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Characterization of rat Glut4 glucose transporter expressed in the yeast *Saccharomyces cerevisiae*: comparison with Glut1 glucose transporter [1]

Toshiko Kasahara, Michihiro Kasahara *

Laboratory of Biophysics, School of Medicine, Teikyo University, Hachioji, Tokyo, 192-03, Japan

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Abstract

Rat Glut4 glucose transporter was expressed in the yeast *Saccharomyces cerevisiae*, but was retained in an intracellular membranous compartment and did not contribute to glucose uptake by intact cells. A crude membrane fraction was prepared and reconstituted in liposome with the use of the freeze–thaw/sonication method. D-glucose-specific, cytochalasin B inhibitable glucose transport activity was observed. Kinetic analysis of D-glucose transport was performed by an integrated rate equation approach. The K_m under zero-trans influx condition was 12 ± 1 mM (mean $\pm$ S.E., $n = 3$) and that under equilibrium exchange condition was 22 ± 3 mM ($n = 4$). D-glucose transport was inhibited by 2-deoxy-D-glucose or 3-*O*-methyl-D-glucose, but not by D-allose, D-fructose or L-glucose. Cytochalasin B, phloretin and phlorizin inhibited D-glucose transport, but neither *p*-chloromercuribenzoic acid (pCMB) (0–0.1 mM) nor *p*-chloromercuribenzene sulfonic acid (pCMBS) (0–1.0 mM) inhibited this activity. High concentrations of $HgCl_2$ were required to inhibit D-glucose transport (IC_{50}, 370 μM). Comparing these properties to those of rat Glut1, we found two notable differences; (1) in Glut1, K_m under zero-trans influx was significantly smaller than that under equilibrium exchange but in Glut4 less than two-fold difference was seen between these two K_m values; and (2) Glut1 was inhibited with pCMB, pCMBS and low concentrations of $HgCl_2$ (IC_{50}, 3.5 μM), whereas Glut4 was almost insensitive to SH reagents. To examine the role of the exofacial cysteine, we replaced Met-455 of Glut4 (corresponding to Cys-429 of Glut1) with cysteine. The mutated Glut4 was inhibited by pCMB or pCMBS and the IC_{50} of $HgCl_2$ decreased to 47 μM, whereas K_m, substrate specificity and the sensitivity to cytochalasin B were not significantly changed, indicating that the existence of exofacial cysteine contributed only to increase SH sensitivity in Glut4.

Keywords: Glut4; Glut1; Glucose transporter; Characterization; (*S. cerevisiae*); (Rat)

1. Introduction

The facilitated diffusion of glucose across the plasma membrane of animal cells is mediated by a family of homologous yet genetically distinct glucose transporters [1–3]. To date, six glucose transporters (Glut1–5,7) have been identified and each has been shown to exhibit tissue-specific expression. Of these

Abbreviations: pCMB, *p*-chloromercuribenzoate; pCMBS, *p*-chloromercuribenzene sulfonate.

* Corresponding author. Fax: +81 426 783262.

[1] The nucleotide sequence data of rat *GLUT4* reported in this paper have been submitted to the GenBank/DDBJ/EMBL database under the accession number D28561.

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Glut isoforms, the expression of Glut4 is restricted in insulin-sensitive tissues, where other isoforms, Glut1 and Glut5, are also expressed [2]. With the administration of insulin, both Glut1 and Glut4 translocate from the intracellular compartment to the cell surface and contribute to the increase of glucose uptake [4–6]. The role of each isoform in these insulin-sensitive tissues has been intensively investigated, but it has been difficult to study the detailed properties of each one in its original tissue due to the co-expression of isoforms and rapid glucose metabolism. To solve this problem, individual isoforms were introduced into heterologous expression systems, such as *Xenopus* oocytes and various tissue culture cells. In these expression systems, however, the activity of Glut4 was often too low to allow detailed examination of its functional properties, since Glut4 has a tendency to be retained in an intracellular compartment [7–9]. Until now, only K_m for 2-deoxy-D-glucose under zero-trans influx, K_m for 3-*O*-methyl-D-glucose and substrate specificity have been measured under lengthy assay times because of insufficient activity [10–14].

We have developed a new heterologous expression system using the yeast *Saccharomyces cerevisiae*, in which major glucose transport related genes were disrupted [15]. Rat Glut1 was expressed and was retained in an intracellular compartment, but upon reconstitution it showed D-glucose transport activity as active as that of human erythrocyte Glut1 [15]. In this study, we used the same reconstitution technique and determined various properties of Glut4, including kinetic parameters under two representative conditions, substrate specificity and sensitivity to various inhibitors. When compared with Glut1, Glut4 showed markedly less difference in K_m values under zero-trans condition and equilibrium exchange condition and was almost insensitive to SH inhibitors.

2. Materials and methods

2.1. Expression of rat GLUT4 in S. cerevisiae

The construction of plasmids for expression of *GLUT1* (called GLUT1-pTV3e), yeast glucose transporter, *HXT2* (HXT2-pTV3e) and yeast galactose transporter, *GAL2* (GAL2-pTV3e) by using a GAL expression system in a multicopy plasmid pTV3e (YEp TRP1 bla) was described previously [15,16].

GLUT4 was cloned from a rat fat cell cDNA library (RL1101b, Clontech). To make an *Eco*RI site, the nucleotide sequence following the initiation codon of *GLUT4*, ATGCCGTCGGGTTTC was modified to ATGCCGTCGGAATTC, which changed the deduced amino acid sequence from Met-Pro-Ser-Gly-Phe to Met-Pro-Ser-Glu-Phe. To make a *Cla*I site, the nucleotide sequence following the termination codon of *GLUT4*, TGAGGGGCA was modified to TAATCGATA. A plasmid, GLUT4-pTV3e, was made by replacing the ORF of *GAL2* of GAL2-pTV3e with the ORF of *GLUT4* using restriction enzymes *Eco*RI and *Cla*I. The nucleotide sequences of *GLUT4* in GLUT4-pTV3e were verified by sequencing the two strands.

These plasmids were introduced to LBY416 (MAT**α** *hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trpl his3 leu2 ura3*), provided by L. Bisson (University of California, Davis, CA) [15–17]. All cells were grown at 30°C in a synthetic medium [18] with 2% galactose as a carbon source and supplemented with uracil, adenine and amino acids except for tryptophan. To evaluate the effects of proteolytic degradation, GLUT4-pTV3e was introduced in BJ3505 (MATa *pep4::HIS3 prb1-Δ1.6R his3 lys2-208 trp1-Δ101 ura3-52 gal2 can1*), provided by Y. Osumi (National Inst. for Physiol. Sci., Japan).

2.2. Preparation of crude membrane fraction of yeast

Cells of LBY416 possessing one of these plasmids were cultured to an early log phase (OD_{650}, 0.2–0.4), washed two times with H_2O and suspended with 10 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA. Cells were disrupted by glass beads (Mini-Beadbeater, Biospec Products) and spun for 1 min at $6200 \times g$. The supernatant was spun for 20 min at $247\,000 \times g$ to recover the pellet as a crude membrane fraction.

2.3. Reconstitution of glucose transport

The modified freeze–thaw/sonication method was used to reconstitute glucose transporter from the crude membrane fraction of yeast, as described [15,19].

2.4. Measurement of glucose transport in reconstituted liposomes

The initial rate of glucose transport was assessed by the transport of 0.1–0.5 mM D-[^{14}C]glucose (CFB96, Amersham) for 5–15 s at 25°C with 0.32–0.48 mg of reconstituted liposomes. In the kinetic studies, the transport of 0.5–16 mM (zero-trans influx) or 1–40 mM (equilibrium exchange) of D-[^{14}C]glucose was measured for 5, 10, 15 and 30 s. For equilibrium exchange condition, liposomes were incubated at the indicated concentration of D-glucose for 90 min prior to the measurement. The uptake reaction was started by the addition of the isotope and terminated by the addition of a cold stopping solution as described [15,16]. Nonspecific uptake of D-glucose was estimated from the transport in the presence of 2 mM $HgCl_2$. Time-course of D-glucose specific transport was fitted to an exponential curve and the initial rate of transport was calculated by a non-linear least-squares method (KaleidaGraph, Synergy Software)

2.5. Immunofluorescence microscopy

Yeast cells grown in an early log phase were fixed with 5% formaldehyde. After methanol and acetone treatment, cells were treated successively with anti-Glut4 Ab and goat anti-rabbit IgG Ab conjugated with tetramethylrhodamine isothiocyanate (111-026-003, Jackson Immuno Research) [20]. The samples were mounted with FluorSave (Calbiochem) and observed with a microscope equipped with epifluorescence optics (BH-2, Olympus).

2.6. Immunoblotting

Immunoblotting of homogenates was performed as described [15]. Polyclonal rabbit Ab to human erythrocyte Glut1 or polyclonal rabbit anti-Glut1 Ab raised against the synthetic peptides corresponding to amino acids 480–492 (13 amino acids of the carboxyl terminus) of the deduced amino acid sequence of rat *GLUT1* was used as an anti-Glut1 Ab [21]. Polyclonal rabbit Ab raised against the synthetic peptides corresponding to amino acids 497–509 (13 amino acids of the carboxyl terminus) of the deduced amino acid sequence of rat *GLUT4* was used as an anti-Glut4 Ab. Neither antibody cross-reacted with the other antigens (see Fig. 1). Autoradiography of ^{125}I-Protein A (IM144, Amersham) was performed with imaging plates (BAS2000, Fuji Film).

2.7. Site-directed mutagenesis

To replace Met-455 of Glut4 with cysteine, the nucleotide sequence ATG was modified to TGT by using PCR [22], which was confirmed by sequencing the two strands.

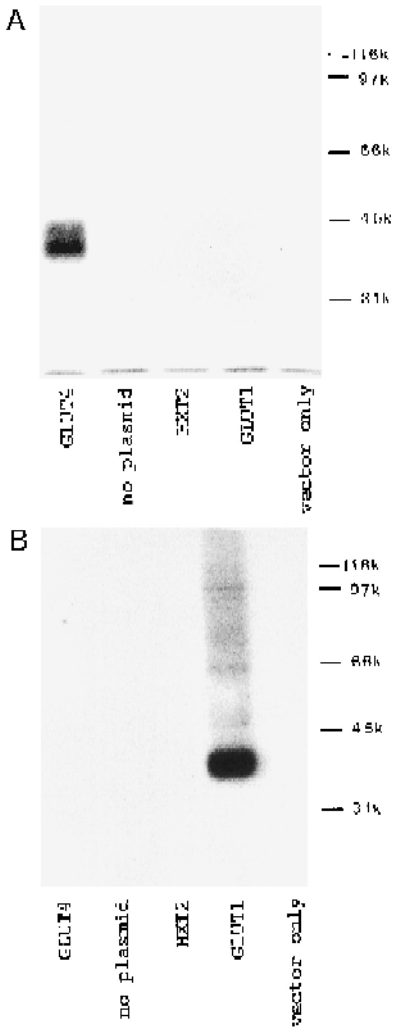

Fig. 1. Immunoblotting of Glut4 or Glut1 expressed in yeast. *GLUT4* cells, *GLUT1* cells, *HXT2* cells, control cells or cells with no plasmid were cultured to an early log-phase and disrupted by glass beads as described in Section 2. Each homogenate (11 μg) was subjected to immunoblotting with anti-Glut4 Ab (A) or anti-Glut1 Ab (B).

2.8. Other assay

Protein was determined with bicinchoninic acid, according to the manufacturer's instruction (Pierce).

3. Results

Rat *GLUT4* was cloned from a rat fat cell cDNA library. A plasmid carrying *GLUT4*, GLUT4-pTV3e, was made and introduced to LBY416 cells, in which two major glucose transport related genes, *HXT2* and *SNF3*, were disrupted. These were termed *GLUT4* cells. In the same way, LBY416 cells were transformed with a plasmid carrying a yeast glucose transporter gene, *HXT2* (HXT2 cells) or a vector, pTV3e (control cells). To examine the expression of Glut4 glucose transporter in LBY416, immunoblotting was performed with anti-Glut4 Ab. A single band corresponding to 43 kDa was observed with *GLUT4* cells, whereas no detectable band was seen with HXT2 cells, *GLUT1* cells, control cells or cells with no plasmid (Fig. 1A) With anti-Glut1 Ab, no detectable band was seen with *GLUT4* cells, *HXT2* cells, control cells or cells with no plasmid, but a single major band corresponding to 40 kDa was seen with *GLUT1* cells (Fig. 1B). These molecular masses are about 10 kDa smaller than those of the calculated molecular mass derived from deduced amino acid sequences (55 kDa for Glut4, 54 kDa for Glut1). Similar observations of apparently lower molecular mass were often seen in other yeast transporters [16]. We used another strain, BJ3505, whose two potent proteinases were deficient, to ascertain there was no significant proteolytic degradation in LBY416. BJ3505 also showed the identical molecular mass of 43 kDa in Glut4 and 40 kDa in Glut1 (data not shown). From these results, we concluded there was no detectable proteolytic degradation of Glut4 in LBY416.

Glucose transport activity in *GLUT4* cells was measured and no increase in glucose transport was noted (data not shown). We used immunofluorescence microscopy to locate Glut4 and found that a majority of Glut4 in *GLUT4* cells was retained in an intracellular compartment probably corresponding to endoplasmic reticulum and was not transferred to the cell surface (Fig. 2). This may be the reason we were unable to see any increase in glucose transport activity in intact *GLUT4* cells. In our previous study, Glut1 in *GLUT1* cells was also retained in an intracellular compartment and *GLUT1* cells showed no increase in glucose transport activity. Distinct D-glucose specific transport was shown, however, when a crude membrane fraction of *GLUT1* cells was reconstituted in liposomes.

A crude membrane fraction was prepared from *GLUT4* cells and reconstituted in liposomes with the use of the freeze–thaw/sonication method. Significant D-glucose specific transport was observed, whereas no appreciable D-glucose specific transport

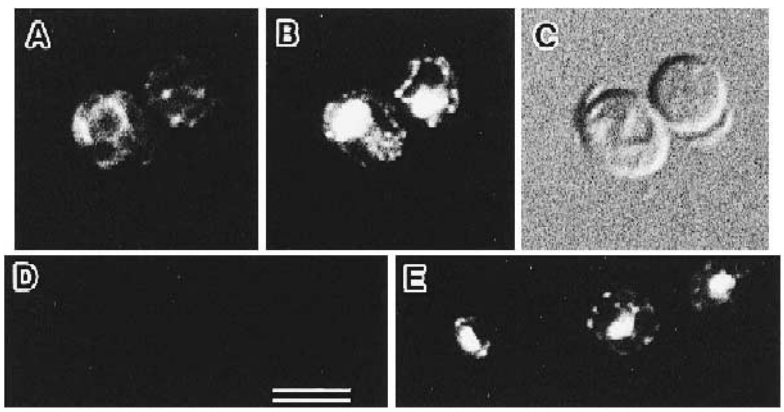

Fig. 2. Immunofluorescence microscopy of Glut4 in *GLUT4* cells. *GLUT4* cells (A, B and C) or control cells (D and E) were cultured to an early log phase and fixed with 5% formaldehyde [20]. The fixed cells on a glass slide were treated with anti-Glut4 Ab and stained with rhodamine-labeled goat-anti-rabbit IgG Ab (A and D). Nuclei were simultaneously stained with DAPI (B and E). Nomarski differential interference-contrast image of the same specimen is also shown (C). Bar: 5 μm.

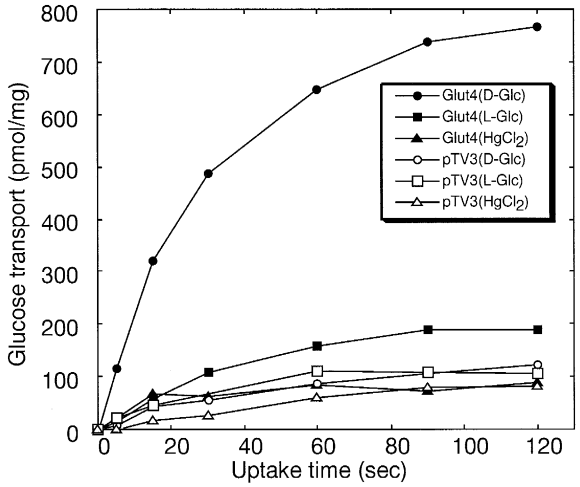

Fig. 3. Time-course of reconstituted glucose transport. The crude membrane fraction of *GLUT4* cells (●,■,▲) or the crude membrane fraction of control cells (○,□,△) was reconstituted in liposomes by the freeze–thaw/sonication method. An aliquot of 87 μg of protein in 0.40 mg of liposomes was used for D-glucose transport (●,○), D-glucose transport in the presence of 2 mM $HgCl_2$ (▲,△) or L-glucose transport (■,□) at 25°C as described in Section 2. When $HgCl_2$ was used, it was preincubated for more than 5 min at 25°C.

was observed when the crude membrane fraction prepared from control cells was reconstituted in liposomes (Fig. 3). In contrast to Glut1, a minor amount of L-glucose transport was always seen, although details were not pursued in this study.

Kinetic parameters of Glut4 and Glut1 for D-glucose transport were obtained using an integrated equation approach under zero-trans influx and equilibrium exchange conditions at 25°C (Table 1). The K_m of Glut4 under each condition was relatively close, 12 ± 1 mM under zero-trans influx and 22 ± 3 mM for equilibrium exchange with the difference in K_m values being less than 2-fold, whereas Glut1 showed quite a different feature. In Glut1 the K_m under equilibrium exchange was 7.4-fold that under zero-trans influx, which corresponds well to that of human erythrocytes [1].

Studies of substrate specificity for D-glucose transport in Glut4 showed there were three groups of sugars (Fig. 4). Those which competed as well as D-glucose were 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose; those showing almost no competition were D-allose, L-glucose and D-fructose; and those which did not compete efficiently as D-glucose were D-galactose, 6-deoxy-D-glucose and D-xylose. Fig. 4 shows that Glut1 exhibits similar substrate specificity. We noted that inhibition by D-glucose, D-galactose, 6-deoxy-D-glucose or D-xylose was less in Glut4 than in Glut1, which may be due to lower affinity of Glut4 for D-glucose and these sugars.

Table 1
Kinetic parameters of glucose transporter

	Yeast-made Glut4	Yeast-made Glut1	Human erythrocyte Glut1 [a]	Mouse Glut4 [b]	Mouse Glut1 [b]
Zero-trans influx	mean ± S.E. ($n = 3$)	mean ± S.E. ($n = 3$)			
K_m (mM)(*a*)	12 ±1	3.5 ±0.6	1.6	9	11
V_{max} (nmol/s per mg)	1.4 ±0.3	1.8 ±0.5	0.6–0.9	76	580
V_{max}/K_m	0.11 ± 0.02	0.56 ± 0.02		8.4	53
Equilibrium exchange	mean ± S.E. ($n = 4$)	mean ± S.E. ($n = 3$)			
K_m (mM)(*b*)	22 ±3	26 ±2	13–20	19	44
V_{max}(nmol/s per mg)	3.7 ±0.9	16 ±3	4.4–6.1	120	1500
V_{max}/K_m	0.16 ± 0.03	0.60 ± 0.08		6.3	34
b/*a*	1.8	7.4	8.1–13	2.1	4.0

[a] Data obtained at 20°C are taken from [1]. V_{max} values are expressed as mM/s.
[b] Data are taken from [14], where 3-*O*-methyl-D-glucose transport via mouse Glut4 or Glut1 in *Xenopus* oocytes was measured at 22°C. V_{max} values are expressed as pmol/min per oocyte.
The crude membrane fraction of *GLUT1* cells or *GLUT4* cells was reconstituted in liposomes. An aliquot of 30–59 μg (*GLUT1* cells) or 69–111 μg (*GLUT4* cells) of protein in 0.34–0.40 mg of liposomes was used. The transport of 0.5–16 mM (zero-trans influx) or 1–40 mM (equilibrium exchange) of D-[^{14}C]glucose was measured for 5, 10, 15 and 30 s and the V_{max} and K_m under each condition were determined as described in Section 2. The mean value ± S.E. is shown with the number of determinations in parentheses.

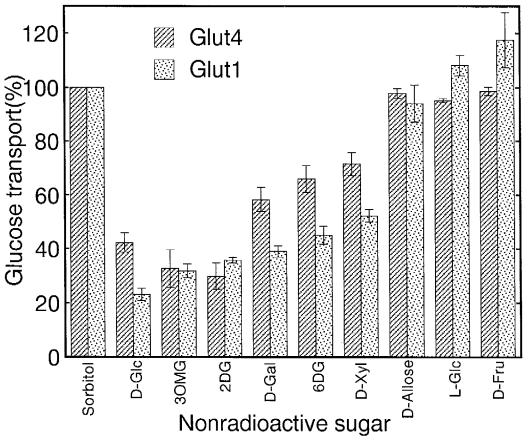

Fig. 4. Substrate specificity of reconstituted glucose transport mediated by Glut1 or Glut4. Liposomes were made with the crude membrane fraction of *GLUT1* cells or *GLUT4* cells. An aliquot of 66–95 μg of protein (*GLUT1* cells) or 83–144 μg of protein (*GLUT4* cells) in 0.32–0.48 mg of liposomes was used for the 15 s transport assay with 0.1 mM D-glucose. The background was estimated by D-glucose transport in the presence of 2 mM $HgCl_2$ and subtracted. Since high concentrations of sugars inhibited glucose transport apparently due to osmotic pressure, the transport activities are expressed as the percentage of the values with 20 mM D-sorbitol addition; these were 6.2–11.6 pmol/15 s (Glut1) and 4.0–11.6 pmol/15 s (Glut4). The vertical bars indicate S.E. Abbreviations used: DG, deoxy-D-glucose; 3OMG, 3-*O*-methyl-D-glucose.

Sensitivity to various inhibitors was determined in Glut4 and Glut1 (Table 2). Cytochalasin B, a potent inhibitor of many mammalian glucose transporters, inhibited glucose transport activity of Glut4 (IC_{50}, 0.33 μM) as well as Glut1 (IC_{50}, 0.44 μM). Both phloretin and phlorizin inhibited Glut4 with an IC_{50} of 9.4 μM and 140 μM, respectively, which were less than those in Glut1. Neither pCMB nor pCMBS inhibited the transport. $HgCl_2$ inhibited it at high concentrations (IC_{50}, 370 ± 40 μM), whereas the IC_{50} of $HgCl_2$ of Glut1 was 3.5 μM, which was about 100-fold less than that of Glut4. Both pCMB and pCMBS efficiently inhibited Glut1 activity. These results showed that there was marked difference between Glut1 and Glut4 in their sensitivity to SH inhibitors. From hydropathy plot analysis, Glut1 has one exofacial cysteine residue (Cys-429) at the extracellular loop between TM 11 and 12 and Glut4 has none (Fig. 5). Using site-directed mutagenesis, we introduced a new cysteine at the position of Met-455 of Glut4 (M455C), which corresponds to Cys-429 of

Table 2
Inhibitor of reconstituted glucose transport

Inhibtor	IC_{50} (μM) (mean $\pm$ S.E.)	
	Glut4	Glut1
Cytochalasin B	0.33 ± 0.03 ($n = 3$)	0.44 ± 0.03 ($n = 3$)
Phloretin	9.4 ± 1.2 ($n = 2$)	49 ± 12 ($n = 2$)
Phlorizin	140 ± 40 ($n = 3$)	360 ± 70 ($n = 2$)
$HgCl_2$	370 ± 40 ($n = 5$)	3.5 ± 0.5 ($n = 3$)
pCMB	N.I. [a]	15 ± 4 ($n = 3$)
pCMBS	N.I. [a]	$44 + 4$ ($n = 3$)

[a] No inhibition (0–0.1 mM with pCMB and 0–1.0 mM with pCMBS)

The crude membrane fraction of *GLUT1* cells or *GLUT4* cells was reconstituted in liposomes. An aliquot of 30–105 μg (*GLUT1* cells) or 34–98 μg (*GLUT4* cells) of protein in 0.32–0.48 mg of liposomes was used. The transport of 0.1 mM of D-[^{14}C]glucose was measured for 15 s. Liposomes were preincubated with each inhibitor for more than 5 min at 25°C. Inhibition doses were obtained with 4–7 assays using varying concentrations of inhibitors. When cytochalasin B or a diphenolic reagent (phloretin or phloridzin) was used as an inhibitor, dimethylsulfoxide or ethanol, respectively, was carried over to the assay solution; this amounted to less than 0.5% and had no apparent effect on glucose transport. Results are expressed as mean $\pm$ S.E. and n is the number of experiments.

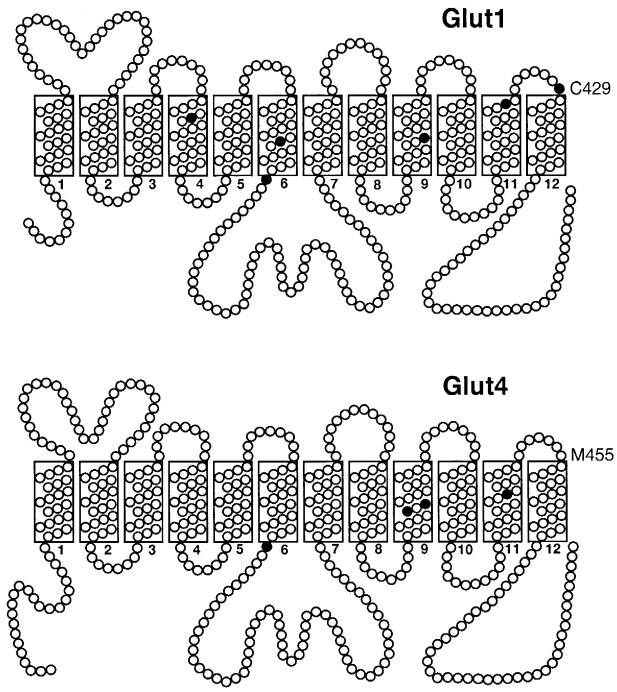

Fig. 5. Predicted membrane topology of Glut1 and Glut4. The cysteine residues are indicated by closed circles. The transmembrane topology of Baldwin [1] is adopted.

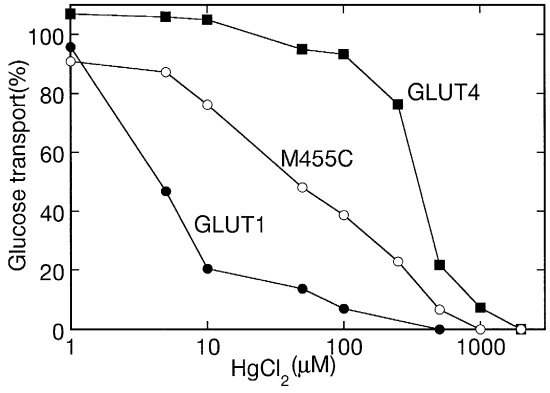

Fig. 6. Inhibition of reconstituted glucose transport by $HgCl_2$ The crude membrane fraction of *GLUT1* cells (●), *GLUT4* cells (■) or M455C cells (○) was reconstituted in liposomes. A portion of 66 μg (●), 75 μg (■) or 46 μg (○) of protein in 0.32–0.48 mg of liposomes was used for 15 s transport. After subtraction of the background estimated by D-glucose transport in the presence of 2 mM $HgCl_2$. D-Glucose-specific transport activities are expressed as the percentage of those without $HgCl_2$; that was 15.4 pmol/15 s (●), 21.9 pmol/15 s (■) or 6.7 pmol/15 s (○). Liposomes were preincubated with indicated concentrations of $HgCl_2$ for more than 5 min at 25°C.

Glut1. The reconstituted glucose transport activity of M455C was similar to native Glut4. The K_m and V_{max} values were 11 mM and 1.2 nmol/s per mg under zero-trans influx and 15 mM and 2.3 nmol/s per mg under equilibrium exchange. These values are within the range of those of native Glut4. Substrate specificity did not significantly change (data not shown) and the sensitivity to cytochalasin B (IC_{50}, 0.24 μM) was almost identical; the sensitivity to SH reagents, however, was drastically increased. The IC_{50} of $HgCl_2$ decreased to 47 ± 7 μM (Fig. 6) and both pCMB and pCMBS inhibited the activity. One mM of pCMBS inhibited 62% of the activity and 100 μM of pCMB inhibited 31%.

4. Discussion

The expression of Glut4 in yeast was confirmed by immunoblotting with anti-Glut4 Ab; however, no appreciable increase of glucose transport was observed with intact *GLUT4* cells. Retention of rat Glut4 in an intracellular structure as seen by immunofluorescence microscopy is consistent with our previous observation that rat Glut1 in yeast was retained in an intracellular compartment [15]. When we expressed yeast sugar transporter (Hxt2 or Gal2) in LBY416 cells in the same manner, the expressed Hxt2 or Gal2 was correctly transferred to the cell surface and worked properly [16]. These results indicate that mammalian glucose transporters lack some sorting signal(s) which is indispensable for the localization in plasma membrane of yeast. Since animal cell transporters, Glut1 and Glut4 are homologous to yeast transporters, Hxt2 and Gal2 and partial replacement of Hxt2 with the corresponding part of Glut1 did not hinder glucose transport in intact cells (Kasahara, T. and Kasahara, M., unpublished data), it is of considerable interest to identify the sorting signal(s) in yeast transporters.

The kinetic parameters of rat Glut4 and Glut1 for D-glucose transport were determined under two representative conditions: zero-trans influx and equilibrium exchange. With reconstitution we were able to use D-glucose as a substrate and exclude the metabolic effects. In human erythrocytes where Glut1 alone is expressed, kinetic parameters for D-glucose were obtained under several conditions and our results correspond well with those (Table 1). K_m values obtained with yeast-made Glut1 are comparable with those obtained with human erythrocyte Glut1. The K_m under equilibrium exchange was 7-fold larger than that under zero-trans influx (expressed as b/a in Table 1), which corresponds to 8.1–13 in human erythrocyte Glut1. Concomitantly, an accelerated exchange was observed in that V_{max} under equilibrium exchange was 9-fold larger than that under zero-trans influx. In other heterologous expression systems such as *Xenopus* oocyte and tissue culture cells, 2-deoxy-D-glucose or 3-*O*-methyl-D-glucose was used as a substrate [11–13], so we were unable to compare our results with theirs because of the difference in substrate. We measured the glucose uptake for 5, 10, 15 and 30 s and used an integrated equation approach to obtain kinetic parameters, since transport rates were accelerated under equilibrium exchange and the 5 s measurement of glucose uptake was not short enough to obtain initial rates. The V_{max}/K_m ratios were almost identical under zero-trans influx and under equilibrium exchange in each glucose transporter, indicating our transport data were appropriately obtained according to Krupka's criteria [23]. We previously observed that the kinetic parameters of rat Glut1 expressed in yeast showed good correspon-

dence to those of human erythrocyte [15]. However, there was no native tissue which expressed Glut4 alone. Only recently, Dauterive et al. reported the kinetic parameters of mouse Glut1 and Glut4 expressed in *Xenopus* oocytes under zero-trans influx and equilibrium exchange using 3-*O*-methyl-D-glucose as a substrate [14]. In spite of the substrate difference, the phenomenon of accelerated exchange was observed with mouse Glut1 expressed in *Xenopus* oocytes. In Glut4, the acceleration was less than 2-fold as with that of mouse. One notable difference between our findings and those of Dauterive et al. [14] is that in our case K_m under zero-trans condition of Glut4 was 3-fold larger than that of Glut1, while Dauterive et al. observed no notable difference; this might be due to the difference in substrate or species used. The fact that Glut1 and Glut4 showed different K_m ratios under two representative assay conditions indicates a difference in molecular mechanism among the Glut family transporters.

Other biochemical properties of rat Glut4 were compared with those of rat Glut1. We found no significant difference in substrate specificity. Our results, however, were quite different from those of Colville et al. [10] using *Xenopus* oocytes and 2-deoxy-D-glucose as a substrate. One reason may be that metabolizable glucose analogues show different substrate specificity from non-metabolizable glucose analogues, as Maher et al. have pointed out [24]. In addition, the difference in uptake time: 15 s in our assays and 60 min in 2-deoxy-D-glucose transport in *Xenopus* oocytes. We noted a significant difference in sensitivity to SH inhibitors. Rat Glut1 was about 100-fold more sensitive to $HgCl_2$ than Glut4. Human erythrocyte Glut1 was inhibited by several μM of $HgCl_2$ [25] and several hundreds of μM of $HgCl_2$ was required to inhibit insulin-stimulated glucose transport in rat adipocytes [26].

The point mutation of Glut4 (Met-455 to Cys) increased the sensitivity to SH reagents, but single cysteine mutation did not give the same sensitivity as Glut1. Other amino acid residue(s) might be coordinated. Although the replacement with cysteine was critical for SH sensitivity, it did not alter functional properties. These results are consistent with the notion that no cysteine residue has an important role in the function of Glut4, as Wellner et al. [27] or Due et al. [28] concluded from their studies of cysteine-less Glut1 or Glut1 whose cysteine residues were individually replaced with other amino acids. The difference of SH sensitivity between Glut1 and Glut4 is notable. Our preliminary studies using reconstituted plasma membrane fraction from insulin-stimulated rat adipocytes in liposomes showed almost the same SH sensitivity as found in this study (Kasahara, T. and Kasahara, M., unpublished data). This may serve as a useful tool to investigate the role of each isoform in insulin-sensitive tissues where both isoforms are expressed.

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