Biochimica et Biophysica Acta, 1066 (1991) 59–62

ADONIS 0005273691002571

BBAMEM 75277

Glycosylation of the human erythrocyte glucose transporter: a minimum structure is required for glucose transport activity

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(Received 27 December 1990)

Key words: Glucose transport; Transport kinetics; Glycosylation; (Human erythrocyte)

The involvement of the carbohydrate moiety of the human erythrocyte glucose transporter in glucose transport activity was previously demonstrated (Feugeas et al. (1990) Biochim. Biophys. Acta 1030, 60–64): *N*-glycanase treatment of the transport glycoprotein reconstituted in proteoliposomes resulted in a dramatic decrease of the V_{max} In this study, kinetic measurements of glucose equilibrium influx confirm our previous results. In order to investigate that a minimum glycosidic structure is required to maintain glucose transport activity, proteoliposomes were respectively treated with either sialidase, or sialidase and endo-β-galactosidase, or a pool of exo-glycosidases which allows the release of all the sugar residues, except the proximal *N*-acetylglucosamine. Kinetic measurements of zero-trans influx made on sialidase- and (sialidase + endo-β-galactosidase)-treated proteoliposomes did not reveal any significant changes in the glucose transport activity. On the contrary, treatment of the same proteoliposomes by a pool of exoglycosidases led to a complete abolition of activity, suggesting that a minimum glycosidic structure is required for glucose transport activity.

Introduction

The glucose transporter of human erythrocytes is one of the most commonly used models to study facilitated diffusion transport with a single substrate [2–4]. This transport is achieved by a fully integrated membrane glycoprotein having only one N-linked glycosidic chain [5].

In a previous work [6], we investigated the structure of this N-linked chain and we showed that it exhibits a large structural microheterogeneity due to the presence of complex-type carbohydrate chains carrying repetitive units of *N*-acetyllactosamine with a broad variability in the number of these units and in fucosyl and sialyl substitution. More over, significant amounts of bi-antennary and hybrid-type structures were also present. More recently, Endo et al. [7] also showed that the glycosidic moiety of the glucose transporter exhibits a structural microheterogeneity with the presence of large complex-type bi-antennary polylactosamine carbohydrate chains.

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Recently, using the native glycoprotein reconstituted in proteoliposomes [1], kinetic measurements of glucose zero-trans influx were carried out and we demonstrated that *N*-glycanase treatment of the proteoliposomes results in a dramatic decrease of the glucose transport activity. In this reconstituted system, glycoprotein molecules exhibit a normal/reverse distribution of approximatively 50:50 in the liposome membrane, and consequently, the 50% decrease in glucose transport activity that we observed after *N*-glycanase treatment of the proteoliposomes corresponds to a complete abolition of the activity for the deglycosylated transport molecules. This result strongly suggested that the carbohydrate moiety of the human erythrocyte glucose transport was involved in transport activity.

The glucose transport glycoprotein exhibited differential kinetic parameters, depending upon experimental conditions; for example, transport rate is faster when glycoproteins are filled with glucose than when they are empty [8]. Thus, it became important to demonstrate that previous results obtained from zero-trans influx measurements were not an exception attributable to some special experimental conditions, but are, indeed, a general rule. For that purpose, glucose influx was measured on native and *N*-glycanase-treated

proteoliposomes under equilibrium-exchange conditions.

Another point must also be clarified: is the structural microheterogeneity, previously observed [6], important for glucose transport activity? In order to investigate this possibility, proteoliposomes were treated with various endo- and exo-glycosidases and glucose transport activities of the resulting proteoliposomes were compared to that of native 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. Chemicals were purchased from Sigma (U.S.A.).

The human erythrocyte glucose transporter was purified and reconstituted in proteoliposomes according to Baldwin et al. [9]. Characterization of the transporter and kinetic measurements of glucose zero-trans influx were made as described previously [1].

Enzymes

N-Glycanase (EC 3.5.1.52) from *Flavobacterium meningosepticum* and endo-β-galactosidase (EC 3.2.1.103) from *Bacteroides fragilis* were obtained from Genzyme (U.S.A.). α-L-Fucosidase (EC 3.2.1.51) from bovine epididymis, β-galactosidase (EC 3.2.1.23), β-*N*-acetylhexosaminidase (EC 3.2.1.52) and α-mannosidase (EC 3.2.1.24) from jack bean were purchased from Sigma (U.S.A.). Sialidase (EC 3.2.1.18) from *Vibrio cholerae* was obtained from Behringwerke (Germany). All these enzymes were used as described previously by us and others [6,10,11]. To ensure that differences in glucose transport observed really originated from the enzymatic treatment, a portion of proteoliposomes was processed similarly, except for the addition of enzyme(s): these will be referred to as native proteoliposomes.

Equilibrium-exchange

Kinetic parameters of equilibrium-exchange influx were measured according to modifications of the procedures described by Carruthers and Melchior [12] and Helgerson and Carruthers [13]. Glucose transport assays were performed at 15 °C. 50 μl of a glucose solution of determined concentration (20, 40, 80, 120 and 160 mM) were mixed with 50 μl of the liposome suspension and set aside for 4 h at 20 °C, in order to obtain the same glucose concentration inside and outside the liposomes. At t_{zero}, 50 μl of a glucose solution at the same final concentration (10, 20, 40, 60 and 80 mM) and containing enough radioactive glucose to obtain a final specific activity of 0.11 GBq/mmol were added to the equilibrated mixture and stirred vigorously. 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 cold 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 determination of L-glucose uptake, under the above conditions.

Zero-trans influx

Measurements of zero-trans influx were performed as previously described [1] on native and enzyme-treated proteoliposomes and S. D. were calculated for each point.

Carbohydrate analysis

Residual carbohydrate structures present on the proteoliposomes after their enzyme treatment were released by *N*-glycanase treatment, labeled by reducing the terminal *N*-acetylglucosamine with tritiated sodium borohydride [14] and purified by descending paper chromatography on Whatman No. 3 paper in the solvent system, *n*-butanol/ethanol/water (4:1:1, v/v), for 48 h. The resulting tritiated alditols were analyzed on a high resolution Bio-Gel P-4 column calibrated with dextran hydrolysate [15].

Results and Discussion

Equilibrium-exchange influx measurements

Glucose equilibrium-exchange was measured for both native and *N*-glycanase-treated proteoliposomes at 10 s using 5 glucose concentrations (Fig. 1). Lineweaver-Burk plots were obtained from these curves

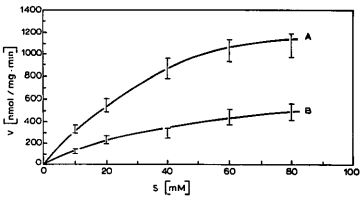

Fig. 1. Kinetics of glucose uptake by native (A) and *N*-glycanase-treated (B) proteoliposomes. Glucose equilibrium-exchange influx was measured at 10 s and 15 °C. S.D. is indicated by error bars.

TABLE I

Kinetic parameters of glucose influx, calculated (I) from equilibrium-exchange measurements for native and N-glycanase-treated proteoliposomes, (II) from zero-trans influx measurements for native and N-glycanase-, siahdase-, (sialidase + endo-β-galactosidase)- and exo-glycosidases-treated proteoliposomes

The relative variations for V_{max} compared to the V_{max} obtained without any treatment are indicated in parentheses.

Proteoliposomes	K_m (mM)	V_{max} (nmol/mg per min)
(I) Equilibrium-exchange		
Native	41 ±4	1700 ±111 (100%)
N-Glycanase-treated	68 ±7	1000 ± 95 (59%)
(II) Zero-trans influx		
Native	3.0±0.5	125 ± 10 (100%)
N-Glycanase-treated	4.5±0.5	62.0± 6 (50%)
Sialidase-treated	3.1±0.5	111 ± 10 (89%)
Sialidase + Endo-β-galactosidase-treated	3.7±0.6	109 ± 11 (87%)
Exo-glycosidase-treated	3.1±0.5	56 ± 6 (45%)

by linear regression using a computer program (data not shown), and K_m and V_{max} were determined graphically for both forms of transporter. From data of Table I, it appears that, for equilibrium-exchange influx measurements, the relative changes observed for K_m and V_{max} values are in the same range of values as those previously observed for zero-trans influx measurements [1]. Consequently, all subsequent experiments were performed using conditions of zero-trans Influx. Moreover, these data confirm that the release of glycosidic chain components from the human erythrocyte glucose transporter induces the loss of glucose transport activity.

Table I shows that the parameters calculated for equilibrium-exchange are about 15-times higher than those obtained for zero-trans influx and thus agree with previous observations [8].

Partial deglycosylation of the proteoliposomes

The conclusions drawn from our structural study of the transporter [6] led us to carry out three types of enzymatic treatment:

(1) Sialidase treatment: since about 30% of the glycosidic chains are sialylated, the release of the sialyl residues should induce important changes in local acidity and electric charges.

(2) Endo-β-galactosidase treatment: since about 90% of the glycosidic chains are constituted of poly(*N*-acetyllactosaminoglycans), their degradation could change glucose transport activity by shortening the length of the sugar chain. We previously showed [6] that this treatment led to carbohydrate chains eluted at 14 to 11 glucose units on a Bio-Gel P-4 column. This result is comparable with those obtained by Endo et al. [7].

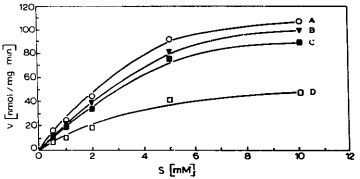

Fig. 2. Kinetics of glucose uptake by native (A), sialidase-treated (B), sialidase + endo-β-galactosidase-treated (C) and exo-glycosidases-treated (D) proteoliposomes. Glucose zero-trans influx was measured at 10 s and 15°C.

(3) Treatment with a pool of exo-glycosidases including sialidase, α-L-fucosidase, β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase. This latter enzyme exhibited a secondary β-mannosidase activity (demonstrated by using its natural substrate, the trisaccharide ManGlcNAc$_2$). Consequently, the expected residual glycosidic structure should be constituted of only one GlcNAc residue. This hypothesis was confirmed by biochemical analyses: after being subjected to the exo-glycosidase pool action, residual glycans present on the transport glycoprotein were released by *N*-glycanase, labeled and analyzed. The elution profile obtained on a Bio-Gel P-4 column showed the presence of a single peak at 2.6 glucose units, having the same elution volume as an authentic sample of tritiated *N*-acetylglucosaminitol (data not shown).

Zero-trans influx on partly deglycosylated proteoliposomes

Glucose zero-trans influx rates of the three types of enzyme-treated proteoliposomes and the native proteoliposomes were measured at 15°C and then compared (Fig. 2). Kinetic parameters of glucose zero-trans Influx were determined from the Lineweaver-Burk plots and

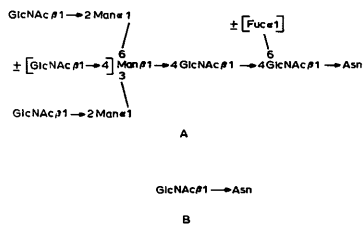

Fig. 3. Glycosidic structures present on the human erythrocyte glucose transporter after treatment with endo-β-galactosidase (7) (A) and a pool of exo-glycosidases (B).

are given in Table I. Curves obtained for native, desialylated and sialidase + endo-β-galactosidase-treated liposomes were very similar; in contrast, the one obtained for liposomes treated with the pool of enzymes showed a dramatically decreased glucose transport. Since only half of the glycoproteins can be reached by the enzymes, the effective $V_{m,x}$ decrease observed after treatments with sialidase and sialidase + endo-β-galactosidase is about 25% of the native rate. After these treatments, the glucose transporter remains functional, but slightly altered. On the other hand, treatment with the pool of enzymes results in the total abolition of glucose transport activity. Such a treatment releases the glycosidic chain, but maintains the integrality of the peptidic moiety, whereas *N*-glycanase-treatment, which was performed previously [1], releases the glycosidic chain and converts the asparagine residue linked to the glycosidic chain into aspartic acid. Data presented in Table I clearly show that both treatments abolish glucose transport activity. Consequently, the loss of activity cannot be attributed to a change in the peptide moiety but rather to the release of the glycosidic chain.

In conclusion, sialidase-treatment, which reduces the negative charges or acidic groups but does not shorter the polylactosaminoglycan chains, has the same effect on glucose transport activity as the combined sialidase + endo-β-galactosidase-treatment. Thus, such a shortening of the glycosidic chain until the minimum structure shown in Fig. 3A [6,7] was attained, did not seem to have any more effect on the transport activity, suggesting that they would be a minimum glycosidic structure sufficient to maintain the glucose transport activity.

Acknowledgements

This work was supported by grants from the Institut National pour la Santé et la Recherche Médicale, La Fondation pour la Recherche Médicale and La Ligue Française contre le Cancer. Thanks are expressed to Drs. Aubery and Wantyghem for their helpful suggestions.

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