More than apical: distribution of SGLT1 in Caco-2 cells

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Kipp, Helmut, Saeed Khoursandi, Daniel Scharlau, and Rolf K. H. Kinne. More than apical: distribution of SGLT1 in Caco-2 cells. Am J Physiol Cell Physiol 285: C737–C749, 2003. First published May 28, 2003; 10.1152/ajpcell.00041.2003.—We investigated the distribution of the endogenous sodium-D-glucose cotransporter (SGLT1) in polarized Caco-2 cells, a model for enterocytes. A cellular organelle fraction was separated by free-flow electrophoresis and subjected to the analysis of endogenous and exogenous marker enzymes for various membrane vesicle components. Furthermore, the presence of SGLT1 was tested by an ELISA assay employing newly developed epitope-specific antibodies. Thereby it was found that the major amount of SGLT1 resided in intracellular compartments and only a minor amount in apical plasma membranes. The distribution ratio between intracellular SGLT1 and apical membrane-associated SGLT1 was ~2:1. Further immunocytochemical investigation of SGLT1 distribution in fixed Caco-2 cells by epifluorescence and confocal microscopy revealed that the intracellular compartments containing SGLT1 were associated with microtubules. Elimination of SGLT1 synthesis by incubation of cells with cycloheximide did not significantly reduce the size of the intracellular SGLT1 pool. Furthermore, the half-life of SGLT1 in Caco-2 cells was determined to be 2.5 days by metabolic labeling followed by immunoprecipitation. Our data suggest that most of the intracellular SGLT1 are not transporters en route from biosynthesis to their cellular destination but represent an intracellular reserve pool. We therefore propose that intracellular compartments containing SGLT1 are involved in the regulation of SGLT1 abundance at the apical cell surface.

Sodium gradient-driven accumulation of solutes into epithelial cells lining the renal proximal tubule and the small intestine is a first critical physiological step by which essential nutrients like D-glucose are reabsorbed from the luminal content of the digestive tract. Absorption of D-glucose in both renal proximal tubule and small intestine epithelial cells is performed by secondary active sodium-D-glucose cotransporters (i.e., SGLT1), which accumulate D-glucose in these epithelial cells against a chemical gradient powered by simultaneous “downhill” transport of sodium ions. According to studies with isolated plasma membrane vesicles (21), functional sodium-D-glucose cotransporters are present in the luminal brush border membrane of the epithelial cell (6) but not in the basolateral membrane. Sodium-D-glucose cotransporters are conserved from lower vertebrates (23) to man (46).

The autosomal recessive disorder glucose galactose malabsorption syndrome is caused by missense mutations in the SGLT1 gene. Heterologous expression of these mutant SGLT1 genes in Xenopus laevis oocytes leads to complete loss of sodium-dependent D-glucose uptake into oocytes compared with oocytes expressing wild-type SGLT1, although in both cases the overall protein levels were comparable. Transport deficiency of the mutants was due to a trafficking defect of the SGLT1 protein, which did not reach the plasma membrane but was accumulated in intracellular compartments (30).

Trafficking of SGLT1 is not only an important issue for the explanation of SGLT1 malfunctions in disease states, but intracellular trafficking also seems to play a critical role in the regulation of sodium-D-glucose co-transport mediated by wild-type SGLT1. Sodium-dependent D-glucose uptake into Xenopus laevis oocytes expressing wild-type SGLT1 was increased by activation of protein kinase A and decreased by the activation of protein kinase C. These reversible changes in the maximum transport rate occurred within minutes and were accompanied by proportional changes in the number of SGLT1 transporters in the plasma membrane (18). These data suggest a mechanism by which sodium-dependent D-glucose transport can be rapidly upregulated by the recruitment of SGLT1 to the plasma membrane and can be downregulated by endocytosis of SGLT1. Such regulatory mechanisms would necessarily require intracellular pools, stores of SGLT1.

There are conflicting data in the literature with regard to the cellular localization of SGLT1 in epithelial cells. An exclusively apical localization is reported in SGLT1-transfected Madin-Darby canine kidney (MDCK) cells (40, 41) and in thin sections of rat small intestine (48). In LLC-PK1 cells, a cell line from the pig kidney proximal tubule, endogenous SGLT1 was not detected in the brush border membrane but “near” the apical plasma membrane (20). In excised loops from rabbit jejunum, SGLT1 was located in the brush border membrane and was also proposed in intracellular sites.

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and RNS71

Generation of Anti-SGLT1 Antibodies QIS30, LYT30, and RNS71

EXPERIMENTAL PROCEDURES

Materials and Methods

All chemicals were of the highest purity available and were purchased from Sigma (Deisenhofen, Germany). Other reagents and antibodies were also from Sigma unless other sources are indicated. Proteins were separated on 10% gels by SDS-PAGE according to Laemmli (25). For Western blotting, polypeptides were electrotransferred (42) on polyvinylidene difluoride membrane (Schleicher & Schuell). Primary antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies followed by detection with an enhanced chemiluminescence system from NEN Life Science Products. Sodium-dependent uptake of α-[U-14C]methyl-D-glucose (300 mCi/mmol; NEN) into Caco-2 cells was carried out as described earlier (5, 31).

Generation of Anti-SGLT1 Antibodies QIS30, LYT30, and RNS71

Glutathione S-transferase (GST) fusion proteins containing fragments of rabbit SGLT1 (QIS30: amino acids 243–272, starting with Q, I, S; LYT30: amino acids 338–367, starting with L, Y, T; and RNS71: amino acids 564–634, starting with R, N, S) were used as antigens to raise antibodies. The corresponding coding DNA fragments were amplified from full-length cDNA (32) by polymerase chain reaction (PCR) using the following oligonucleotides: QIS30, 5'-ATT GGA TCC TGG ACA CCT AGT CCT AGT G-3' (containing a BamHI site) and 5'-ATT GTC GAC TAC CCA GTG ATG GCA TC-3' (containing a SalI site); LYT30, 5'-ATT GGA TCC TGC TGT ACA CAG ACA AA-3' (containing a BamHI site) and 5'-ATT GTC GAC TAT GGG AAG GCA ATG TT-3' (containing a SalI site); and RNS71, 5'-ATT GGA TCC GTC GTA ATA GCA AAG AG-3' (containing a BamHI site) and 5'-ATT GTC GAC TAG GAG TGT TCT GTG AG-3' (containing a SalI site). The PCR products were digested with BamHI and SalI and ligated to the BamHI/SalI sites of pGEX-5X-3 vector (Amersham Pharmacia Biotechnology). In-frame cloning was confirmed by DNA sequencing. Expression of the GST fusion proteins in Escherichia coli BL21 cells and purification using glutathione-Sepharose beads were performed according to protocols provided by Amersham Pharmacia Biotechnology. A commercial service was employed to raise polyclonal antibodies in rabbits (Biotrend, Köln, Germany) with the use of a standard immunization and bleeding schedule protocol. Antibodies directed against GST were removed from the antisera by a passage through a Sepharose-GST column. Peptide-specific antibodies were then purified using Sepharose-fusion protein columns.

Polymerase Chain Reaction

mRNA was isolated from Caco-2 cells by using the RNasea kit from Qiagen (Hilden, Germany). cDNA was reverse transcribed from mRNA with oligo(dT)15 primer (Promega) and Superscript II reverse transcriptase (Invitrogen). Reversed transcribed cDNA was used as template to identify message for SGLT1 and the two SGLT2 sequences (see Table 1) in Caco-2 cells by PCR. The following sequence-specific primers were used: SGLT1 (sense), 5'-GCC CTG GTT TTG GTG GTT G-3'; SGLT1 (antisense), 5'-CGA GAT CTT GGT GAA AAT GTA GAG C-3'; SGLT2a (sense), 5'-CAG CAT AGC TGA GAC CCC AGA G-3'; SGLT2a (antisense), 5'-TAT AGT ACC TCG GTT GGT CTT CAG C-3'; SGLT2b (sense), GAG CAC ACA GAG GCA GGC TC-3'; and SGLT2b (antisense), GCC TCT GTT GGT TCT GCA CAT-3'. Genomic DNA was employed as template for the positive control, which was prepared from Caco-2 cells with the QiAamp DNA kit from Qiagen.

Cell Culture

Caco-2 cells were acquired from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were grown in 75-cm² tubes (Falcon, Heidelberg, Germany) at 37°C and 5% CO₂ in minimal essential medium (Life Technologies) that was supplemented with

Table 1. **BLAST search for human proteins in the NCBI protein data base with possible epitopes for anti-SGLT1 antibodies QIS30, LYT30, and RNS71**

<table>
<thead>
<tr>
<th>Protein</th>
<th>QIS30 Match</th>
<th>LYT30 Match</th>
<th>RNS71 Match</th>
<th>NCBI Accession No.</th>
<th>Protein Identity</th>
<th>Expression in Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NP_000334</td>
<td>SGLT1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NP_055042</td>
<td>SGLT2a</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NP_003092</td>
<td>SGLT2b</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>XP_064487</td>
<td>Similar to SGLT1</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>CAC00574</td>
<td>Similar to SGLT1</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>XP_166767</td>
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<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>NP_443176</td>
<td>RKST1</td>
<td>nd</td>
</tr>
</tbody>
</table>

NCBI, National Center for Biotechnology Information; SGLT1, sodium-d-glucose cotransporter 1; nd, not determined.
10% fetal calf serum, 1% nonessential amino acids, and 1% glutamine. Every 2 days, the culture medium was renewed. For the preparation of the cellular organelle fraction (COF) and immunoprecipitation, Caco-2 cells (passages 15–25) were seeded (2 × 10^5 cells/dish) on 5-cm petriPerm hydrophilic dishes (Vivascience, Hannover, Germany) and grown until a confluent, polarized monolayer was formed (>14 days). Alternatively, Caco-2 cells were grown (1 × 10^6 cells/filter) on 0.9-cm² polyethylene terephthalate (PET) filter inserts (0.4-μm average pore size; Becton Dickinson, Heidelberg, Germany).

Endosome Labeling and Preparation of Cellular Organelle Fraction

Endosomes of Caco-2 cells were labeled according to a pulse-chase protocol, which was used in previous studies to label early and late endosomes of HeLa cells (3). Late endosomes were labeled by incubation of Caco-2 cells with medium containing 20 mg/ml FITC-dextran (70 kDa, dialyzed against PBS) for 3 min at 37°C, followed by a chase of 12 min in marker-free medium. HRP was then added to a final concentration of 10 mg/ml, and cells were incubated for 3 min at 37°C to label early endosomes. Typically, three petriPerm dishes with Caco-2 cells pretreated with endosome markers were used for the preparation of a COF. Cells were washed four times with ice-cold FFE buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, and 0.25 M sucrose, pH 7.4 adjusted with NaOH), removed with a rubber policeman, and suspended in 12 ml of FFE buffer. The suspension was equilibrated in a high-pressure chamber (Parr Instrument model 4639) with 900 lb./in.² nitrogen at 4°C for 15 min. The cell suspension was rapidly expanded against atmospheric pressure and centrifuged at 850 g for 10 min to remove cell debris and nuclei. The supernatant was layered on top of a 4-ml sucrose cushion (40% sucrose in FFE buffer) and centrifuged at 100,000 g for 45 min at 4°C in a swinging bucket rotor. The interphase between the 40% sucrose and the 0.25 M sucrose layers was collected (COF). Protein in an aliquot of the COF was precipitated with trichloroacetic acid, and the precipitate was washed twice with PBS and metabolically labeled with 0.1 mCi/ml Tran35S-Label (1175 Ci/mmol; ICN Biomedicals) in methionine- and cysteine-free minimal essential medium (Life Technologies) supplemented with 10% dialyzed fetal calf serum for 1 h at 37°C. The cells were washed twice with PBS and maintained at 37°C with regular growth medium for the chase times indicated. Immunoprecipitation of SGLT1, using 10 μg of anti-QIS30 antibody, was carried out as described elsewhere (22).

Conventional and Confocal Microscopy

Caco-2 cells grown on 5-cm petriPerm dishes were washed twice with PBS and metabolically labeled with 0.1 μCi/ml Tran35S-Label (1175 Ci/mmol; ICN Biomedicals) in methionine- and cysteine-free minimal essential medium (Life Technologies) supplemented with 10% dialyzed fetal calf serum for 1 h at 37°C. The cells were washed twice with PBS and maintained at 37°C with regular growth medium for the chase times indicated. Immunoprecipitation of SGLT1, using 10 μg of anti-QIS30 antibody, was carried out as described elsewhere (22).

ELISA Assay

A 40-μl aliquot of each fraction obtained from FFE was coated to individual wells of a 96-well plate by mixing with 40-μl coating buffer (NaHCO3/Na2CO3 buffer, pH 10.3) and incubation overnight at 4°C. After blocking with 2% bovine serum albumin in TBS (170 mM NaCl, 0.05% Tween 20, 20 mM Tris, pH 7.4 adjusted with HCl) for 60 min at room temperature, the wells were washed four times with TBS and then incubated with anti-QIS30 antibody (1:2,000) in 0.2% bovine serum albumin in TBS for 90 min at room temperature. The wells were washed four times with TBS and were then incubated with goat anti-rabbit IgG linked to biotin (1:4,000) in 0.2% bovine serum albumin in TBS for 60 min at room temperature. The wells were washed four times with TBS and were then incubated with avidin linked to alkaline phosphatase (1:10,000) for 30 min at room temperature. The wells were washed four times with TBS, and 200 μl of alkaline phosphatase assay mixture (100 mM glycine, 1 mM MgCl2, 0.1 mM ZnSO4, 5 mM p-nitrophenolphosphate, pH 10.5 adjusted with NaOH) were added per well. The plate was incubated for 1 h at 37°C, and the absorption at 405 nm was read.

Fig. 1. Specificity of anti-sodium/glucose cotransporter (SGLT1) antibodies QIS30, LYT30, and RNS71. Caco-2 cell homogenate (20 μg/lane) was separated by SDS-PAGE on 10% gels and blotted onto polyvinylidene difluoride membrane. The membranes were then probed in Western blots with peptide-specific QIS30, LYT30, and RNS71 antibodies (Anti) and preimmune sera (Pre).
After being washed with PBS, DNA was stained by incubation with 4',6-diamidino-2-phenylindole (DAPI; 25 ng/ml in PBS) for 10 min. Labeling of the plasma membrane was achieved by incubation of the cells with 2 mg/ml Sulfo-NHS-Biotin (Pierce) in PBS, pH 8.0, for 10 min at 4°C. After being washed with the same PBS, the cells were incubated with Cy2-labeled Streptavidin (1:500; Amersham Life Science) for 60 min to detect the biotin bound to integral membrane proteins. The specimens were mounted with Mowiol (CalBiochem) and analyzed using conventional and confocal microscopy. For conventional fluorescence microscopy, cells were viewed under a Zeiss Axiophot microscope equipped with a

Fig. 2. Expression of SGLT1 and SGLT2 in Caco-2 cells. Polymerase chain reaction (PCR) using specific primers for SGLT1 and the 2 SGLT2 sequences present in the NCBI database (SGLT2a and SGLT2b, see Table 1). The PCR reactions were carried out with no template (negative control) or by using genomic DNA from Caco-2 cells (positive control) or cDNA obtained from reverse transcribed Caco-2 cell mRNA as template. Asterisks indicate the expected sizes of the PCR products. The identity of PCR products was confirmed by subcloning and DNA sequencing.

Fig. 3. Distribution of SGLT1 in Caco-2 cells: free-flow electrophoresis (FFE). Early endosomal (horseradish peroxidase) and late endosomal (FITC-dextran) intracellular compartments of Caco-2 cells were labeled according to a pulse-chase protocol (see EXPERIMENTAL PROCEDURES). A cellular organelle fraction (COF) was then prepared from Caco-2 cells and subjected to separation by FFE without (A–C) or with prior trypsin treatment of COF (D–F). The fractions obtained from FFE were assayed for (A and D) protein, alkaline phosphatase (AP; apical plasma membrane), and Na/K-ATPase (Na/K; basolateral plasma membrane) (A and D), for HRP (early endosomes) and FITC-dextran fluorescence (late endosomes) (B and E), and for SGLT1, using an ELISA assay (C and F). Subcellular domains (apical and basolateral plasma membranes and intracellular compartments) were assigned to the FFE fractions from separations carried out with trypsinized COF samples (D–F). SGLT1 was located in the apical plasma membrane and in FFE fractions, which comigrated with early endosomes (F). The intracellular-apical SGLT1 distribution ratio was estimated by measurement of the areas below the respective curves from nontrypsinized samples (C); the intracellular-apical distribution ratio of SGLT1 was 2:1. All data are expressed as percentages of the total amount recovered after separation by FFE. Results are shown that are representative of results observed in at least 4 independent experiments.
Zeiss ×40/0.9 objective lens, a Zeiss ×63/1.25 oil-immersion objective lens, and a Zeiss AxioCam charge-coupled device camera. Images from triple or double staining were obtained at the same focal planes. Image acquisition and analysis were performed using Axiovision 2.05 and Image Pro Plus 4.5 software, respectively. For confocal microscopy, a Noran OZ laser scanning confocal imaging system connected to a Nikon Eclipse TE200 inverted microscope was used. Magnification was set to ×400 under a Nikon ×40/1.3 oil-immersion objective lens. The microscope was connected to an argon laser (Omnichrome series 43; 457/488/514 nm), a diode pumped solid state Nd:YAG laser (Coherent DPSS 532; 532 nm), and a Coherent Enterprise UV laser (352/361 nm). The system was controlled by a Silicon Graphics workstation. Images of Cy2, Cy3, and DAPI were captured from the same optical section using the Intervision acquisition software. The captured images were then pseudocolored: green for Cy2, red for Cy3, and blue for DAPI. Regions of colocalization of Cy2 and Cy3 appear in yellow.

RESULTS

Generation and Specificity of Anti-SGLT1 Antibodies

Three peptides (QIS30, LYT30, and RNS71) representing extramembranous loops (34) of the rabbit SGLT1 (32) amino acid sequence were expressed as GST fusion proteins in E. coli and subsequently used to immunize rabbits for the production of antisera. Peptide-specific antibodies were purified from the respective antisera by affinity chromatography. Because of the high homology between rabbit and human SGLT1, the peptide-specific antibodies QIS30, LYT30, and RNS71 also recognized the human isoform of SGLT1 present in Caco-2 cells. In Western blots, all three peptide-specific antibodies reacted with a single antigen present in Caco-2 cell homogenates at 73 kDa, the expected size of mature SGLT1. IgG from the respective preimmune sera did not recognize antigens in Caco-2 cell homogenates at all (Fig. 1). Use of all anti-SGLT1 antibodies (QIS30, LYT30, RNS71) led to essentially the same results in the experiments presented. However, most of the experiments were carried out using QIS30 peptide-specific antibody, simply because affinity chromatography of QIS30 antiserum gave the highest yield in peptide-specific antibodies and anti-QIS30 antibody was, therefore, available in the largest amount.

The specificity of the anti-peptide antibodies was further analyzed by a BLAST search for human amino acid sequences similar to the QIS30, LYT30 and RNS71 peptides in the National Center for Biotechnology Information (NCBI) protein database. Six proteins, in addition to SGLT1, were identified that may be recognized by the antibodies (Table 1). Candidate proteins 4–7 share only 5–10 consecutive matching amino acids with one of the SGLT1 peptides, which is probably a minimum requirement for an appropriate antibody epitope. Furthermore, the sequence of each of the candidate proteins 4–7 matched with only one of the three antibody peptides QIS30, LYT30, and RNS71, respectively. Because all three anti-peptide antibodies recognized only a single antigen at 73 kDa in Western blots, it is unlikely that one of the candidate proteins 4–7 is recognized by one of the antibodies. Likely proteins to be recognized by the antibodies are the two low-affinity sodium-D-glucose cotransporters (SGLT2) in the database (proteins 2 and 3); therefore, we tested for the expression of SGLT2 in Caco-2 cells by RT-PCR. As shown in Fig. 2, mRNA for neither of the two SGLT2 transporters was detected in Caco-2 cells; thus in Caco-2 cells our antibodies are specific for SGLT1.
SGLT1 Distribution in Caco-2 Cells

Free-flow electrophoresis. SGLT1 distribution was analyzed by separation of a COF prepared from Caco-2 cells. Cells were disrupted by nitrogen decompression, by equilibration with nitrogen at 950 lb./in.² and rapid expansion against atmospheric pressure (39). The cellular organelles and plasma membranes were then collected by centrifugation on a sucrose cushion. The Caco-2 cell preparation obtained by this procedure was depleted of nuclei, mitochondria, and cytosol and was termed the COF. The COF was resolved in an electric field by FFE. This method utilizes differences in the surface charges of organelles and plasma membrane vesicles for the separation of subcellular material and was successfully applied to the separation of apical and basolateral plasma membranes from proximal tubule cells of rat kidney (16) and to the preparation of lysosomes (15) and functional endosomes from cultured cells (29, 36) and animal tissue (13).

After separation of the COF by FFE, the obtained fractions were analyzed for the endogenous marker enzymes activities of alkaline phosphatase, which is only located at the apical plasma membrane of Caco-2 cells (17), and Na/K-ATPase, which is only located at the basolateral plasma membrane of Caco-2 cells (43). To identify endosomal compartments, the exogenous markers HRP (early endosomes) and FITC-dextran (late endosomes) were determined, which had been applied to the cells according to a pulse-chase protocol (see EXPERIMENTAL PROCEDURES) before subcellular fractionation. It was observed in earlier studies that separation of endosomal compartments from the bulk of plasma membranes required gentle trypsin digestion of the cell preparation subjected to FFE, probably to remove cytoskeletal interconnections between cellular organelles (29, 37). Therefore, we performed FFE of the COF with and without prior trypsin treatment; typical results are summarized in Fig. 3. Apical and basolateral plasma membranes were readily separated by FFE without trypsin treatment of the sample, as indicated by the distribution of alkaline phosphatase and Na/K-ATPase (Fig. 3A), whereas basolateral membranes (Na/K-ATPase), early endosomes (HRP), and late endosomes (FITC-dextran) were not resolved under these conditions (Fig. 3, A and B). Separation of the endosomal compartments and the basolateral membrane was achieved by gentle trypsin treatment (0.5% trypsin/mg protein for 5 min) of the COF before FFE. Trypsin-digested samples were resolved into clearly distinguishable peaks of alkaline phosphatase activity (apical membrane), Na/K-ATPase activity (basolateral membrane), HRP activity (early endosomes), and FITC-dextran fluorescence (late endosomes) (Fig. 3, D and E). With the distribution of marker enzymes among the fractions separated by FFE having been established, SGLT1 was detected by an ELISA assay using the peptide-specific antibody QIS30. Only a minor fraction of the total amount of SGLT1 present in Caco-2 cells was located in the apical membrane; most of the SGLT1 resided in intracellular compartments, which copurified in FFE with early endosomes (Fig. 3, C and F). The distribution ratio between intracellular compartments and apical plasma membrane was estimated by comparison of the areas below the respective peaks and was ~2:1. Two peaks for SGLT1-distribution were observed in trypsin-treated and nontreated samples, indicating that the second peak is not an artifact caused by trypsin treatment.

Epifluorescence and confocal microscopy. The subcellular fractionation studies using FFE indicated that a substantial amount of SGLT1 was located in intracellular compartments. We next attempted to visualize and further characterize this intracellular population in whole Caco-2 cells by immunofluorescence micros-
copy using epitope-specific antibodies against SGLT1. In nonpermeabilized Caco-2 cells, SGLT1 was detected as sparse punctuated structures, which represent transporters (or clusters of transporters) at the apical cell surface (Fig. 4A). By contrast, in permeabilized cells, in which the antibody also accessed intracellular compartments containing SGLT1, significantly more immunostaining was visible (Fig. 4B). These results are in good accordance with the results from the biochemical cell fractionation studies, which suggested the same SGLT1 distribution: a small amount in the apical plasma membrane and a larger pool of transporters inside the cell.

In fixed and permeabilized Caco-2 cells, SGLT1 was detected throughout the cell with prominent perinuclear staining (Fig. 5A). However, SGLT1 seemed to be not randomly distributed, which was particularly obvious in regions where Caco-2 cells were torn apart due to the preparation process (Fig. 5B and C). In these regions, SGLT1-containing intracellular compartments had an appearance of small vesicles, which were assembled like “pearls on an invisible string.” This observation led to the hypothesis that SGLT1-containing vesicles were associated with cytoskeletal elements, probably microtubules.

Fig. 6. Intracellular SGLT1-containing compartments are associated with microtubules. Caco-2 cells were grown to confluence on poly-l-lysine-coated coverslips, fixed with paraformaldehyde, and perforated with Triton X-100. The cells were then incubated with peptide-specific anti-SGLT1 antibody QIS30 and a mouse monoclonal antibody directed against β-tubulin, followed by incubation with anti-rabbit IgG linked to Cy3 (red) fluorophore and anti-mouse IgG linked to Cy2 (green) fluorophore. Incubation of the specimens with only the secondary antibodies revealed no observable fluorescence (data not shown). A–C: examples of 3 different preparations. Nuclei are shown in blue (DAPI staining). The specimens were observed with epifluorescence microscopy (A–C) and confocal microscopy (D–F). D: β-tubulin; E: SGLT1; F: merged picture. Scale bars, 10 μm.
To test this hypothesis, we double-stained Caco-2 cells for SGLT1 (red) and β-tubulin (microtubules, green). SGLT1 was located in structures that were lined up along microtubules (Fig. 6, A–C). Again, association of SGLT1-containing compartments was best investigated in cells that were torn apart during the preparation process and is most impressively demonstrated in Fig. 6B. Interestingly, on microtubules that were mechanically stretched (Fig. 6C, arrow), associated SGLT1-containing compartments were also stretched simultaneously and became tubular-shaped themselves, indicating a very tight association of SGLT1 compartments with microtubules. Colocalization of SGLT1 compartments and microtubules was further confirmed by analysis of the same specimens with a confocal laser scanning microscope (Fig. 6, D–F). When images of labeled β-tubulin (green) and SGLT1 (red) were merged, the vesicles containing SGLT1 appeared yellow, reflecting the additive effect of superimposing red and green pixels (Fig. 6F).

**Polarity Status of Caco-2 Cell Cultures**

Intracellular compartments containing membrane transporters may be due to epithelial cells not yet being polarized. Thus, membrane proteins associated with the apical or basolateral plasma membrane of polarized MDCK cells were found in intracellular compartments in nonpolarized MDCK cells (26). Therefore, we investigated the development of polarity of Caco-2 cells under our particular growth conditions (seeding cell density, growth substratum). Polarity status of Caco-2 cells was estimated by measuring the sodium-dependent α-methyl-d-glucose uptake into Caco-2 cells, because sodium-dependent d-glucose uptake is a property typical of only polarized Caco-2 cells (5). Caco-2 cell cultures grown on petriPerm support reach polarity 8–11 days after seeding (Fig. 7). For FFE studies, Caco-2 cells that were grown for >14 days were used, indicating that these studies were all performed on polarized Caco-2 cells. The fact that these cells reach polarity earlier than those grown on filters is probably due to the 200-fold higher amount of cells used for seeding.

Caco-2 cells grown on PET filter inserts reach polarity 11–12 days after seeding (Fig. 7). The distribution of SGLT1 in polarized Caco-2 cells, which were grown for >14 days on PET filter inserts, was analyzed by confocal laser scanning microscopy. Integral proteins of the apical membrane were labeled with Sulfo-NHS-Biotin and Streptavidin-Cy2 (green). A series of images from confocal planes (0.1-μm thickness) perpendicular to the z-axis starting from the apical membrane (0 μm) below the nucleus (6.5 μm) are shown in Fig. 8. SGLT1 (red) can be detected not only in the apical membrane (green) but also in images from intracellular confocal planes (4.0, 5.0, and 6.5 μm), where the staining of the cell surface is no longer visible. These data indicate that an intracellular pool of SGLT1 is a property of the polarized Caco-2 cell.

**Distribution of SGLT1 in Caco-2 Cells With Switched Off Protein Synthesis**

Our biochemical experiments and histological observations identified a large intracellular population of SGLT1 in Caco-2 cells that is dispersed in microtubule-associated vesicles. What is a large population of a plasma membrane transporter, typically fulfilling its physiological task in the apical plasma membrane, doing in the cell interior? Two scenarios are to be considered in this regard: 1) intracellular SGLT1-containing vesicles may be en route from biosynthesis to their cellular destination or 2) may represent an intracellular reserve pool, from which transporters can be recruited or stored to regulate apical abundance of SGLT1 by an endo/exocytosis mechanism. To discriminate between these two possibilities, we studied intracellular distribution and function of SGLT1 in Caco-2 cells in which protein biosynthesis was inhibited by cycloheximide.

De novo protein synthesis was efficiently blocked by incubation of Caco-2 cells with cycloheximide (10 μg/ml) for 60 min. Treatment with cycloheximide completely abolished metabolic labeling with [35S]methionine/cysteine of proteins in Caco-2 cell homogenates. Homogenates from [35S]-labeled control cells and from cells incubated with cycloheximide showed similar protein patterns (Fig. 9A). However, proteins from the homogenate of control cells were intensively labeled with [35S], whereas no radioactivity was detected in proteins from the homogenate of cycloheximide-treated cells (Fig. 9B). These data indicate that the conditions for cycloheximide treatment of Caco-2 cells were sufficient to completely block de novo protein synthesis.

Specificity of anti-QIS30 antibodies for the immunoprecipitation of SGLT1 was established by comparison of immunoprecipitations with anti-QIS30 antibodies and the respective preimmune IgG from [35S]methionine/cysteine-labeled Caco-2 cells. As shown in Fig.
$^{35}\text{S}$-labeled SGLT1 was readily precipitated with anti-QIS30 antibodies, whereas the preimmune serum failed to precipitate SGLT1. Evidence that biosynthesis of SGLT1 in particular was also inhibited by cycloheximide was established by immunoprecipitation. No radioactivity was associated with SGLT1 in immunoprecipitates after cycloheximide treatment, whereas significant amounts of $^{35}\text{S}$-labeled SGLT1 were detected in metabolically labeled control cells (Fig. 9D).

Cycloheximide inhibits protein biosynthesis by inhibiting peptidyltransferase in ribosomes of eucaryotic cells; however, in some instances cycloheximide can also cause superinduction of certain proteins (10, 28). In homogenates from Caco-2 cells, no significant change in the total amount of proteins (Fig. 9A) and in the amount of SGLT1 detected by Western blot (Fig. 9E) was observed after treatment of the cells with cycloheximide, indicating that superinduction of proteins by cycloheximide is not a concern in our present study regarding SGLT1 distribution in Caco-2 cells.

Western blot analysis of homogenates from cycloheximide-treated cells and control cells revealed no detectable change in the total amount of SGLT1 present in Caco-2 cells (Fig. 9E). Taking into account that our
conditions for cycloheximide treatment are sufficient to deplete proteins from the biosynthetic pathway, these data indicated that newly synthesized SGLT1 contributed only a very small extent, which remained below detection limits, to the total amount of SGLT1 present in Caco-2 cells. This scenario is in good accordance with our further investigations. Depletion of proteins from the biosynthetic pathway did not significantly alter SGLT1 distribution in Caco-2 cells as observed using immunofluorescence microscopy (data not shown), indicating that the intracellular compartments containing SGLT1 are not endoplasmic reticulum or Golgi. No significant differences between control cells and cycloheximide-treated cells were observed when SGLT1 distribution was examined by FFE (Fig. 10A). Furthermore, cycloheximide treatment for 2 h had no significant effect on sodium-dependent ß-methyl-D-glucose uptake into Caco-2 cells, a measure for transport active SGLT1 protein present in the apical surface of the cell (Fig. 10B).

**Half-Life of SGLT1 in Caco-2 Cells**

Our experiments regarding SGLT1 distribution in cycloheximide-treated Caco-2 cells suggest that newly synthesized SGLT1 did not significantly contribute to the total amount of SGLT1 present in Caco-2 cells. If this assumption is true, the overall half-life of SGLT1 in Caco-2 cells should be relatively “long” compared with the process of protein biosynthesis. Therefore, we measured the half-life of SGLT1 in Caco-2 cells. Caco-2 cells were pulse labeled with [35S]methionine/cysteine for 1 h and than chased for 1, 2, 3, 4, or 5 days, and the content of newly synthesized SGLT1 was determined by measuring the amount of radioactivity associated with immunoprecipitated SGLT1 (Fig. 11A). The half-life of SGLT1 was estimated from a semilogarithmic plot of [35S] intensity vs. labeling time (Fig. 11B). Assuming first-order decay, SGLT1 had an apparent half-life of 2.5 days in Caco-2 cells.

**DISCUSSION**

Although originally derived from a human colon carcinoma, Caco-2 cells spontaneously undergo an enterocyte differentiation characterized by a polarization of the cell layer with the formation of domes and the presence of an apical brush border, the membrane of which is endowed with enterocyte-specific enzymes and solute transport systems (4, 5, 7). Moreover, Caco-2 cell monolayers grown on permeable support exhibit several other characteristics of the ileum (11, 12, 19). We used Caco-2 cells as a cell culture model to study the distribution of endogenous SGLT1 in human enterocytes.

In Caco-2 cells, SGLT1 has an apparent half-life of 2.5 days as determined by metabolic labeling. The overall residence time of SGLT1 in Caco-2 cells is rather long compared with the time required for synthesis, processing, and membrane targeting of typical membrane proteins. The processing and passage of newly synthesized membrane proteins through endoplasmic reticulum and Golgi have been shown to be complete after 30–60 min (2, 22) in rat hepatocytes. Therefore, the relative long overall residence time of SGLT1 in Caco-2 cells suggests that most of the intracellular SGLT1 transporters are not en route from biosynthesis to their cellular destination. This assumption is supported by the observation that elimination of newly synthesized proteins from the biosynthetic path-
way by cycloheximide did not significantly alter the size and appearance of the SGLT1 pool present in Caco-2 cells. Assuming a steady state at which protein synthesis and degradation proceed with the same rates, only a small SGLT1 amount is added to and retrieved from a large existing SGLT1 pool in a given time interval. The large existing pool of SGLT1 is distributed between a SGLT1 population, which is located in the apical plasma membrane, and a SGLT1 population, which is dispersed in intracellular, microtubule-associated vesicular structures. The quantification of immunodetectable SGLT1 in Caco-2 cells revealed a distribution ratio between apical plasma membrane and intracellular compartments of 1:2. Most of the SGLT1 actually resides inside the enterocyte!

What is SGLT1 doing inside the cell? Intracellular pools have been described for several membrane transporters: 1) the insulin-responsive glucose transporter 4 in rat adipocytes (35); 2) the aquaporin 2 water channel in LLC-PK1 cells (14); 3) the cystic fibrosis transmembrane conductance regulator (CFTR) in rat duodenal epithelium (1); 4) the proton/potassium ATPase in gastric parietal cells (47); and 5) canalicular ATP-binding cassette (ABC) transporters in rat hepatocytes (24). Common to all these examples is that these transporters are regulated in their plasma membrane abundance by the shifting of transporters between intracellular sites and the plasma membrane.

There are also indications for posttranslational regulation of SGLT1. SGLT1-mediated D-glucose uptake into cells was rapidly altered by prior treatment with forskolin (45), cAMP or the cAMP-raising agent cholera toxin (33), high D-glucose (38), and the peptide hormone glucagon-like peptide 2 (GLP-2) (8). In some of

Fig. 10. Distribution of SGLT1 in Caco-2 cells with switched-off protein synthesis. A: Caco-2 cells were incubated with CHX, and the cellular distribution of SGLT1 was determined by ELISA assay after separation of Caco-2 cell organelles with FFE. The results were compared with control cells, which received no CHX treatment. The quantification of the areas below the curves representing the intracellular-apical SGLT1 pools, respectively, revealed an intracellular-apical SGLT1 distribution ratio of 2:1 for both CHX-treated and control cells. B: Caco-2 cells were incubated with 10 µg/ml CHX for 2 h, and the sodium-dependent uptake of α-methyl-D-glucose, a specific substrate for SGLT1, was measured for 30 min and compared with similar uptake experiments in control cells (100%), which was 4.39 ± 0.93 pmol/mg protein 1/30 min 1/2. Data are means ± SE, n = 4. No significant difference could be detected with regard to α-methyl-D-glucose uptake between CHX-treated and control cells. To further demonstrate the specificity for SGLT1, we also performed the uptake experiments in the presence of 0.5 mM phlorizin, a specific inhibitor of SGLT1. For both experiments, typical results are presented that were observed in at least 3 independent experiments.

Fig. 11. Half-life of SGLT1 in Caco-2 cells. Caco-2 cells were metabolically labeled by incubation with [35S]methionine/cysteine for 2 h and chased with marker-free medium for 1, 2, 3, 4, and 5 days. SGLT1 was then immunoprecipitated from the cell lysates (1.5 mg protein of each lysate). Immunoprecipitates were separated by SDS-PAGE, and radiolabeled bands were detected (A) and quantified with a PhosphorImager (B). The half-life of SGLT1 was estimated from a semilogarithmic plot (B) of the relative [35S] intensity (highest value = 100) vs. chase time. Assuming first-order decay, data points were fitted linearly by the least-squares method. The half-life of SGLT1 was determined to be 2.5 days. The half-life of SGLT1 was established in 1 set of 5 immunoprecipitation experiments.
these examples, changes in D-glucose absorption have been shown to be accompanied by simultaneous changes in the plasma membrane abundance of SGLT1. Shifting of SGLT1 between cellular pools was recently directly demonstrated. Upon treatment of isolated blind loops of rabbit jejunum with epidermal growth factor, SGLT1 was increased in prepared brush border membrane vesicles and decreased in a prepared microsomal membrane fraction (9). We propose that the intracellular population of SGLT1 that we identified in Caco-2 cells is critically involved in the regulation of SGLT1 abundance at the cell surface. This is also supported by our finding that intracellular compartments containing SGLT1 are associated with microtubules. Because microtubules are intracellular “railroad tracks,” this observation implies mobility of the intracellular SGLT1 pool.

We have introduced a powerful method for the investigation of cellular distribution of membrane transports in cultured epithelial cells. In contrast to classic biochemical subcellular fractionation techniques, preparation of a COF, followed by FFE, allowed the separation of a whole variety of membrane-enclosed cellular compartments in a single step, ready for analysis. Further analysis of the FFE fractions with immunochromical methods (ELISA) then revealed the distribution of SGLT1 (or any other protein of interest, against which a suitable antibody is available) over a large spectrum of cellular compartments. Because this method is also quantitative, it is suitable for detecting changes in the subcellular distribution of SGLT1 after exposure of cells to various stimuli before separation and analysis. In other words, this method has the potential to identify physiological signals affecting the intracellular distribution and trafficking of SGLT1 in epithelial cells, a project presently pursued in our laboratory.

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