Serine-294 and threonine-295 in the exofacial loop domain between helices 7 and 8 of glucose transporters (GLUT) are involved in the conformational alterations during the transport process

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The role of a conserved polar motif (STS) in the exofacial loop between helices 7 and 8 of GLUT4 for transporter function was investigated by site-directed mutagenesis and expression of the constructs in COS-7 cells. Reconstituted glucose-transport activity, cytochalasin B binding and photolabelling with the exofacial label 2-*N*⁴-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannosyloxy)-2-propylamine (ATB-BMPA) were assayed in membranes from transfected cells and corrected for immunoreactivity of expressed transporters. Replacement of Ser-294 with Ala or Thr suppressed transport activity and cytochalasin B

binding. ATB-BMPA photolabelling was normal in S294A mutants, and even increased in S294T mutants. Replacement of Thr-295 with Ala suppressed transport activity and cytochalasin B binding, whereas ATB-BMPA photolabelling was normal; substitution of Ser failed to alter the investigated parameters. Similarly, exchanging Ser-296 for Ala generated a normally functioning protein. The data suggest that Ser-294 and Thr-295 are involved in the conformational change in GLUT during the transport process, and that their substitution may arrest the transporter in an outward-facing conformation.

INTRODUCTION

Glucose transport into mammalian cells is catalysed by a family of transport-facilitator proteins, GLUT1–4, which consist of 12 membrane-spanning helices, a large highly charged intracellular loop domain, a large glycosylated extracellular loop domain and several conserved amino acid motifs [1–4]. It is generally assumed that the 12 helices form a membrane pore which alters its conformation on binding of glucose to its recognition site, or continuously oscillates between an outward- and an inward-facing conformation [5,6]. This model has been corroborated in experiments with mutants of GLUT1 in which the exofacial recognition site appeared intact, as determined by binding of a selective photolabel, 2-*N*⁴-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannosyloxy)-2-propylamine (ATB-BMPA), whereas the endofacial site, as assessed by binding of cytochalasin B, was obliterated [7]. Thus the mutagenesis appeared to have locked the transporter in the outward-facing conformation. Furthermore, in mutants of W412 [8–10] or RR333/334 [11], and after deletion of the C-terminal tail [12], glucose transport, but not cytochalasin B binding, was reduced, suggesting that the transporter is retained in an inactive inward-facing conformation.

Several lines of evidence indicate that the membrane-spanning helix 7 and its adjacent loop domains are crucial for the function of the protein. The biochemical characterization of fragments from GLUT1 labelled with a photoreactive glucose derivative suggests that the arrangement of helices 7 and 8 is altered on glucose binding, secluding a thermolysin-cleavage site [5]. Furthermore mutagenesis of conserved residues in helix 7 selectively reduced ATB-BMPA labelling (Q282L [13]) or cytochalasin B binding (Y293I [7]). Thus this region of the glucose transporter appears to be involved in the recognition of glucose and/or the conformational alteration triggered by glucose binding. A sequence comparison of hexose-transport facilitators (Figure 1) indicates that a polar motif (STS) in the exofacial loop domain between helices 7 and 8 is conserved in the mammalian glucose

transporters. (N.B. To facilitate comparisons numbering of amino acid residues corresponds to that of GLUT1.) This comparison led us to hypothesize that the motif is involved in the recognition of glucose, and to study its role by site-directed mutagenesis. The results indicate that substitution of Ser-294 and Thr-295 can lock the glucose transporter in an exofacial conformation which is unable to transport glucose and bind cytochalasin B, but binds the exofacial photolabel ATB-BMPA with normal or even increased affinity. It is suggested that the STS motif is involved in the conformational alterations of the transporter during the transport process.

MATERIALS AND METHODS

Construction of mutant DNA and expression vectors

Mutants were generated by oligonucleotide-directed mutagenesis as described [14]. Single-stranded DNA of rat GLUT4 [15] subcloned into pBluescript was used as template. Mutated constructs were identified by sequencing and subcloned as *Xba*I–*Kpn*I fragments from pBluescript into the mammalian expression vector pCMV which harbours a simian virus 40 origin, a cytomegalovirus promoter and a polyadenylation site [16].

Transfection of COS-7 cells

Transfection of COS-7 cells with glucose transporter cDNA was performed by a modification of the previously described procedure [16]. COS-7 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and grown in Dulbecco's modified Eagle's medium, pH 7.4, containing 10% (v/v) fetal calf serum, 20 mM Hepes and 2 mM glutamine. The cells were harvested by trypsin treatment 24 h before the transfection and were seeded on to 153 cm² culture dishes at a final density of 6.25 × 10⁵ cells/dish. At 4 h before the transfection,

Abbreviations used: ATB-BMPA, 2-*N*⁴-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannosyloxy)-2-propylamine.

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	Helix 7	Helix 8
GLUT1	ILIAVVLQLSQQLSGINAVFY YSTS IFEKAGV--QQPVYATIGSGIVNTAFTVVSFLFVVER	
GLUT2	ILVALMLHVAQQFSGINGIFY YSTS IFQTAGI--SKPVYATIGVGAVNMVFTAVSVFLVEK	
GLUT3	IIISIVLQLSQQLSGINAVFY YSTG IFKDAGV--QEPIYATIGAGVNTIFTVVSFLFVER	
GLUT4	LIIAVVLQLSQQLSGINAVFY YSTS IFETAGV--QGPAYATIGAGVNTVFTLVSVLLVER	
GLUT5	LLSIIIVLMGGQQLSGVNAIYYADQIYLSAGVPEEHVQYVTAGTGAVNVVMTFCVAVFVEL	
TSU62917	LFVAVVAHLAQQFSGINAALFY STSL FEISIGLTSQAV-YATLGVGSMIVVITVASIFLIER	
SMGTP1A	VLIACLIQVLQQLSGINAVITY SSLM LELAGIPDVLYQCVFAIGVLNVIVTVVSLPLIER	
CEF53H8	LAVSIIVMIAQQFTGCTAVFAF STDM FLNAGLTPVIARFSTLAIGIVYFLFACTSPFLIHK	
HXT1	TMMGIMIQLSQQLTGDNYYFFYYGTIVFQAVGLSD--SFETSIVFGVVNFFSTCCSLYTVDR	
HXT2	VIMGIMIQLSQQLTGNNYFFYYGT TF INAVGMKD--SFQTSIVLGIVNFASTFVALYTVDK	

Figure 1 Sequence comparison of helix 7 and the exofacial loop domain comprising the polar STS motif

Comparison of the loop domains between helices 7 and 8 in hexose-transport facilitators. The mutated motif and its conservation in other glucose-transport facilitators is highlighted by bold type. TSU62917, putative hexose transporter from *Taenia solium*; SMGTP1A, glucose transporter from *Schistosoma mansoni*; CEF53H8, putative glucose transporter from *Caenorhabditis elegans*; HXT1 and 2, hexose transporter from *Saccharomyces cerevisiae*.

25 ml of fresh culture medium was added to the monolayer. DNA (25 µg/plate) was co-precipitated with 125 mM CaCl₂ in HBS buffer (140 mM NaCl, 0.75 mM Na₂HPO₄, 25 mM Hepes, pH 7.1) for 15 min in a final volume of 2.5 ml. The calcium phosphate-DNA suspension was added to the monolayers, and cells were incubated for 16 h at 37 °C. Thereafter the medium was exchanged for 7.5 ml of 15 % glycerol in HBS buffer, and the cells were incubated for 3 min. The buffer was removed by aspiration, and monolayers were washed twice with prewarmed PBS (138 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The cells were then incubated with fresh culture medium in an atmosphere of 5 % CO₂ at 37 °C for 48 h.

Preparation of membrane fractions from transfected cells

The transfected cells were homogenized and fractionated as previously described [16] with a modification of a protocol previously employed in 3T3-L1 cells [17]. Most of the glucose-transporter protein was found in the plasma membrane (13 000 g) and high-density microsomal (45 000 g) fractions which were used for the subsequent assays.

Immunochemical detection of glucose transporters

Immunochemical detection and quantification of the GLUT4 was performed as described previously with antiserum against human erythrocyte glucose transporter or a dodecapeptide corresponding to the C-terminus of GLUT4 [17]. The relative abundance of overexpressed GLUT4 and constitutive GLUT1 in COS-7 cells was assessed with protein standards (adipocyte and erythrocyte membranes respectively) of known glucose-transporter abundance (cytochalasin B-binding sites). Levels of GLUT4 were routinely 10–20-fold higher than those of the constitutive GLUT1.

Reconstitution of glucose-transport activity from membrane fractions

Glucose-transport activity reconstituted into phosphatidylcholine liposomes was assayed as described previously [18] with minor modifications [19]. The results were normalized for the abundance of transporters in the membrane fractions as determined by Western blotting. In control experiments, the number of transporters present in the phosphatidylcholine liposomes was

determined and was found to correspond to those in the native membrane fractions.

Assay of equilibrium cytochalasin B binding

Samples of plasma membranes (20 µg protein) in 200 µl Tris/HCl buffer (20 mM, pH 7.4) were incubated with 0.05 µCi [³H]cytochalasin B (NEN; specific radioactivity 9.8 Ci/mmol), 0.04 µCi of [¹⁴C]urea (Amersham; 56 mCi/mmol), and the desired concentrations of unlabelled cytochalasin B for 10 min on ice. Membranes were separated by centrifugation in a refrigerated Microfuge [10 000 g (13 500 rev./min); 30 min]. After removal of the supernatants, the tips of the tubes were cut off, and the pellets were solubilized in 0.5 ml of tissue solubilizer (BTS-450; Beckman, München, Germany). Bound and free radioactivity were determined in a water-compatible scintillation cocktail (Ready Protein; Beckman). Scatchard plots were evaluated graphically as described previously [20,21], and binding to the constitutive GLUT1, as determined with controls transfected with blank vector, was subtracted accordingly.

Photolabelling of glucose transporters with ATB-BMPA

ATB-BMPA was prepared as described [22]. Photolabelling was performed by a modification of a previously published procedure [7]. Samples of plasma membranes (100 µl) containing 30 µg of membrane protein in Tris/HCl buffer (20 mM, pH 7.4) were incubated with ATB-BMPA (30 µl; 150 µCi; specific radioactivity 10 Ci/mmol) for 2 min on ice. The samples were photolysed for 1 min in a Rayonet RPR-100 photochemical reactor with 300 nm lamps. Membranes were separated by centrifugation in a refrigerated Microfuge (13 500 rev./min; 20 min), and the resulting pellets were solubilized in 2 % Thesit. GLUT4 was immunoprecipitated [23], and the immunoprecipitates were separated by SDS/PAGE (10 % gels). The gels were sliced, solubilized, and the incorporated radioactivity was determined by scintillation counting.

RESULTS

Expression of wild-type GLUT4 and mutants in COS-7 cells

We have chosen the glucose transporter isoform GLUT4 for mutagenesis and expression, because it can easily be detected and isolated with specific antiserum in the presence of the constitutive GLUT1 [16], thereby allowing an exact normalization of glucose-

Figure 2 Immunoblotting detection of overexpressed GLUT4 constructs in membrane fractions of transfected COS-7 cells

Samples (20 μ g of protein) of the indicated membrane fractions were separated by SDS/PAGE and immunoblotted as described in the Materials and methods section. PM, plasma membranes; HD, high-density microsomes; LD, low-density microsomes; pCMV, transfection with blank vector; WT, wild-type GLUT4.

transport activity for the amount of expressed transporter. Transfection of COS-7 cells with cDNA of GLUT4 in a vector driven by the CMV promoter results in high copy expression of glucose transporters [16] which are further enriched by isolation of membrane fractions (plasma membranes and high density microsomes). When the activity of these transporters is determined by reconstitution of solubilized transporters into phosphatidylcholine liposomes, the assay yields a proportional relationship between the immunoreactivity of expressed transporters and the glucose-transport activity [24]. All constructs were expressed in COS-7 cells in good yield; there was no significant difference in the expression of the GLUT4 and the different constructs, or in the subcellular distribution of the mutants (Figure 2).

Exchange of Ser-294 for threonine, alanine or glycine

Figure 3(A) illustrates a comparison of the glucose-transport activities reconstituted from COS cells transfected with wild-type GLUT4 or mutants of Ser-294. Transfection with wild-type GLUT4 resulted in a 7-fold increase in transport activity. In contrast, transport rates obtained with the constructs S294A or S294T were indistinguishable from that obtained with bland vector, indicating that the transport activity of these mutants was essentially abolished (Figure 3A). Similarly, no specific cytochalasin B binding was detectable in cells transfected with mutants S294A and S294T (Table 1). In contrast, photolabelling with ATB-BMPA was normal or increased in S294A and S294T respectively (Figure 3B). Means $\pm$ S.D. of bound photolabel in three different membrane preparations were: wild-type,

Figure 3 Glucose-transport activity and ATB-BMPA labelling in mutants of Ser-294 of the GLUT4

(A) Glucose transporters from COS-7 cells transfected with the indicated constructs were solubilized and reconstituted into phosphatidylcholine liposomes as described. Transport activity was assayed, and data were corrected for differences in the immunoreactivity of GLUT4 as assayed by Western blotting. Co, Samples from cells transfected with bland vector. (B) Membranes from cells transfected with the indicated construct were labelled with ATB-BMPA as described, and the labelled transporters were isolated by immunoprecipitation and separated by SDS/PAGE.

Table 1 Affinity of binding of cytochalasin B in membranes of transfected COS-7 cells

The binding of cytochalasin B was assayed by a centrifugation assay as described in the Materials and methods section. Data were corrected for background binding as determined with cells transfected with bland vector, and for small differences in the abundance of the expressed foreign transporter. K_D values were determined as described previously [21] and represent means $\pm$ S.D. Because the K_D of wild-type cytochalasin B was somewhat variable (90–150 nM), separate controls were given for each of the two series.

Mutant	Cytochalasin B binding K_D (nM)
Wild-type GLUT4	159 $\pm$ 5
S294A	No detectable binding
S294T	No detectable binding
S294G	251 $\pm$ 80
Wild-type GLUT4	141 $\pm$ 34
T295A	2283 $\pm$ 227
T295S	214 $\pm$ 37
T295G	173 $\pm$ 9
S296A	221 $\pm$ 24

4400 $\pm$ 510 c.p.m.; S294A, 5716 $\pm$ 690 c.p.m.; S294T, 8738 $\pm$ 2095 c.p.m.; S294G, 2342 $\pm$ 238 c.p.m.). Thus exchanging Ser-294 for Ala or Thr seems to selectively affect transport activity and

Table 2 Summary of the alterations observed in mutants of the STS motif in the exofacial loop domain between helices 7 and 8

The symbols summarize the effects shown in Figures 3–5 (↓, 90–100% reduction of glucose transport activity or cytochalasin B binding; ↑, 100% increase in ATB-BMPA labelling; ↓, 50% reduction in ATB-BMPA labelling; (↓), 50% increase in K_D of cytochalasin B binding. Mutants that appear locked in the exofacial conformation are highlighted by bold type.

	Transport activity	Affinity of recognition site	
		Exofacial	Endofacial
S294A	↓ ↓	Normal	↓ ↓
S294T	↓ ↓	↑ ↑	↓ ↓
S294G	Normal	↓	(↓)
T295A	↓ ↓	Normal	↓ ↓
T295S	Normal	Normal	(↓)
T295G	Normal	Normal	Normal
S296A	Normal	Normal	(↓)

Figure 4 Effect of substitution of Thr-295 on glucose-transport activity and ATB-BMPA labelling

(A) Glucose transporters from COS-7 cells transfected with wild-type GLUT4 or the indicated construct were solubilized and reconstituted into phosphatidylcholine liposomes as described in the Materials and methods section. Transport activity was assayed, and data were corrected for differences in the immunoreactivity of GLUT4 as assayed by Western blotting. Co, Samples from cells transfected with bland vector. (B) Membranes from cells transfected with the indicated construct were labelled with ATB-BMPA as described in the Materials and methods section. The labelled transporters were immunoprecipitated with specific antiserum and separated by SDS/PAGE. Gels were cut, and slices were solubilized and counted.

cytochalasin B binding to the endofacial ligand-binding site, but not glucose binding to the exofacial recognition site. Exchanging serine for glycine (S294G) apparently failed to affect transport activity. However, S294G exhibited significantly reduced ATB-BMPA labelling (Figure 3) and moderately reduced cytochalasin B binding (Table 2).

Figure 5 Glucose-transport activity and ATB-BMPA labelling after exchange of Ser-296 for alanine

(A) Glucose transporters from COS-7 cells transfected with the indicated constructs were solubilized and reconstituted into phosphatidylcholine liposomes as described. Transport activity was assayed, and data were corrected for differences in the immunoreactivity of GLUT4 as assayed by Western blotting. Co, Samples from cells transfected with bland vector. (B) Membranes from cells transfected with the indicated construct were labelled with ATB-BMPA as described, and the labelled transporters were isolated by immunoprecipitation and separated by SDS/PAGE.

Replacement of Thr-295 with alanine, serine or glycine

Figure 4 illustrates the effects of exchanging Thr-295 for alanine, serine or glycine. Exchanging Thr-295 for alanine essentially abolished the reconstituted glucose-transport activity (Figure 4A) and markedly reduced the affinity of cytochalasin B binding (Table 1; 2.28 μ M as compared with 140 nM in the wild-type GLUT4), but failed to affect photolabelling with ATB-BMPA. Thus, as in the S294A and S294T mutants, the amino acid exchange selectively affected transport function and the endofacial binding site, but not the exofacial glucose-binding site. When Thr-295 was exchanged for glycine or serine, transport activity and ATB-BMPA labelling were normal (Figure 4). Cytochalasin B binding (Table 1) was marginally reduced or normal (T295G).

Substitution of Ser-296 for alanine

Exchanging Ser-296 for alanine failed to affect reconstituted glucose-transport activity or ATB-BMPA labelling (Figure 5) and only produced a minimal reduction in cytochalasin B binding (Table 1).

DISCUSSION

The present results indicate that mutagenesis of the exofacial STS motif between helices 7 and 8 can selectively obliterate glucose-transport activity and the internal ligand-binding site,

without affecting the external glucose-binding site. In three of the mutants investigated (S294A, S294T and T295A), the photolabelling with the exofacial ligand ATB-BMPA appeared normal or even increased, whereas glucose-transport activity and cytochalasin B binding were essentially abolished. A similar pattern of alterations has previously been observed after exchange of Tyr-293 for isoleucine in GLUT4 [7]. The present data comply with the current hypothesis that the transport facilitators alternate between inward- and outward-facing conformations (C_o and C_i), exposing either the exofacial or the endofacial hexose-binding site [4]. According to this model, residues 293, 294 and 295 are crucial for the switching of the transporter from inward- to outward-facing conformation and back. It is commonly held that two different equilibria determine the conformational orientation of the carrier: the $C_o \rightleftharpoons C_i$ equilibrium which reflects the kinetics of the substrate-induced conformational alteration, and the substrate-independent equilibrium $C_o \rightleftharpoons C_i$ [25]. The effects of the mutations appear to affect mainly the $C_o \rightleftharpoons C_i$ equilibrium for the following reasons. The apparent affinities for internal (K_{in} , here cytochalasin B) and external (K_{out} , here ATB-BMPA) binding site are given by the equations $K_{in} = K_d(1 + h/g)$ and $K_{out} = K_d(1 + g/h)$, where K_d is the true binding constant for the binding of the substrate to the carrier, and g and h are the rate constants for $C_o \rightarrow C_i$ and the $C_o \leftarrow C_i$ respectively [25]. If the h/g ratio increases from 1 (in the wild-type transporter) to a very large number, the apparent affinity of the internal site (cytochalasin B) becomes very low (high K_{in}). According to the second equation, the apparent affinity of the external site (K_{out}) would increase only twofold. The alterations seen with the mutant S293T (obliteration of the cytochalasin B binding, twofold increase in ATB-BMPA labelling) comply well with this model. However, we cannot exclude additional effects on the $C_o \rightleftharpoons C_i$ and $C_o \rightleftharpoons C_o$ equilibria, which might explain why a normal or moderately reduced photolabelling was observed in mutants S294A and S294G respectively.

The present results reveal a complex pattern of requirements, steric and polar, for positions 294 and 295. Steric requirements seem to predominate, since both positions are markedly affected by the introduction of alanines but tolerate glycines without gross functional alteration. This finding suggests that hydroxy groups are involved in the conformational alteration of the loop and that hydrophobic side chains in positions 294 and 295 can hinder these alterations. However, glycines appear to be tolerated in the critical positions 294 and 295 because they provide maximal flexibility of the peptide chain. It should be noted that a similarly complex pattern results from mutagenesis of Tyr-293 [7,24]: transport activity and cytochalasin B binding are normal in Y293F, but markedly reduced in Y293I.

We initiated the present study under the assumption that the STS motif may be involved in the recognition of glucose. The finding that ATB-BMPA photolabelling was normal in all mutants argues against this assumption, and seems to indicate that the loop does not contact the hexose directly. However, it should be noted that the comparison of different hexose transporters (Figure 1) shows some correlation of the motif with their function as glucose transporters. In the fructose transporter GLUT5 the STS motif is substituted by ADQ, and the loop is extended by two additional residues (Figure 1). In contrast, glucose transporters from phylogenetically very remote organisms, e.g. *Taenia solium*, *Schistosoma mansoni* [26] and *Caenorhabditis elegans*, comprise the motifs STS, SSL and STD respectively. Therefore we feel that the residues in positions

294–296 may be characteristic for the hexose transported by the carrier, although they are unlikely to contact the hexose directly.

An inherent problem of the mutagenesis approach is the possibility that amino acid exchanges might disrupt the correct folding of a peptide chain in a non-specific manner. The following arguments support our view that the alterations observed here reflect specific effects, i.e. that the exchanged amino acid is directly involved in the arrangement of the helices and/or the recognition of glucose. First, very small alterations such as the replacement of S with T in S294T affected the function of the protein; this result is hard to explain with a non-specific disruption of the protein folding. Secondly, all mutants retain residual or even full activity in at least one of the investigated parameters. Thus it is concluded that residues 294 and 295 are specifically involved in the recognition of the hexose by the carrier protein, and in the subsequent conformational alteration of the latter.

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