Characterization of GLUT3 protein expressed in Chinese hamster ovary cells

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We have expressed GLUT3 protein, an isoform of a facilitative glucose transporter, in Chinese hamster ovary cells by transfection of its cDNA using an expression vector. The expressed GLUT3 protein was detected by Western-blot analysis as a broad band of 45–65 kDa, indicating intensive glycosylation of the protein. The expressed GLUT3 protein was observed, by immunofluorescence staining, to be located mainly at the plasma membrane, and its expression was associated with a marked increase in glucose-transport activity. Kinetic analysis revealed that the K_m value of GLUT3 protein for 3-*O*-methylglucose uptake was approx. 35 % of that of GLUT1 protein, whereas the K_m value of GLUT3 protein for 2-deoxy-D-glucose uptake was very similar to that of GLUT1 protein. The V_{max} value of GLUT3 protein for 3-*O*-methylglucose and 2-deoxyglucose uptake was approx. 20–50 % of that of GLUT1 protein. GLUT3 protein was well photolabelled with [³H]cytochalasin B or a mannose derivative, 2-*N*-4-[³H](1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine. Thus GLUT3 protein has very similar characteristics to GLUT1 protein including its subcellular localization, but exhibits lower K_m and V_{max} values for 3-*O*-methylglucose uptake.

INTRODUCTION

All mammalian cells possess, on their cell surface, membrane proteins that transport glucose across the plasma membrane [1,2]. Recently, five isoforms of facilitative glucose transporters have been identified and sequenced [3]. Although they share a very similar structure, they are encoded by different genes and exhibit different tissue distributions. The transcripts of GLUT3 transporter gene are widely distributed in most kinds of tissues and are very abundant in the brain in human and monkey [4], whereas the expression of the GLUT3 gene is restricted to the brain in rodents such as rat and mouse [5]. Thus GLUT3 transporter might be involved in glucose utilization in the brain which depends preferentially on glucose for its metabolic energy. However, the properties of GLUT3 protein remain unknown. In this study, we have characterized the function and subcellular distribution of GLUT3 protein expressed in Chinese hamster ovary (CHO) cells transfected with GLUT3 cDNA and compared its characteristics with those of GLUT1 protein, the most well-defined glucose transporter among the five glucose-transporter isoforms.

MATERIALS AND METHODS

Screening of the human GLUT3 cDNA library

Human GLUT3 cDNA was isolated from the human brain cDNA library by the oligonucleotide hybridization technique. The oligonucleotide probe used to screen the cDNA library was 34 DNA nucleotides long. It was custom-synthesized with the configuration: 5'-d(CAGAGCTGGGGTGACCTTCTGTGTC-CCCATCGCT)-3'. This sequence corresponds to nucleotides 240–273 of human GLUT3 cDNA as recently reported [4]. The oligonucleotide was radiolabelled at the 5' end with a specific radioactivity of approx. 2×10^8 c.p.m./ μ g by the transfer of [γ -³²P]ATP using bacteriophage T4 polynucleotide kinase [6]. Phage was plated at a density of 5×10^{10} phage/150 mm plate, and plaques were transferred to 'Plaque Screen' filters (DuPont–

New England Nuclear). Approx. 5×10^5 plaques were screened with the ³²P-labelled oligonucleotide probe.

Expression of GLUT3 protein in CHO cells

The *Bam*HI–*Ssp*I fragment of human GLUT3 cDNA (nucleotide numbers 115–2317) was ligated into the expression vector pMTHneo and transfected into CHO cells by calcium phosphate precipitation as described previously [7]. Two cell lines were selected for their resistance to the neomycin derivative G418 (600 μ g/ml; Gibco), and were termed T-19 and T-49 in this study. Clones A and B, both of which express rabbit GLUT1 protein [8], were prepared in a similar manner [7]. The cells were maintained in Ham's F-12 containing 10 % (v/v) calf serum.

Western-blot analysis

Cells were homogenized in 10 mM-Tris/1 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride/250 mM-sucrose, pH 7.4, in a Potter–Elvehjem glass–Teflon-type homogenizer at 4 °C. The homogenates were centrifuged at 900 *g* for 10 min to sediment the fraction containing mainly the nuclei, and the resulting supernatant was centrifuged at 170 000 *g* for 75 min at 4 °C. The pellet was subjected to SDS/PAGE (10 % gel) and transferred on to nitrocellulose filters. The filters were incubated with the antiserum raised against the synthesized peptide corresponding to the C-terminal domain of GLUT1 (residues 478–492) [9], the C-terminal domain of GLUT3 (residues 482–496), or the conserved domains common to GLUT1 and GLUT3 (GLUT1: residues 388–401; GLUT3: residues 386–399). The filters were then incubated with ¹²⁵I-protein A (ICN, Costa Mesa, CA, U.S.A.) and subjected to autoradiography.

Immunofluorescence staining

CHO cells were fixed in 3 % formaldehyde in phosphate-buffered saline (PBS; 138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄ and 1.8 mM-KH₂PO₄), scraped off from the dish with a rubber blade, and embedded in 10 % (w/v) gelatin/PBS.

Abbreviations used: CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine.

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Semithin frozen sections (1 μm thick) were made and incubated with an antibody against the C-terminal domain of GLUT1 for the detection of GLUT1 or with an antibody against the C-terminal domain of GLUT3 for the detection of GLUT3. These antibodies were prepared from the antisera by using an affinity column prepared with a corresponding peptide as previously described [9]. The sections were then incubated with rhodamine-labelled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.) [10].

Photoaffinity labelling of cell-surface glucose transporter with 2-N-4-(1-azido-2,2,2-trifluoroethyl)[^3H]benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-[^3H]BMPA)

Photoaffinity labelling of glucose transporter on the cell surface was performed as previously described [7,11]. Briefly, CHO cells cultured in dishes (diameter 35 mm) were incubated with 20 μM -ATB-[^3H]BMPA (a generous gift from Dr. G. D. Holman) in 250 μl of PBS, pH 7.4, for 1 min at 22 $^{\circ}\text{C}$ and then irradiated three times, for 10 s each time, with a 625 W u.v. lamp (American Ultraviolet, Santa Ana, CA, U.S.A.). The cells were washed three times with PBS, homogenized and centrifuged at 170 000 g for 75 min at 4 $^{\circ}\text{C}$. The pellet was electrophoresed on SDS/polyacrylamide (10%) gel. The gel lanes were sliced, and their radioactivity was determined.

Photoaffinity labelling of glucose transporter in membranes with [^3H]cytochalasin B

The membranes (250 μg) were placed on to a plastic dish (32 mm diameter) and incubated with 0.25 μM -[^3H]cytochalasin B (18.5 Ci/mmol; DuPont-New England Nuclear) plus 20 μM -unlabelled cytochalasin E. Samples (250 μl) were irradiated twice for 10 s each time and diluted with 5 ml of ice-cold 5 mM- NaH_2PO_4 /1 mM-EDTA/250 mM-sucrose, pH 7.4, and centrifuged at 146 000 g for 75 min, yielding pellets [11,12], which were subjected to SDS/PAGE (10% gel) as described by Laemmli [13]. The gel lanes were sliced, dissolved with Protosol (Du Pont-New England Nuclear), combined with a liquid-scintillation mixture (Econofluor; Du Pont-New England Nuclear), and the radioactivity in each gel slice was determined.

2-Deoxy-D-glucose uptake

The measurement of 2-deoxy-D-glucose uptake in CHO cells was performed as previously described [7]. Nearly confluent monolayers of CHO cells in 24-well plates were rinsed with Krebs-Ringer phosphate buffer. Uptake was initiated by the addition of 250 μl of Krebs-Ringer phosphate buffer containing the indicated concentrations of 2-deoxy-D-[1,2- ^3H]glucose (ICN) for 2 min at 37 $^{\circ}\text{C}$, and stopped by the addition of ice-cold Krebs-Ringer phosphate buffer with 0.3 mM-phloretin. The cells were washed three times with ice-cold PBS, solubilized with 0.1% Triton X-100, and their radioactivity was measured.

3-O-Methylglucose uptake

The measurement of 3-O-methylglucose uptake was performed basically by the same procedure as for 2-deoxy-D-glucose uptake except that the uptake was measured at 16 $^{\circ}\text{C}$. Uptake was terminated by the same method, and the radioactivity was determined.

RESULTS

Isolation of GLUT3 cDNA clones

Twelve positive clones were isolated from 5×10^5 recombinant phages through three or four rounds of plaque hybridization. The *Eco*RI-digested fragments from these clones were electrophoresed through 1% agarose gel, and the longest fragment

Fig. 1. Western-blot analysis of GLUT3 (a) and GLUT1 (b) protein expressed in CHO cells

Human GLUT3 cDNA and rabbit GLUT1 cDNA were ligated into the expression vector pMTHneo and transfected into CHO cells. Lane 1 is control CHO cells. Clones B (lane 2) and A (lane 3) were cell lines prepared for the expression of GLUT1, and clones T-19 (lane 4) and T-49 (lane 5) were prepared for the expression of GLUT3. Membranes from these cell lines were subjected to SDS/PAGE (10% gel) and transferred on to nitrocellulose filters. The filters were incubated with antiserum raised against the C-terminal domain of GLUT3 (a) or with antiserum against the C-terminal domain of GLUT1 (b). The filters were then incubated with ^{125}I -Protein A and subjected to autoradiography.

(3.9 kb) was subcloned into the *Eco*RI site of the plasmid pUC19. The sequence analysis revealed that this 3.9 kb fragment contained a full-length human GLUT3 cDNA.

Western-blot analysis of GLUT1 and GLUT3 proteins expressed in CHO cells

GLUT3 cDNA was ligated into an expression vector termed pMTHneo and transfected into CHO cells. Fig. 1(a) demonstrates the Western-blot analysis using the antiserum against the C-terminal domain of GLUT3. GLUT3 protein was not observed in control CHO cells, clones A or B, but expressed GLUT3 protein was observed as a broad band of 45–65 kDa in clones T-19 and T-49. Western-blot analysis with the antiserum against GLUT1 (Fig. 1b) revealed that clones A and B expressed a large amount of GLUT1. In contrast, clones T-19 and T-49 contained a small amount of GLUT1 as did the control CHO cell, which indicates that the GLUT1 protein expressed in clones T-19 and T-49 is endogenous GLUT1 in CHO cells. We then compared the amount of GLUT3 protein expressed in clone T-49 and the amount of GLUT1 protein expressed in clone B, by using the antiserum against the conserved domain common to GLUT1 and GLUT3, which recognize both transporter isoforms. Fig. 2 represents Western-blot analysis of GLUT3 protein in clone T-49 and GLUT1 protein in clone B at various dilutions of this antiserum. An increase in the radioactivity detected by Western-blot analysis was observed to be parallel with an increase in this antibody concentration, and no significant difference was observed in concentration-dependent curves between GLUT3 and GLUT1. These results suggest that this antibody bound to GLUT1 and GLUT3 with the same affinity and that the amount

Fig. 2. Western-blot analysis of GLUT3 protein and GLUT1 protein with the antibody that recognizes both proteins

Membranes from clone B and clone T-49 were subjected to SDS/PAGE (10% gel) and transferred to nitrocellulose filters. The filters were incubated with antiserum raised against the conserved domain common to GLUT1 and GLUT3 at the indicated dilution. The filters were then incubated with ^{125}I -protein A and subjected to autoradiography (b: A, clone B; B, clone T-49). The radioactivities of the transporter bands were measured and plotted against the dilution of the antiserum (a: $\circ$ — $\circ$, clone B; $\bullet$ — $\bullet$, clone T-49).

of GLUT3 expressed in clone T-49 was nearly equal to that of GLUT1 expressed in clone B.

Immunofluorescence staining of GLUT3 protein expressed in CHO cells

To determine the subcellular localization of GLUT3 protein expressed in CHO cells, we performed immunofluorescence staining using the affinity-purified antibody against the C-terminal domain of GLUT3. The expressed GLUT3 protein was observed to be located mainly at the cell surface of clone T-49, and a small amount was observed in the intracellular region (Fig. 3a). No significant staining was observed in control CHO cells (Fig. 3b). The staining in clone T-49 was completely abolished by co-incubation with the GLUT3 C-terminal peptide and antibody, indicating that the staining was specific for GLUT3 protein (results not shown). No significant difference was observed between the staining of GLUT3 in clone T-49 and that of GLUT1 in clone B (Fig. 3c). These results suggest that the subcellular distribution of GLUT3 protein in CHO cells is essentially the same as that of GLUT1 protein.

Photoaffinity labelling of GLUT3 protein on the cell surface with ATB- ^{3}H BMBA, or GLUT3 protein in the membranes with ^{3}H cytochalasin B

We studied whether or not GLUT3 could be photolabelled with the photoreactive agents such as ATB- ^{3}H BMBA and ^{3}H cytochalasin B. Fig. 4(a) represents the photoaffinity labelling of glucose transporter on the cell surface of clone T-49 and control CHO cells. The radioactivity of the gel lane derived from clone T-49 was demonstrated to be markedly increased in the region of the gel between 45 and 65 kDa, compared with that from control CHO cells (Fig. 4a). Fig. 4(b) represents the

Fig. 3. Immunofluorescence staining of GLUT3 and GLUT1 proteins expressed in CHO cells

The sections were prepared from clone T-49 (a), control CHO cells (b) and clone B (c) as described in the Materials and methods section. These sections were incubated with an anti-peptide antibody against the C-terminal domain of GLUT3 for the detection of GLUT3 in clone T-49 (a) and control CHO cells (b) or with an anti-peptide antibody against the C-terminal domain of GLUT1 in clone B (c). These sections were then incubated with rhodamine-labelled goat anti-rabbit IgG. The arrowheads show the transporters at the plasma membranes.

photoaffinity labelling of glucose transporter in the membrane fraction of clone T-49 and control CHO cells. The marked increase in the labelling was observed in the region of 45–65 kDa in clone T-49 compared with the labelling in control CHO cells with ^{3}H cytochalasin B.

2-Deoxy-D-glucose uptake

Fig. 5(a) demonstrates the 2- ^{3}H deoxy-D-glucose uptake (0.1 mM) into control CHO cells, clones B, A, T-19 and T-49. The clones expressing GLUT1 (clones A and B) and GLUT3 (clones T-19 and T-49) exhibited a significant increase in glucose-transport activity, compared with control CHO cells. We performed a kinetic analysis of 2-deoxy-D-glucose uptake into these cells. The calculation on the basis of a double-reciprocal plot ($1/v$ versus $1/[S]$) revealed that the K_m and V_{max} values of the expressed human GLUT3 were 0.92 mM and 0.35 mmol/min per 1.5×10^5 cells in clone T-19 and 0.81 mM and 0.62 mmol/min per 1.5×10^5 cells in clone T-49 respectively and the K_m and the V_{max} values of the expressed rabbit GLUT1 were 0.94 mM and 1.32 mmol/min per 1.5×10^5 cells in clone B and 0.87 mM and 0.71 mmol/min per 1.5×10^5 cells in clone A respectively. It is possible that hexokinase capacity becomes rate-limiting for 2-deoxyglucose uptake in the cell with a dramatically elevated rate of glucose transport. However, this does not seem to be the case under our assay conditions, since 2-deoxyglucose uptake was proportional to the amount of expressed glucose transporter and double-reciprocal plots lie on a single straight line.

3-O-Methylglucose uptake

To further confirm the results on glucose-transport activity obtained with 2-deoxyglucose, we also measured glucose-transport activity using 3-O-methylglucose. Fig. 6(a) demonstrates the uptake of 3-O- ^{3}H methylglucose uptake (0.2 mM) into control CHO cells, clones B, A, T-19 and T-49. The clones

Fig. 4. Photoaffinity labelling of GLUT3 protein on the cell surface with ATB-[^3H]BMPA (a) or of GLUT3 protein in the membranes with [^3H]cytochalasin B (b)

(a) CHO cells cultured in dishes (diameter 35 mm) were incubated with 20 μCi of ATB-[^3H]BMPA in 250 μl of PBS and irradiated with a u.v. lamp. The cells were washed three times with PBS, homogenized and centrifuged at 170000 g for 75 min at 4 $^{\circ}\text{C}$. The pellet was electrophoresed on 10% polyacrylamide gel. The gel lanes were sliced, and their radioactivities determined. (b) Membranes (250 μg) prepared from CHO cells were incubated with 0.25 μM [^3H]cytochalasin B plus 20 μM unlabelled cytochalasin E and then irradiated with a u.v. lamp. The samples were centrifuged at 146000 g for 75 min and the pellets were electrophoresed on 10% polyacrylamide gel. The gel lanes were sliced and the radioactivity was determined. Positions of molecular mass markers (in kDa) are indicated by the arrows.

Fig. 6. Increase in 3-*O*-methylglucose uptake induced by the expression of GLUT3 and GLUT1 proteins (a) and analysis of the uptake kinetics (b)

(a) Uptake of 3-*O*-methylglucose uptake at 0.2 mM was measured as described in the Materials and methods section. Lane 1, control CHO cells; lane 2, clone B; lane 3, clone A; lane 4, clone T-19; lane 5, clone T-49. The data are means $\pm$ S.D. of three separate experiments. (b) The increase induced by expression of GLUT3 and GLUT1 proteins in 3-*O*-methylglucose uptake at various concentrations was calculated by subtracting the values of the uptake in control CHO cells from those in clone B ($\circ$), those in clone A ($\bullet$), those in clone T-19 ($\blacktriangle$) and those in clone T-49 ($\triangle$).

Fig. 5. Increase in 2-deoxy-D-glucose uptake induced by the expression of GLUT3 and GLUT1 proteins (a) and analysis of the uptake kinetics (b)

(a) Uptake of 2-deoxy-D-glucose at 0.1 mM was measured as described in the Materials and methods section. Lane 1, control CHO cells; lane 2, clone B; lane 3, clone A; lane 4, clone T-19; lane 5, clone T-49. The data presented are means $\pm$ S.D. of three separate experiments. (b) The increase induced by expression of GLUT3 and GLUT1 proteins in 2-deoxy-D-glucose uptake at various concentrations was calculated by subtracting the values of the uptake in control CHO cells from those in clone B ($\circ$), those in clone A ($\bullet$), those in clone T-19 ($\blacktriangle$) and those in clone T-49 ($\triangle$).

expressing GLUT1 (clones A and B) and GLUT3 (clones T-19 and T-49) exhibited a significant increase in glucose-transport activity, compared with control CHO cells. Kinetic analysis of 3-*O*-methylglucose uptake was shown in Fig. 6(b). The K_m and the V_{max} values of the expressed human GLUT3 were 3.44 mM

and 46.9 pmol/min per 1.5×10^5 cells in clone T-19 and 3.57 mM and 77.8 pmol/min per 1.5×10^5 cells in clone T-49 respectively, and the K_m and the V_{max} values of the expressed rabbit GLUT1 were 10.2 mM and 364.3 pmol/min per 1.5×10^5 cells in clone B and 11.1 mM and 150.2 pmol/min per 1.5×10^5 cells in clone A respectively.

DISCUSSION

In this study, we succeeded in a stable expression of GLUT3 protein in CHO cells by the transfection of its cDNA ligated with an expression vector. The expressed GLUT3 could be photo-labelled with ATB-BMPA and cytochalasin B (Figs. 4a and 4b), indicating that these photoreactive agents are useful probes for the analysis of GLUT3 protein, as used for GLUT1 and GLUT4 proteins [14]. The subcellular localization of GLUT3 protein expressed in CHO cells appeared to be very similar to, practically identical with, that of GLUT1 protein. The presence of GLUT3 on the cell surface was further supported by the results of photoaffinity labelling of glucose transporter with ATB-[^3H]BMPA on the cell surface (Fig. 4a). Most of the GLUT4 protein in mammalian cells, one of the facilitative glucose transporters, resides in the intracellular vesicles under basal conditions, whereas most of the GLUT1 transporter resides at the plasma membrane, irrespective of the type of cell including CHO cells [15,16]. The retention of GLUT4 transporter in the intracellular vesicles under basal conditions is thought to contribute to the high responsiveness of glucose-transport activity on stimulation by insulin. The GLUT1-like rather than GLUT4-like subcellular localization of GLUT3 transporter suggests that it, like the GLUT1 transporter, is likely to be responsible for basal or constitutive glucose uptake and that it would not contribute greatly to insulin-stimulated glucose uptake, even if it responded to insulin.

It has been reported that GLUT1 protein has a low K_m value for glucose uptake, whereas GLUT2 has a high K_m value [3,17,18]. Kinetic analysis revealed that the K_m value of GLUT3 protein for 3-*O*-methylglucose uptake in CHO cells was approx. 35% of that of GLUT1, whereas the K_m value of GLUT3 protein for 2-deoxyglucose uptake was very similar to that of GLUT1 protein. It has previously been demonstrated that the expressed GLUT3 protein in oocytes exhibited a lower K_m value for uptake of D-glucose and 3-*O*-methylglucose than GLUT1 [3,19]. Our results on 3-*O*-methylglucose uptake in mammalian cells are very similar to the results obtained in *Xenopus* oocytes. Glucose-transport characteristics including K_m and V_{max} , as well as substrate specificity vary among isoforms. In addition, previous reports have demonstrated that the characteristics of 2-deoxyglucose uptake are different from those of 3-*O*-methylglucose uptake in various kinds of cell [20–24]. Thus it is not surprising that the K_m value for 3-*O*-methylglucose uptake is different but that for 2-deoxyglucose uptake is similar between GLUT1 and GLUT3 in mammalian cells.

Although V_{max} values are obtained for expressed GLUT3 in clone T-49 and for expressed GLUT1 in clone B, it is essential to estimate the number of cell-surface transporters in order to calculate the V_{max} value of one GLUT3 and that of one GLUT1 transporter protein. The GLUT1 expressed in clone B and GLUT3 expressed in clone T-49 exhibited a similar level of cellular expression (Fig. 2) and a similar subcellular distribution (Fig. 3), suggesting that the amount of cell-surface GLUT1 protein in clone B and that of cell-surface GLUT3 protein in clone T-49 were very similar. On the basis of these results, the V_{max} value of GLUT3 protein for uptake of 3-*O*-methylglucose and 2-deoxyglucose is estimated to be approx. 20–50% of that of GLUT1. Thus we can conclude that GLUT3 protein is designated as a low- K_m and low- V_{max} glucose transporter compared with other glucose-transporter isoforms.

GLUT3 isoform is thought to be a brain-type glucose transporter, although brain tissues also express a significant amount of GLUT1 transporter [4,5]. The brain depends greatly on glucose for energy and as the major carbon source for a wide variety of molecules [25]. Glucose crosses the blood-brain barrier by a transport mechanism which is thought to be mediated by GLUT1 transporter [26–28]. Beyond the blood-brain barrier, glucose can be taken up into neurons by carrier processes, which might be mediated by GLUT3 transporter, since the expression of GLUT1 in brain tissue was reported to be restricted mainly to the blood-brain barrier [26–28]. In the brain, the capacity of hexokinase for glucose is much greater than the apparent capacity of the transport system, and glycolysis is regulated by hexokinase activity and not by glucose transport into brain cells under normal conditions [25]. However, the glucose-transport process might become important under conditions of increased demand and hypoglycaemia, since brain utilizes glucose preferentially as a source of metabolic energy. Under such conditions, the low- K_m property of GLUT3 may contribute to preventing disruption of brain function.

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