Replacement of both tryptophan residues at 388 and 412 completely abolished cytochalasin B photolabelling of the GLUT1 glucose transporter

Kouichi INUKAI,* Tomoichiro ASANO,* Hideki KATAGIRI,* Motonobu ANAI,* Makoto FUNAKI,* Hisamitsu ISHIHARA,† Katsunori TSUKUDA,† Masatoshi KIKUCHI,† Yoshio YAZAKI* and Yoshitomo OKA*‡

*Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan, and †Institute for Adult Disease, Asahi Life Foundation, Nishishinjuku, Shinjuku, Tokyo 160, Japan

A mutated GLUT1 glucose transporter, a Trp-388, 412 mutant whose tryptophans 388 and 412 were both replaced by leucines, was constructed by site-directed mutagenesis and expressed in Chinese hamster ovary cells. Glucose transport activity was decreased to approx. 30% in the Trp-388, 412 mutant compared with that in the wild type, a similar decrease in transport activity had been observed previously in the Trp-388 mutant and the Trp-412 mutant which had leucine at 388 and 412 respectively. Cytochalasin B labelling of the Trp-388 mutant was only decreased rather than abolished, a result similar to that obtained

previously for the Trp-412 mutant. Cytochalasin B labelling was finally abolished completely in the Trp-388, 412 mutant, while cytochalasin B binding to this mutant was decreased to approx. 30% of that of the wild-type GLUT1 at the concentration used for photolabelling. This level of binding is thought to be adequate to detect labelling, assuming that the labelling efficiency of these transporters is similar. These findings suggest that cytochalasin B binds to the transmembrane domain of the glucose transporter in the vicinity of helix 10–11, and is inserted covalently by photoactivation at either the 388 or the 412 site.

INTRODUCTION

Facilitative glucose transport in mammalian cells is mediated by a family of proteins comprised of at least five members, named GLUT1–GLUT5 based on the order in which they were identified by cDNA cloning [1–9]. The GLUT1 glucose transporter isoform, which is likely to be responsible for basal or constitutive glucose uptake [2], was cloned from the human HepG2 cell line [1], and its structure and function have been studied extensively [10–14].

Facilitated glucose activity of GLUT1 is competitively inhibited by cytochalasin B [15], which is expected to bind at or near the glucose-binding site of the transporter [16]. With u.v. irradiation, cytochalasin B is covalently inserted into glucose transporter molecules in a D-glucose inhibitable manner [17–19]. Proteolytic digestion experiments performed on the erythrocyte transporter have localized the cytochalasin B photolabelling site in the vicinity of transmembrane helix segments 10–11 [20,21]. In addition, the wavelength needed for photolabelling is 280 nm, which suggests that the photolabelling proceeds via photoactivation of an aromatic amino acid residue on the transporter rather than the cytochalasin B molecule itself [22,23]. These results lead to the hypothesis that the photolabelling site of GLUT1 is localized on either Trp-388 or Trp-412. Previously, the tryptophan residue in position 412 of GLUT1 was altered to leucine by site-directed mutagenesis to investigate the cytochalasin B photolabelling site. The photolabelling of this mutated GLUT1 was not completely abolished, but labelling was decreased by 40% compared with that of the wild-type GLUT1 [24].

By expressing a mutated glucose transporter, we found that amino acid substitution at Trp-388 reduces but does not abolish cytochalasin B photolabelling, whereas substitution of the amino acids at both Trp-388 and Trp-412 of GLUT1 completely abolishes this photolabelling.

MATERIALS AND METHODS

Expression of mutated glucose transporter

The single amino acid substitution of leucine for Trp-388 of GLUT1 was performed as previously described [25]. In brief, the G to T point mutation was introduced at nucleotide 1286 of the wild-type rabbit GLUT1 glucose transporter cDNA by PCR [26], using two synthesized oligonucleotides, 2230–2245 and 1255–1295, whose G at nucleotide 1286 had been replaced by T. This point mutation resulted in substitution of leucine (TTG) for tryptophan (TGG) at residue 388 of the encoded GLUT1 glucose transporter (Trp-388 mutant).

In order to introduce two amino acid substitutions of leucines for Trp-388 and Trp-412, a two-step PCR was performed. First, using two synthesized oligonucleotides, 884–903 and 1351–1387 whose G at nucleotide 1358 had been substituted with T, the G to T point mutation was introduced at nucleotide 1358 of the wild-type glucose transporter cDNA. Similarly, the second G to T point mutation was introduced at nucleotide 1286 as described above. These two point mutations result in substitutions of leucine (TTG) for tryptophan (TGG) at residues 388 and 412 of the encoded GLUT1 glucose transporter (Trp-388, 412 mutant).

These two fragments, prepared by PCR, as well as the ligation sites were sequenced and observed to have no mutations other than those at the desired nucleotides. The *Mae*I fragments of the mutated rabbit GLUT1 glucose transporter cDNA (nucleotides 111–2230) were ligated into an expression vector termed pMTHneo and transfected into Chinese hamster ovary (CHO) cells by calcium phosphate precipitation as previously described [10]. Several cell lines were selected on the basis of their resistance to 600 µg/ml of a neomycin derivative, G418 (Gibco), and were termed W388-L1 and W388-L2 (Trp-388 mutant) and W388,412-L1, W388,412-L2 (Trp-388, 412 mutant) in this study.

Abbreviation used: CHO, Chinese hamster ovary.

‡ To whom correspondence should be addressed.

Western-blot analysis

CHO cells were maintained in Ham's F-12 medium containing 10% (v/v) calf serum and Western blotting of all membrane particles from CHO cells was performed as previously described [10]. Cells were homogenized in 10 mM Tris, 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 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 nuclei and the resulting supernatant was centrifuged at 170 000 *g* for 75 min at 4 °C. The pellet was subjected to SDS/(10%) polyacrylamide gel electrophoresis and transferred on 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 the antibody have been described previously [27]. The filters were then incubated with ¹²⁵I-Protein A (Amersham) and subjected to autoradiography.

[³H]Cytochalasin B labelling

The membranes of CHO cells were prepared as described above. A 500 µg quantity of membranes was photoaffinity-labelled with 0.5 µM [³H]cytochalasin B in the presence of 20 µM unlabelled cytochalasin E as previously described [27]. The membranes were suspended in 1% (w/v) SDS/50 mM dithiothreitol and electrophoresed on a 10% (w/v) polyacrylamide gel as described by Laemmli [28]. The gel lanes were sliced, solubilized with Protosol (DuPont–New England Nuclear), combined with a liquid-scintillation mixture (Econofluor, DuPont–New England Nuclear), and the radioactivity in each gel was determined.

[³H]Cytochalasin B binding

[³H]Cytochalasin B binding to the glucose transporter in the membrane was determined according to previously described methods with some modifications [29]. In brief, the membranes (2.5 µg/µl) were suspended in ice-cold TES buffer (10 mM Tris, 1 mM EDTA, 250 mM sucrose) in the presence of 20 µM cytochalasin E. Aliquots (60 µl) of this mixture were incubated for 10 min at 4 °C with various concentrations of [³H]cytochalasin B. The samples were then rapidly poured on to a dry GF filter (Whatman) in a filtration-manifold well, under vacuum conditions. The tube was washed twice with 3 ml of ice-cold TES buffer and the resulting solution was poured on the filter. These washing procedures took approx. 3 s. Cytochalasin B bound to the membranes was determined by measuring the radioactivity of filters in the scintillation liquid (Aquasol II, DuPont–New England Nuclear). Cytochalasin B binding was not decreased when the duration of the washing procedure was extended to 15 s, indicating that dissociation of cytochalasin B from the transporter is negligible under these assay conditions. Cytochalasin B binding to the expressed glucose transporter in the membranes was calculated by subtracting cytochalasin B binding to control CHO cell membranes, which represents the amount of non-specific cytochalasin B binding to CHO membrane proteins and that of cytochalasin B binding to the intrinsic glucose transporter of CHO cells. The binding data were analysed as described by Scatchard [30].

Measurement of glucose uptake

Glucose uptake activity in CHO cells was determined by measuring the uptake of 2-deoxy-D-glucose, as previously described with some modifications [10]. Near confluent monolayers of

CHO cells in 24-well plates were incubated with 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 with 0.3 mM phloretin. The cells were washed and solubilized with 0.1% Triton X-100 and the radioactivity was measured.

Cell surface labelling of glucose transporter

Labelling of cell-surface glycoproteins was performed according to previously described methods [31] with some modifications. The CHO cells in the culture dish (diameter 60 mm) were incubated with 1 unit/ml of neuraminidase at 22 °C in PBS, pH 6.0, for 15 min. The following procedures for labelling were performed at 15 °C. The cells were pre-reduced with 2 mM sodium borohydride in PBS, pH 8.0, for 5 min. They were incubated with 40 units/ml of galactose oxidase in PBS, pH 7.4, for 30 min and then reduced with sodium borotritide (1 mCi) in 1 ml of PBS, pH 8.0. After 10 min, 1 ml of PBS, pH 7.4, was added and the cells were incubated for another 5 min, then washed and solubilized with 2% dodecyl octaethyleneglycol ether (C₁₂E₈) in 50 mM Hepes/150 mM NaCl, pH 7.6, containing 1 mM phenylmethanesulphonyl fluoride. The cell lysate was centrifuged at 13 000 *g* for 30 min at 4 °C. The labelled glucose transporter in the supernatant (200 µl) was immunoprecipitated with 6 µg of anti-peptide antibody raised against the C-terminal domain (residues 478–492) and 25 µl of Protein A Cellulofine. The immunoprecipitates were suspended with 2% (w/v) SDS (lauryl grade, Pierce), 20 mM dithiothreitol and 6 M urea and then subjected to SDS/(10%) polyacrylamide gel electrophoresis. The gel lanes were sliced and the incorporated ³H in each gel slice was determined as previously described [27].

RESULTS

The Trp-388 mutated glucose transporter protein was expressed, as shown in lanes 3 and 4 of Figure 1(a). Western blotting of two stable transfectants (clones W388-L1 and W388-L2) with an anti-peptide antibody directed against the C-terminal domain (residues 478–492) of GLUT1 revealed significant quantities of the expressed 50 000-*M_r* glucose transporter in these cells. Only a small amount of endogenous GLUT1 is observed in parental CHO cells (Figure 1, lane 1). The amount of mutated glucose transporter expression in clone W388-L1 was 1.3-fold greater than that of wild-type glucose transporter expression in the cells termed clone C (Figure 1, lane 2). Figure 1(b) shows cytochalasin B labelling of the expressed transporters. As previously observed, a large quantity of 50 000-*M_r* protein was photolabelled with 0.5 µM cytochalasin B in the membranes of clone C. This labelling was largely inhibited by the presence of 500 mM D-glucose (results not shown), indicating that this labelled protein is the glucose transporter. Contrary to our expectation, the Trp-388-mutated glucose transporter was photoaffinity-labelled with cytochalasin B, although the quantity of mutated glucose transporter labelled was decreased by approx. 30% compared with that of the wild-type glucose transporter, after normalization of the differing amounts of expressed glucose transporters.

As cytochalasin B labelling was not abolished in either the Trp-412 mutant [24] or the Trp-388 mutant (Figure 1b), we next studied the mutated glucose transporter whose Trp-388 and Trp-412 were substituted with leucine (Trp-388, 412 mutant). This mutated glucose transporter was also successfully expressed in CHO cells (Figure 2a). Western blotting of two stable transfectant cell lines (clones W388,412-L1 and W388,412-L2) with an antibody directed against the C-terminal domain of GLUT1

Figure 1 Western blotting (a) and cytochalasin B photoaffinity-labelling (b) of the expressed Trp-388 mutant GLUT1 protein in CHO cells

(a) The Trp-388 mutant GLUT1 cDNA was transfected into CHO cells, as previously described [25], and two clones were selected and termed clones W388-L1 and W388-L2. The wild-type GLUT1 cDNA was transfected into other CHO cells, termed clone C. Cells were homogenized at 4 °C in 10 mM Tris, 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 250 mM sucrose, pH 7.4. The homogenates were centrifuged at 900 *g* for 10 min and the resulting supernatant was centrifuged at 170 000 *g* for 75 min at 4 °C. The pellet was subjected to SDS/(10%) polyacrylamide gel electrophoresis 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 filters were then incubated with ^{125}I -Protein A (Amersham) and subjected to autoradiography. Lane 1, control CHO cells; lane 2, clone C; lane 3, clone W388-L1; lane 4, clone W388-L2. (b) Membranes of CHO cells (500 μg) were photoaffinity-labelled with 0.5 μM [^3H]cytochalasin B, as described previously [27], and subjected to SDS/(10%) polyacrylamide gel electrophoresis. The gel lanes were sliced, solubilized with Protosol (DuPont–New England Nuclear), and the radioactivity in each gel slice was determined. These experiments were repeated three times and yielded similar results. Control CHO (Δ), clone C ($\circ$), clone W388-L1 ($\bullet$), clone W388-L2 ($\blacktriangle$).

Figure 2 Western blotting (a) and photoaffinity-labelling with cytochalasin B (b) of the expressed Trp-388, 412 mutant GLUT1 protein in CHO cells

(a) The Trp-388,412 mutant GLUT1 cDNA was prepared and transfected into CHO cells as previously described [10]. Two clones were selected and termed clone W388,412-L1 (lane 2) and clone W388,412-L2 (lane 3). The wild-type GLUT1 was expressed in two clones designated clone D (lane 4) and clone C (lane 5). Western blotting was performed as described in the legend of Figure 1(a). The relative amounts of expressed glucose transporters are approx. 2:1:1:3 (from lanes 2 to 5). Lane 1, control CHO cells; lane 2, clone W388,412-L1; lane 3, clone W388,412-L2; lane 4, clone D; lane 5, clone C. (b) Membranes of CHO cells (500 μg) were photoaffinity-labelled with 0.5 μM [^3H]cytochalasin B as described previously [27] and were subjected to SDS/(10%) polyacrylamide gel electrophoresis. The gel lanes were sliced, solubilized with Protosol, and the radioactivity in each gel slice was determined. These experiments were repeated three times, with similar results. Control CHO (Δ), clone C ($\circ$), clone D ($\blacktriangle$), clone W388,412-L1 ($\bullet$), clone W388,412-L2 ($\square$).

revealed significant expression of the 50000- M_r glucose transporter in these cells, as shown in lanes 2 and 3 of Figure 2(a). Two clones expressing a large amount of the wild-type GLUT1, which were prepared as previously described, were also studied. The quantities of the wild-type GLUT1 expressed in these clones, termed clones D and C, are shown in Figure 2 (lanes 4 and 5 respectively). The expression of mutated GLUT1 in clone W388,412-L1 (Figure 2, lane 2) was two-thirds that of the wild-type GLUT1 in clone C (Figure 2, lane 5), based on measurement

Figure 3 Scatchard analysis of cytochalasin B binding to the expressed wild-type and mutant GLUT1

Membranes were prepared from control CHO cells, clone C, clone W388,412-L1 and cytochalasin B binding was measured at 0.05, 0.15, 0.5, and 1.5 μ M cytochalasin B concentrations. The data shown are the means of duplicate determinations and are corrected for binding to the membranes of control CHO cells. Thus the data represent binding to the expressed wild-type and mutant GLUT1. These experiments were repeated three times and yielded similar results. Clone C ($\circ$), clone W388,412-L1 ($\bullet$).

Table 1 Cytochalasin B binding to the expressed wild-type and mutant GLUT1 at 0.5 μ M cytochalasin B

The data presented are corrected for binding to membranes of control CHO cells and are means $\pm$ S.D. of three separate experiments.

Clone	Cytochalasin B binding (pmol/mg of protein)
Clone W388,412-L2	2.10 $\pm$ 0.20
Clone W388,412-L1	3.22 $\pm$ 0.49
Clone C	14.18 $\pm$ 0.92

of the radioactivity in corresponding bands. The amount of mutant GLUT1 in clone W388,412-L2 (Figure 2, lane 3) is comparable with that of the wild-type GLUT1 in clone D (Figure 2, lane 4). Labelling of glucose transporter with 0.5 μ M cytochalasin B showed that the amount of labelled glucose transporter in clone D was approx. 35% of that in clone C (Figure 2b). In contrast, cytochalasin B transporter labelling in clone W388,412-L1 was essentially the same as that in control CHO cells, indicating that the mutant glucose transporter expressed in clone W388,412-L1 was not labelled with cytochalasin B. Absence of cytochalasin B labelling of the Trp-388, 412 mutant was further confirmed in clone W388,412-L2.

Cytochalasin B binding to the expressed glucose transporters was then measured and analysed by Scatchard's method (Figure 3). It should be noted that the present results are corrected for binding to endogenous GLUT1 and thus represent binding to the

Figure 4 Cell-surface labelling of the expressed GLUT1 protein in CHO cells with galactose oxidase and borotritide

Cell-surface glycoproteins were labelled, using previously described methods, with some modifications. The CHO cells were incubated with 1 unit/ml of neuraminidase at 22 $^{\circ}$ C in PBS, pH 6.0, for 15 min. The following labelling procedures were performed at 15 $^{\circ}$ C. The cells were pre-reduced with 2 mM sodium borohydride in PBS, pH 8.0, for 5 min. The cells were incubated with 40 units/ml of galactose oxidase in PBS, pH 7.4, for 30 min and then reduced with sodium borotritide (1 mCi) in 1 ml of PBS, pH 8.0. After 10 min, 1 ml of PBS, pH 7.4, was added and the cells were incubated for another 5 min. The cells were then washed and solubilized with 2% $C_{12}E_8$ in 50 mM Hepes, 150 mM NaCl, pH 7.6. The cell lysate was centrifuged at 13000 g for 30 min at 4 $^{\circ}$ C. The labelled glucose transporter in the supernatant (200 μ l) was immunoprecipitated with 6 μ g of anti-peptide antibody raised against the C-terminal domain and 25 μ l of Protein A Cellulofine. The immunoprecipitates were suspended in 2% (w/v) SDS, 20 mM dithiothreitol and 6 M urea and then subjected to SDS/(10%) polyacrylamide gel electrophoresis. The gel lanes were sliced and the incorporated tritium in each gel slice was determined. Control CHO cells (Δ), clone C ($\circ$), clone W388,412-L1 ($\bullet$).

expressed mutant GLUT1 or wild-type GLUT1. Interestingly, cytochalasin B binding to the mutant GLUT1 in clones W388,412-L2 and W388,412-L1 was not completely abolished at 0.5 μ M cytochalasin B, the same as the concentration employed in photolabelling (Table 1). Binding in clone W388,412-L1 was, however, decreased by 80% compared with that of the wild-type GLUT1 in clone C. A further decrease in cytochalasin B binding was observed in clone W388,412-L2. Taking into account the differences in the expressed amounts of mutant GLUT1 and wild-type GLUT1 between clone C and clone W388,412-L1, cytochalasin B binding to the Trp-388, 412 mutant was decreased to approx. 30% of that of wild-type GLUT1. Scatchard analysis of the binding data demonstrated that the K_d values of the wild-type GLUT1 and the Trp-388, 412 mutant were 115 and 650 nM respectively. Thus the Trp-388, 412 mutant has a lower affinity for cytochalasin B than does the wild-type GLUT1. The R_0

Table 2 2-Deoxy-D-glucose uptake by the expressed mutated and wild-type GLUT1 at 1 mM

The data presented are the means $\pm$ S.D. of three separate experiments.

Clone	2-Deoxy-D-glucose uptake (pmol/3 min per 1.5×10^5 cells)
Control CHO cells	50 ± 6
Clone W388,412-L2	71 ± 9
Clone W388,412-L1	84 ± 10
Clone C	255 ± 32

Figure 5 Inhibition of 2-deoxy-D-glucose uptake by cytochalasin B

Near confluent monolayers of CHO cells in 24-well plates were incubated with 1 mM 2-deoxy-D-[1,2- 3 H]glucose for 3 min at 37 °C in the absence and the presence of indicated concentrations of cytochalasin B. The reaction was terminated by the addition of ice-cold KRP buffer with 0.3 mM phloretin. The data presented are the mean $\pm$ S.D. of three separate experiments, which are corrected for uptake in control CHO cells and thus, represent 2-deoxy-D-glucose uptake by the expressed wild-type (clone C, $\circ$) and mutant GLUT1 (clone W388,412-L1, $\bullet$).

values of these membranes for cytochalasin B were 24 and 13 pmol/mg of membrane protein respectively. After correcting differences in the amounts of glucose transporter expressed, the number of apparent binding sites on the Trp-388, 412 mutant was approx. 80 % of that of the wild-type GLUT1. The reason for the apparent decrease in the number of binding sites in the Trp-388, 412 mutant is not clear. It can be argued that the binding-site reduction of approx. 20 %, deduced from Scatchard analysis of the binding data, is greater than the actually value. Another possibility is that some of the mutated glucose transporters were not inserted into membranes in an appropriate manner, which prevented cytochalasin B binding to these transporters.

To estimate the amounts of wild-type and mutated glucose transporters on the cell surface, cell-surface glycoproteins of clone C and clone W388,412-L1 were labelled in intact cells by oxidation with galactose oxidase and subsequent reduction with borotritide (Figure 4). The amount of labelled 50000- M_r protein in clone W388,412-L1, which was specifically immunoprecipitated with an antibody against the C-terminal domain of GLUT1, was approx. 55 % of that in clone C. Taking into account the difference in the amounts of glucose transporter expressed, the relative distribution of the glucose transporter on the plasma membrane was slightly decreased in the mutated GLUT1 glucose transporter compared with the wild-type transporter. Labelling was barely detectable in control CHO cells. These results indicate that insertion of the Trp-388, 412 mutant into the plasma membrane was similar to that of the wild-type GLUT1 protein, although a slight decrease in plasma membrane targeting was observed.

Transporter function of the expressed GLUT1 was assessed by uptake of 1 mM 2-deoxy-D-glucose and is shown in Table 2. Expression of the wild-type GLUT1 resulted in a marked increase (approx. 5-fold) in transport activity, as compared with control CHO cells. In contrast, only a small increase in the uptake of 2-deoxy-D-glucose was observed in clones W388,412-L1 and W388,412-L2. Although the increases observed in clones W388,412-L1 and W388,412-L2 were small, transport activity was consistently greater than that observed in control CHO cells when glucose uptake measurements were performed in parallel in these clones. In addition, the increase in clone W388,412-L2 was smaller than that in clone W388,412-L1, probably due to differences in the amounts of mutant glucose transporter expressed between these two clones. Taking into account these differences in expressed amounts and plasma membrane distribution, the glucose transport activity of the Trp-388, 412 mutant was estimated to be decreased to approx. 30 % of that of wild-type GLUT1. Consistent with the results that cytochalasin B binds to the Trp-388, 412 mutant glucose transporter, 2-deoxy-D-glucose uptake was inhibited by cytochalasin B in clone W388,412-L1 (Figure 5). The results of 2-deoxy-D-glucose uptake by clone C and clone W388,412-L1 were corrected for uptake by parental CHO cells such that the corrected values represent uptake by the expressed Trp-388, 412 mutant transporter and the wild-type transporter respectively.

DISCUSSION

Proteolytic digestion of the cytochalasin B-labelled GLUT1 transporter has been employed to identify the photolabelling site [20,21]. Previously, cleavage of the labelled protein with *N*-bromosuccinimide suggested that the cytochalasin B photolabelling site might be Trp-412 of the GLUT1 glucose transporter. Recently, we demonstrated the unexpected result that substitution of leucine for Trp-412 of the rabbit GLUT1 does not abolish cytochalasin B labelling, although the amount of labelled mutated GLUT1 was approx. 60 % that of wild-type GLUT1 [24].

The present study revealed that, in contrast to our expectations, substitution of leucine for Trp-388 of GLUT1 does not abolish, but rather only decreases, cytochalasin B labelling. This result is similar to that obtained with the Trp-412 mutant. Cytochalasin B labelling was finally abolished completely with substitution of leucine for both Trp-388 and Trp-412. These results raise the possibility that two cytochalasin B photolabelling sites, Trp-388 and Trp-412, exist within the GLUT1 protein, and that cytochalasin B is inserted covalently into either Trp-388 or Trp-412 by photo-activation. The proposed mechanism of cytochalasin B

Figure 6 Proposed schematic representation of the mechanism of cytochalasin B binding and labelling

Trp-388 and Trp-412 are necessary to achieve the appropriate conformation for cytochalasin B binding (upper). Photoactivation of aromatic amino acid residues, Trp-388 and Trp-412, leads to incorporation of cytochalasin B into one of these activated residues (below).

photolabelling is schematically represented in Figure 6. This proposal is not inconsistent with previous results obtained by cleavage of the photolabelled glucose transporter with *N*-bromosuccinimide [20]. Although it was concluded that the labelled 3000-*M_r* fragment was composed of residues 388–412 [20], this fragment must be comprised of residues 389–412 only [21], because *N*-bromosuccinimide cleaves at the C-terminal side of tryptophan [32]. However, its assignment to residues 364–388 is another possibility, which seems to have been dismissed in previous reports [20]. Each of these two cleaved segments of *M_r* 3000 contains one tryptophan residue (Trp-388 and Trp-412).

The completely abolished cytochalasin B labelling in the Trp-388, 412 mutant does not appear to be attributable to decreased cytochalasin B binding for the following reasons: cytochalasin B binding to the Trp-388, 412 mutant was decreased to approx. 30% of that of wild-type GLUT1 at the concentration used for photolabelling. Considering that the amount of the Trp-388, 412 mutant in clone W388,412-L1 was double that of the wild-type glucose transporter in clone D, the photolabelled amount of the Trp-388, 412 mutant would be 60% that of wild-type, assuming the same photolabelling efficiency for these two transporters. As glucose transporter labelling was clearly demonstrated in clone D, 60% of the observed labelling should have been detectable. However, labelling of clone W388,412-L1 membranes was essentially the same as that of control CHO cell membranes. Thus it is highly unlikely that the abolished cytochalasin B labelling of the Trp-388, 412 mutant is due to reduced cytochalasin B binding. However, the lack of sufficient binding affinity of cytochalasin B to the Trp-388, 412 mutant still remains another explanation for the absence of cytochalasin B labelling, because the photolabelling efficiency might not always parallel with the binding of cytochalasin B. Scatchard analysis of cytochalasin B binding demonstrated that the cytochalasin B binding affinity for the Trp-388, 412 mutant was significantly lower than that for wild-type GLUT1. In contrast, either the Trp-388 mutant or the Trp-412 mutant had a binding affinity similar to that of wild-type GLUT1 [24,25]. These results suggest that one tryptophan residue, either at 388 or 412, is necessary to maintain a high affinity for cytochalasin B.

Glucose transport activity of the Trp-388, 412 mutant was markedly (by 70%) diminished. Similar marked decreases in

glucose transporter activity were also observed in the Trp-412 [24] and the Trp-388 mutants [25]. Thus introduction of two mutations in Trp-388 and Trp-412 did not further affect transport activity compared with a single mutation at 388 or 412 position. These results further suggest that the domain responsible for glucose transport and that for cytochalasin B binding are not identical in the GLUT1 glucose transporter. Garcia et al. recently reported that the reduced activity of the Trp-388 mutant expressed in *Xenopus* oocytes resulted from the decreased targeting of plasma membranes and a modest decrease in intrinsic activity, whereas the reduced transport activity of the Trp-412 mutant was due entirely to a decrease in the intrinsic activity of mutant proteins [33]. The Trp-388, 412 mutant expressed in CHO cells appeared to exhibit features of both the Trp-388 mutant and the Trp-412 mutant expressed in *Xenopus* oocytes. However, the Trp-388 mutant expressed in CHO cells was observed to target plasma membranes in a manner similar to that of wild-type GLUT1 [25], a result different from that in *Xenopus* oocytes, suggesting that targeting machinery differs between *Xenopus* oocytes and CHO cells.

In conclusion, taken together with the previous results of proteolytic digestion of labelled transporter [20,21], the wavelength needed for photolabelling [22,23], and decreased cytochalasin B labelling in the Trp-412 mutant [24], our present data indicate that Trp-388 and Trp-412 play important roles in cytochalasin B binding to the glucose transporter and that these two tryptophan residues are likely to be the two putative cytochalasin B labelling sites on GLUT1 protein. However, further studies, such as direct sequencing of tryptic peptides, are required to obtain some experimental verification.

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