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The role of N-glycosylation in the targeting and stability of GLUT1 glucose transporter

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The cDNAs encoding the GLUT1 glucose transporter protein were altered by site-directed mutagenesis at consensus sites for the addition of N-linked glycosylation These cDNAs were transfected into CHO cells with an expression vector and the subcellular distribution and stability of the expressed glycosylation-defective GLUT1 protein were analyzed. Immunohistochemical analysis with a specific antibody demonstrated that a significant portion of glycosylation-defective GLUT1 protein remained in the intracellular compartment By contrast, most of the wild-type GLUT1 proteins expressed with the same procedures resided in the plasma membranes Metabolic labeling studies revealed that the half-life of the glycosylation-defective GLUT1 protein was significantly shorter than that of wild-type GLUT1 protein. These results indicate that N-glycosylation of the glucose transporter affects its intracellular targeting and protein stability

Glucose transporter; GLUT1, Glycosylation; Subcellular distribution

1. INTRODUCTION

The N-glycosylation of peptides has been postulated to serve several biological functions. Among five isoforms of mammalian facilitative glucose transporter protein, one potential N-linked glycosylation site (Asn-Xaa-Thr/Ser) is conserved [1,2]. We have shown, using a site-directed mutagenesis method, that N-glycosylation of the GLUT1 glucose transporter increases its affinity for glucose and thus increases hexose transport activity [3]. In this study, we have investigated the roles of N-glycosylation of GLUT1 protein in targeting the cell surface and in protein stability.

2. MATERIALS AND METHODS

2.1. *Expression of glycosylation-defective GLUT1 protein in CHO cells*

Three mutated GLUT1 proteins were designed to replace the codon for asparagine at amino acid position 45 with the codon for aspartate, tyrosine, or glutamine. The CHO cells expressing each of these three glycosylation-defective GLUT1 proteins were prepared as described previously [3] These cells were grown in Ham F-12 medium containing 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin sulfate (0.1 mg/ml) in 5% CO_2

2.2. *Western blot analysis*

The membrane fractions prepared as described previously [4] were suspended in 1% SDS, 50 mM dithiothreitol, electrophoresed, and transferred onto a nitrocellulose filter The filters were incubated with the antisera raised against the C-terminal domain of GLUT1 [5] at a 1:40 dilution The filters were then incubated with [^{125}I]-protein A (Amersham) and washed. The dried blots were autoradiographed using Kodak AR X-ray film and an intensifying screen at −70°C.

2.3. *Immunofluorescene staining*

CHO cells were fixed in 3% formaldehyde in phosphate-buffered saline (PBS), scraped off the dish with a rubber blade, and embedded in 10% gelatin-PBS. Semithin frozen sections (1 μm thick) were made and incubated with the affinity-purified antibody against the C-terminal domain of GLUT1 [5]. The sections were then incubated with rhodamine-labelled goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) [6]

2.4. *Stability of the wild-type and mutated glucose transporter proteins*

The CHO cells expressing wild-type GLUT1 or mutated GLUT1 were plated onto 6-cm dishes and incubated with 5 mM cadmium chloride for 12 h to increase the expression of the transfected cDNAs. After washing 4 times with methionine free medium, cells were incubated with 0.8 ml of labelling medium containing 0.1 mCi of Tran^{35}S-label (ICN) for 6 h at 37°C. The labelling medium was removed and the cells were rinsed twice and incubated with Ham F-12 medium containing 10% fetal calf serum for the indicated periods. The cells were then washed, solubilized with 2% dodecyl octaethyleneglycol ether, and centrifuged at 13,000 × *g* for 30 min at 4°C. The labelled glucose transporter in the supernatant (200 μl) was immunoprecipitated with 6 μg of the affinity-purified antibody against the C-terminal domain of GLUT1 and 25 μl of protein A Cellulofine as described previously [4]. The immunoprecipitates were subjected to SDS-PAGE (10%). The gels were soaked in Enlightening (NEN) for 30 min, dried, and subjected to autoradiography [7].

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3. RESULTS AND DISCUSSION

N-glycosylation of proteins has been demonstrated to play a variety of roles including modulation of biological activity, intracellular targeting, protein folding, and maintenance of protein stability [8,9]. The mammalian facilitative glucose transporter family consists of five isoforms, all of which have one N-linked glycosylation site (Asn-Xaa-Thr/Ser), located between the membrane-spanning segments M1 and M2 in common [1,2]. In the case of GLUT1, which is abundant in human red blood cells, this N-linked carbohydrate accounts for approximately one-fourth of the apparent molecular weight of this protein, as assessed by gel electrophoresis [10–12]. In order to study the functional role of N-glycosylation of GLUT1 protein in glucose transporter activity, we previously expressed the mutated GLUT1 proteins without glycosylation in CHO cells. Lack of N-glycosylation of the GLUT1 protein decreased its affinity for a mannose-derivative, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(*D*-mannos-4-yloxy)-2-propylamine, and cytochalasin B, associated with an elevated K_m value for glucose uptake. Thus, N-glycosylation of GLUT1 is important for its transport activity [3]. The results in this study indicate that N-glycosylation also plays an important role in the targeting and stability of GLUT1 protein.

Immunoblot analysis with an anti-peptide antibody against the C-terminal domain of GLUT1 confirmed expression of the three glycosylation-defective GLUT1 proteins (Fig. 1). These proteins formed sharp bands and had molecular weights significantly smaller than that of the wild-type GLUT1 on SDS-PAGE. We performed immunofluorescence staining using the same antibody, in order to determine the subcellular localization of mutated GLUT1 protein. Slight staining was observed on the surfaces of control CHO cells, indicating the presence of the endogenous GLUT1 protein (data not shown). Marked staining was observed in cells transfected with an expression vector containing wild-type GLUT1 cDNA. The expressed wild-type GLUT1 protein was located mainly at the cell surface, while a small amount was observed intracellularly (Fig. 2, panel A). This staining was completely abolished by co-incubation with the GLUT1 C-terminal peptide and the antibody, indicating that the staining was specific for GLUT1 protein (data not shown). Some of the glycosylation-defective GLUT1 proteins expressed in CHO cells were observed on the cell surface, a result consistent with that of previous study which demonstrated that expression of glycosylation-defective GLUT1 increases glucose transport activity in CHO cells [3]. However, in contrast to the findings of wild-type GLUT1 protein expression, the majority of glycosylation-defective GLUT1 protein was observed intracellularly (Fig. 2, panels B, C and D).

We previously showed that expressed GLUT4 protein remained in intracellular sites, revealed to be the trans-Golgi reticulum and adjacent vesicles by immunoelectron microscopy, in CHO cells [13]. Thus, it is likely that an intracellular targeting system is present in CHO cells and retains GLUT4 protein within intracellular vesicles. The intracellular localization of glycosylation-defective GLUT1 protein was, however, quite different. The expressed glycosylation-defective GLUT1 proteins were scattered in the cell interior (Fig. 2). Immunoelectron microscopy revealed that the mutated GLUT1 proteins resided in unidentified vesicles (data not shown). Thus, lack of N-glycosylation appeared to cause improper targeting of the protein to the cell interior. This alteration in targeting might be due to the lack of glycosylation itself or a resulting structural alteration of the GLUT1 protein.

In order to determine whether the lack of N-glycosylation affects the stability of the transporter protein, the cells were labelled metabolically with ^{35}S. After the indicated periods of incubation, the cells were solubilized and the wild-type and mutated GLUT1 proteins were immunoprecipitated. The amount of labelled glucose

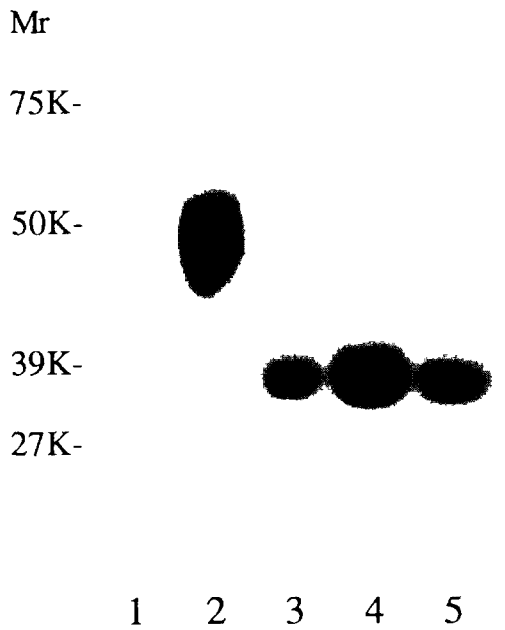

Fig. 1. Western blotting of wild-type GLUT1 and glycosylation-defective GLUT1 proteins expressed in CHO cells. The wild-type and three glycosylation-defective GLUT1 proteins were expressed in CHO cells by transfection with the corresponding cDNAs. Membranes prepared from these cell lines were electrophoresed and then transferred onto nitrocellulose filters. The filters were incubated with antisera against the C-terminal domain of GLUT1 protein, and then incubated with [^{125}I]-protein A and subjected to autoradiography. Lane 1, control CHO cells; lane 2, CHO cells expressing wild-type GLUT1; lane 3, CHO cells expressing mutated ^{45}Asp GLUT1; lane 4, CHO cells expressing mutated ^{45}Tyr GLUT1; lane 5, CHO cells expressing mutated ^{45}Gln GLUT1.

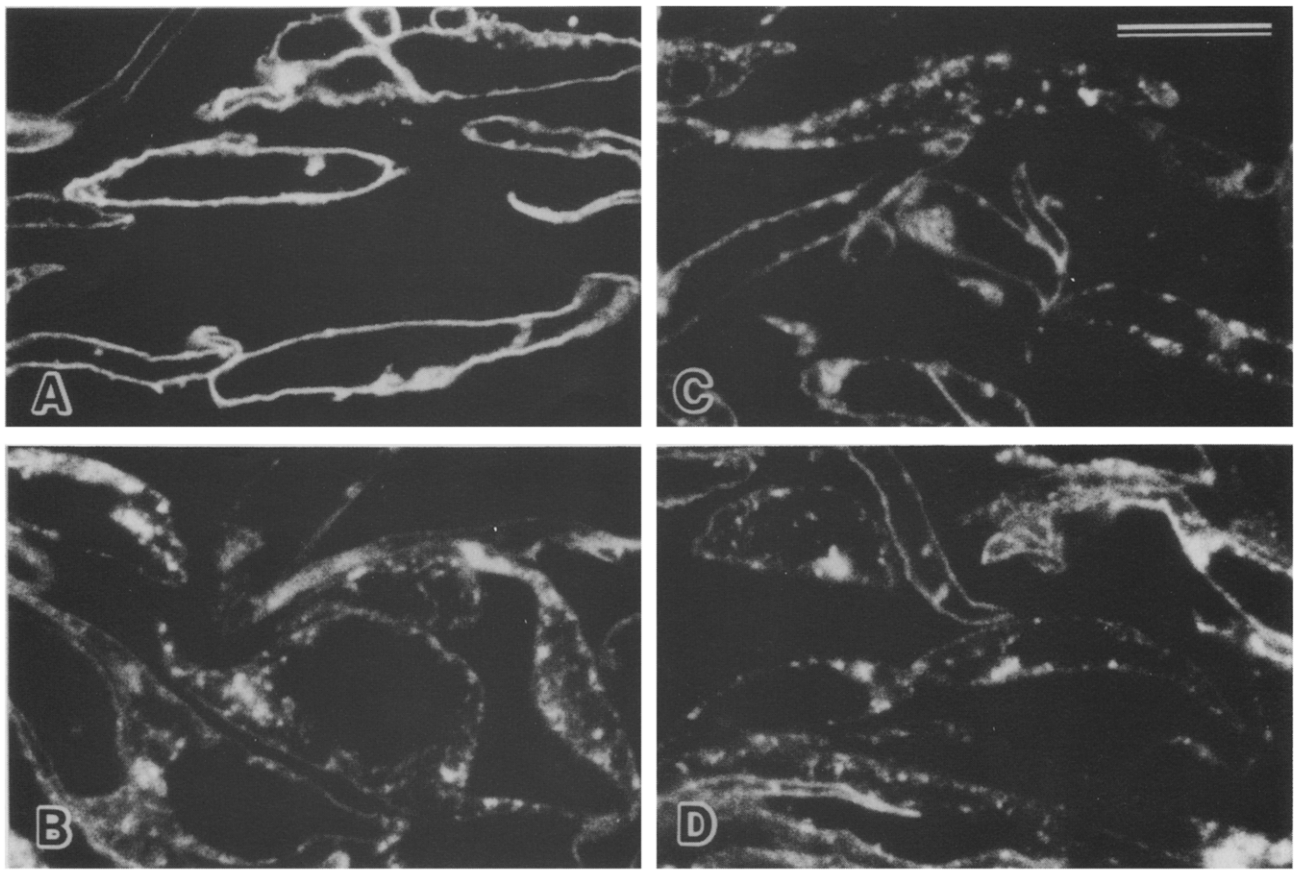

Fig. 2. Immunofluorecence staining of wild-type GLUT1 and glycosylation-defective GLUT1 proteins expressed in CHO cells. The semithin frozen sections (1 μm thick) were prepared from the cells and were incubated with the anti-peptide antibody against the C-terminal domain of GLUT1 protein, subsequently with rhodamine-labeled goat anti-rabbit IgG. Panel A. CHO cells expressing wild-type GLUT1. Panel B. CHO cells expressing mutated ^{45}Asp GLUT1. Panel C. CHO cells expressing mutated ^{45}Tyr GLUT1; Panel D. CHO cells expressing mutated ^{45}Gln GLUT1 Bar = 10 μm.

transporter was subsequently determined by quantitating fluorograms and was plotted as a function of the incubation period (Fig. 3). The half-life of wild-type GLUT1 protein was observed to be 25–28 h, while that of the three glycosylation-defective GLUT1 proteins was 16–18 h, indicating that the lack of N-glycosylation reduced protein stability. Although the cause of the decrease in protein stability is not clear, several mechanisms are possible. These possibilities include a structural alteration, induced by lack of N-glycosylation, which increases the access of proteolytic enzymes to the GLUT1 protein. Alternatively, the incorrect localization of unglycosylated GLUT1 protein within the intracellular compartment may lead to its rapid degradation.

In conclusion, our results indicate that N-glycosylation of GLUT1 protein plays an important role, not only as a structural component which increases transport activity [3], but also in intracellular targeting and protein stability.

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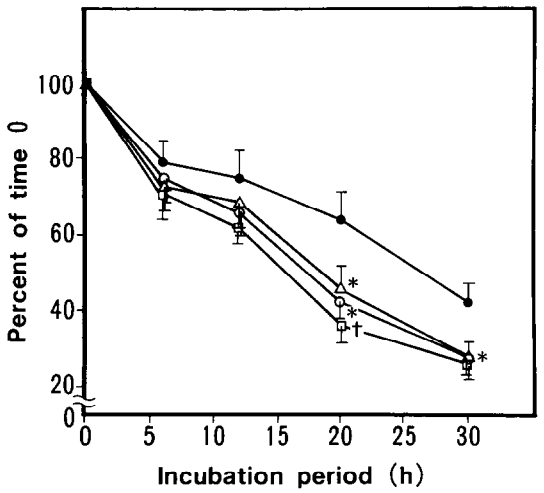

Fig. 3. Time course of degradation of wild-type and glycosylation-defective GLUT1 proteins. Cells were labelled with medium containing 0.1 mCi of Tran^{35}S-label for 6 h at 37°C. The labelling medium was removed and the cells were incubated for the indicated periods, washed and lysed. The labelled glucose transporter was immunoprecipitated and then subjected to SDS-PAGE (10%). The gels were soaked in Enlightening (NEN) for 30 min, dried, and subjected to autoradiography. The amount of labelled glucose transporters is plotted as a function of the incubation period. The results presented are the mean±S.E.M. of four separate experiments. Closed circle, CHO cells expressing wild-type GLUT1; open circle, CHO cells expressing mutated ^{45}Asp GLUT1; open square, CHO cells expressing mutated ^{45}Tyr GLUT1; open triangle, CHO cells expressing mutated ^{45}Gln GLUT1. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with the wild-type GLUT1.

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