lett* **407:** 267–270, 1997.
24. White MK, Bramwell ME and Harris H, Kinetic parameters of hexose transport in hybrids between malignant and nonmalignant cells. *J Cell Sci* **62:** 49–80, 1983.
25. Smith RM, Tiesinga JJ, Shah N, Smith JA and Jarett L,

Genistein inhibits insulin-stimulated glucose transport and decreases immunocytochemical labeling of GLUT4 carboxyl-terminus without affecting translocation of GLUT4 in isolated rat adipocytes: Additional evidence of GLUT4 activation by insulin. *Arch Biochem Biophys* **300:** 238–246, 1993.
26. Nishimura H and Simpson IA, Staurosporine inhibits phorbol 12-myristate 13-acetate and insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters in rat adipose cells. *Biochem J* **302:** 271–277, 1994.
27. Clarke JF, Young PW, Yonezawa K, Kasuga M and Holman GD, Inhibition of the translocation of GLUT1 and GLUT4 in 3T3–L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem J* **300:** 631–635, 1994.
28. Carey JO, Azevedo JJ, Morris PG, Pories WJ and Dohm GL, Okadaic acid, vanadate and phenylarsine oxide stimulate 2-deoxyglucose transport in insulin-resistant human skeletal muscle. *Diabetes* **44:** 682–688, 1995.
29. Rondinone CM and Smith U, Okadaic acid exerts a full insulin-like effect on glucose transport and glucose transporter 4 translocation in human adipocytes. Evidence for a phosphatidylinositol 3-kinase-independent pathway. *J Biol Chem* **271:** 18148–18153, 1995.
30. McKoy K, Ahmed N, Tan A and Berridge MV, Interleukin-3 increases the specific activity of plasma membrane glucose transporters in a bone-marrow derived cell line: Possible involvement of phosphorylation in the activation of transporters. *J Biol Chem* **272:** 17276–17282, 1997.
31. Ahmed N, Kansara M and Berridge MV, Acute regulation of glucose transport in a monocyte-macrophage cell line: Glut-3 affinity for glucose is enhanced during the respiratory burst. *Biochem J* **327:** 369–375, 1997.
32. Ahmed N and Berridge MV, Regulation of glucose transport by interleukin-3 in growth factor-dependent and oncogene-transformed bone marrow-derived cell lines. *Leuk Res* **21:** 609–618, 1997.
33. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ and Eckner RJ, Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic cell lines. *Proc Natl Acad Sci USA* **80:** 2931–2935, 1983.
34. Stein RB, Tai JY and Scolnick EM, Molecular cloning of the temperature-sensitive 371 Kirsten murine sarcoma virus and expression in *Escherichia coli* of the mutant and wild-type Kirsten ras p21 proteins. *J Virol* **60:** 782–786, 1986.
35. Kipreos ET, Lee GJ and Wang JYJ, Isolation of temperature-sensitive tyrosine kinase mutants of v-*abl* oncogene by screening with antibodies for phosphotyrosine. *Proc Natl Acad Sci USA* **84:** 1345–1349, 1987.
36. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
37. Bramwell ME, Davies A and Baldwin SA, Heterogeneity of the glucose transporter in malignant and suppressed hybrid cells. *Exp Cell Res* **188:** 97–104, 1990.
38. Kitagawa T, Tsuruhara Y, Hayashi M, Endo T and Stanbridge EJ, Tumor-associated glycosylation change in the glucose transporter Glut-1 controlled by tumour suppressor function in human cell hybrids. *J Cell Sci* **108:** 3735–3743, 1995.
39. Shi Y, Liu H, Vanderburg G, Samuel SJ, Ismail BF and Jung CY, Modulation of GLUT1 intrinsic activity in clone 9 cells by inhibition of oxidative phosphorylation. *J Biol Chem* **270:** 21772–21778, 1995.
40. Liu H, Xiong S, Shi Y, Samuel SJ, Lachaal M and Jung CY, ATP-sensitive binding of a 70-kDa cytosolic protein to the glucose transporter in rat adipocytes. *J Biol Chem* **270:** 7869–7875, 1995.
41. Cheatham B, Volchuk A, Kahn CR, Wang L, Rhodes CJ and Klip A, Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins. *Proc Natl Acad Sci USA* **93:** 15169–15173, 1996.
42. Conricode KM, Involvement of phosphatidylinositol 3-kinase in stimulation of glucose transport by growth factors in 3T3–L1 adipocytes. *Biochem Mol Biol Int* **36:** 835–843, 1995.
43. Vera JC, Reyes AM, Carcamo JG, Velasquez FV, Rivas CI, Zhang RH, Strobel P, Iribarren R, Scher HI, Slebe JC and Golde DW, Genistein is a natural inhibitor of hexose and dehydroascorbic acid transport through the glucose transporter, GLUT1. *J Biol Chem* **271:** 8719–8724, 1996.