Increased glucose transport in *ras*-transformed fibroblasts: a possible role for *N*-glycosylation of GLUT1

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Abstract 2-Deoxyglucose uptake was enhanced in ts371 KiMuSV-NRK cells when growing at the permissive temperature to allow the expression of a transforming p21 *ras* protein. This change is due to a decrease in the K_m by approximately 2.5-fold without affecting the V_{max} of the transporter. The amount of the GLUT1 glucose transporter dit not increase as deduced from immunoblot experiments on total membranes. Nevertheless, *ras*-transformed GLUT1 displays a higher molecular mass due to an increased *N*-glycosylation of the protein. Experiments made in tunicamycin-treated cells indicates that a higher glycosylation is responsible for the increase in 2-deoxyglucose uptake in *ras*-transformed cells.

Key words: Glucose transport; *ras* Transformation; GLUT1; *N*-Glycosylation; Rat fibroblast

1. Introduction

One of the most characteristic and early recognized biochemical markers of the transformed phenotype is the high glucose utilization [1–3]. The facilitated uptake of glucose across the plasma membrane of mammalian cells is mediated by a family of integral membrane glycoproteins, namely, the GLUT transporters [4,5]. Among them, the ubiquitous GLUT1 glucose transporter is responsible for the increased glucose transport induced by oncogenes and growth factors. In some cases, the mechanism of this activation has been attributed to an increase in GLUT1 mRNA levels. This finding has been described in fibroblasts [6–8] as well as in adipocytes [9] after *ras* transformation and it appears as a result of the transcriptional activation of the gene. Two enhancer elements have been described to be involved in the response to oncogenes and growth factors [10]. However, others have found that the higher glucose transport accounted in *ras*-transformed 3T3-L1 fibroblasts or adipocytes is not due to a higher expression of the gene, but to a translocation of GLUT1 from an intracellular pool to the cell surface [11].

The intrinsic activity of GLUT1 may be also modulated through changes in the glycosylation level of the protein [12,13] and this seems to be the mechanism by which TGF-β induces glucose transport in Swiss 3T3 cells [14].

Here we present evidence that changes in GLUT1 glycosylation can be responsible of the *ras* oncogene stimulation of 2-deoxyglucose uptake in KiMuSV-NRK cells.

2. Materials and methods

2.1. Materials

Tunicamycin was from Boehringer Mannheim. Reagents for cell culture were from Gibco. 2-Deoxy-D-[U-^{14}C]glucose was from New England Nuclear. The Bb polyclonal antibody against brain GLUT1 was a gift from Dr. A. Zorzano (Dept. Bioquímica, Universitat de Barcelona, Spain). All the others chemicals and were purchased from Sigma or Boehringer Mannheim.

2.2. Cell culture

NRK-44F and ts371 KiMuSV-NRK cells were gently supplied by Dr. J. Massagué (Sloan Kettering Cancer Institute, New York). Ki-MuSV-NRK cells originate from NRK cells which carry the KiMuSV with a point mutation in the Ki-*ras* oncogene (*ts*371 KiMuSV) that confers thermolability to the oncogenic protein p21^{Ki-ras}, which is rapidly degraded at 37°C while it remains stable at the permissive temperature of 32°C [15,16]. Thus, when these cells were grown at the permissive temperature of 32°C they displayed a transformed phenotype with a rounded morphology and high refringency, whereas they showed a flat morphology when grown at the non-permissive temperature of 37°C. Cells were cultured in a humidified atmosphere with 5% CO_2, in Dulbecco's Modified Eagle Medium (DME) containing 10% foetal calf serum with 100 IU penicillin/ml and 100 μg/ml streptomycin, at 32°C or 37°C. After 5 days in these conditions, cells were incubated in a glutamine-free medium (incubation medium: 20 mM Hepes, pH 7.2, 126 mM NaCl, 15 mM $NaHCO_3$, 3.8 mM KCl, 0.9 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.3 mM $CaCl_2$, 6 mM glucose) for 20 h [17]. In these conditions, cells are quiescent and the glycolytic flux is low. The cell density was 1.15–1.30×10^5 cells/cm^2 with a 80% confluence. In experiments with tunicamycin, this agent was added to achieve the desired concentration and the controls received the same volume of dimethyl sulfoxide as vehicle (0.01% v:v).

2.3. Determination of 2-deoxyglucose uptake

The uptake of 2-deoxyglucose was determined essentially as described previously [18], using 2-deoxy-D-[U-^{14}C]glucose. This assay is based in the fact that 2-deoxyglucose is readily taken up and phosphorylated by the cells, but it is not further metabolized. Confluent cells (35 mm plaques) were incubated at 37°C for the 5 h previous to the assay to eliminate the effects of temperature on cell growth and glucose concentration in the medium. After washing 3 times in glucose-free medium, the assay was performed at room temperature in the same medium without glucose in the presence of 100 μM 2-deoxy-D-[U-^{14}C]glucose (0.3 μCi/ml). After incubation, 1 ml of 0.1% SDS was added to each plaque and the radioactivity in the extract determined by liquid scintillation counting. Total uptake was corrected for simple diffusion by substracting the amount of label taken up in the presence of 20 μM cytochalasin B.

2.4. Western blot experiments

Cells were harvested with cold phosphate-buffered saline and centrifuged at $12000\times g$ for 3 min. Then, cell pellets were solubilized in extraction buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, leupeptin 20 μg/ml) and centrifuged at $1200\times g$ for 20 min. Supernatants were subjected to SDS-PAGE. Samples were then transferred to a Immobilon-P membrane (Millipore), incubated with the Bb antibody and visualized by autoradiography by using ^{125}I-labeled protein A (Amersham).

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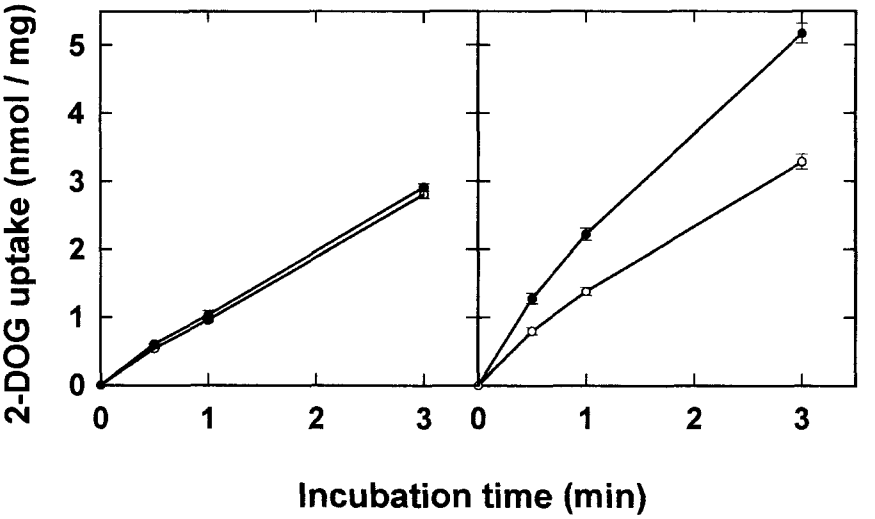

Fig. 1. 2-Deoxyglucose uptake in NRK (left panel) and KiMuSV-NRK cells (right panel). 2-Deoxyglucose uptake was studied in NRK and KiMuSV-NRK cells grown at 37°C (○) or 32°C (●). Cells were maintained in incubation medium for 20 h at the appropriate temperature, the medium was renewed and all cells were incubated at 37°C for 5 h. The assay was performed at room temperature as described in Section 2. Values are means ± SEM for three independent experiments performed by triplicate.

2.5. Digestion with glycosidase

Solubilized cells were incubated at 37°C for 16 h with 10 U/ml of *N*-glycosidase F (Boehringer Mannheim) as described in the commercial protocol. Fetuin was used as control of complete digestion.

2.6. Statistical analysis and presentation of results

Results are given as means ± SEM for the number of independent experiments (i.e. distinct cell culture dishes) specified. Significance of differences between treatment was determined by Student's *t*-test.

3. Results

To investigate the role of glucose transport in *ras*-mediated transformation, 2-deoxyglucose uptake was measured in KiMuSV-NRK cells grown at 37°C or at the permissive temperature of 32°C. Fig. 1 shows a time course assay where an increase in the sugar uptake is observed in transformed cells compared to non-transformed KiMuSV-NRK cells. The uptake of 2-deoxyglucose was not modified in the parental NRK cells, indicating that growth temperature does not affect the uptake. The kinetic parameters of this transport were also studied in normal and *ras*-transformed cells, showing a decrease in the K_m values (5.13 ± 0.77 vs. 2.02 ± 0.35 mM, respectively) and no changes in V_{max} values (24.6 ± 2.96 vs. 26.4 ± 1.09 nmol/mg × min, respectively), indicating a higher affinity for the substrate in transformed cells.

When cells were grown at the permissive temperature for the maintenance of the transformed phenotype, no apparent changes were observed in the amount of GLUT1 in a Western blot assay using the polyclonal antibody Bb (Fig. 2). This figure shows that an heterogeneous 47–75 kDa broad band was detected in cell lysates from non-transformed cells, similarly to the results obtained in other cell types [7,14,19]. In contrast, when cells were grown previously at the permissive temperature of 32°C to achieve the transformed phenotype, the molecular mass of GLUT1 was markedly increased to about 60–90 kDa. These differences in size are due to changes in the pattern of *N*-glycosylation of GLUT1 since when cell lysates were digested with *N*-glycosidase F prior to the Western blot, the differences in the molecular mass of GLUT1 from normal and transformed cells disappeared (Fig. 2). Likewise, a 38 kDa well-defined band was observed in normal and *ras*-transformed cells, supporting our hypothesis that differences in molecular mass were due to changes in the *N*-glycosylation pattern of the molecule. This band correspond to the previously described unglycosylated form of GLUT1 [13].

In order to verify whether changes in *N*-glycosylation were responsible for the changes in 2-deoxyglucose uptake in *ras*-transformed cells, tunicamycin was used as inhibitor of *N*-

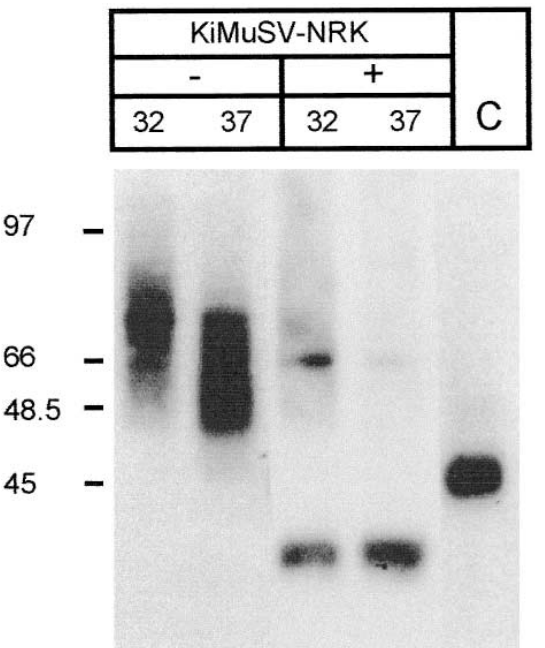

Fig. 2. Immunodetection of GLUT1 from normal and transformed KiMuSV-NRK cells. Effect of deglycosylation. Cells lysates from KiMuSV-NRK cells grown at 37°C and 32°C were analyzed by Western blot using the Bb polyclonal antibody (10 μg protein/lane) before (−) and after (+) digestion with *N*-glycosydase F. Molecular mass markers are shown in the left (kDa). The positive control (C) corresponds to 10 μg of rat brain extract.

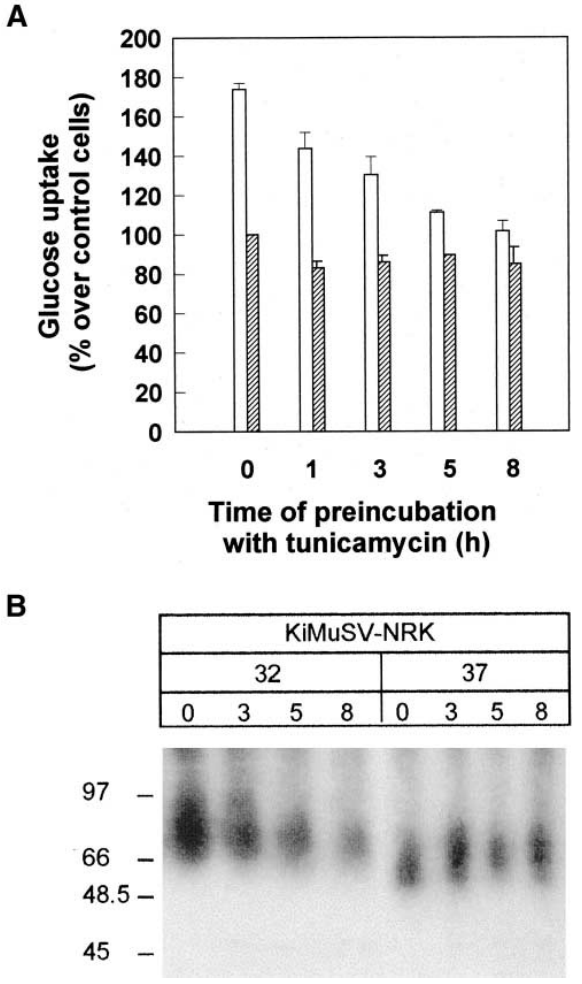

Fig. 3. A: Effects of tunicamycin on 2-deoxyglucose uptake in normal and *ras*-transformed KiMuSV-NRK cells. KiMuSV-NRK cells were grown at 32°C (open bars) or 37°C (hatched bars). Cells were maintained in incubation medium for 20 h at the appropriate temperature, the medium was renewed and all cells were incubated at 37°C in the presence of 25 μM tunicamycin for the indicated period of time. 2-Deoxyglucose uptake assay was performed at room temperature with an incubation time of 3 min as described in Section 2. Values are means ± SEM for 3 independent experiments performed by triplicate. B: Immunodetection of GLUT1 in normal and *ras*-transformed KiMuSV-NRK cells treated with tunicamycin. KiMuSV-NRK cells were grown at 32°C or 37°C. Cells were maintained in incubation medium for 20 h at the appropriate temperature, the medium was renewed and all cells were incubated at 37°C in the presence of 25 μM tunicamycin for the indicated period of time. Cells lysates were analyzed by Western blot using the Bb polyclonal antibody (10 μg protein/lane). Molecular mass markers are shown in the left (kDa).

glycosylation. Fig. 3a shows that treatment of cell cultures with 25 μM tunicamycin produced a decrease in the sugar uptake in transformed cells in a time-dependent manner, reaching similar values that those observed in non-transformed cells after 8 h of incubation with the agent. On the other hand, this agent does not have significant effects when added to non-transformed cells. A parallel Western blot experiment of GLUT1 in tunicamycin treated cells shows that this compound does induce a decrease in the molecular mass of the transporter in transformed cells, supporting the hypothesis that changes in the level of *N*-glycosylation are responsible for the increase in 2-deoxyglucose uptake in *ras*-transformed KiMuSV-NRK cells (Fig. 3b).

4. Discussion

Cell transformation is accompanied of changes in many cellular functions, perturbing normal cellular growth and their control mechanisms. One such alteration is a marked increase in glucose consumption, also accounted in mitogen-stimulated cells [2,3,20,21]. On the other hand, another characteristic of transformed cells is an increase in *N*-glycosylation of cell surface carbohydrates (also known as the 'Warren phenomenon' [22]) and it has been described that specific changes such as an increase in β1-6 branched oligosaccharides are associated with malignancy [23,24].

We have found a raise in 2-deoxyglucose uptake in KiMuSV-NRK cells when growing at the permissive temperature to maintain the *ras*-transformed phenotype, as compared with non-transformed cells. This finding is consistent with other published works, since an increase in 2-deoxyglucose uptake has been described in different cell types transformed by *ras* oncogenes. Several mechanisms have been described as responsible for the increase in sugar uptake, such as a higher concentration of GLUT1 protein [6–9] or the translocation of GLUT1 from an intracellular pool to the cell surface [11]. In fact, several evidences suggest that the specific cellular system may be determinant in the mechanism by which oncogenes trigger glucose uptake. Thus, in chicken embryo fibroblasts transformed by *src*, an increase in the number of GLUT1 molecules due to a lower turnover seems to be responsible of this change [20], whereas in *src*-transformed mouse fibroblasts an increase in mRNA expression has been described [21].

In our system, we have shown that the increase in 2-deoxyglucose uptake is not consequence of an increase in the amount of GLUT1, but of a change in the *N*-glycosylation pattern of the transporter, which is evidenced as a shift from a broad band of 47–75 kDa in non-transformed KiMuSV-NRK cells to a band of 60–90 kDa in their transformed counterpart. This is relevant because, although it has been described that the *N*-glycosylation of whole membrane glycoproteins is increased in *ras*-transformed cells [25–27], this is the first time that *ras* oncogene has been shown to affect the *N*-glycosylation state of GLUT1. Oligosaccharides, especially *N*-linked chains, contribute to the stability and biological function of the molecules [28] and changes have been identified in specific proteins, which alter in one way or another their properties. Although functions such as recognition and binding many times are down-regulated by glycosylation, increased *N*-glycosylation has been also associated with a higher biological activity: this is a case, for example, of E-cadherin [29] or the α5β1 integrin receptor [30].

Several studies suggest a role for *N*-glycosylation of GLUT1 in glucose transport: a *N*-linked carbohydrate addition occurs at Asn-45 [13,31] but there is discrepancy on how this glycosylation affects kinetic parameters. Thus, Haspel and coworkers [12] have shown an increase in transport activity after depletion of asparagine-linked oligosaccharides, while a decrease in the V_{max} for glucose uptake has been reported in 3T3 cells after inhibition of glycosylation with tunicamycin [32]. More recently, a loss of transport activity after *N*-glycanase treatment of GLUT1 has been reported [33]. Glycosyla-

tion-defective mutants show a higher K_m and it has been postulated a role of glycosylation in maintaining a structure of GLUT1 with high affinity for glucose [13]. Furthermore, these mutants are defective in the targeting to the plasma membrane and present a shorter half-life than wild-type cells [34].

In KiMuSV-NRK cells, changes in *N*-glycosylation lead to a higher affinity for glucose as a result of a decrease in the K_m without apparent changes in the V_{max}. This finding is in agreement with the results reported in tumorigenic human hybrids, where a decrease in the K_m occurs associated to an increase in *N*-glycosylation of GLUT1 [35], in Swiss 3T3 cells treated with TGF-β [14] or in a respiratory-deficient mutant of the V79 hamster fibroblast cell line [36]. In these two cases, an increase in glucose transport occurs concomitant to an hyperglycosylation of the molecule in the absence of changes in the amount of protein.

In summary, our results support the motion that, together with regulation of gene expression and cell localization, changes in *N*-glycosylation could be a physiological mechanism of glucose transport control.

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