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Glycosylation of the human erythrocyte glucose transporter is essential for glucose transport activity

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The human erythrocyte glucose transporter is a fully integrated membrane glycoprotein having only one *N*-linked carbohydrate chain on the extracellular part of the molecule. Several authors have suggested the involvement of the carbohydrate moiety in glucose transport, but no definitive results have been published to date. Using transport glycoproteins reconstituted in proteoliposomes, kinetic studies of zero-trans influx were performed before and after *N*-glycanase treatment of the proteoliposomes: this enzymatic treatment results in a 50 % decrease of the V_{max}. The orientation of transport glycoproteins in the lipid bilayer of liposomes was investigated and it appears that about half of the reconstituted transporter molecules are oriented properly. Finally, it could be concluded that the release of the carbohydrate moiety from the transport glycoproteins leads to the loss of their transport activity.

Introduction

The glucose transporter of human erythrocytes is one of the most used model to study facilitated-diffusion transport with a single substrate and kinetics and biological aspects of this transport have thus been extensively reviewed [1–3]. The protein responsible for glucose transport in human erythrocytes runs as band 4.5 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of red blood cell membranes [4]. Mueckler et al. [5] described the complete structure of the glucose transporter in HepG2 hepatoma cells that has a high homology with the human red cell glucose transporter: it is a fully integrated membrane glycoprotein having 12 membrane-spanning α-helices, carrying one *N*-linked glycosidic chain on Asn-45. Kinetic studies performed on erythrocytes [6] indicated that glucose transport could be described by an asymmetrical carrier model in which the glucose efflux was higher than the glucose influx. Such an asymmetry in the glucose transport could originate from cytosolic factors [7] as ATP, but a recent study [8] performed on a reconstituted system demonstrated that ATP does not regulate the glucose transport.

Previously, we showed [9] that the carbohydrate chain of the erythrocyte glucose transporter exhibited a large structural microheterogeneity and may possibly be involved in glucose transport. Such a possibility was also considered by several authors. Kitagawa et al. [10] demonstrated that tunicamycin inhibition of protein glycosylation in Swiss 3T3 cells resulted in the decrease of hexose uptake in dose- and time-dependent manners. In contrast, Haspel et al. [11] reported that glucose deprivation altered the glycosylation of the glucose transporter in murine fibroblasts and, consequently, these cells exhibited 5-fold increases in hexose transport and glucose-displaceable cytochalasin B binding. This increase was attributed to a higher amount of normally glycosylated glucose transporter in the cell membrane, non-glycosylated glucose transporter in this case, being either not functional or not localized on the cell surface. These analyses suggest that alterations in asparagine-linked oligosaccharides may affect the function and the biosynthesis of the glucose transporter. More recently, the same group [12] showed that the glucose transporter produced by a cell mutant, which expressed a transport glycoprotein having a high-mannose instead of the complex-type glycosidic chain present in normal cells, exhibits glucose transport kinetic parameters similar to

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those of normal cells. From these conflicting results, it appears that the biological role specifically attributable to the carbohydrate moiety of the glucose transporter remains hypothetical.

In this paper, we present new information concerning the possible biological role of the glycosidic chain of the human erythrocyte glucose transporter. For that purpose, kinetic parameters of glucose transport were measured on native and enzymatically deglycosylated proteins. In order to overcome side effects generated by the presence of cytosolic factors or/and by differences in glycoprotein turnover, all determinations were made on purified transporters reconstituted in proteoliposomes.

Materials and Methods

Materials

Freshly outdated human blood was kindly provided by a blood bank. L-[1-^{14}C]Glucose and D-[U-^{14}C]glucose, with respective specific activities of 2 and 10 GBq/mmol, were obtained from Amersham-France. *N*-Glycanase (EC 3.5.1.52) from *Flavobacterium meningosepticum* was purchased from Genzyme (U.S.A) and *L*-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) trypsin from bovine pancreas (EC 3.4.21.4) was obtained from Worthington (U.S.A.). Electrophoresis reagents were purchased from Bio-Rad (U.S.A), except for sodium dodecyl sulfate which was obtained from Pierce (U.S.A.). Other chemicals were obtained from Sigma (U.S.A.).

Purification of the glucose transporter and preparation of proteoliposomes

The human erythrocyte glucose transporter was purified according to Baldwin et al. [13]. It was reconstituted in proteoliposomes by addition of egg lecithin and dialysed for two days against the following buffer (dialysis buffer): 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM of sodium chloride and 1 mM of ethylenediaminotetracetic acid (EDTA).

N-Glycanase treatment of proteoliposomes

Two 20 ml portions of proteoliposome suspension were centrifuged at 130 000 × *g* for 60 min at 4° C. One of pellet was mixed in 20 µl of *N*-glycanase solution (250 units/ml) and 230 µl of 200 mM sodium phosphate buffer (pH 8.6) (sample 1). The other one was resuspended in 250 µl of sodium phosphate buffer (sample 2). Both samples were incubated at 37° C for 24 h under nitrogen, then 10 µl of enzyme solution and 10 µl of phosphate buffer were added to samples 1 and 2, respectively, and incubated again under the same conditions for 12 h. Each sample was then diluted with 20 ml of dialysis buffer. After vigorous stirring, both suspensions were centrifuged at 130 000 × *g* for 60 min at 4° C. The pellets were resuspended in 5 ml of dialysis buffer and used for kinetic studies.

Trypsin treatment

Native or deglycosylated proteoliposomes were treated under mild conditions with TPCK trypsin from bovine pancreas [14]. Enzyme at a final concentration of 5 µg/ml was added to the liposome suspension in dialysis buffer and the mixture was incubated for 1 h at 37° C. The same amount of enzyme was then added and the degradation continued for 2 h. After treatment, samples were handled in the same way as indicated for *N*-glycanase treatment.

Zero-trans influx

Kinetic parameters were measured according to modifications of the procedures described by Helgerson and Carruthers [15] and Carruthers and Melchior [16]. All glucose transport assays were performed at 15° C. At t_0, 50 µl of a glucose solution of determined concentration (1, 2, 4, 10 and 20 mM), containing radioactive glucose in order to obtain a final specific activity of 0.11 GBq/mmol, were added to 50 µl of the liposome suspension and simultaneously well stirred. At time t_1, 500 µl of a cold 2 mM mercuric chloride solution (4° C) were rapidly added and immediately stirred in order to stop glucose transport. The resulting mixture was ultracentrifuged at 400 000 × *g* for 3 min in a Beckman TL 100 centrifuge. The supernatant was discarded and the pellet was washed twice with 500 µl of the mercuric chloride solution and ultracentrifuged under the same conditions. Finally, the pellet was dissolved in 100 µl of 1% Triton X-100 solution and the radioactivity was measured by liquid scintillation counting. At least five determinations were made for each point.

The non-specific adsorption was estimated by measuring the D-glucose transported at t_0. Alternatively, the take-up of L-glucose was also used as blank [17].

Measurement of the glucose transport in the presence of competitive inhibitors

Inhibition by cytochalasin B. 5µl of cytochalasin B solution (2.3 mg in 1 ml of ethanol) were placed in a 5 ml vial and evaporated under nitrogen. 500 µl of glucose solution were then added to the residue and the mixture was vigorously shaken. Glucose transport assays were made as described above using a cytochalasin B-containing glucose solution.

Other specific inhibitors [6]. 1-Propyl-D-glucose and 4,6-*O*-ethylidene-D-glucose were used and, as for cytochalasin B, they were added to the glucose solution before determinations were made.

Other methods

The protein content was determined using the Kaplan and Pedersen's procedure [18] and phospholipids were measured according to Bartlett's method [19]. The purity of glycoproteins was checked by electrophoresis on a 12% polyacrylamide gel in the presence of SDS (Pierce)

according to Laemmli [20]. Native transport glycoprotein was characterized by specific photolabeling using tritiated cytochalasin B, as previously described [21].

Results and Discussion

1. Isolation and characterization of the human erythrocyte glucose transporter

In order to prevent sample variations, each set of experiments was performed with the glucose transporter isolated from the erythrocytes of a single donor. Five different preparations were used which gave similar relative changes. Purified glycoprotein was characterized by SDS-PAGE before and after photolabeling by tritiated cytochalasin B [21]. Deglycosylation of the glucose transport glycoprotein by *N*-glycanase was previously investigated [9] and SDS-PAGE analysis of the deglycosylated material shows that no degradation of the polypeptidic chain occurred during enzyme treatment. Independently of the enzyme action, a 36 h incubation at 37 °C could result in a decrease of the transport activity. To ensure that differences in glucose transport observed between the native and deglycosylated forms of the transporter really originated from the release of the glycosidic chain, both samples were subjected to the same treatment, except for the addition of the enzyme to the native sample.

The sizes of the native and deglycosylated proteoliposomes were estimated by a nanosizer and both types of liposomes exhibited homogeneous size distribution with a maximum at 180 nm.

2. Kinetic parameters of native and deglycosylated glucose transporter

Fig. 1 shows the time course of glucose uptake in the reconstituted system at two glucose concentrations. From these data, we conclude that until $t = 20$ s, glucose uptake remains linear, when the temperature is kept at 15 °C. Moreover, comparison of Fig. 1A and B suggests that glucose uptake was lower after deglycosylation.

The specificity for glucose transport activity was checked by making measurements in the presence of cytochalasin B, a specific inhibitor of glucose transport. Using concentrations of 25 μM cytochalasin B and 5 mM glucose, and L-glucose as the reference, transport in both native and deglycosylated proteoliposomes was completely inhibited.

Measurements of glucose zero-trans influx were made at 10 s using five glucose concentrations (Fig. 2A). K_m and V_{max} for both forms of transporter were determined from the Lineweaver-Burk plot (Fig. 2B) and it appears that after deglycosylation, K_m of the transporter slightly increased, while V_{max} was dramatically decreased (Table I). Similar kinetic changes were also observed with the deglycosylated Na^+/H^+ antiporter [22] which were at-

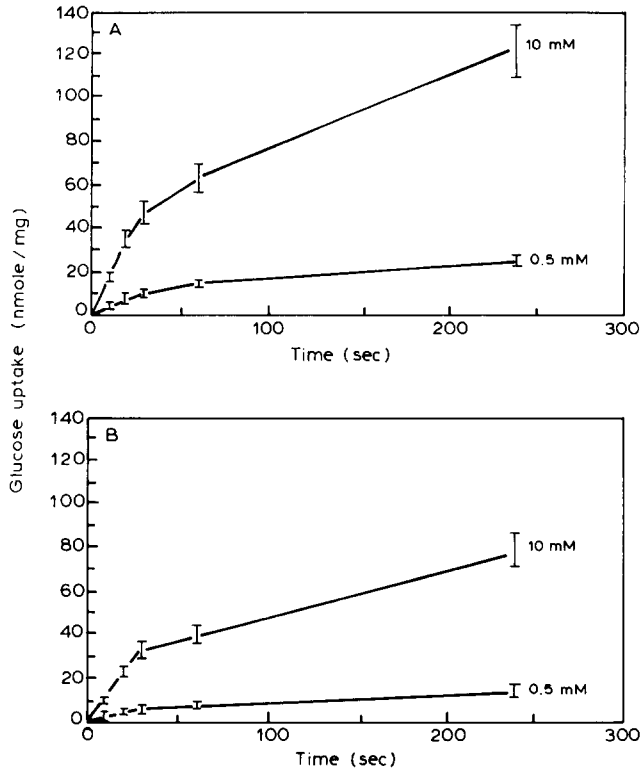

Fig. 1. Time-course of glucose uptake by native (A) and deglycosylated (B) proteoliposomes. Assays contained 0.5 mM and 10 mM D-glucose. The D-glucose uptake at t_0 was subtracted from the D-glucose uptake at each time, to give the corrected uptake.

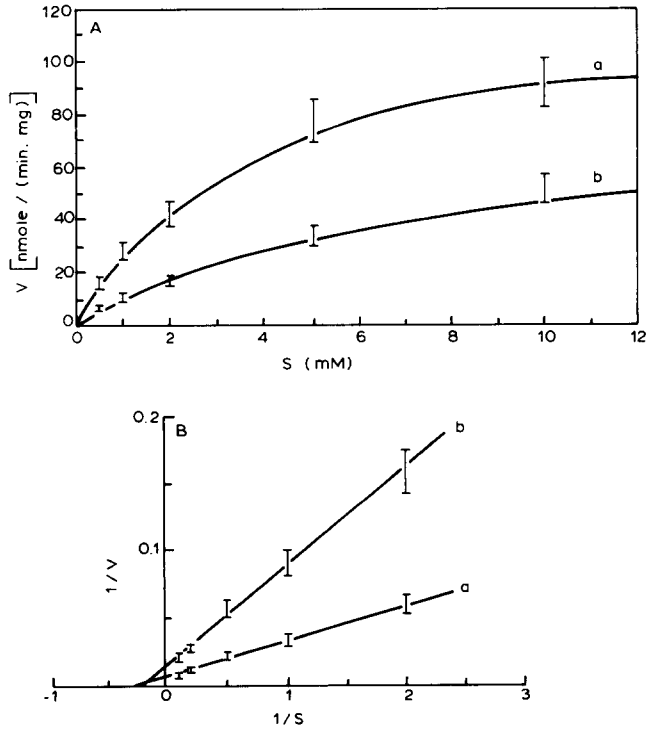

Fig. 2. Kinetics of glucose uptake by native (a) and deglycosylated (b) proteoliposomes: (A) Zero trans-influx was measured at 10 s. (B) Lineweaver-Burk plot.

TABLE I

Kinetic parameters calculated for zero-trans influx of the glucose transport in native and deglycosylated proteoliposomes, before and after trypsin treatment

The S.D. was calculated from a minimum of five independent measurements. The relative variations for V_{max} compared to the V_{max} obtained without any treatment are indicated in parentheses.

Parameter	Proteoliposomes	
	native	deglycosylated
K_m (mM)		
Before trypsin treatment	3.0± 0.5	4.5±0.5
After trypsin treatment	2.0± 0.2	1.8±0.5
V_{max} (nmol/mg per min)		
Before trypsin treatment	125 ±10 (100%)	62 ±6 (50%)
After trypsin treatment	69 ± 6 (55%)	10 ±5 (8%)

tributed, in this case, either to a decrease of the efficiency of the transporter or to a decrease of the number of active transporters. However, before any conclusion can be drawn from these hypotheses, the orientation of the transport glycoprotein in the liposome lipid bilayer must be considered.

3. Orientation of the transport glycoproteins in the proteoliposomes

After reconstitution in proteoliposomes, the transport glycoprotein could be oriented either in a normal or in a reverse position (Fig. 3): the influx observed will be a combination of influx resulting from transporters in the normal orientation working in the influx mode and influx resulting from transporters in the reverse orientation working in the efflux mode. The orientation of the glycoprotein was first investigated using specific

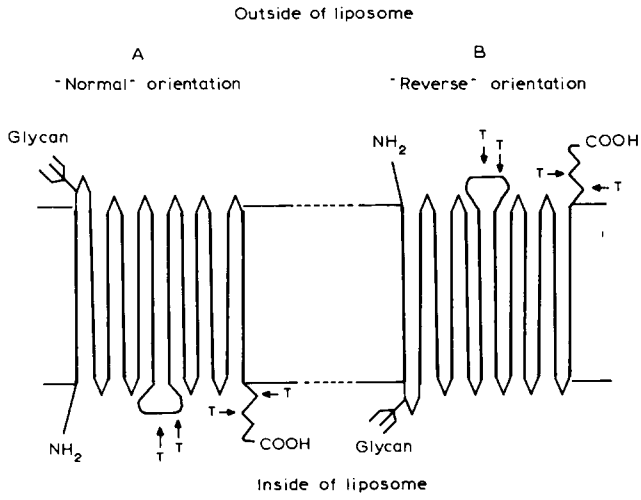

Fig. 3. Orientation of the transport glycoprotein in the lipid bilayer of proteoliposomes. (A) 'Normal' orientation in which the N-linked carbohydrate chain is on the outside face of the liposome. (B) 'Reverse' orientation in which this chain is on the inside face of the liposome. The sites of trypsin cleavage given by Mueckler et al. [5] are indicated by T →.

inhibitors of glucose transport [6]: 1-propyl-D-glucose which recognizes the inside face of the glycoprotein (efflux inhibitor) and 4,6-ethylidene-D-glucose, which recognizes the outside face of the glycoprotein (influx inhibitor). Transport of glucose at the concentration of 10 mM was measured in the presence of 90 and 400 mM of these blocking agents. For both inhibitors, a 50 % decrease in glucose transport was observed at 90 mM and a total inhibition at 400 mM. This later result is unexpected since at 400 mM a weak glucose transport activity should remain, if inhibition was competitive. It is possible that each inhibitor could diffuse into the proteoliposomes in a non-specific way and thus no conclusion as regards the orientation of the transport glycoprotein can be deduced.

Then, the transporter orientation was estimated by the method proposed by Baldwin et al. [14], i.e. the mild action of trypsin. Optimal conditions were determined and proteoliposomes subjected to this treatment. Since the sites of trypsin cleavage are all located on the internal face of the glycoprotein in its normal orientation [5], only proteoliposomes having the transporter in a reverse orientation would be degraded and the remaining glucose transport activity would be due to proteoliposomes having their transport glycoproteins in the normal orientation (Fig. 3). After trypsin treatment, the V_{max} of the native proteoliposomes was decreased of 45% and the K_m of 33% (Table I): these results are comparable to those observed by Wheeler and Hinkle [17] for similar reconstituted system, suggesting that glycoproteins in our reconstituted system exhibit a normal/reverse distribution of about 50/50.

4. Role of the glycosidic chain

Since glycoproteins in the reconstituted system are oriented about equally in both directions, at best 50% of them could be degraded by *N*-glycanase. Therefore, the expected decrease of V_{max} might vary between 0 and 50% whether the enzyme action is partial or total. As a 50% decrease was observed, we conclude that *N*-glycanase treatment abolishes the glucose transport activity of proteoliposomes normally oriented. This was confirmed by combined enzymatic treatments *N*-glycanase followed by trypsin (Table I): a residual glucose transport activity close to zero was observed.

Haspel et al. [12] showed that glucose transporters having a high-mannose carbohydrate chain instead of the complex one remain functional with moderate variation in their glucose transport activity. Likewise, it was reported [17] that endo-β-galactosidase treatment did not reduce reconstituted transport activity. It thus appears that only the complete release of the glycosidic chain could abolish the glucose transport. Nevertheless, the presence of a minimum glycosidic structure could be sufficient to maintain the glucose transport activity and we propose to explore this hypothesis. Some of these

results could be used for researchs on other transporters also present in cell membranes, such as the nucleoside transporter described by Plagemann et al. [23].

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