Role of tryptophan-388 of GLUT1 glucose transporter in glucose-transport activity and photoaffinity-labelling with forskolin

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GLUT1 glucose-transporter cDNA was modified to substitute leucine for Trp-388 and transfected into Chinese hamster ovary cells using the expression vector termed pMTHneo. This tryptophan residue is conserved among most of the facilitative glucose-transporter isoforms and has been proposed to be the photolabelling site of forskolin, a competitive inhibitor of glucose transport. In addition, this residue is located on membrane-spanning helix 10 which is suggested to contain the dynamic segment of the transporter. The mutated glucose transporter was expressed and inserted into the plasma membrane in a fashion similar to the wild-type. Unexpectedly, this mutation did not abolish photolabelling with forskolin. However, the mutation induced a marked decrease in 2-deoxyglucose uptake with a 4-fold decrease in turnover number and a 1.25-fold increase in K_m

compared with the wild-type GLUT1. A similar decrease in zero-*trans* influx activity was also observed for 3-*O*-methylglucose. In contrast, no apparent decrease was observed in zero *trans* efflux activity for 3-*O*-methylglucose. The mutation decreased the turnover number of the glucose transporter in equilibrium exchange influx for 3-*O*-methylglucose by 33 % without any change in K_m . These results indicate that (1) Trp-388 is not the photolabelling site for forskolin, if we assume that the labelling occurs at a single site and (2) Trp-388 is more likely to be involved in interconversion between the inward-facing and outward-facing conformers of GLUT1 than binding of glucose, and thus, substitution of leucine for Trp-388 in this dynamic segment would decrease the rate of alternating conformation, which would preferentially affect the influx activity.

INTRODUCTION

Recent cDNA cloning has disclosed the presence of a structurally related family of proteins that catalyse facilitated glucose transport across the plasma membrane [1–12]. A marked structural similarity exists within this family. All five isoforms of mammalian facilitative glucose transporters have 12 membrane-spanning α -helical domains with both N- and C-termini located on the cytoplasmic face. In addition, several amino acid residues are conserved among isoforms especially in transmembrane domains, implying that these residues may play an important role in the function of these membrane proteins. Trp-388 of GLUT1 is one of these conserved amino acid residues and is observed in GLUT1, 2, 3 and 4 [1–11] as well as the *Escherichia coli* H⁺/arabinose transporter [13] and yeast glucose transporter [14]. Interestingly, ligand-dependent quenching of tryptophan fluorescence has suggested that helix 10 contains the dynamic segment close to Trp-388 [15,16]. This segment may move as a result of the conformational change between an outward-facing form and an inward-facing form which accompanies the transport of glucose. Furthermore, Trp-388 of GLUT1 is proposed to be a candidate for the photoaffinity-labelling site of 3-iodo-4-azidophenethylamino-7-*O*-succinyldeacetyl (IAPS)-forskolin, a photoactive derivative of forskolin [17]. Facilitated glucose transport of GLUT1 is competitively inhibited by forskolin [18] which may bind to, or close to, the glucose-binding site of the transporter.

These findings suggest that Trp-388 may play a very important role in ligand binding as well as glucose transport. Thus it is of great interest to investigate the effect of amino acid substitution of Trp-388 on the transport activity and on photolabelling with

IAPS-forskolin. By means of expression of mutated glucose transporter, we here report that the single amino acid substitution of leucine for Trp-388 of GLUT1 does not abolish photolabelling with IAPS-forskolin but markedly decreases the zero-*trans* influx of glucose without significant alteration to the zero-*trans* efflux of glucose. Our results suggest that Trp-388 does not constitute the binding site for glucose but rather this residue is likely to be involved in the inward movement of glucose through the transporter.

MATERIALS AND METHODS

Expression of mutated glucose transporter

The point mutation of G to T was introduced at nucleotide 1286 of rabbit GLUT1 glucose-transporter cDNA [3] by PCR [19] using synthesized oligonucleotides corresponding to nucleotides 2230–2245 and 1255–1295 of rabbit GLUT1 cDNA except that G at nucleotide 1286 was replaced by T. This oligonucleotide contains the *Apa*I site (GGGCCC) at position 1266. This point mutation results in substitution of leucine (TTG) for tryptophan (TGG) at residue 388 of the encoded rabbit GLUT1 glucose transporter. The *Apa*I–*Mae*I fragment (nucleotide 1266–2230) prepared by PCR was ligated to the *Mae*I–*Apa*I fragment (nucleotide 111–1266) of the rabbit GLUT1 glucose transporter. The entire fragment prepared by PCR as well as the ligation sites were sequenced and observed to have no other mutations except at nucleotide 1286. The *Mae*I fragment of the mutated GLUT1 glucose transporter cDNA (nucleotide 111–2230) was ligated into the expression vector termed pMTHneo and transfected into Chinese hamster ovary (CHO) cells by calcium phosphate

Abbreviations used: IAPS, 3-iodo-4-azidophenethylamino-7-*O*-succinyldeacetyl; TES, 10 mM Tris, 1 mM EDTA, 250 mM sucrose plus 1 mM phenylmethanesulphonyl fluoride, pH 7.4; CHO, Chinese-hamster ovary; KRP, Krebs–Ringer phosphate.

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precipitation as previously described [20]. Two cell lines were selected by their resistance to 600 µg/ml neomycin derivative, G418 (Gibco). One that expressed more glucose transporters than the other was termed clone L388 in this study.

Immunoblot analysis

CHO cells were maintained in Ham's F-12 containing 10% calf serum, and immunoblotting of the total membrane particles from CHO cells was performed as previously described [20]. Cells were homogenized in buffer containing 10 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, and 1 mM phenylmethanesulphonyl fluoride (termed TES buffer in this study) 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 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% gels) and transferred to a nitrocellulose filter. The filters were incubated with antisera raised against the synthesized peptide corresponding to residues 478–492 at a 1:40 dilution. The production and characterization of these antibodies have been previously described [21]. The filters were then incubated with ¹²⁵I-Protein A (Amersham Corp.) and subjected to autoradiography.

Cell-surface labelling of glucose transporter

Labelling of the cell-surface glycoproteins was performed by previously described methods [22] with some modifications [23]. The CHO cells in the culture dish (diameter 60 mm) were incubated before labelling with 1 unit/ml neuraminidase at 22 °C in PBS, pH 6 for 15 min. The following procedures for labelling were performed at 15 °C. Cells were pre-reduced with 2 mM NaBH₄ in PBS, pH 8, for 5 min. Next, the cells were incubated with 40 units/ml galactose oxidase in PBS, pH 7.4, for 30 min and then reduced with NaB³H₄ (1 mCi) in 1 ml of PBS, pH 8. After 10 min, 1 ml of PBS, pH 7.4, was added and the cells were further incubated for 5 min. The cells were then washed and solubilized with 2% dodecyl octaethyleneglycol ether in a buffer containing 50 mM Hepes, pH 7.6, 150 mM NaCl and 1 mM phenylmethanesulphonyl fluoride. The cell lysate was centrifuged at 13 000 g for 30 min at 4 °C. Immunoprecipitation of the labelled glucose transporter in the supernatant was performed with 30 µg of anti-peptide antibody against the C-terminal domain (residues 478–492) and 25 µl of Protein A Cellulofine (Seikagaku-Kogyo, Tokyo, Japan). The immunoprecipitates were then suspended in a solubilizing solution of 2% SDS (lauryl grade; Pierce), 20 mM dithiothreitol and 6 M urea and then subjected to SDS/PAGE (10% gel). The gel lanes were sliced and the incorporated ³H in each gel slice was determined as previously described [21].

IAPS-forskolin labelling of glucose transporter

The membranes from CHO cells were prepared as described above and were photoaffinity-labelled with [¹²⁵I]IAPS-forskolin as previously described [24] with some modifications. The membranes (400 µg in 400 µl of TES buffer) were preincubated in the presence or absence of 10 µM cytochalasin B for 30 min on ice. [¹²⁵I]IAPS-forskolin in ethanol (final concentration 2 nM) was then added and the membranes were incubated for an addition 30 min on ice in the dark. The membranes were subsequently centrifuged at 146 000 g for 75 min at 4 °C, and the pellets were resuspended in 125 µl of TES buffer. The resuspended

membranes were then diluted by addition of 1 ml of TES buffer and were photolysed. After photolysis, 4 ml of 1% 2-mercaptoethanol was added, and the membranes were centrifuged again at 146 000 g for 75 min at 4 °C. The pellets were solubilized, immunoprecipitated and electrophoresed as described above. Gels were dried, and subjected to autoradiography.

Glucose influx and efflux measurements

2-Deoxy-D-glucose uptake

The uptake of 2-deoxy-D-glucose was measured as previously described [20]. Near-confluent monolayers of CHO cells in 24-well plates were incubated with the indicated concentrations of 2-deoxy-D-[1,2-³H]glucose (ICN) for 3 min at 37 °C. The reaction was terminated by the addition of ice-cold Krebs-Ringer phosphate (KRP) buffer containing 0.3 mM phloretin. The cells were washed and solubilized with 0.1% Triton X-100 and the radioactivity was measured.

Zero-trans influx for 3-O-methyl-D-glucose

These experiments were carried out as for 2-deoxy-D-glucose uptake except that CHO cells were incubated with the indicated concentrations of 3-O-[³H]methylglucose (du Pont-New England Nuclear) for 15 s at 22 °C.

Zero-trans efflux for 3-O-methyl-D-glucose

Zero-trans efflux activity was determined by measuring the efflux of 3-O-[³H]methylglucose as previously described [20] with some modifications. Near-confluent monolayers of CHO cells in 24-well plates were preincubated with the indicated concentrations of 3-O-[³H]methylglucose for 30 min at 22 °C. After aspiration of the medium, the plates were washed in 1 litre of KRP buffer for 15 s at 22 °C. The cells were solubilized with 0.1% Triton X-100 and the radioactivity remaining in the cells was measured. The efflux activity was calculated by subtraction of this radioactivity from that at time 0. The radioactivity in the cells at time 0 was determined by the addition of 0.3 mM phloretin before washing the cells and washing was performed with 1 litre of ice-cold KRP buffer containing 0.3 mM phloretin. The efflux was observed to be linear as a function of washing period up to at least 30 s under these assay conditions.

Equilibrium exchange influx for 3-O-methylglucose

These experiments were carried out as for zero trans influx, except that CHO cells were pre-equilibrated with unlabelled 3-O-methylglucose at the same concentrations as that used for influx of 3-O-[³H]methylglucose.

RESULTS

The mutated GLUT1 glucose transporter with leucine substituted for tryptophan at residue 388 was successfully expressed as shown in lanes 4 and 5 in Figure 1(a). Immunoblotting of two stable transfectant cell lines with the anti-peptide antibody directed against the C-terminal domain (residues 478–492) of GLUT1 revealed a large amount of the expressed *M_r*-50 000 glucose transporter in these cells. Only a small amount of endogenous GLUT1 protein of CHO cells was observed in lanes 1 and 2 after longer exposure. Quantification of the radioactivity in the glucose transporter bands isolated from the nitrocellulose paper revealed that the amount of expressed mutated glucose transporter in the cells designated clone L388 (lane 4) was 1.3-

Figure 1 Immunoblotting (a) and cell-surface labelling with galactose oxidase and NaB^3H_4 (b) of the expressed mutated rabbit GLUT1 glucose-transporter protein in CHO cells

(a) The point mutation was introduced at nucleotide 1286 of rabbit GLUT1 glucose-transporter cDNA to substitute leucine for tryptophan at residue 388. The mutated glucose-transporter cDNA was ligated into the expression vector termed pMTHneo [20], transfected into CHO cells, and two clones were selected by the resistance to G418. Cells were homogenized and centrifuged for membrane preparation. The prepared membranes (100 μg) were subjected to SDS/PAGE (10% gels) and transferred to nitrocellulose filter. The filters were incubated with antisera raised against the C-terminal domain of GLUT1, then incubated with ^{125}I -Protein A and subjected to autoradiography. Membranes were prepared from control CHO cells (lane 1), CHO cells transfected with expression vector alone without GLUT1 cDNA (lane 2), CHO cells expressing normal rabbit GLUT1 glucose transporter, termed clone A (lane 3), two CHO cell lines expressing mutated glucose transporter (lanes 4 and 5, the cells in lane 4 were designated clone L388). (b) Labelling of the cell-surface glycoproteins was performed by the methods described previously [22] with some modifications [23]. The CHO cells were pre-reduced with NaBH_4 , oxidized with galactose oxidase, and then labelled by reduction with 1 mCi of NaB^3H_4 . The cells were then solubilized and immunoprecipitated with anti-peptide antibody against the C-terminal domain of GLUT1 and Protein A Cellulofine. The immunoprecipitates were then subjected to SDS/PAGE (10% gel). The gel lanes were sliced, and the incorporated ^3H in each gel slice (5 mm long) was determined. $\circ$, Clone A; $\bullet$, clone L388; $\triangle$, control cells.

Figure 2 Photoaffinity-labelling with IAPS-forskolin of the expressed mutated GLUT1 glucose-transporter protein in CHO cells

Preparation of the membranes from CHO cells was performed as described in the legend for Figure 1. After preincubation in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of 10 μM cytochalasin B, the membranes (400 μg in 400 μl of TES buffer) were incubated with 2.0 nM [^{125}I]IAPS-forskolin. The membranes were centrifuged at 146 000 g for 75 min at 4 $^\circ\text{C}$, and the pellets were resuspended, diluted and photolysed as described previously [21]. Then 4 ml of 1% 2-mercaptoethanol was added, and the membranes were centrifuged again. The pellets were solubilized, immunoprecipitated and electrophoresed as described in the legend for Figure 1. Gels were dried, and subjected to autoradiography. Membranes were prepared from clone A (lanes 1 and 2), clone L388 (lanes 3 and 4) and control CHO cells (lanes 5 and 6).

fold that of the expressed wild-type GLUT1 glucose transporter in the cells termed previously clone A (lane 3) [20].

To estimate the amount of wild-type and mutated glucose transporters on the cell surface, the glycoproteins on the cell

Table 1 Zero-trans influx for 2-deoxy-D-glucose

Near-confluent monolayers of CHO cells in 24-well plates were incubated with 0.1 mM 2-deoxy-D-[1,2- ^3H]glucose for 3 min at 37 $^\circ\text{C}$. The reaction was terminated by the addition of ice-cold KRP buffer containing 0.3 mM phloretin. The cells were washed and solubilized and the radioactivity was measured. The data presented were means $\pm$ S.D. for four separate experiments. Cells expressing mutated glucose transporter shown in lane 5 in Figure 1(a) were designated clone L388-2.

Clone	2-Deoxy-D-glucose uptake (nmol/3 min per 1.5×10^5 cells)
Control CHO cells	0.083 ± 0.005
Clone A	0.504 ± 0.014
Clone L388	0.164 ± 0.014
Clone L388-2	0.122 ± 0.005

surface of clone A and clone L388 were labelled in intact cells by oxidation with galactose oxidase and subsequent reduction with NaB^3H_4 [23]. The amount of labelled M_r -50000 protein in clone L388 that was specifically immunoprecipitated by the antibody against the C-terminal domain of GLUT1 glucose transporter was 1.3-fold that in clone A (Figure 1b), whereas almost no significant labelling was observed in control CHO cells. The amount of expressed wild-type and mutated glucose transporter on the cell surface was parallel to the amount of these glucose transporters expressed in the cells (Figures 1a and 1b), indicating that the mutated glucose transporter was inserted into the plasma membrane in a fashion similar to the wild-type GLUT1 protein.

We next performed photoaffinity-labelling of the glucose transporters with IAPS-forskolin in the membranes from clone A and clone L388, because Trp-388 has been proposed to be the IAPS-forskolin-labelling site [17]. The wild-type GLUT1 glucose transporter expressed in CHO cells (clone A) was photoaffinity-labelled with [^{125}I]IAPS-forskolin (Figure 2, lane 1) and this labelling was inhibited in the presence of cytochalasin B (lane 2) as previously observed for GLUT1 in human erythrocytes [24]. However, we obtained the unexpected result that the mutated glucose transporter expressed in clone L388 retained the ability to photoincorporate [^{125}I]IAPS-forskolin in a cytochalasin B-inhibitable manner (lanes 3 and 4), although the amount of the labelled protein in clone L388 is approx. 60% of that in clone A, taking into account the difference in the amount of expressed glucose transporters (Figure 1a).

As previously observed [20], expression of the wild-type rabbit GLUT1 glucose-transporter protein resulted in a marked increase (approximately 6-fold) in the uptake of 2-deoxy-D-glucose (0.1 mM) in clone A compared with control CHO cells (Table 1). In contrast, only a small increase (approximately 2-fold) in 2-deoxy-D-glucose uptake was observed in clone L388. Another transfected clone which is shown in lane 5 in Figure 1(a) also had a slight but consistent increase (approximately 1.5-fold) in transport activity of 2-deoxy-D-glucose compared with parent control cells. The increase in transport activity observed in this other clone was always smaller than that observed in clone L388, which was probably due to lower expression of mutant transporter than in clone L388. No significant differences were observed between control CHO cells and the clonal line of CHO cells transfected with expression vector alone [23]. Thus the increase in transport activity observed in clone L388 is due to the expression of mutant glucose transporter and not simply to the difference among the clones. On the basis of the difference in the amount of the expressed glucose transporters on the cell surface,

Table 2 Zero-trans influx for 3-O-methyl-D-glucose

The measurement of 3-O-methyl-D-glucose uptake at 0.1 mM was carried out as for 2-deoxy-D-glucose uptake except that CHO cells were incubated with the indicated concentrations of 3-O-[³H]methylglucose for 30 s at 22 °C. Results are means $\pm$ S.D. for four separate experiments.

Clone	3-O-Methyl-D-glucose uptake (pmol/15 s per 1.5×10^5 cells)
Control CHO cells	1.06 ± 0.07
Clone A	5.40 ± 1.21
Clone L388	2.71 ± 0.14
Clone L412	2.40 ± 0.11

the transport activity of the mutated glucose transporter was estimated to be only 25% of that of the wild-type glucose transporter. A similar decrease in glucose-transport activity was also observed in clone L388 when transport activity was measured using 3-O-methyl-D-glucose (Table 2). As previously reported [25], 3-O-methylglucose uptake was also decreased in the GLUT1 mutant in which leucine was substituted for Trp-412 (Table 2).

To characterize further the zero-trans glucose influx activity of the mutated glucose transporter, 2-deoxyglucose uptake was measured at several concentrations and the increase induced by expression was analysed in clone A and clone L388 (Figures 3a and 3b). A 3-fold decrease in $V_{\max}$ and a slight (1.25-fold) increase in K_m were observed in the mutated glucose transporter

compared with the wild-type. After normalizing for the amount of expressed glucose transporters on the plasma membrane of clone A and clone L388, a 3-fold decrease in $V_{\max}$ indicated a 4-fold decrease in the turnover number of the mutated glucose transporter compared with the wild-type glucose transporter. Similar characteristics of the mutated glucose transporter were observed in another clone which is shown in lane 5 of Figure 1(a) (results not shown).

Additional interesting results were obtained in the zero trans efflux activity of the mutated glucose transporter. At two concentrations of 3-O-methylglucose, the mean value of efflux of clone L388 was slightly higher than that of clone A (Table 3). Taking into account the difference in the amount of expressed glucose transporters, the efflux activity of the mutated glucose transporter is not significantly different from that of the wild-type. This result is in marked contrast with the zero-trans influx activity of the mutated GLUT1, which is markedly decreased compared with that of the wild-type (Tables 1 and 2 and Figure 3). In contrast with the results obtained with the Trp-388 mutant, substitution of leucine for Trp-412 decreased not only the zero-trans glucose influx activity (Table 2) but also the zero-trans glucose efflux activity (Table 3) as previously observed [25]. It should be noted that the amount of the expressed glucose transporter on the cell surface in L412 cells is greater (1.4-fold) than that in L388 cells (results not shown).

In addition to the measurement of zero-trans influx and efflux activity, we also measured the equilibrium exchange of glucose-transport activity. At 3-O-methylglucose concentrations of both 0.1 mM and 10 mM (Table 4), the equilibrium exchange influx

Figure 3 Increase in 2-deoxy-D-glucose uptake induced by expression of wild-type and mutated glucose transporter (a) and an analysis of the uptake kinetics (b)

2-Deoxy-D-glucose uptake at 0.1, 0.2, 1 and 10 mM concentrations were performed as described in the legend for Table 1. The increase in 2-deoxy-D-glucose uptake induced by the expression of the wild-type and mutated glucose transporter was calculated by subtraction of the uptake value in control CHO cells from that in clone A (○) and clone L388 (●) respectively. The data presented are means $\pm$ S.D. of four separate experiments (a).

Table 3 Zero-trans efflux for 3-O-methyl-D-glucose

Near-confluent monolayers of CHO cells in 24-well plates were preincubated with 0.1 mM and 10 mM 3-*O*-[³H]methylglucose for 30 min at 22 °C. After aspiration of the medium, the plates were washed in 1 litre of KRP buffer for 15 s at 22 °C. The cells were then solubilized and the radioactivity was measured. The efflux activity was calculated by subtraction of this radioactivity from that at time 0. The radioactivity in the cells at time 0 was determined by the addition of 0.3 mM phloretin before washing the cells and washing was performed with 1 litre of ice-cold KRP buffer containing 0.3 mM phloretin. The data presented are means $\pm$ S.D. for four separate experiments and expressed as the fold increase compared with the value obtained in control CHO cells.

Clone	3- <i>O</i> -Methyl-D-glucose efflux (fold increase compared with parent CHO cells)	
	0.1 mM	10 mM
Clone A	4.37 $\pm$ 0.82	3.79 $\pm$ 0.79
Clone L388	4.72 $\pm$ 0.52	4.13 $\pm$ 0.27
Clone L412	2.82 $\pm$ 0.54	2.75 $\pm$ 0.47

Table 4 Equilibrium exchange influx for 3-O-methylglucose

These experiments were carried out as for the zero trans influx, except that CHO cells were pre-equilibrated with unlabelled 3-*O*-methylglucose at the same concentrations as that used for influx of 3-*O*-[³H]methylglucose. The data presented are means $\pm$ S.D. for three separate experiments and expressed as fold increase compared with the value obtained in control CHO cells.

Clone	3- <i>O</i> -Methyl-D-glucose efflux (fold increase compared with parent CHO cells)	
	0.1 mM	10 mM
Clone A	5.78 $\pm$ 0.40	4.89 $\pm$ 0.47
Clone L388	4.72 $\pm$ 0.31	4.22 $\pm$ 0.32

activity of expressed glucose transporters of clone L388 was 80–85% of that of clone A. When the kinetics of glucose transport were analysed, it appeared that the decrease in transport activity was mainly due to a decrease in $V_{\max}$, since no apparent change in K_m was observed. After normalizing for the amount of expressed glucose transporters on the cell surface, the turnover number of the mutated transporter was estimated to be 67% that of the wild-type in equilibrium exchange influx with 3-*O*-methylglucose.

DISCUSSION

The GLUT1 glucose transporter has been reported to be photoaffinity-labelled with [³H]forskolin [26] as well as [¹²⁵I]IAPS-forskolin with a higher affinity [24]. Chemical and proteolytic digestion and isoelectric focusing studies have suggested that the labelling site with IAPS-forskolin is located on helix 10 (residues 369–389). Among these residues Trp-388 is proposed to be the best candidate for this labelling site [17], because this residue is conserved in the *E. coli* H⁺/arabinose transporter [13] which is also labelled with [¹²⁵I]IAPS-forskolin [17]. However, the results in the present study of photolabelling of the Trp-388 mutant with IAPS-forskolin do not support this hypothesis, if we assume that photoaffinity labelling occurs only at a single residue. It is likely that the forskolin-labelling site is another amino acid residue

which is located on helix 10 and is also conserved in the *E. coli* H⁺/arabinose transporter, such as Pro-385 and Ala-377, if we assume that the labelling site is located on helix 10 [17]. Another possibility is that the labelling site is not single and Trp-388 is one of these sites. Nevertheless, Trp-388 is not essential for forskolin binding and labelling. Precise localization of the point(s) of photoincorporation of IAPS-forskolin into the GLUT1 protein will require purification and sequencing of the cleaved fragment of the labelled glucose transporter.

Glucose-influx activity was markedly decreased in the Trp-388 mutant. Kinetic analysis demonstrated a 4-fold decrease in turnover number and a slight increase in K_m in the Trp-388 mutant compared with the wild-type glucose transporter (Figure 3). Thus substitution of leucine for Trp-388 decreased the intrinsic activity of the glucose transporter by 5-fold, if one defines this as the turnover number/ K_m [27]. The decreased glucose uptake in the Trp-388 mutant is consistent to some extent with the findings of Garcia et al. [28] who reported that amino acid substitution at Trp-388 decreased the transport activity of GLUT1 in *Xenopus* oocytes, although they did not perform the kinetic analysis of glucose uptake in the Trp-388 mutant. They reported that the apparently reduced transport activity of the Trp-388 mutant in oocytes was the result of a combination of a decreased level of the protein, decreased targeting to the plasma membrane and a modest decrease in the intrinsic activity. However, our results did not indicate decreased targeting of the mutant to the plasma membrane in CHO cells. The amount of mutated transporters on the cell surface in clone L388 was greater (1.3-fold) than the wild-type in clone A as demonstrated by labelling of the cell-surface glycosidic chain of GLUT1 (Figure 1b), which is likely to reflect the fact that the expression level of the Trp-388 mutant in clone L388 was greater (1.3-fold) than that of the wild-type GLUT1 in clone A (Figure 1a). These findings suggested that targeting of the transporter to the plasma membrane was not impaired in the Trp-388 mutant, and decreased transport activity in clone L388 was mainly due to a decrease in the intrinsic activity itself of the mutated transporter. It is suggested that the difference between their and our results might be due to the difference in the cell type, i.e. that the machinery for intracellular targeting is different between mammalian cells and *Xenopus* oocytes.

Of great interest is the result demonstrating that no significant decrease was observed in the glucose efflux activity despite a marked decrease in the influx activity of the Trp-388 mutant. This result was in contrast with the alterations of glucose-transport characteristics induced by the mutation at 412. As previously reported [25], substitution of leucine for Trp-412 significantly decreased the zero-trans glucose-influx activity of the transporter (Table 2) as well as the zero-trans glucose-efflux activity (Table 3). It should be noted that the amount of expressed glucose transporter on the cell surface in L412 cells was greater (1.4-fold) than that on L388 cells (results not shown), when we compared the zero-trans efflux activity between L388 cells and L412 cells (Table 3). Thus, in contrast with the substitution at Trp-412, substitution at Trp-388 had little effect on the efflux activity of 3-*O*-methylglucose, although both mutations caused a similar marked decrease in the influx activity.

An alternating conformation model has been proposed for the mechanism of facilitated glucose transport [29–34]. We have proposed a model for the mechanism of facilitated glucose transport that operates like a gated channel for glucose [23]. In this model, the glucose transporter has both outer and inner gates as well as a pocket for glucose binding between the gates. The outer and inner gates open and close alternately such that only glucose is allowed to pass through the glucose transporter. We feel it is important to account for the difference between zero-

Scheme 1 Schematic representation of a kinetic model of the facilitative glucose transporter

G refers to glucose, and Ti and To represent the inward- and outward-facing form of the glucose transporter respectively.

trans influx and efflux after amino acid substitution at residue 388 in terms of this alternating conformation model (Scheme 1). There appear to be at least two possibilities. One possibility is that the mutation may have induced a decrease in the rate of conformational change of the loaded mutated glucose transporter from the outward-facing form to the inward-facing form (To-G→Ti-G). Another possibility is that the mutation may have induced a decrease in the rate of conformational change of unloaded glucose transporter from the inward-facing form to the outward-facing form (Ti→To).

In order to distinguish between these two possibilities experimentally, 3-*O*-methylglucose equilibrium exchange influx was measured in L388 cells. In the case of *zero-trans* influx, return of unloaded transporter from the inward-facing form to the outward-facing form (Ti→To) should be rate-limiting, because the rate of equilibrium exchange influx has been demonstrated to be faster than the *zero-trans* influx in GLUT1 [35,36]. This would indicate that the conformational change of the loaded transporter is faster than that of the unloaded transporter. If the first possibility was the case, i.e. the mutation induced a decrease only in the rate of conformational change from To-G to Ti-G, then equilibrium exchange influx activity should decrease to a degree similar to that observed in *zero-trans* influx activity. If the second possibility were the case, i.e. the mutation decreased only the rate of conformational change from Ti to To, then there should be no decrease in equilibrium exchange influx in the L388 mutant compared with the wild-type. However, the kinetic analysis revealed that the turnover number of the mutated transporter was 67% that of the wild-type in equilibrium exchange influx of 3-*O*-methylglucose. This implies that the mutation induced a decrease in the rate of conformational change of the loaded mutated glucose transporter from the outward-facing form to the inward-facing (To-G→Ti-G). However, this decrease does not account for the more profound decrease in turnover number (75%) of the *zero-trans* influx compared with that of equilibrium exchange influx (33%), suggesting that the rate of conformational change of the unloaded mutated glucose transporter from the inward to the outward-facing (Ti→To) must also be decreased compared with the wild-type. The combined decrease in the rate of these two steps (To-G→Ti-G, and Ti→To) is likely to induce a marked decrease in the intrinsic activity of the *zero-trans* influx.

K_m values for *zero-trans* and equilibrium exchange influx of glucose were only slightly affected by this mutation. This may imply that Trp-388 does not constitute the binding site for glucose. Rather, this residue is more likely to be involved in the movement of glucose through the transporter, i.e. Trp-388 plays an important role in the conformational change between outward-facing and inward-facing forms. Thus the results in this study further support the notion that helix 10, where Trp-388 is located, contains the dynamic segment which moves during transport of glucose. Helix 10 is rich in proline residues and membrane-buried proline residues have been proposed to play an important role in such conformational changes [37]. These possibilities should provide an interesting area of investigation for future studies on the glucose transporter.

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