From triple cysteine mutants to the cysteine-less glucose transporter GLUT1: a functional analysis

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Abstract Two triple cysteine mutants containing Cys-less N- or C-terminal halves and the Cys-less GLUT1 were generated by site-directed mutagenesis. Following expression in *Xenopus* oocytes, the intrinsic transport activities of the multiple cysteine mutants were slightly decreased when either the cysteine residues of the C-terminal half or all six residues were changed; substitution of serine for cysteine residues located at the N-terminal half was without consequence for the catalytic activity. The exofacial ligand ethylidene glucose inhibited 2-deoxy-D-glucose uptake of wild-type and Cys-less GLUT1-expressing *Xenopus* oocytes with comparable half-saturation constants (11.5 and 13.2 mM). However, each of the multiple cysteine mutants exhibited an increase in affinity for the endofacial inhibitor cytochalasin B, with the greatest effect being observed for the Cys-less construct (decrease in K_i by the factor 5–6).

Key words: GLUT1 glucose transporter; Multiple cysteine mutants; *Xenopus* oocyte

1. Introduction

The family of glucose transporters that mediates facilitative diffusion contains six members, which have been isolated, cloned and sequenced. These GLUT1–GLUT5 and GLUT7 share an almost identical topological organization with 12 transmembrane α-helices and a 42–65% amino acid identity exists between GLUT1 compared with GLUTs 2–5 [1,2]. Derived from the nucleotide sequence GLUT1 is known to possess six cysteine residues. Several reviews [3–5] have addressed the role of cysteine residues in glucose transport and transporter conformation. As proposed by Hebert and Carruthers [6], cysteine residues may be involved in oligomerization or may be important for stabilization of a local tertiary structure by disulfide bonds. Sulfhydryl reagents are known to inhibit glucose transport, probably by impairment of the interconversion of transporter conformation. Our previous results have demonstrated that after individual replacement of each single cysteine the glucose transport is not diminished [7]. Therefore, individual cysteine residues, if involved in oligomerization or intramolecular stabilization by a single disulfide bond, do not seem to be essential for the catalytic activity per se.

In order to further characterize the role of cysteine residues in transport function, we constructed two tripel mutants with Cys-less halves (C133S-C201G-C07S and C347S-C421S-C429S) as well as a six-fold mutant, i.e. the Cys-less GLUT1. Using the *Xenopus* oocyte expression system, the transport activities of these mutants were determined and the uptake

rates normalized to the relative plasma membrane contents in order to calculate the intrinsic transport activities. Furthermore, the affinities of both the exofacial ligand ethylidene glucose and the endofacial transport inhibitor cytochalasin B to the multiple cysteine mutants were calculated and compared with wild-type GLUT1.

2. Materials and methods

2.1. Engineering the triple and and six-fold cysteine mutations

A 2.4-kb fragment, derived from pSPGT [8], was subcloned into the expression vector pSP64T [9] and site-directed mutagenesis performed according to the procedure of Deng and Nickoloff [10] using a Clontech kit (Clontech Laboratories, Palo Alto, CA). Using the mutated C207S GLUT1, the tripel mutant (N-terminal half: C133S-C201G-C207S) was constructed. In the same manner, derived from the C347S mutant the other tripel cysteine mutant (C-terminal half: C347-C421S-C429S) was created. The Cys-less GLUT1 was generated using the *Stu*I restriction site in the middle of the GLUT1 cDNA. The resulting changes of the mutagenic oligonucleotides and the amino acids are as follows:

Amino acid no.	Nucleotide change	Amino acid change
C133	5′ TGC 3′ → 5′ AGC 3′	C → S
C201	5′ TGC 3′ → 5′ GGC 3′	C → G
C207	5′ TGC 3′ → 5′ AGT 3′	C → S
C347	5′ TGC 3′ → 5′ AGT 3′	C → S
C421	5′ TGC 3′ → 5′ AGC 3′	C → S
C429	5′ TGT 3′ → 5′ TCT 3′	C → S

Mutagenesis was confirmed by DNA sequence analysis. After in vitro transcription, proof of full-length cRNA and quantitation were performed as described previously [11]. The final concentration of all cRNA preparations was adjusted to 0.5 mg/ml by counting the incorporated [^{35}S]UTP and by comparison of the wild-type and mutant cRNAs on a denaturing agarose gel, also including 28S and 18S rRNA as standards (Pharmacia, Freiburg, Germany).

2.2. Transport measurements

Collection, defolliculation and culture of *Xenopus* oocytes were carried out routinely as described previously [13]. Three days after microinjection of the respective cRNAs, tritium-labeled 2-deoxy-D-glucose (50 μM, 1 μC/0.5 ml) uptake was conducted for comparison of hexose transport between wild-type and mutant GLUT1-expressing oocytes. In brief, after 30 min incubation of oocytes in 0.5 ml of modified Barth's solution (MBS) containing tritium-labeled 2-deoxy-D-glucose the oocytes were washed 3 × with ice-cold MBS containing 0.1 mM phloretin, then dissolved in 1% SDS before the radioactivity was determined.

2.3. Determination of the relative GLUT1 content in plasma membrane fractions

In order to isolate plasma membranes, single oocytes (routinely 100 of each group) were triturated in MBS by use of an adjustable 10-μl Eppendorf pipette. For dot-blot analyses, these membrane preparations were further treated as described previously [13]. A rabbit antiserum (1 : 250 dilution) raised against a synthetic peptide corresponding to the C-terminal 15 residues of human GLUT1 (F350; a kind gift from Dr. M. Mueckler, Washington Unversity, St Louis, MO) and ^{125}I-

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labeled goat anti-rabbit IgG secondary antibody (NEN; specific activity 6.8 Ci/g) were used for detection. The glucose transporter proteins in plasma membrane fractions were compared with a purified glucose transporter (a kind gift from Dr. G. Lienhard, Dartmouth Medical School, Hanover, NH). After cutting the respective areas, the radioactivity was determined in a gamma counter (Cobra Auto-Gamma, Packard).

The mutant oligonucleotides were from Molbiol (Berlin, Germany); the radiolabeled glucose analogue (2-deoxy-D-[2,6-^{3}H] glucose) from Amersham-Buchler (Braunschweig, Germany). The Sequenase version 2 DNA sequencing kit was from USB (Cleveland, OH) and phloretin as well as cytochalasin B was purchased from Sigma (Deisenhofen, Germany). 4,6-O-Ethylidene-α-D-glucose was from Aldrich (Steinheim, Germany). All other substances used were of the highest grade. *Xenopus laevis* frogs were obtained from Xenopus Laborzucht (H. Kähler, Hamburg, Germany). Statistical evaluation was carried out using Student's *t* test for paired values (conducted with Stat View version 4, Abacus Concepts); Ultra Fit (Biosoft) was used for curve-fitting of the data points and calculating the K_i values.

3. Results

The GLUT1 glucose transporter contains 6 cysteine residues at positions 133, 201 and 207 (N-terminus half) and 347, 421 and 429 (C-terminus half). In recent investigations [7,14], single cysteine mutants were tested whether any individual residue was critical for function. The results indicate that none of the cysteine residues were essential for transport activity. Therefore, we constructed glucose transporters with Cys-less N- and C-terminal halves, and the Cys-less GLUT1 protein. The proce-

Table 1
Uptake rates, relative plasma membrane (PM) contents and relative intrinsic activities (IA) of multiple cysteine mutants-containing GLUT1

(A) Uptake and relative PM content

Mutants	Uptake (pmol/oocyte × 30 min) mean ± S.D.	Relative PM content (cpm/20 μg protein) mean ± S.D.
Wild-type GLUT1	34.8 ± 8.8	8174 ± 2000
Cys-less N-terminal half	31.8 ± 8.3	7306 ± 2094
Wild-type GLUT1	34.8 ± 8.8	9881 ± 4424
Cys-less C-terminal half	26.6 ± 8.6	8273 ± 2169
Wild-type GLUT1	28.8 ± 12.1	1666 ± 71
Cys-less GLUT 1	27.2 ± 8.2	1668 ± 663

(B) Relative intrinsic activity (rIA): % of wild-type uptake % of wild type PM content

Mutants	Relative IA (arbitrary units) mean ± S.E.	Number of independent experiments
Wild-type GLUT1	1	
Cys-less N-terminal half	1.10 ± 0.06	3
Cys-less C-terminal half	0.87 ± 0.05	4
Cys-less GLUT1	0.85 ± 0.07	3

(A) Tritium-labeled 2-deoxy-D-glucose uptake was determined and the mean ± S.D. of representative experiments are presented comparing wild-type GLUT1 with each of the multiple cyteine mutants. The relative contents of plasma membrane (PM) GLUT1 protein were calculated from radioactivity of the ^{125}I-labeled goat anti-rabbit IgG secondary antibody of Western dot-blots (mean ± S.D. of two to four dots) counted in a gamma counter.
(B) Relative intrinsic activities were calculated by normalizing the uptake values to the relative PM contents for the wild-type and the multiple cysteine mutants of GLUT1. For comparison the rIA of wild-type GLUT1 was set to 1. The values represent the mean ± S.E. of relative intrinsic activities obtained from three to four independent experiments.
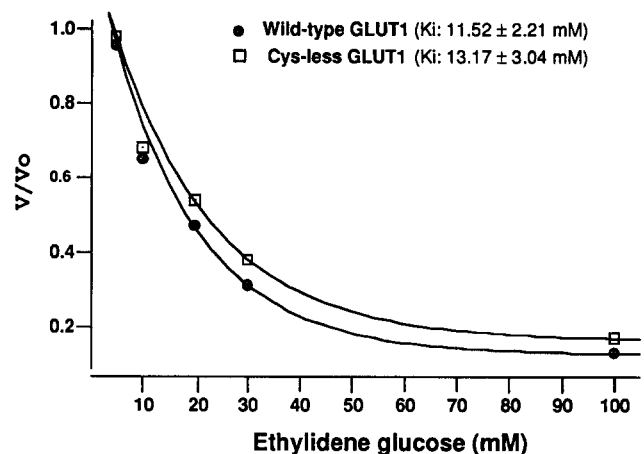

Fig. 1. Ethylidene glucose inhibition of 2-deoxy-D-glucose uptake of wild-type and Cys-less GLUT1-expressing oocytes. Fractional uptake rates (v/vo = inhibited/non-inhibited uptake rates) from four independent experiments comparing wild-type with Cys-less GLUT1 were included in the curve-fitting performed by UltraFit (Biosoft). Except for 100 mM ethylidene glucose, the % remaining 2-deoxy-D-glucose uptake was not statistically different ($P > 0.05$) at each of the indicated inhibitor concentrations. The best fits of the data points were obtained following the equation of single exponential decay with offset and weighting using the Marquardt algorithm.

dure to determine the relative intrinsic transporter activities was as follows. (1) The uptake rates of the mutants were related to the wild-type transport activity. (2) The relative contents of the plasma membrane were determined and expressed as % of wild-type. (3) Uptake rates were normalized to the plasma membrane expression. Table 1A presents uptake rates and relative plasma membrane contents from representative experiments for each of the multiple cysteine mutants. Data points obtained from three to four independent experiments were compiled to calculate the relative intrinsic activities for each group. A modest decrease in transport activity (Table 1B) was found after changing the three C-terminus cysteine residues ($P = 0.035$) and upon replacement of all six cysteine residues ($P = 0.069$), whereas the substitution of serine for cysteine residues at the N-terminal half was without effect.

Since multiple cysteine residues might be involved in glucose transport regulation by oligomerization or stabilization of a particular local tertiary structure, we also investigated whether the lack of cysteine residues causes changes in 2-deoxy-D-glucose uptake inhibition by ethylidene glucose (exofacial inhibitor) or cytochalasin B (endofacial inhibitor). Fig. 1 presents the ethylidene glucose inhibition of 2-deoxy-D-glucose uptake (50 μM) at four inhibitor concentrations. The K_i value obtained for the wild-type GLUT1 (approximately 12 mM) was identical with that reported by Hashiramoto et al. [15] in CHO cells and was similar to those reported in *Xenopus* oocytes by Mueckler et al. (16 mM) [16] and Due et al. (14 mM) [17]. Although the fractional uptake rates (inhibited/non-inhibited uptake rates calculated from four independent experiments) were slightly greater for the Cys-less construct at each inhibitor concentration, the inhibition was statistically different ($P < 0.05$) only at the highest ethylidene glucose concentration (100 mM).

To further characterize the kinetic behavior of the multiple cysteine mutants, cytochalasin B was applied as an inward-binding glucose transport inhibitor. Including the data points
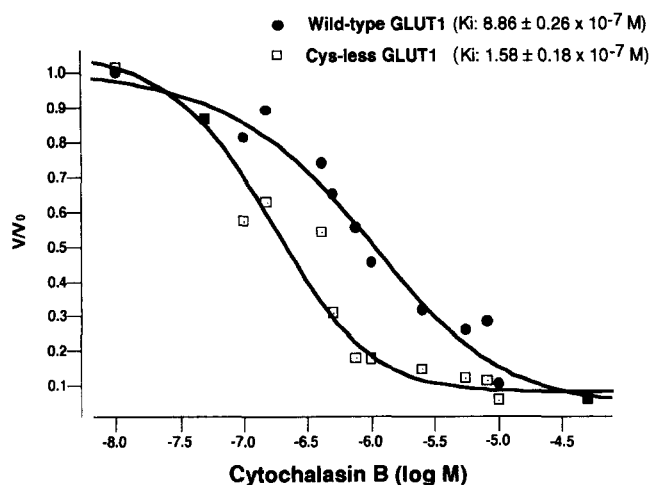
Fig. 2. Cytochalasin B inhibition of 2-deoxy-D-glucose uptake of wild-type and Cys-less GLUT1-expressing oocytes. Fractional uptake rates (v/vo = inhibited/non-inhibited uptake rates) obtained from three independent experiments comparing wild-type with Cys-less GLUT1 were included in the curve-fitting performed by UltraFit (Biosoft). The best fits of the data points were obtained following the equation: v/vo = min + (max − min)/(1 + (IC$_{50}$/dose N) with 'robust' weighting using the Marquardt algorithm.

of three independent experiments, the 2-deoxy-D-glucose uptake was found to be inhibited with a half-saturation constant of approximately 0.9 μM (Fig. 2). The calculated value was in the range (0.5–1.1 μM) reported for GLUT1 after expression in Xenopus oocytes [11,17]. The affinity for cytochalasin B, however, clearly increased, when all six cysteine residues were replaced by serine (K_i values: 0.16 μM of Cys-less vs. 0.89 μM of wild-type GLUT1). This effect prompted us to further investigate if cysteine residues of a particular half were responsible for the lower K_i values, since only the substitution of the C-terminal cysteine residues resulted in a decrease of intrinsic activity. Upon comparing 2-deoxy-D-glucose uptake inhibition between oocytes expressing the Cys-less N- or C-terminal half, the affinities for cytochalasin B were rather similar between both groups, with a slightly greater inhibitory effect on the triple mutant of the C-terminus (Fig. 3).

4. Discussion

Recent studies have begun to address the role of cysteine residues in catalytic activity and conformation of GLUT1. The general importance can be deduced from their potential involvement in oligomerization, stabilization of local tertiary structures and transport inhibition by sulfhydryl reagents. Results of Wellner et al. [7] that no single cysteine residue is essential for the catalytic activity of GLUT1 were confirmed very recently by Due et al. [17]. This group also published for the first time 3-O-methyl-D-glucose transport rates of a cysteine-less construct which were slightly greater than those observed in wild-type GLUT1-expressing oocytes. They concluded that cysteines are not required for 3-O-methyl-D-glucose transport, thus, excluding oligomerization by disulfide-bond formation as a prerequesite of maximum catalytic activity. In addition, we calculated the relative intrinsic activities by normalizing the 2-deoxy-D-glucose uptake rates to the relative plasma membrane contents of each of the multiple cysteine

mutants. Substitutions of cysteine residues that are located at the C-terminal half or at all six positions lead to a modest reduction of catalytic activities (remaining intrinsic activity was 85–87% of wild-type GLUT1). A great number of experiments confirmed that the simultaneous change of the three cysteine residues harbored in the N-terminal half was without consequence to the 2-deoxy-D-glucose uptake rates. However, it cannot be excluded that transporter oligomerization involves several pairs of cysteine residues without functional consequences or that multimeric assembly takes place by means different from disulfide bonding (e.g. leucine zipper motif)

Since replacement of cysteine residues could theoretically lead to changes in the local tertiary structure resulting in altered affinities for transport inhibitors, ethylidene glucose and cytochalasin B were applied to inhibit 2-deoxy-D-glucose uptake either from the exofacial or endofacial side of the plasma membrane. Consistent with results of Due et al. [17], only minimal differences in inhibition of 2-deoxy-D-glucose uptake rates by ethylidene glucose could be detected when wild-type and Cys-less GLUT1 were compared. As it was known from our previous findings [14] that substitution of arginine for cysteine at position 421 decreases the affinity of cytochalasin B to GLUT1, serine was introduced instead when the six-fold or the triple C-terminal half cysteine mutants was created. The increase in affinity of the cysteine-less GLUT1 for cytochalasin B lead to the speculation that changes in the local tertiary microstructure may enhance the accessibility of the protein to the inhibitory substrate from the endofacial side of the membrane, but leaving unchanged the outward-facing binding site of ethylidene glucose. Our results failed to give a definite answer as to whether cysteine residues of a particular half of the glucose transporter preferentially contribute to this effect. It rather seems that change of cysteine residues from both opposite halves are involved in lowering the K_i values of cytochalasin B. We cannot explain at this time the discrepancy between our results and those obtained by Due et al. [17] who reported a half-saturation
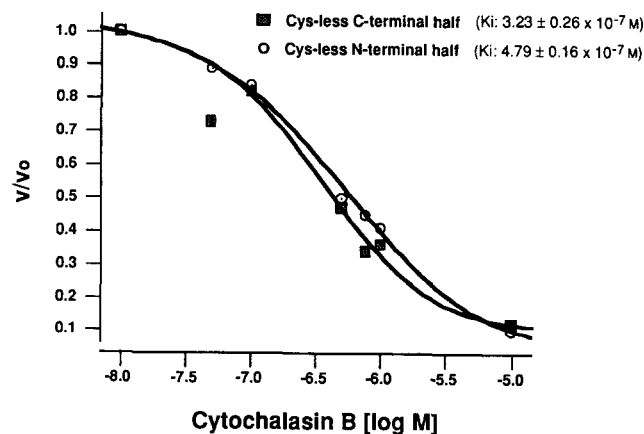

Fig. 3. Cytochalasin B inhibition of 2-deoxy-D-glucose uptake of multiple cysteine mutants containing the Cys-less N-terminus of C-terminus halves determined after expression in Xenopus oocytes. Mean fractional uptake rates (v/vo = inhibited/non-inhibited uptake rates) calculated for each of the indicated inhibitor concentrations from one experiment were included in the curve-fitting performed by UltraFit (Biosoft). The means at each inhibitor concentration were determined from the uptake rates of 15–20 oocytes. The best fits of the data points were obtained following the equation: v/vo = min + (max − min)/(1 + (IC$_{50}$/dose) N) with 'robust' weighting using the Marquardt algorithm.

constant of cytochalasin B (1.1 μM) for the Cys-less GLUT1 which is considered not to be different from the value of wild-type transporter. Both groups, however, agree in the notion that no single cysteine residue is critical for glucose transport and, if oligomerization by disulfide bonds takes place in the membrane, it does not contribute much, if at all, to the intrinsic activity of GLUT1.

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