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Glucose binding enhances the papain susceptibility of the intracellular loop of the GLUT1 glucose transporter

Tomoichiro Asano, Hideki Katagiri, Katsunori Tsukuda, Jiann-Liang Lin, Hisamitsu Ishihara, Kouichi Inukai, Yoshio Yazaki and Yoshitomo Oka

Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan

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Digestion of human GLUT1 protein in erythrocytes with 5 μg/ml papain for 5 min yielded several fragments. By using several site-specific antibodies, two of these fragments containing the intracellular loop domain between M6 and M7 were demonstrated to be further digested by a prolonged incubation with papain. The addition of 0.2 M D-glucose enhanced this digestion between M6 and M7 by approximately 3.5-fold, while the addition of 0.2 M D-sorbitol exhibited no effects. These results strongly suggest that D-glucose binding induces the conformational change of the intracellular loop domain between M6 and M7 of GLUT1 protein. Since the homology of the amino acid sequence was low in this intracellular domain among the five facilitative glucose transporter isoforms, this intracellular loop might contribute to the difference in their K_m and V_{max} values for glucose uptake.

Glucose transporter; GLUT1; Glucose; Papain

1. INTRODUCTION

Recently, five types of the facilitative glucose transporter protein have been identified [1,2], yet many aspects of its mode of action and regulation remain unexplained. These five isoforms share a very similar structure containing 12 transmembrane domains, suggesting that the mechanism of glucose transport would be essentially the same among these isoforms. Previous papers have suggested that glucose transporter protein has two glucose binding sites which face to the outside and to the inside of the cell, because certain sugars were shown to bind specifically to either the inside or the outside of the transporter protein [3–5]. D-Glucose, which binds to the outer glucose binding site of the transporter is hypothesized to move to the inner binding site of the transporter where it is subsequently released into the cell interior. Conformational change of the glucose transporter needs to take place during this process. However, the domains responsible for this conformational change have not yet been elucidated.

In this study, we have demonstrated that co-incubation with D-glucose enhances the susceptibility to papain of the intracellular loop domain between M6 and M7 of the GLUT1 glucose transporter, suggesting that the structure of this domain is altered by the binding of D-glucose.

Correspondence address: Y. Oka, Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Fax: (81) (3) 5684-3987.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]Cytochalasin B (15.5 Ci/mmol) was obtained from Amersham, cytochalasin E, D-glucose, D-sorbitol and papain from Sigma, prestained protein molecular weight standards from Bethesda Research Laboratories, and outdated human packed erythrocytes were provided by Tokyo University Hospital Blood Bank.

2.2. *Papain digestion of GLUT1 protein in the membranes of human erythrocytes*

Membranes were prepared from human erythrocytes by a modification of the method of Steck and Kant [6]. For papain digestion, erythrocyte membranes at 0.625 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) were incubated with 5 μg/ml papain. The mixture was frozen and then thawed in a water bath at 37°C. After 5 min incubation in the water bath, a $^1/_{10}$ volume of either 2 M D-glucose or 2 M D-sorbitol was added to the buffer, and further papain digestion was performed for the indicated periods. The digestion was terminated by the addition of 0.1 mM E-64, and the membranes were washed twice in ice-cold buffer containing 5 mM HEPES and 154 mM NaCl (pH 7.4) in order to remove papain.

2.3. *Photoaffinity labeling*

[^{3}H]Cytochalasin B in 100% ethanol was dried under nitrogen and suspended with 10 mM sodium phosphate buffer (pH 7.4). The incubation mixture contained 1 mg of papain-treated or untreated membranes per ml, 0.25 μM [^{3}H]cytochalasin B, 10 μM cytochalasin E, 10 mM sodium phosphate buffer (pH 7.4), and either 0.2 M D-glucose or 0.2 M D-sorbitol. The mixture (250 μl) was placed on ice in the dark and then irradiated three times for 10 s each with a 625 W ultraviolet lamp (American Ultraviolet Co.) [7,8]. The samples were immediately centrifuged for 30 min at 54,200 × *g* to remove free [^{3}H]cytochalasin B, and electrophoresed on SDS-polyacrylamide (14%) gel as described by Laemmli [9]. The gel lanes were sliced, solubilized with Protosol (Du Pont-New England Nuclear), and the radioactivity in each gel was determined. For the autoradiographic detection of the papain-digested fragments labeled with [^{3}H]cytocha-

lasin B, the gel was soaked in Enlightening (Du Pont-New England Nuclear), dried, and subjected to autoradiography.

2.4. *Immunoblotting analysis*

Anti-GLUT1 antibodies were raised in rabbits against the synthetic peptides corresponding to amino acid resisues 215–226, 253–267 and 388–401 of GLUT1, using the peptide-keyhole limpet hemocyanin conjugates as previously described [10]. The papain-treated membranes were subjected to SDS-polyacrylamide (14%) gel electrophoresis and transferred onto nitrocellulose filters. The filters were incubated with antisera at a 1:40 dilution. The filters were then incubated with ^{125}I-labelled protein A (Amersham Corp.) and subjected to autoradiography.

3. RESULTS AND DISCUSSION

Glucose transporter protein is a membrane protein with 12 transmembrane domains. Spectroscopic findings have suggested that glucose transporter protein consists of a mainly α-helical structure and some β-turn structures, both of which are retained after digestion with papain [11]. Here we have demonstrated that incubation with 5 μg/ml papain for 5 min digested GLUT1 protein to several fragments leaving no intact GLUT1 protein (Figs. 1 and 2). In spite of the fragmentation of the protein, GLUT1 retained the ability to be photoaffinity-labeled with [^{3}H]cytochalasin B (Fig. 1). The apparent molecular weights of the fragments labeled with [^{3}H]cytochalasin B were approximately 18, 13 and less than 6 kDa on autoradiographic detection (Fig. 1b). In addition, the cytochalasin B binding to these papain-digested GLUT1 fragments was revealed to be inhibited by the co-incubation with D-glucose as observed in intact GLUT1 protein (Fig. 1a and b). Digestion with 5 μg/ml papain for 5 min at 37°C did not abolish the ability of GLUT1 glucose transporter to bind both cytochalasin B and D-glucose, suggesting that the conformation of the fragments was not drastically different from that of intact GLUT1 protein in the membrane.

Subsequently, the fragments derived from papain-digested GLUT1 protein were depicted by immunoblot analysis using antibodies against the synthetic peptides corresponding to amino acid residues 215–226, 253–267 and 388–401 of the GLUT1 sequence (Fig. 2). The 12 and 17 kDa fragments of GLUT1 were detected by immunoblotting using the antipeptide antibodies against amino acid residues 388–401 and 253–267 of GLUT1, respectively (Fig. 2a and b). Immunoblotting using the antipeptide antibody against amino acid residues 215–226 showed two bands of approximately 6 and 14 kDa, the latter band of which was identical to that detected by immunoblotting using the antipeptide antibody against amino acid residues 253–267 of GLUT1 (Fig. 2c). In contrast, no protein band was observed by immunoblotting using the antipeptide antibody against amino acid residues 466–479 or 478–492 of GLUT1 (data not shown), suggesting that the C-terminal domain of GLUT1 was digested with papain

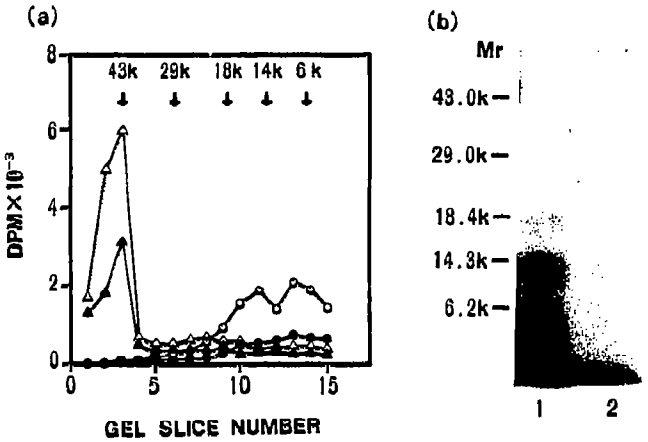

Fig. 1. Photoaffinity labeling of the intact and papain-digested GLUT1 in the membranes of human red blood cells. The membranes were frozen in the presence of 5 μg/ml papain and thawed. The samples were quickly transferred to a water bath at 37°C and incubated for 5 min. The intact and papain-digested membranes were photolabeled with [^{3}H]cytochalasin B in the presence of either 0.2 M D-glucose or 0.2 M D-sorbitol. The membranes were electrophoresed on a SDS-polyacrylamide (14%) gel. (a) The radioactivity from [^{3}H]cytochalasin B in each lane. The gel lanes were sliced, solubilized and the radioactivity was determined. Intact membrane in the presence of D-sorbitol (△) or D-glucose (▲); papain-digested membrane in the presence of D-sorbitol (○) or D-glucose (●). (b) Autoradiographic detection of the papain-digested membrane labeled with [^{3}H]cytochalasin B in the presence of D-sorbitol (lane 1) or D-glucose (lane 2).

and lost as soluble peptides during centrifugation step used to remove papain from the membranes. Fragmentation of GLUT1 described above was not observed when papain was added to the intact, sealed red blood cells (data not shown). Thus, papain digestion of the GLUT1 protein is likely to take place at the cytoplasmic side of the membrane. Although the low specificity of protease activity of papain makes it difficult to point out the accurate sites of the digestion, the orientations of the 12 kDa fragment in Fig. 2a, the 14 kDa fragment in Fig. 2b, and the 14 and 6 kDa fragments in Fig. 2c were predicted as shown in Fig. 2d, e, f and g, respectively. Prolonged incubation with papain induced further digestion of the 14 kDa fragment in Fig. 2b and c, and the 6 kDa fragments in Fig. 2c, but not that of the 15 kDa fragment in Fig. 2a. Following the further digestion with papain of the 14 and 6 kDa fragments no apparent fragments were detected with the antibodies against amino acid residues 215–226 or 253–267, suggesting that digestion takes place in the intracellular loop between M6 and M7 of the GLUT1, and that the domains containing amino acid residues 215–226 and 253–267 were nicked and lost as soluble peptides. The presence of 0.2 M D-glucose enhanced the digestion of this intracellular loop between M6 and M7 of GLUT1 protein compared with the absence of glucose, while the presence of 0.2 M D-sorbitol had no significant effects on this digestion. The time-course study revealed that the digestion proceeded approximately 3.5-fold

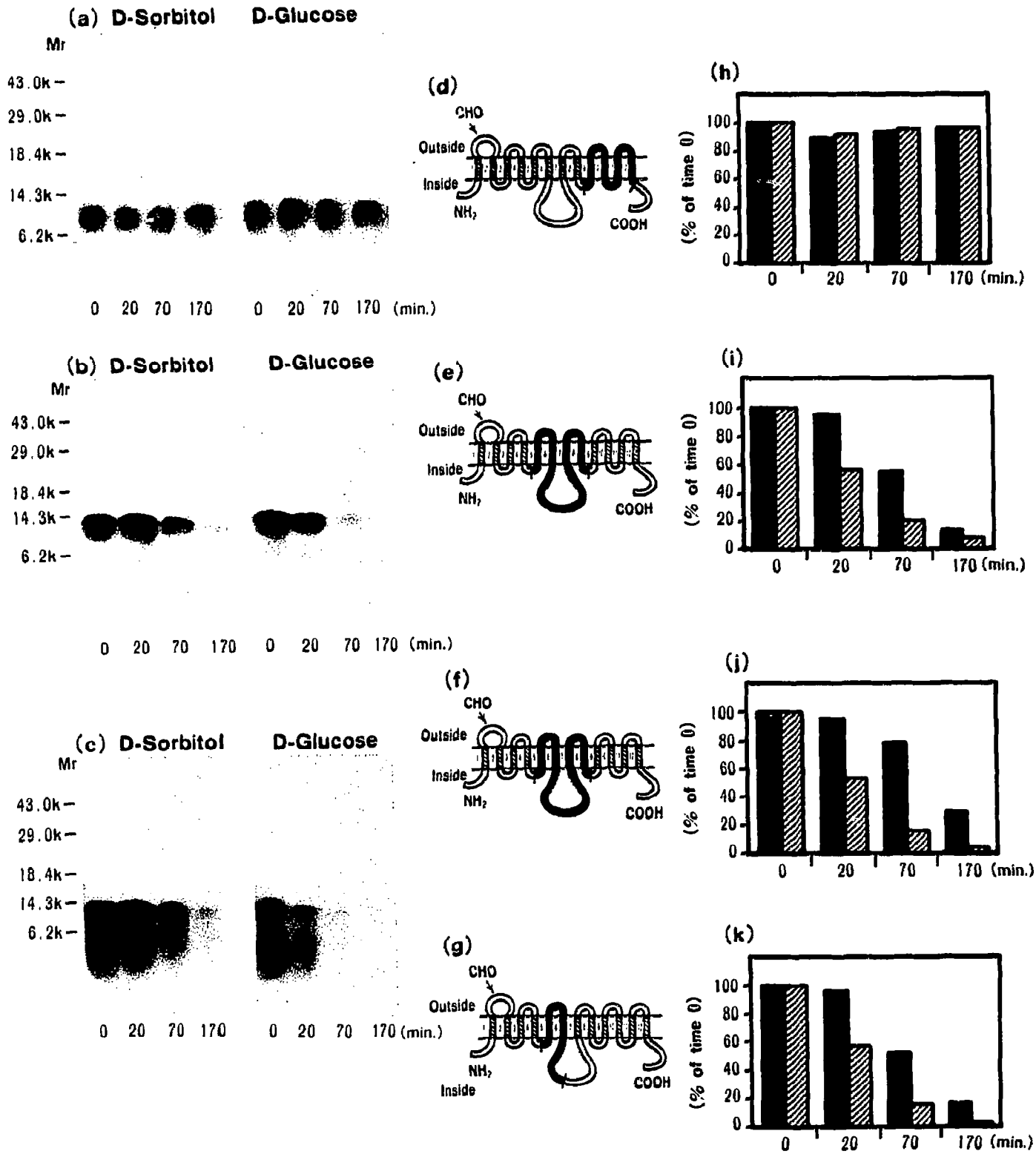

Fig. 2. Immunoblot analysis of the papain-digested GLUT1. The membranes were frozen in the presence of 5 μg/ml papain and thawed. After 5 min incubation in a water bath at 37°C, further digestion with papain was carried out in the presence of either 0.2 M D-glucose or 0.2 M D-sorbitol for the indicated periods as described in Materials and Methods. After the indicated periods of the further incubation, the digestion was terminated and the samples were electrophoresed on a 14% acrylamide gel and transferred onto nitrocellulose filters. The filters were then incubated with the anti-GLUT1 antibodies against each of the synthetic peptides corresponding to amino acid residues (a) 215–226 (b) 253–267 and (c) 388–401 of the GLUT1 sequence at a 1:40 dilution. The filters were then incubated with ^{125}I-labelled protein A and subjected to autoradiography. The predicted orientation of the 12 kDa fragment in a, the 14 kDa fragment in b, and the 14 and 6 kDa fragments in c are shown as thick lines in d, e, f and g, respectively. The radioactivity in each band was measured and the time–courses of papain digestion of these four fragments were plotted in h, i, j and k, respectively. D-sorbitol, filled bars; D-glucose, hatched bars.

faster in the presence of 0.2 M D-glucose compared with that in the presence of 0.2 M D-sorbitol (Fig. 2i, j, k). In addition, 2-deoxy-D-glucose could be substituted for D-glucose for the enhancement of this papain digestion (data not shown).

Previous studies using GLUT1 glucose transporter in human erythrocytes have proposed an alternating conformational model for the mechanism of facilitative glucose transport [3,12–17]. In this model, glucose transporters can possess glucose binding sites either on the outer side or on the inner side of the cell membrane, because certain sugars were shown to bind specifically to either the inside or the outside of the transporter protein. D-Glucose bound to the outer glucose binding site of the transporter seems to be transferred to the inner glucose binding site of the transporter and released into the cell interior. During this process, conformational change of the glucose transporter must take place. A previous study reported that 0.2 M D-glucose increased the rate of tryptic cleavage of GLUT1 transporter in human erythrocytes by about 1.5-fold [17], which is supposed to take place at the C-terminal side of residues 269 and 456, suggesting that the conformational change occurs in glucose transporter. Using site-specific antibodies our results have provided further concrete evidence that the conformational change occurs in the intracellular large loop domain between M6 and M7. The amino acid sequence of this domain is not highly conserved among the five isoforms of the facilitative glucose transporter family compared with most of the other domains [1,2]. These five isoforms have different K_m and V_{max} values for glucose transport activity. If the conformational change of the intracellular loop between M6 and M7 plays an important role in the transport activity, we may speculate that the difference in their intracellular domains between M6 and M7 induces the difference in their K_m and V_{max} values for glucose uptake. A study using the chimeric transporter whose intracellular loop domain is exchanged will be needed to clarify this issue.

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