Monoclonal antibodies possibly recognize conformational changes in the human erythrocyte glucose transporter

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Two monoclonal antibodies (MAG17 and MAG20) were raised against the human erythrocyte glucose transporter, which was purified on an immunoaffinity column using a polyclonal antibody to the C-terminal peptide (residues 477–492) of the glucose transporter of HepG2 cells. To obtain antibodies which recognize the native glucose transporter integrated in the membrane, hybridomas were screened both by e.l.i.s.a. with purified glucose transporter and by dot-blotting with erythrocyte membranes. The antibodies immunoprecipitated D-glucose-inhibitable [³H]cytochalasin B-photoaffinity-labelled glucose transporters, but did not recognize the transporter on Western blotting. The presence of the C-terminal peptide did not inhibit the binding of these antibodies to the glucose transporter, suggesting that the antibodies recognized sites different from the transporter C-terminus. D-Glucose (0.1–100 μ M) inhibited the binding of MAG17 and MAG20 to the transporter by 50 %, indicating that the conformation of the epitopes was altered allosterically by D-glucose. Cytochalasin B inhibited the binding of MAG17 to the transporter, but enhanced the binding of MAG20 at low concentrations (less than 0.02 μ M). These data suggest that the glucose transporter has high- and low-affinity binding sites for D-glucose and cytochalasin B, and that binding of D-glucose and cytochalasin B induces conformational changes in the transporter. Monoclonal antibodies which recognize the tertiary structure of the glucose transporter can be used for investigating its function and structure when integrated in the membrane.

INTRODUCTION

Glucose is transported into cells across the plasma membrane by glucose transporters [1–3]. The erythrocyte glucose transporter has been extensively studied in terms of its structure, substrate specificity, inhibitors and kinetics of glucose transport. Mueckler *et al.* [4] and Birnbaum *et al.* [5] have presented the cDNA-deduced amino acid sequence of the erythrocyte/HepG2/brain-type glucose transporter (GLUT1). More recently, cDNAs of the liver-type (GLUT2) [6,7], the fetal skeletal muscle-type (GLUT3) [8], the insulin-responsive (fat/muscle)-type (GLUT4) [9–11] and the jejunal (GLUT5) glucose transporters [12] have been cloned. These five types of glucose transporters have 55–65 % amino acid sequence identity and each contains twelve putative transmembrane domains, and they are considered to be a structurally related family of proteins. However, the relationship between the structure of the glucose transporter and its function is still unknown.

Erythrocytes represent a good system with which to examine the mechanism of glucose entry into the cell [2]. They have no glucose transporter in the cytosol, and glucose transport is not regulated by insulin. Erythrocyte plasma membranes have a relatively simple composition and are easily isolated without contamination by intracellular organelles. The glucose transporter in the isolated plasma membrane shows glucose transport kinetics indistinguishable from those obtained with intact cells [2]. As to the mechanism of glucose transport across the erythrocyte membrane, models suggesting one [13] or two substrate-binding sites [14] have been proposed. Accumulating evidence [13–19] suggests that a conformational change to the glucose transporter molecule is involved in the mechanism of glucose transport. It is not known, however, which portion of the glucose transporter is subjected to conformational change when glucose binds to transporter molecule.

We have raised two monoclonal antibodies which recognize the native form of the human erythrocyte glucose transporter integrated into the membrane. We have studied the effects of D-glucose and cytochalasin B, a potent reversible inhibitor of glucose transport, on the binding of the antibodies to the transporter, and obtained findings suggesting that D-glucose and cytochalasin B alter the tertiary structure of the glucose transporter in the membrane. These antibodies may provide a useful tool to investigate the structure and function of the glucose transporter.

EXPERIMENTAL

Materials

[4-³H]Cytochalasin B (18.5 mCi/mmol) and D-[1-¹⁴C]glucose (54.6 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Control mouse IgG1- κ (P3/X63-R118 myeloma protein) was kindly given by Dr. K. Iwai (Kyoto University). Dot-blotting apparatus was from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.).

Photoaffinity labelling of erythrocyte ghosts

Photoaffinity labelling of the glucose transporter in the ghosts [16] with 0.5 μ M-[³H]cytochalasin B was performed as described by Carter-Su *et al.* [20] in the presence of 10 μ M-cytochalasin E and 500 mM-D-glucose or D-sorbitol using a u.v. lamp (PCQ Photochemical Lamp, UVP, at a distance of 5 cm for 5 min).

Immunoaffinity purification of glucose transporters

A peptide corresponding to residues 477–492 (C-terminal portion) of the HepG2-type glucose transporter was synthesized by the solid-phase method [21]. Antibody against the C-terminal peptide (CGT) was obtained in a rabbit by the method of Gentry

Abbreviation used: PBS, Dulbecco's phosphate-buffered saline (Ca²⁺/Mg²⁺-free, pH 7.4).

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et al. [22]. Serum was examined as to whether it immuno-precipitated ^{125}I -labelled Tyr-CGT and $[^3\text{H}]$ cytochalasin B photoaffinity-labelled glucose transporter as described below. The antibody to the CGT (αCGT) was affinity-purified using a Cys-CGT-coupled Sepharose 4B column. Glucose transporters were affinity-purified using an αCGT -coupled Sepharose 4B column from solubilized erythrocyte ghosts.

Production of monoclonal antibodies to the glucose transporter

Female BALB/c mice (2 months old) were immunized intradermally on the back with a mixture of 50 μg of purified glucose transporter protein and 150 μl of complete Freund's adjuvant, and 4 weeks later with 25 μg of glucose transporter in incomplete Freund's adjuvant. After a further 4 weeks, blood was sampled from the tail vein and the serum was analysed by both e.l.i.s.a. and dot-blotting as described below. To mice which produced antibodies, an additional 25 μg of glucose transporter was given intraperitoneally in incomplete Freund's adjuvant, and 3 days later, the mice were killed and the spleens were removed. Spleen cells were washed and fused with P3/X63-Ag8.653 by the method of Kennet [23]. Positive clones, which showed strong antibody binding to the glucose transporter by e.l.i.s.a. and dot-blotting, underwent three runs of cloning by the limiting dilution technique. Monoclonal antibodies (MAGs, IgG1) were purified using a Protein A-Sepharose column under the same conditions as for eluting the IgG1 subclass.

Solid-phase binding assay to the glucose transporter

For e.l.i.s.a., antigen (purified glucose transporter), diluted with bicarbonate buffer (pH 9.6), was applied to each well of a plate (100 μl ; 5 μg of protein) and incubated overnight at 4 °C. For dot-blotting, erythrocyte membranes (4 $\mu\text{g}/\text{well}$) were applied to each well of the microfiltration apparatus (Bio-Rad) with nitrocellulose paper and the assay was performed according to the manufacturer's instructions. All procedures were carried out at room temperature. Each well was washed with $3 \times 200 \mu\text{l}$ of phosphate-buffered saline (PBS) and then blocked by 200 μl of 3% BSA in PBS. After 1 h the wells were washed once with PBS containing 0.05% Tween 20 (T-PBS) and twice with PBS. The purified antibodies (2.5 $\mu\text{g}/\text{ml}$) in PBS containing 3% BSA were applied to each well (100 $\mu\text{l}/\text{well}$). After 1 h the wells were washed with $2 \times 200 \mu\text{l}$ of T-PBS and once with PBS. Nitrocellulose paper was taken off the apparatus, blocked with 3% BSA/PBS for 1 h and washed three times as described above. The paper was incubated with ^{125}I -labelled anti-rabbit or anti-mouse IgG antibody $[(3-5) \times 10^5 \text{ c.p.m.}/\text{ml}]$ in PBS/3% BSA for 1 h, and washed three times. The dots were cut from the paper and radioactivity was counted using a γ -radiation counter.

Effect of C-terminal peptide, cytochalasin B and D- or L-glucose on antibody binding to the glucose transporter

The C-terminal peptide of the glucose transporter (20 ng/ml in PBS), cytochalasin B (0–20 μM in PBS with 0.5% ethanol), or D- or L-glucose (0–100 μM in PBS) was incubated with the antibody (2.5 $\mu\text{g}/\text{ml}$) in 100 μl of PBS in wells of the dot-blot apparatus. Erythrocyte membranes were obtained from the ghosts by homogenizing in a Polytron (50% of max. speed; $2 \times 30 \text{ s}$) in PBS. Antibody binding to glucose transporters in the erythrocyte membranes was determined by dot-blotting as described above.

Immunoprecipitation

$[^3\text{H}]$ Cytochalasin B-photoaffinity-labelled erythrocyte ghosts (1 mg/ml) were solubilized with 1% Triton X-100 and 1 mM-phenylmethanesulphonyl fluoride in PBS at 4 °C for 40 min. Solubilized materials were incubated with Protein A-Sepharose 4B to adsorb non-specific binding protein. The supernatant

(100 μl) was then incubated with 50 μl of either Protein A-Sepharose 4B to which 5 μg of αCGT or control rabbit IgG was bound, or rabbit anti-(mouse IgG)-coupled Sepharose 4B to which 5 μg of the monoclonal antibody or control mouse IgG1- κ was bound. After incubation at 23 °C for 1 h with constant mixing, the Sepharose was washed three times in ice-cold PBS/0.1% Triton X-100. Bound protein was eluted from the Sepharose by boiling in SDS/PAGE Laemmli buffer for 1 min at 100 °C and subjected to SDS/10%-PAGE [24].

Measurement of protein

The concentration of IgG was calculated from the absorbance at 280 nm (1.38 absorbance units = 1 mg/ml). Protein concentrations were determined by the method of Bradford [25].

Expression of data

Statistical significance was tested using the paired or unpaired student's *t* test, and differences were accepted as significant at the $P < 0.05$ level. Results are expressed as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

Monoclonal antibodies against the glucose transporter

Twenty-eight out of 480 wells containing hybridomas were positive, and 11 clones were obtained. We finally purified and characterized two monoclonal antibodies (MAG17 and MAG20), both of which were of the IgG1- κ subclass. $[^3\text{H}]$ Cytochalasin B-photoaffinity-labelled erythrocyte ghosts were solubilized and photoaffinity-labelled proteins were immunoprecipitated with the antibodies. The precipitates were then

Fig. 1. Immunoprecipitation of $[^3\text{H}]$ cytochalasin B-photoaffinity-labelled glucose transporter

Human erythrocyte ghosts (100 μg) were photoaffinity-labelled with 0.5 μM - $[^3\text{H}]$ cytochalasin B in the presence of 10 μM -cytochalasin E and 500 mM-D-glucose (●, Δ) or D-sorbitol (○, ■, □) and solubilized with 1% Triton X-100 in PBS. Detergent extracts were immunoprecipitated with 5 μg of antibody [αCGT (○), MAG17 (□, Δ) or MAG20 (■, ●)] and Protein A-Sepharose 4B (for rabbit IgG) or anti-(mouse IgG)-antibody-coupled Sepharose 4B. The precipitates were subjected to SDS/PAGE (10% gel), the gel lanes were sliced and the radioactivity was determined. In the presence of D-glucose the results obtained with αCGT were essentially the same as those with MAG17 and MAG20, and were omitted from this Figure. Values at the top of the Figure indicate molecular mass in kDa.

Table 1. Effect of synthetic C-terminal peptide on antibody binding to the glucose transporter integrated into the membrane

Erythrocyte membrane (4 µg/ml) was adsorbed on to nitrocellulose paper using microfiltration apparatus (Bio-Dot) as described in legends to Fig. 2. Binding of the antibodies (2.5 µg of αCGT, MAG17 and MAG20/ml) was measured in the absence or the presence of C-terminal peptide (CTP; 25 ng/ml) with ¹²⁵I-anti-rabbit or ¹²⁵I-anti-mouse IgG. Dots were cut and radioactivity was counted in a γ-radiation counter. Each result is the mean of duplicate samples. Non-specific binding of each antibody to the nitrocellulose paper in the absence of membranes was subtracted from the total count. Inhibition (%) = (c.p.m. with C-terminal peptide)/(c.p.m. without C-terminal peptide) × 100

Antibody	Binding (c.p.m.)		Inhibition (%)
	–CTP	+CTP	
αCGT	3996	630	15.7
MAG17	3743	3652	97.0
MAG20	3888	4440	114.2

Fig. 2. Effects of D- and L-glucose on antibody binding to the glucose transporter integrated into the membrane

Erythrocyte membranes were applied to a sheet of nitrocellulose paper in the microfiltration apparatus (Bio-Dot). Dots were incubated with the antibodies (2.5 µg/ml) in the presence of D-glucose (a) or L-glucose (b) (0–100 µM), and then with ¹²⁵I-anti-rabbit [for αCGT (○)] or ¹²⁵I-anti-mouse [for MAG17 (□) and MAG20 (■)] IgG. Radioactivities of the dots were counted in a γ-radiation counter. Results were expressed as percentages of the binding in the absence of D- or L-glucose. Each point is the mean of duplicate samples. Non-specific binding, determined as binding of control IgG1-κ to the membranes, was low and was not affected by D- or L-glucose. Experiments were carried out three times with similar results.

analysed by SDS/PAGE. αCGT, MAG17 and MAG20 immunoprecipitated proteins with molecular masses of 55 and 110 kDa (Fig. 1). These two peaks were D-glucose-inhibitable and appeared to be monomeric and aggregated forms of the glucose transporter respectively. We next examined, by dot-blot analysis, if the antibodies reacted with the native glucose transporter integrated in the membrane. MAG17, MAG20 and αCGT bound glucose transporter in the membrane in a dose-dependent manner (results not shown). We also studied the effect of the C-terminal peptide on the binding of these antibodies. The presence of C-terminal peptide inhibited αCGT binding to the transporter, but failed to inhibit the binding of the two monoclonal antibodies (Table 1). Thus the monoclonal antibodies recognized sites different from the C-terminus (residues 447–492) of the glucose transporter. To see if the antibodies recognized the extracellular domain of the transporter, intact erythrocytes were incubated

with ¹²⁵I-labelled antibodies, washed, and the radioactivity was counted. Both MAG17 and MAG20 failed to bind to the cells (results not shown). Furthermore, these antibodies did not significantly affect [¹⁴C]glucose uptake by erythrocytes (results not shown). In contrast to αCGT, the monoclonal antibodies did not detect the glucose transporter by Western blotting, when the erythrocyte membranes were subjected to SDS/PAGE with or without heating (results not shown). Thus epitopes for the monoclonal antibodies seemed to be denatured by the Western blotting procedure.

Interaction of antibodies with D-glucose or cytochalasin B

We studied the effect of D- and L-glucose on antibody binding to the glucose transporter integrated in the membrane. The binding of MAG17 and MAG20 was inhibited by 50% by D-glucose, even at a concentration as low as 0.1 µM (Fig. 2a). There was no further inhibition by higher concentrations of D-glucose. The binding of αCGT was not affected. The non-transported sugar L-glucose had no effect on the antibody binding (Fig. 2b). In additional experiments we studied the effects of D-xylose (0–100 µM) and D-fructose (0–100 µM) on antibody binding. These D-sugars, which are known not to be permeants for the carrier, were without effect on antibody binding (results not shown). Thus the effect of D-glucose seems to be specific. The ability of D-glucose to inhibit MAG binding at 0.1 µM is remarkable. This value is about 3–4 orders of magnitude below the K_m for glucose transport [16]. The same phenomenon has been reported by Koepsell *et al.* [26] for the Na⁺/D-glucose co-transporter. They prepared monoclonal antibodies against the renal Na⁺/D-glucose co-transporter and found that binding of the antibodies to the native membrane proteins was altered by D-glucose at concentrations less than 10 nM. The affinity of the D-glucose-binding site was at least 1000 times higher than the apparent K_m of Na⁺-gradient-dependent D-glucose uptake. From these findings they have concluded that a high-affinity D-glucose-binding site is present on the transporter. Similarly, our data may also be explained by the existence of two binding sites for glucose with low and high affinities. D-Glucose binding to the high-affinity site may change the conformation of the epitope by allosteric modification so that the affinity of the monoclonal antibodies to the glucose transporter is decreased. On the other hand, binding to the low-affinity site is without effect on the epitopes. The K_m for glucose transport may thus reflect the K_m of the low-affinity site.

We next examined the effect of cytochalasin B on antibody binding to the membrane-integrated glucose transporter (Table 2). Interestingly, a low concentration (0.02 µM) of cytochalasin B enhanced the binding of MAG20. With higher concentrations of cytochalasin B the binding of MAG17 was inhibited, whereas the binding of MAG20 did not change. The binding of αCGT was not affected by any concentration (results not shown). Thus, as for D-glucose, there may also be two cytochalasin B-binding sites on the glucose transporter molecule with high and low affinities. Binding of cytochalasin B to the high-affinity site may change the conformation of the glucose transporter to a form which is favourable to the binding of MAG20, while binding to the low-affinity site may change the conformation to inhibit binding of MAG17. We also studied the effect of cytochalasin E, an analogue of cytochalasin B which binds to the glucose transporter with a lower affinity ($K_d \sim 20$ µM). Cytochalasin E (0–2 µM) did not affect the binding of MAG20 to the membrane-integrated glucose transporter, but the binding of MAG17 was significantly inhibited by cytochalasin E at a concentration of 2 µM (to $56 \pm 4\%$ of maximum, $P < 0.05$). Increasing the cytochalasin E concentration further had a minimal effect ($51 \pm 1\%$ binding at 20 µM). The IC_{50} (concn. causing 50% of maximal inhibition) of

Table 2. Effect of cytochalasin B on antibody binding to the glucose transporter integrated into the membrane

Erythrocyte membrane was applied to a sheet of nitrocellulose paper in the Bio-Dot apparatus. Binding of the antibodies (2.5 µg/ml) to the glucose transporter was measured in the presence of cytochalasin B (0–20 µM) as described in the legend to Fig. 2. The final concentration of ethanol in each well was adjusted to 0.8%. The experiments were performed three times, and results are shown as means ± S.E.M. Values in parentheses indicate binding as a percentage of that in the absence of cytochalasin B. Non-specific binding, determined as binding of control IgG1-κ to the membrane, was low and was not affected by cytochalasin B. **P* < 0.05 versus radioactivities in the absence of cytochalasin B.

Cytochalasin B (µM)	Binding (c.p.m.)	
	MAG17	MAG20
0	3902 ± 80 (100)	3930 ± 35 (100)
0.002	4169 ± 181 (107 ± 4)	4824 ± 144* (123 ± 3)
0.02	3904 ± 19 (100 ± 2)	4871 ± 98* (124 ± 2)
0.2	2617 ± 87* (67 ± 3)	3870 ± 155 (98 ± 4)
2	2257 ± 154* (58 ± 5)	3933 ± 66 (100 ± 3)
20	2344 ± 57* (60 ± 1)	3963 ± 30 (101 ± 1)

cytochalasin E was about 1 µM, which was greater than that of cytochalasin B (0.1 µM).

Obviously these proposed high-affinity binding sites for D-glucose and cytochalasin B require characterization. Since we used erythrocyte membranes for the dot-blot study, we should consider the possibility that the high-affinity binding sites are located in another membrane component which is closely related to and thus could affect glucose transporter molecules. If glucose transporters exist as a dimeric form in the membrane [27], there could be intermolecular interactions with D-glucose or cytochalasin B binding.

Both cytochalasin B (20 µM) and D-glucose (even at 10 mM; results not shown) failed to inhibit completely antibody binding. It is not clear why cytochalasin B only inhibited 40% of MAG17 binding. In these experiments we determined non-specific binding as binding of control IgG1-κ to the erythrocyte membrane rather than binding of the antibodies to membranes not containing the glucose transporter. However, it is unlikely that this procedure underestimated non-specific binding, contributing to the incomplete inhibition.

Andersson & Lundahl [28] have also produced monoclonal antibodies against the glucose transporter. They detected the antibody by competitive e.l.i.s.a. using purified glucose transporter as an antigen, and concluded from displacement studies that they obtained only C-terminal-specific antibodies. Their method might have failed to detect antibodies which recognize the tertiary structure of the glucose transporter, especially epitopes built up by discrete segments of the molecule. We detected antibodies using both purified glucose transporter and the native form in the membrane as antigens. It is highly likely that our antibodies recognize the tertiary structure of the glucose transporter, and thus they fail to demonstrate the transporter by Western blotting techniques. Monoclonal antibodies which recognize the tertiary structure have been reported in many

proteins, and the epitope is built up by several discrete segments of the polypeptide chain [29]. The present study suggests that the glucose transporter integrated in the membrane can take several forms with different tertiary structures. These monoclonal antibodies will be useful in the study of the structure and function of the glucose transporter.

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