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Probing Transmembrane Topology of the High-Affinity Sodium/Glucose Cotransporter (SGLT1) with Histidine-Tagged Mutants

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Abstract. To reexamine the existing predictions about the general membrane topology of the high-affinity Na^+/glucose cotransporter (SGLT1) and in particular of the large loop at the C-terminal region, a small 6 × Histidine-tag was introduced at different positions of the SGLT1 sequence by site-directed mutagenesis. Eleven His-SGLT1 mutants were constructed and were transiently transfected into COS-7 cells. As demonstrated by immunofluorescent labeling with antipeptide antibodies against SGLT1, all mutants were expressed and inserted into the plasma membrane. Only mutants with the tag in the N-terminal region and the C-terminal region retained Na^+/glucose cotransport activity at 0.1 mM D-glucose. The arrangement of the His-tag in the membrane was analyzed by indirect immunofluorescence, using a monoclonal antihistidine antibody. In nonpermeabilized cells the His-tag could be detected at the N-terminal end (insertion at aa 5) and at the C-terminal end (replacement between aa 584-589 and between aa 622-627), suggesting that these portions of the polypeptide are accessible from the extracellular space. Furthermore, an epitope-specific antibody directed against aa 606-630 reacted strongly with the cell surface. To support this topology intact stably transfected SGLT1 competent CHO cells were partially digested with an immobilized trypsin and subsequently subjected to electrophoresis and Western blot analysis. The size of the digestion product suggests that extravesicular trypsin removed the extracellular loop that contains the amino acid residues 549-664. Thus our results indicate that the last large loop (about aa 541–aa 639) towards the C-terminal end faces the cell exterior where it might be involved in substrate recognition.

Key words: SGLT1 — His-tag — Immunofluorescence — Transient transfection — Trypsin digestion

Introduction

The Na^+/D-glucose cotransporter SGLT1 plays an important role in the reabsorption of filtered D-glucose in the kidney proximal tubule. Characteristics of this transporter and kinetics of the transport have been studied in great detail (Hopfer, 1977; Murer & Kinne, 1980; Lin, Stroh & Kinne, 1982; Turner & Moran, 1982; Semenza et al., 1984; Kipp, Lin & Kinne, 1996). The SGLT1 from the rabbit kidney has 662 amino acid residues, as revealed by cloning techniques (Hediger et al., 1987; Morrison et al., 1991), belongs to the superfamily of sodium/solute symporter (Reizer, Reizer & Saier, 1994), and is highly homologous to SGLT1 from other tissues and species (Hediger, Turk & Wright, 1989; Hediger et al., 1995; Kong, Yet & Lever, 1993; Wood et al., 1994). Like other transport proteins, SGLT1 is a membrane protein with multiple membrane spanning domains (Ball & Loftice, 1987; Allard & Bertrand, 1992; Baldwin, 1993; Pourcher et al., 1996).

An important contribution for clarification of the structure and function relationship of the transporter would be if the topology of the extramembraneous loops that flank the membrane spans could be clearly assigned. To establish a topology for a membrane protein, strategies combining computer calculation, modeling and experimental tests are usually employed. The initial steps include use of the hydropathy analysis (Kyte & Doolittle, 1982), the multiple sequence alignment algorithm (Vingron & Argos, 1988) and the approximation of the loop's

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orientation using the positive-inside rule of von Heijne (1992).

Several approaches have been employed to experimentally test the models developed on this basis. N-glycosylation scanning mutagenesis has been used to determine the topology of the Glut 1 glucose transport protein (Hresko et al., 1994). The same strategy was later also employed for the human SGLT1 (Turk et al., 1996); thereby a glycosylation sequence with four amino acid residues, NNSS extended with a 42-amino acid consensus sequence was introduced at several positions of SGLT1. Further strategies are use of side-specific enzymes for fusion with the gene of interest (Allard & Bertrand, 1992; Fiedler & Scheiner-Bobis, 1996; Schülein et al., 1997) or introduction of an epitope tag into the gene (Yoon & Guidotti, 1994; Howard et al., 1995; Canfield, Norbeck & Levenson, 1996; Pan et al., 1998). The tag can then be detected with a specific antibody. One of the special tag-sequences used for this purpose is the histidine tag. Initially used for the purification of large amounts of proteins (Sutton et al., 1995; O'Connell & Stults, 1997; Reiber, Grover & Brown, 1998) the 6×His-tag can now be inserted into an internal protein sequence and can be detected with an antibody. In this paper we report the successful introduction of the 6×His-tag into various positions of SGLT1 using a pHook™-2 plasmid under control of a CMV promoter.

The localization of the loop residues, particularly the large loop close to the C-terminal end (from aa 541–aa 639) could be examined by means of immunofluorescence using the monoclonal antibody against the 6×His-tag. In addition, a polyclonal antibody against aa 606–aa 630 was used to investigate synthesis and sorting of SGLT1 into the plasma membranes of cells and to detect oligopeptide fragments obtained during partial enzymatic digestion. In summary, our results suggest that the last large extramembraneous loop with more than 80 amino acid residues faces the extracellular space.

Materials and Methods

Production of Antibodies

Immunopurified polyclonal antipeptide antibodies Ali-1, Pan-3 and Pan-2 were raised against oligopeptides near the N-terminus (aa 3-aa 17), the C-terminus (aa 606 to aa 630) and aa 339–aa 356 of the SGLT1, respectively, as described previously (Lin et al., 1998).

The peptides were conjugated with Keyhole Limpet Hemocyanine and two sheep were immunized with each peptide. All antibodies were commercially produced and immunopurified using the respective peptide as the ligand (Cambridge Research Biochemicals, Cheshire, England).

Plasmid Construction

pHook™-2 was initially selected because an enrichment of transiently transfected COS-7 cells based on magnetic selection of antigen-coated magnetic beads was considered (Invitrogen, San Diego, CA).

For construction of a wild-type plasmid pBluescript SK^+ plasmid containing the coding range of the rabbit renal SGLT1 (2207 bp) was digested with restriction enzymes, *Hin*dIII and *Xba*I, and ligated with the eukaryotic expression vector pHook™-2 that was also digested with the same restriction enzymes. The pHook vector contains a Rous sarcoma virus (RSV) promoter and a human cytomegalovirus (CMV) promoter upstream of a multiple cloning site. The former one is for expression of a single chain antibody (sFv) on the transfected cell surface against specific hapten (e.g., 4-ethoxy-methylen-2-phenyl-2-oxazolin-5-one) and the latter one is for expression of the gene of interest (Invitrogen). To construct the SGLT1-His mutants, oligonucleotides encoding six histidines were flanked by overlapping ends containing the appropriate restriction sites used for mutant screening (5′- XXX CAC CAC CAC CAC CAC CAC XXX -3′) and placed into eleven different positions of the SGLT1 gene either to substitute (for internal fragments) the original sequences or to extend the sequences at N- and C-terminus via site-directed mutagenesis using Chameleon™ kit (Stratagen, La Jolla, CA). Each oligonucleotide used for mutagenesis was about 60 nucleotides long with the 6×His-tag encoding part in the center, flanked by 20 nucleotide homologous regions. A higher expression was achieved when only CAC sequences, but without CAT, were selected for our expression system. All plasmids were checked by sequencing, purified through Qiagen endotoxin-free Plasmid Maxi Kit (Qiagen, Hilden, Germany), and subcloned into the pHook™-2 vector with the identical approaches except that for Mut 5, that means mutants were subcloned in a similar way as described above for the wild-type *sglt1* gene. Mut 5 was subcloned from pSGLT1-Mut 5, digested with *Xho*I, filled with dNTPs and Klenow Fragment of DNA-polymerase, and *Xba*I. A 2207-bp fragment of the SGLT1-Mut 5 was ligated with pHook™-2 digested with *Hin*dIII, filled with dNTPs and Klenow of DNA polymerase and *Xba*I.

Northern Blot Analysis

Total RNA from COS-7 cells was isolated by using the RNeasy kit (Qiagen) according to the manufacturer's procedures. RNA (5 μg) was electrophoretically separated on a 1.2% agarose gel in the presence of 2.2 M formaldehyde. After electrophoresis RNA was transferred onto a Nylon N membrane (Amersham, UK) and hybridized with a 1200-bp *N*coI-fragment from the *sglt*1 gene, labeled with digoxigenin (Boehringer, Mannheim, Germany).

Transient Transfection

COS-7 cells were grown to 70 to 80% confluency before transfection. Transient transfection was performed by means of lipofection using either the positively charged lipid, DOSPER (Boehringer Mannheim) or the dendrimer-based SuperFect™ Reagent (Qiagen). In the first case, 4 to 5 μg of lipid was previously mixed with 1 μg DNA prior to addition to a well that contained approximately 3×10^5 cells. In the latter case, 1.5 μg DNA in 75 μl Opti-DMEM (DMEM supplemented with glutamate) were pre-mixed with 7 μl of SuperFect™ Reagent. The mixture was then added to a well containing 1.25×10^5 cells in 500 μl DMEM including D-glucose (4.5 g/l). After incubation of 3 to 4 hr the cells were washed once with fresh DMEM medium and allowed to grow for 48 hr.

The efficiency of transfection was tested in COS-7 cells simultaneously transfected with the pHook™-2*lac*Z control plasmid expressing β-galactosidase under identical conditions. The post-transfection period for an optimal expression of β-galactosidase was 48 hr, as determined with the standard enzymatic assay using O-nitrophenyl-β-D-galactoside as substrate at pH 7.0 according to the manufacturer's

manual (Invitrogen, Carlsbad, CA). The activity was measured from the cell lysate in the presence of 1% Triton X-100 (TX-100). In 8 independent transfections, the activity of the expressed galactosidase was 5.6 ± 3.6 mmoles/h/mg protein ($n = 8$) after correction for endogenous activities, measured in nontransfected cells. Furthermore, the transiently transfected COS-7 cells were stained with the β-Gal staining kit (Invitrogen). The staining procedures were performed according to the manufacturer's manual using X-gal as substrate. After fixing and washing, total number of cells and the number of stained cells in several randomly selected view fields (5 to 6) were counted. The percent of cells expressing β-galactosidase (% transfection) was calculated by the ratio between the number of blue cells and the total number of cells. In pilot experiments the transfection efficiency estimated by this method and the magnitude of [^{14}C] α-methyl D-glucoside uptake were found to be parallel.

Western Blot Analysis

Protein samples were denatured under reducing conditions, and subjected to SDS-PAGE using pre-cast 8–16% gradient gel (Novax, San Diego, CA). The separated polypeptides were then electrotransferred onto a nitrocellulose membrane. The blot was either stained with colloidal gold to visualize the protein pattern or incubated with a polyclonal antipeptide antibody. An enhanced chemiluminescence system (ECL™, Amersham Life Science) was used to detect the immunological reaction. After incubation with Ali-1, Pan-2 or Pan-3, the blot was washed and incubated with an anti-sheep IgG horseradish peroxidase complex. The chemiluminescence was then measured using a Hyperfilm™ or recorded with a Molecular Phosphor Imaging System (Bio-Rad Laboratories, CA).

α-Methylglucoside Uptake

Before measuring α-methylglucoside (AMG) uptake the transfected COS-7 cells were incubated in a glucose-free DMEM medium for 2 hr to reduce the intracellular glucose concentration to a nonsignificant level. Uptakes of AMG were carried out as described previously for CHO cells (Lin et al., 1998).

Antibody Labeling in COS-7 Cells

COS-7 mutants grown in a 12-well plate were chilled on ice and washed three times with ice-cold KRH-Na solution supplemented with 2 mM EGTA, 0.5% BSA and protease inhibitors (a mixture of 20 μg leupeptin, 20 μg aprotinin, 5 μg chymostatin, and 5 μg pepstatin per ml). A monoclonal anti-histidine antibody (CLONTECH Laboratories, Palo Alto, CA) or an immunopurified polyclonal anti-SGLT1 antibody [Ali-1 (0.45 mg/ml), Pan-2 (0.73 mg/ml), or Pan-3 (0.73 mg/ml)] at a dilution of 200-fold with the same medium was added to cover the cells and incubated for 1 hr. After removal of the antibody the cells were washed three times. The cells were then incubated with Anti-mouse IgG-FITC or Anti-sheep IgG-FITC in the same medium (1:200) followed by thorough washing. To verify a specific staining with Pan-3, peptide antigen aa 606–aa 630 was present in Pan-3 solutions in parallel experiments.

For labeling experiments with permeabilized cells, cells were treated with ice-cold acetone/methanol mixture (1:1) for 10 min on ice. After removal of the organic solvent, cells were washed once with the KRH-Na medium and the same labeling procedures followed. Cells were kept on ice throughout the entire experiments. Fluorescence of labeled cells was observed and photographed using an inverted microscope (DM IRB, Leica Mikroskopie und System GmbH, Wetzlar, Germany) attached with a photographic system (Wild MDS 52, Leica).

Partial Trypsin Digestion of CHO Cells

CHO cells or G6D3 cells (5 petri dishes, $d = 10$ cm) grown to confluence were carefully scraped into a plastic tube and washed with ice-cold KRH buffer and resuspended in the same buffer. The suspension was diluted to about 1 mg/ml with osmotically balanced buffer (100 mM Hepes/Tris supplemented with 75 mM NaCl) at pH 8. The suspension was pretreated with 10 mM $HgCl_2$ for 30 min at room temperature. Cells were spun down and washed with the same buffer at pH 8.0. 100 μl of trypsin beads (on agarose basis) (50% slurry) was added to the cell suspension. The mixture was allowed to incubate in a plastic tube at room temperature for 2 hr with gentle rotation. The digestion was terminated by addition of trypsin inhibitors. Cell mass was then separated by centrifugation at 5,000 × g for 30 min. The supernatant was centrifuged, concentrated to dryness using an ultramicrofilter with a cutoff of 0.1 kD, and denatured under reducing conditions. The cell mass was washed with 20 mM Hepes-Tris buffer and solubilized with 1% Triton-X100. The solubilized proteins were then denatured.

Results

Transient Expression of His-tagged SGLT1 Proteins in COS-7 Cells

Eleven Histidine-tagged SGLT1 constructs were transiently expressed in COS-7 cells. Judged by the expression of β-galactosidase in transfected COS-7 cells, the efficiency of transfection reached between 30 and 40%. Transcription of all mutants was examined by Northern blot analysis using a 1200-bp *Nco*I-fragment from the *sglt*1 gene as a probe for hybridization. As shown in Fig. 1, all *sglt*1-containing plasmids induced the transcription of similar levels of mRNA.

Evidence for a Plasma Membrane Localization of His-tagged SGLT1 Proteins

Immunofluorescence was used to detect whether His-tagged SGLT1 proteins are present on the surface of transfected COS-7 cells. It has been shown that a protein residing on the surface of cells can be recognized easily with a specific antibody (Canfield et al., 1996; Howard et al., 1995). We therefore labeled the intact cells with Pan-3 and with anti-sheep IgG, conjugated with a fluorescent probe, FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine isothiocyanate). The antipeptide antibody, Pan-3 has been proven as a useful tool to identify the SGLT1-related polypeptide in tissues and transfected cells (Lin et al., 1998).

As depicted in Fig. 2*A*, all mutants including the wild type induced a clear immunological staining on the cell surface while cells transfected with the vector alone

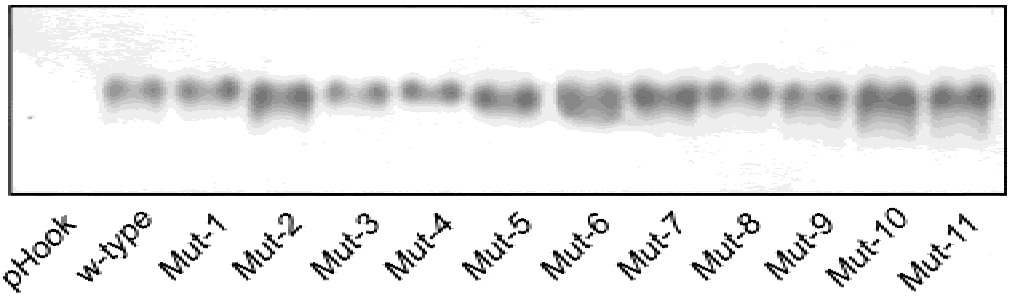

Fig. 1. Northern blot analysis of COS-7 cells transfected with various His-SGLT1 mutants. Equal amounts of total RNA from transfected cells (5 μg) were separated electrophoretically, blotted to a nylon sheet, and hybridized with the SGLT1 DNA probe as described in Materials and Methods. pHook: COS-7 cells transfected with a plasmid containing no *sglt1* gene. Wild type: COS-7 cells transfected with the pHook plasmid including the *sglt1* gene.

showed no sign of staining. The staining disappeared when the peptide aa 606–aa 630 was present simultaneously with the antibody Pan-3. These results suggest that the SGLT1 with inserted 6×His-tags at different positions, when expressed in COS-7 cells, is inserted into the plasma membranes (*see also* Fig. 2*B*).

Transport Properties of His-tagged SGLT1 Proteins

To determine whether the inserted 6×His-tag changes the cotransport activity sodium-dependent α-methylglucoside uptake in COS-7 cells transfected with mutant 1 to mutant 11 was determined. There was virtually no endogenous Na^+/glucose cotransport activity in nontransfected COS-7 cells (Fig. 3). Figure 3 also shows that the cotransport activities were retained by 45 to 65% when the 6×His-tag was inserted at the N-terminal region (Mut 1), and C-terminal region (Mut 9 and Mut 11). Less than 20% of the residual activity was observed for Mutant 10 where 6×His-tag replaced the region close to the end of C-terminus; Na^+-dependent AMG-uptake by the other mutants is virtually absent.

Topology of the Epitopes of Antipeptide Antibodies

Three deduced peptides from distinct regions of SGLT1 were selected and the corresponding polyclonal antibodies were produced. The antibody Pan-3 raised against the amino acid residues 606 to 630 has been used for characterizing the SGLT1 in stably transfected Chinese hamster ovary (CHO) cells (Lin et al., 1998). Ali-1 was raised against residues close to the N-terminus (aa 3–aa 17), and Pan-2 against aa 339–aa 356. As demonstrated in Fig. 4 in intact COS-7 cells transfected with the wild-type SGLT1 only the antibodies Pan-2 and Pan-3 clearly labeled the SGLT1 protein on the cell surface. The reaction of Ali-1 was equivocal. The signals were intensified when the cells were permeabilized with acetone/methanol (Schröppel et al., 1998). When the respective peptide antigens were present in the incubation media, the immunological signals were suppressed. These results, summarized in Table 1, suggest that the immunological staining was antibody-specific, and that two epitopes, aa 339–aa 356 and aa 606–aa 630 are accessible to antibodies in the intact cell and thus located in extracellular loops.

Immunofluorescent Labeling of His-tagged SGLT1 Proteins in Transfected COS-7 Cells

To determine the location of inserted His-tags COS-7 cells were transfected with all mutants in parallel experiments. Figure 5 illustrates that a significant and consistent labeling on intact cells could be observed when mutants 1, 2, 9 and 10 were expressed. Mutants 3 and 11 showed a low and often erratic fluorescence. No detectable labeling was observed with the other mutants. However, when cells were permeabilized prior to addition of the primary antibody (*data not shown*), fluorescence could be observed in all mutants. As control, the nontransfected cells were also incubated with the anti-His antibody under identical conditions. Neither normal nor permeabilized cells showed any staining. Observations of immunological labeling of COS-7 cells from three independent experiments are summarized in Table 1.

Partial Digestion of Intact Cells

The results from labeling of COS-7 cells suggested that the three peptides (aa 606–aa 630, aa 584–aa 589, and aa 622–aa 627) are located most likely in an extracellular loop. Thus experiments were designed to see if this loop

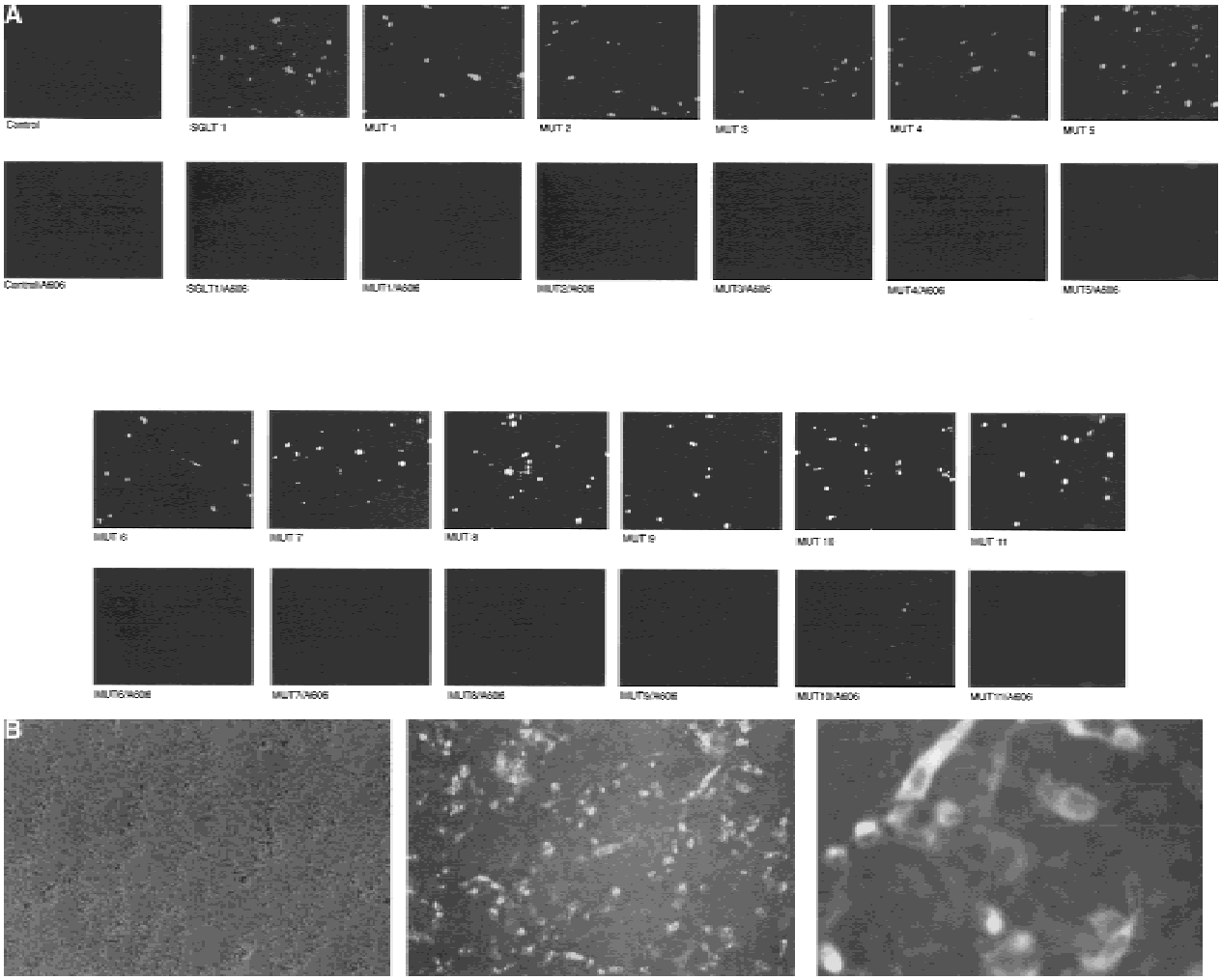

Fig. 2. (*A*) Detection of His-tagged SGLT1 proteins on the surface of transfected COS-7 cells using the peptide antibody Pan-3. Transfected COS-7 cells were grown in 12-well plastic plates and were incubated with Pan-3, alone or with the peptide antigen aa 606–aa 630 as described in Materials and Methods. As secondary antibody, anti-sheep IgG conjugated with FITC, was used for the subsequent staining. Fluorescence labeling of cells was photographed at 125-fold magnification. The first row shows cells incubated with Pan-3 alone; the second row cells incubated with Pan-3 and aa 606–aa 630. Control indicates transfection with vector alone. (*B*) An example of immunofluorescence labeling (Mutant 2) at higher magnification. Left: Cells viewed under light microscope. Middle: Fluorescent image at 125-fold magnification. Right: Fluorescent image at 500-fold magnification.

could be partially digested in intact cells. For digestion, we used a trypsin immobilized on beads to ensure that the enzyme acted only from the outside. The results obtained from limited digestion of intact CHO (nontransfected) and G6D3 (stably transfected) cells are depicted in Fig. 6. When G6D3 cells were treated with immobilized trypsin, a polypeptide with a size of ~14 kD was recovered from the supernatant after spinning down the cell mass, which showed a positive reaction with Pan-3. In contrast, no such band was observed from the digested control CHO cells.

These results are compatible with the assumption that trypsin splits the protein between aa 549 and aa 662, generating a fragment with a molecular weight observed in the experiments. This fragment includes the epitope aa 606–aa 630 that is recognized by the antibody Pan-3.

Discussion

Insertion of a small and hydrophilic epitope-tag and immunofluorescence have been widely used for a variety of cytoplasmic and membrane proteins to determine their cellular localization and sidedness of the loops that link transmembraneous regions (Yoon & Guidotti, 1994; Howard et al., 1995; Canfield et al., 1996). In this study we have used 6×His-tag as a reporter group to distinguish the freely exposed protein segments that form extracellular loops from segments that locate in transmembrane domains or face the cytoplasm. The coding re-

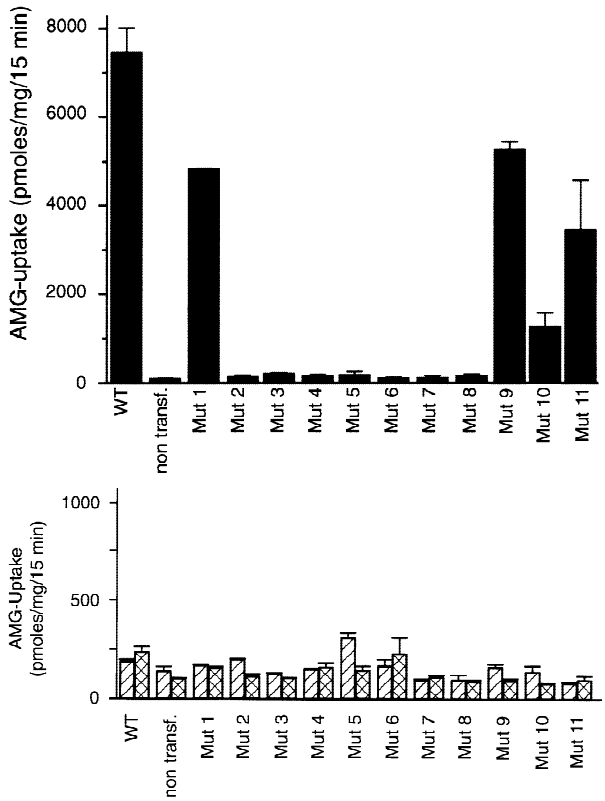

Fig. 3. Sodium-dependent α-methylglucoside uptake by COS-7 cells transfected with various His-tagged SGLT1 mutants. Nontransfected COS-7 cells, COS-7 cells transfected with the wild type, or mutant 1 to 11 were cultured in 12-well plastic plates for 48 hr. [^{14}C]AMG uptake was measured either in the presence of NaCl (■, upper panel) or NaCl + 10^{-4} M phlorizin (▨, lower panel), or choline chloride (▩, lower panel).

gions of 6× histidine were introduced into several different positions of the *sglt*1 gene either by insertion, extension or substitution. All mutants were successfully transcribed in COS-7 cells based on the Northern blot analysis. Transient transfection was selected because a large number of mutants can be handled in a short period of time. In addition, based on experiences of several authors, immunofluorescence labeling of transiently transfected cells is often much stronger than that of stably transfected cells. Although COS-7 cells were only transiently transfected by His-tagged SGLT1 mutants, the transfection efficiency of 40% was sufficient for the qualitative comparison of cotransport activities and fluorescence imaging.

The first question addressed was whether the SGLT1 mutants were inserted into the plasma membrane. In pilot experiments we demonstrated that the wild-type SGLT1 could be directly detected with the polyclonal antibody Pan-3 by immunofluorescence labeling in intact cells. This result suggests that Pan-3 recognizes the peptide sequence aa 606–aa 630 in the intact membrane as a part of an extracellular loop of the transporter. We therefore believe that investigating the membrane insertion of mutants with the same technique is feasible. Indeed, expression of all the His-tagged SGLT1 mutants could be detected with Pan-3 in intact cells. This result implies that all mutants are properly inserted into the plasma membrane with the epitope aa 606–aa 630 facing the extracellular side.

It has been reported that the transport functions of SGLT1 can be influenced by inserted tags, depending on the type of tag used and on the positions of insertion (Howard et al., 1995; Canfield et al., 1996). Variations of protein function of a gene induced by fusion with a reporter enzyme or insertion with an epitope tag have also been shown in other cases (Ball & Loftice, 1987; Allard & Bertrand, 1992; Yang et al., 1997). The transport activity by different mutants varied also in our experiments. Mutants 1, 9 and 11 retained almost the activity of the wild type. This finding might suggest that these segments or at least, the immediate vicinity of the His-tags are not directly involved in substrate binding or translocation. For the other mutants, though inserted into the plasma membrane, no transport activity, at least in the high-affinity range, could be detected. Possible explanations for this loss are: (i) direct involvement of the 6×histidine containing segments in substrate binding and/or translocation; (ii) distortion of the tertiary structure of SGLT1 by the insertion of or replacement with His-tags.

Such distortion might interfere with the tight three-dimensional interactions between transmembrane-domains and/or between homo-oligomers (Lin et al., 1984; Stevens et al., 1990) that are necessary to grant a specific binding and translocation of glucose molecules.

Despite their presence in the plasma membrane some of the His-tag mutants are not recognized in the intact cell by anti-His-tag antibodies. This might be due to the fact that the His-tags are not properly accessible or recognized in some segments of the transporter. The observation that all His-tag mutants show a positive immunoreactivity after cell permeabilization makes this possibility unlikely.

Table 2 compares the conclusions derived from our studies with models based either on exon correlation or glycosylation scanning. All three topology predictions agree for the stretches aa 306–aa 311 (intracellular), aa 336–aa 356 (extracellular) aa 402–aa 407 (intracellular), and aa 514-519 (intracellular). These areas of the molecule thus appear to be rather well defined in their topology. They seem also to be functionally important since labeling with the His-tag abolished the transport activity.

With regard to the N-terminus, our results agree with those obtained by glycosylation scanning. The functionally active, and therefore probably correctly folded and inserted Mut 1 reacts in intact cells with the anti-His antibody. The extracellular location of the N-terminus thus appears also relatively certain.

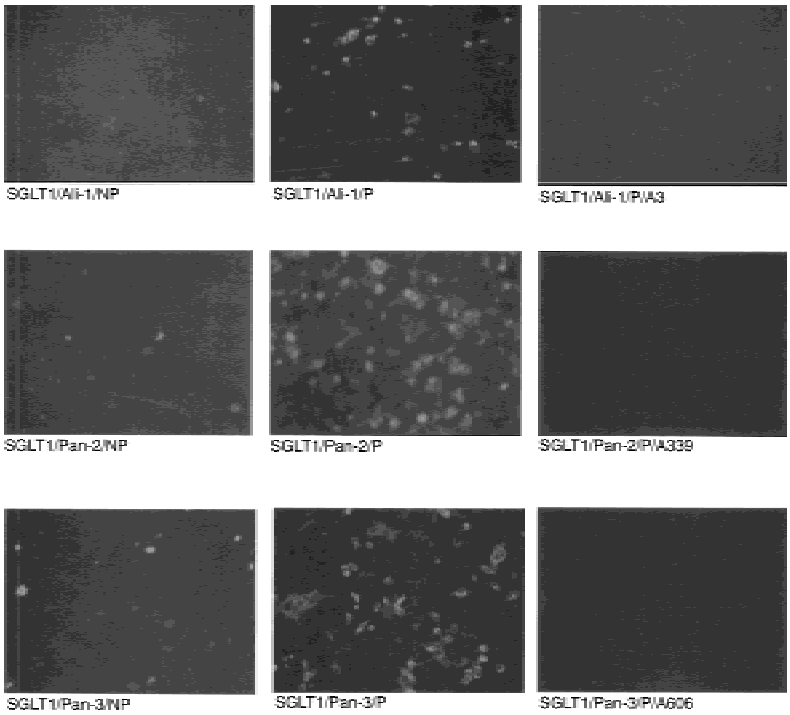

Fig. 4. Labeling of COS-7 cells transfected with the wild-type SGLT1 using antipeptide antibodies. COS-7 cells transfected with the wild-type pSGLT1 were labeled with the antipeptide antibodies, Ali-1, Pan-2 and Pan-3 against SGLT1. To produce immunological fluorescence staining, a second anti-IgG-FITC conjugate was used. 1st column: cells were directly incubated with antibodies. 2nd column: cells were permeabilized prior to the addition of the antibodies. 3rd column: cells were permeabilized but concomittantly incubated with the respective peptide antigen. Magnification 125-fold.

Table 1. Summary of immunofluorescence of SGLT1 or His-SGLT1 in transiently transfected COS-7 cells using either immunopurified polyclonal antipeptide antibodies or monoclonal anti-His-tag antibody

1. Immunofluorescent labeling of COS-7 cells transfected with SGLT1: polyclonal antipeptide antibodies

	Ali-1	Pan-2	Pan-3
Residues			
Recognized	3-17	339-356	606-630
Intact cell	?	+	+
Permeabilized cells	+	+	+

2. Immunofluorescent labeling with monoclonal antibody against 6×His-tag

Mutant	Modification in positions	Type	Immunofluorescent labeling intact cell	Immunofluorescent labeling perm. cell	Orientation of loops*
Mut 1	5	insertion	+	+	Extra
Mut 2	54-59	substitution	+	+	Extra
Mut 3	90-95	substitution	?	+	?
Mut 4	248-253	substitution	−	+	?
Mut 5	306-311	substitution	?	+	?
Mut 6	358-363	substitution	?	+	?
Mut 7	402-407	substitution	−	+	Intra/TMD
Mut 8	514-519	substitution	−	+	Intra/TMD
Mut 9	584-589	substitution	+	+	Extra
Mut 10	622-627	substitution	+	+	Extra
Mut 11	662	extension	−	+	Intra/TMD

* Extra: assigned as a potential extracellular position; Intra/TMD: assigned as a potential intracellular position or a transmembrane domain.

Major emphasis was placed in our studies on the question of how the large hydrophilic loop close to the C-terminus is arranged, since experiments by us and other authors imply a decisive role of this loop in determining sugar binding and translocation (Panayotova-Heiermann et al., 1996; Wielert-Badt, 1998). Mut 9 and Mut 10 clearly expose their His-tag to the cell exterior and Pan 3, an epitope-specific antibody, reacts with the

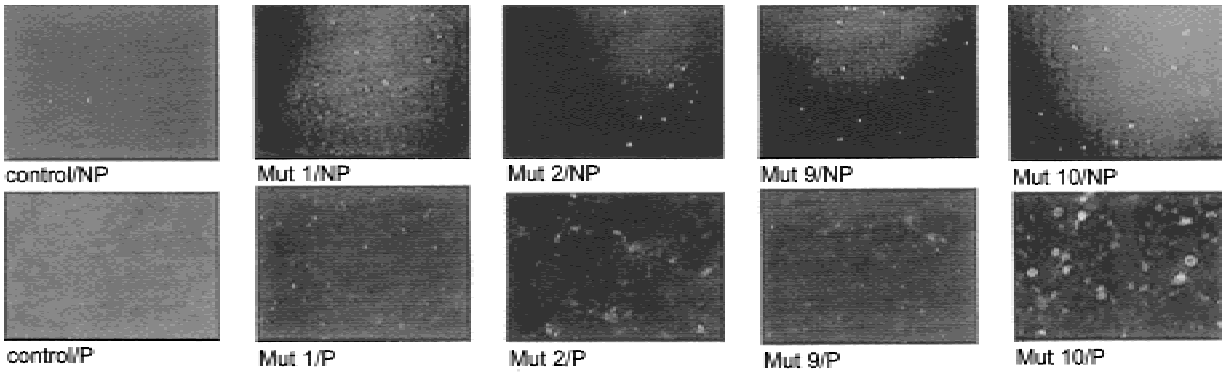

Fig. 5. Labeling of COS-7 cells transfected with various His-tagged SGLT1 mutants using the anti-His monoclonal antibody. COS-7 cells transfected with wild-type (SGLT1) or with various mutants (Mut 1 to Mut 11) were incubated with monoclonal anti-His-tag antibody (*see* Materials and Methods) 1st row; intact cells, 2nd row: permeabilized cells. Magnification 125-fold. Control represents nontransfected COS-7 cells. *Note:* Mut 1–Mut 6 and Mut 7–Mut 11 were two separate cell experiments due to technical considerations.

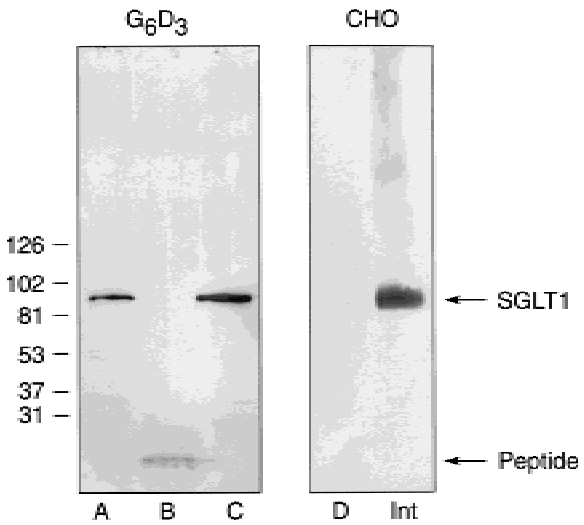

Fig. 6. Proteolytic cleavage of extracellular polypeptide loops. (*A*) Stably transfected G6D3 cells, no digestion. (*B*) Supernatant of stably transfected G6D3 cells after trypsin beads digestion. (*C*) Pellet of stably transfected G6D3 cells after trypsin beads digestion. (*D*) Supernatant of nontransfected CHO cells after trypsin beads digestions. Int: Antibody reaction with immunopurified SGLT1 as reference.

surface in intact cells. These two independent observations led us to assume an extracellular location. This conclusion agrees with the prediction from the exon correlation method but not with the glycosylation scanning method.

The discrepancies compared to previous models raise the question to what extent the insertion of tags can change the folding of the protein in the membrane due to alterations in polarity and hydrophilicity. Such changes cannot be excluded but are probably minor since both Mut 9 and Mut 10 are functionally active.

To circumvent the problem with insertion of His-tags we tried to partly digest stably transfected CHO cells using immobilized trypsin. We then tested the immunological reactivity of fragments of SGLT1 after the digestion. In our pretest and in Western blot analysis (Lin et al., 1998), Pan-3 has been clearly shown to interact with the oligopeptide that includes the residues from aa 606 to aa 630. Pan-3 recognizes a polypeptide band around 15 kD when stably transfected CHO cells are partly digested with immobilized trypsin. Since trypsin is immobilized on the sepharose gel, it does not cleave peptide fragments embedded in the membrane or the loops that are facing the intracellular space in right-side out oriented brush border membrane vesicles (Haase et al., 1978) or cells. Trypsin preferentially cleaves polypeptides at positions of lysine (K) or arginine (R). It is possible to predict the sidedness of the last polar loop (aa 542–aa 639) by analyzing the size of cleaved peptide fragments which contain the epitope for Pan-3 (aa 606-aa 630). By disregarding lysine and arginine residues that are present in transmembrane domains of SGLT1 the fragments that could have positive immunological reactions with Pan-3 can be predicted, these are listed in Table 3. The largest fragment should have a molecular weight of approximately 13 kD, when cleavage occurs in the last polar loop. Results of Western blot analysis of the product released by trypsin, as depicted in Fig. 6, are consistent with the notion obtained from the experiments with inserted His-tags and clearly suggest that the last loop is, indeed, facing the extracellular space. With regard to the residual transport molecule further studies are required to define the exact topology, thus we opted in this paper not to show a model of the whole protein.

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Table 2. Comparison of membrane topology of SGLT1 using different prediction methods

Residues	His-tag or wild type	Exon correl. (Turk et al., 1994)	Glycosylation scan (Turk et al., 1996)
Mut 1–5	Extracellular	Intracellular	Extracellular
Ali 1–3-17*	?	Intracellular	Extracellular
Mut 2–54-59	Extracellular	Extracellular	Intracellular
Mut 3–90-95	?	Intracellular	Extracellular
Mut 4–248-253	?	Extracellular	Extracellular
Mut 5–306-311	Intracellular or TMD	Intracellular	Intracellular
Pan-2–336-356*	Extracellular	Extracellular	Extracellular
Mut 6–358-363	?	Extracellular	Extracellular
Mut 7–402-407	Intracellular or TMD	Intracellular	Intracellular
Mut 8–514-519	Intracellular or TMD	Intracellular	Intracellular
Mut 9–584-589	Extracellular	Extracellular	Intracellular
Pan-3–606-630*	Extracellular	Extracellular	Intracellular
Mut 10–622-627	Extracellular	Extracellular	Intracellular
Mut 11–662 +	Intracellular or TMD	Intracellular	Extracellular

* Peptide-specific antibodies

Table 3. Hypothetical size of fragments that contain the Pan-3 epitope after partial digestion with trypsin beads

Within the large polar loop that contains the Pan-3 epitope

Fragment position	Size (kD)
K549–A 664	13106
K549–K628	9299
R601–K628	3244
R601–A 664	7052

Other possible positions for cleavage but with transmembrane domains: R499, K478, R479

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