

Young Histochemist of the Year

## Apical Localization of Sodium-Dependent Glucose Transporter SGLT1 is Maintained by Cholesterol and Microtubules

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Received October 2, 2006; accepted October 23, 2006; published online December 6, 2006

A GFP-labeled sodium-dependent glucose transporter SGLT1 (SGLT-GFP) was transfected into MDCK cells. SGLT-GFP was localized at the apical membrane in confluent cells. When cellular cholesterol was depleted by methyl- $\beta$ -cyclodextrin (M $\beta$ CD) treatment, the localization of SGLT-GFP gradually switched from apical to whole plasma membrane. Time-lapse microscopy revealed that the effect of M $\beta$ CD appeared within 30 min, and that the transition of SGLT-GFP to the whole plasma membrane was completed within 2 hr after the administration. Immunofluorescence microscopy revealed that the tight junction framework remained steady during this process. The effect of M $\beta$ CD on SGLT-GFP localization was counterbalanced by the addition of cholesterol into the culture medium. Disruption of microtubules by colcemid also perturbed SGLT-GFP localization. SGLT-GFP localized to the whole plasma membrane by colcemid treatment, and apical localization was restored within 1 hr after removal of colcemid. Inhibition of protein synthesis by cycloheximide had no effect on the transition of SGLT-GFP induced by the M $\beta$ CD or colcemid. These results indicated that the apical localization of SGLT-GFP is maintained by cellular cholesterol and microtubules, possibly with an apical recycling machinery.

**Key words:** SGLT, apical membrane, cell polarity, cholesterol, microtubule

### I. Introduction

The sodium-dependent glucose transporter, SGLT1, is localized at the apical plasma membrane in absorptive epithelial cells of small intestine and proximal tubule cells of kidney, where it plays pivotal roles in the absorption and reabsorption of sugars, respectively [14, 16–18, 20, 21]. Localization of SGLT1 to apical membrane is crucial in the vectorial transport of sugars across the epithelial cell layer. The mechanism of apical localization of SGLT1 has yet to be fully elucidated.

It has been reported that SGLT1 associates with detergent-resistant membrane microdomains [10]. Disruption of these detergent-resistant membrane microdomains by acute depletion of cellular cholesterol by treatment with methyl- $\beta$ -

cyclodextrin (M $\beta$ CD) decreased SGLT1 protein abundance in these microdomains, and was paralleled by a decrease of sodium-dependent glucose transport activity. Exposure of kidney proximal tubule cells to sphingomyelinase, which depletes both sphingomyelin and cholesterol in plasma membrane, also decreased glucose transport activity [19]. This effect was counterbalanced by replenishment of cholesterol with cholesterol-enriched liposomes.

Polarized targeting to the apical plasma membrane of integral membrane proteins is associated with various intracellular factors. The tight junction separating the apical and basolateral membrane domains prevents the lateral diffusion of integral membrane proteins. It has also been reported that cytoskeletal elements participate in the constitution of lipid microdomains [1, 5–7, 9], and that microtubules play an essential role in the trafficking of lipid microdomain-associated proteins to the apical membrane [4, 12].

To examine whether such intracellular factors, i.e., tight junction borders, cholesterol, and microtubules, participate in the apical localization of SGLT1, we constructed

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MDCK cells transfected with a GFP-labeled SGLT1 gene (SGLT-GFP). The localization of SGLT-GFP gradually switched from apical to whole plasma membrane after M $\beta$ CD treatment. Disruption of microtubules by colcemid also perturbed SGLT-GFP localization. The tight junction framework remained steady during the course of these transitions of SGLT-GFP. These data suggest that the apical localization of SGLT-GFP is maintained by cholesterol and microtubules, possible with an apical recycling machinery.

## II. Materials and Methods

### *Construction of SGLT-GFP-expressing MDCK cells*

Rat cDNA of SGLT1 was a gift from Dr. M. Kasahara (Kasahara and Mori, GenBank D16101). To generate the GFP-labeled SGLT1, a PCR fragment from rat SGLT1 cDNA was inserted into the XhoI-EcoRI sites of pEGFP-N3 vector (BD Biosciences Clontech Japan, Tokyo Japan). The forward primer includes the XhoI site just before the start codon of the SGLT1 open reading frame, and the reverse primer includes the EcoRI site instead of the stop codon. The DNA sequence was verified with a DNA sequencing. MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM; D5796, Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) under an atmosphere of 5% CO<sub>2</sub> and air at 37°C. SGLT-GFP cDNA was transfected into MDCK cells by means of LipofectAmine Plus transfection reagent (Gibco BRL, Life Technologies, Tokyo, Japan).

### *Time-lapse fluorescence microscopy*

MDCK cells were cultured on 35-mm glass-based dishes (Asahi Techno Glass, Tokyo, Japan). The dishes were set in a culture chamber on the microscope with an atmosphere of 5% CO<sub>2</sub> and air at 37°C. Cells were observed with an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) equipped with a cooled-CCD camera (Cool-snap fx, Nippon Roper, Chiba, Japan). Images were captured and processed with IP Lab (Scanalytics, Fairfax, VA, USA).

### *Ca<sup>2+</sup>-switch experiment*

MDCK cells were cultured confluent on the 35-mm glass-based dishes and treated overnight (20~24 hr) in Ca<sup>2+</sup>-free medium (DMEM without calcium chloride, 21068-028, Gibco BRL) containing 0.2 mM EDTA, 10% FBS. The dishes were then mounted in a microculture chamber on the microscope and observed. During the observation, the medium was changed to the conventional medium containing Ca<sup>2+</sup>.

### *M $\beta$ CD treatment*

M $\beta$ CD and water-soluble cholesterol (cholesterol balanced with M $\beta$ CD) were purchased from Sigma-Aldrich (C4555 and C4951). MDCK cells were cultured confluent, and time-lapse images were captured after the medium

change to the medium containing 15 mM M $\beta$ CD, or 15 mM M $\beta$ CD and 30  $\mu$ g/ml cholesterol.

### *Colcemid treatment*

Colcemid was purchased from Gibco-BRL (Karyo-MAX colcemid solution). MDCK cells were cultured confluent. Colcemid (200 ng/ml) was added, and cells were sampled and fixed for immunofluorescence microscopy.

### *Immunofluorescence staining and laser confocal microscopy*

Anti-peptide antisera were raised in a rabbit or a guinea pig against rat SGLT1 [15, 16]. Mouse monoclonal anti-occludin antibody was purchased from Zymed Laboratories (San Francisco, CA, USA). Rat monoclonal anti-ZO-1 antibody was purchased from Chemicon International (Temecula, CA, USA). Mouse monoclonal anti-gp135 antibody (3F2) was a gift from Dr. G. K. Ojakian [8]. Mouse monoclonal antibody against chicken  $\alpha$ -tubulin was purchased from Sigma-Aldrich. FITC (fluorescein isothiocyanate)-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-guinea pig IgG, LRSC (lissamin rhodamine sulfonyl chloride)-conjugated donkey anti-rat IgG, Rhodamine Red-X-conjugated donkey anti-rabbit IgG, Rhodamine Red-X-conjugated donkey anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Immunofluorescence staining of MDCK cells was carried out basically as previously described [13, 15]. Specimens were mounted in an anti-bleaching embedding medium [11], and observed with an Axioplan 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an MRC-1024 laser confocal system (Nippon Bio-Rad Laboratories, Tokyo, Japan). Images were captured and processed with LaserSharp (Bio-Rad) and PhotoShop (Adobe Systems, San Jose, CA) software.

### *Measurement of transepithelial electric resistance (TER)*

MDCK cells were cultured confluent on the Transwell filters (12 mm diameter, Cat. No. 3401; Corning Coster Japan, Tokyo, Japan). TER was measured using a Millicell-ERS (Nihon Millipore Ltd., Tokyo, Japan) as described elsewhere [3]. The TER values were calculated by subtracting the background values for blank filters and indicated as percentage of values at  $t_0$ .

## III. Results

### *Localization of SGLT-GFP during the formation of cell polarity*

When the cells were cultured confluent, SGLT-GFP was localized at the apical plasma membrane as seen in wild type SGLT1 (Fig. 1A) [13]. Since the apical localization of SGLT1 depends on the formation of the tight junction framework between the apical and basolateral plasma membranes [13], we first checked the localization of SGLT-GFP during the course of formation of cell polarity and the tight junction framework. When MDCK cells whose number was sufficient for confluent culture were seeded in the Ca<sup>2+</sup>-free

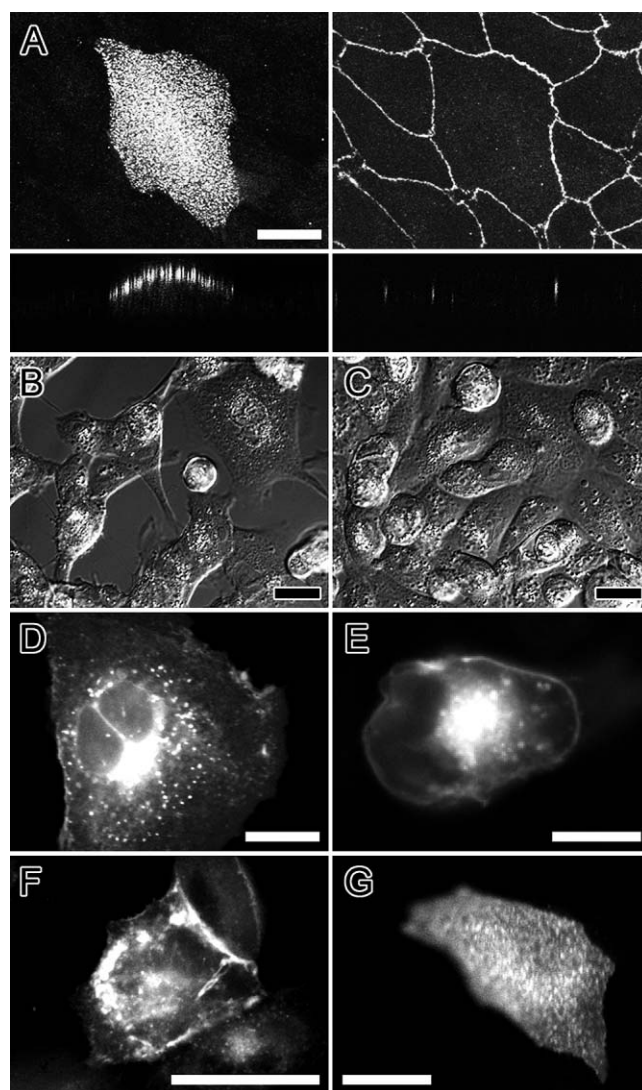
medium for 24 hr, cells exhibited fibroblast-like shapes and epithelial cell sheets were not formed (Fig. 1B). SGLT-GFP was primarily localized to the perinuclear cytoplasmic compartments (Fig. 1D). When the medium was changed to  $\text{Ca}^{2+}$ -containing conventional medium, the cell-to-cell contact was gradually formed by 30 min after the medium change. By 2 hr, epithelial sheets were formed (Fig. 1C), and the tight junction framework was also gradually constructed (data not shown). After subsequent overnight culture, polarized epithelial cell sheets were completed. During the course, the localization of SGLT-GFP gradually changed, and one hour after the medium change, SGLT-GFP appeared along the entire plasma membrane (Fig. 1E). By 2 hr, SGLT-GFP gradually accumulated at the apical membrane (Fig. 1F). When the cells were cultured overnight in conventional medium after the  $\text{Ca}^{2+}$ -switch, SGLT-GFP was restricted to the apical membrane (Fig. 1G).

#### Effects of M $\beta$ CD on apical localization of SGLT-GFP

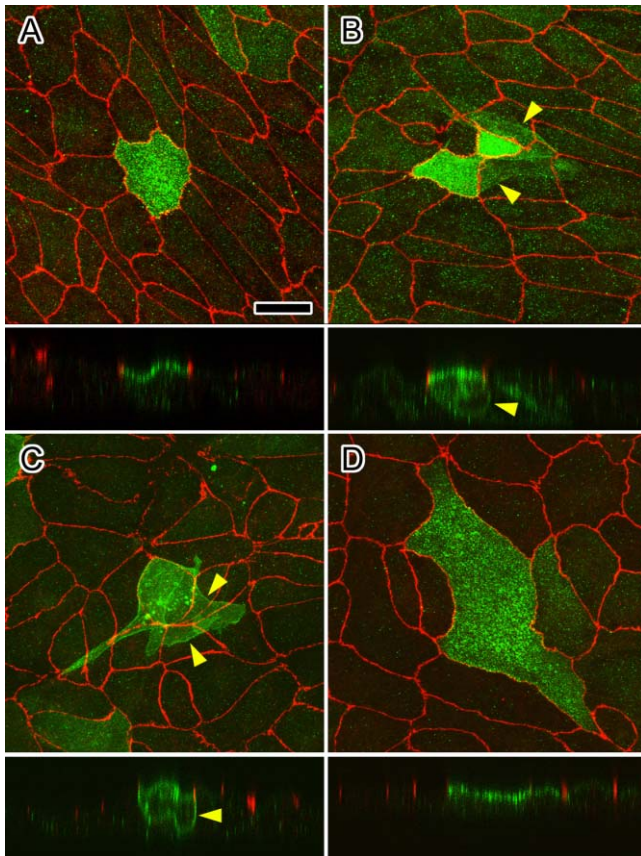
To examine whether cholesterol participates in the apical localization machinery of SGLT-GFP, cellular cholesterol was completely removed by M $\beta$ CD treatment. When the confluent MDCK cells were treated with 15 mM M $\beta$ CD for 2 hr, the localization of SGLT-GFP was perturbed and gradually switched from the apical to the whole plasma membrane. Immunofluorescence microscopy revealed that the staining pattern of the tight junction framework remained steady throughout the course of the transition of SGLT-GFP localization (Fig. 2A–C). The effect of M $\beta$ CD on SGLT-GFP localization was completely counterbalanced by the addition of cholesterol (30  $\mu\text{g}/\text{ml}$ ) to the culture medium (Fig. 2D). These results suggested that the apical localization of SGLT-GFP is maintained by a system associated with cellular cholesterol.

#### Effects of microtubule disruption on apical localization of SGLT-GFP

To clarify whether the apical localization of the SGLT-GFP is associated with the microtubule cytoskeleton, microtubules were disrupted by colcemid treatment. When the confluent cells were treated with colcemid (200 ng/ml), microtubules in the cells were disrupted within 1 hr (Fig. 3A–C). The apical localization of SGLT-GFP, on the other hand, was maintained at 1 hr (Figs. 3B, 4B). After 2 hr of colcemid administration, basolateral signals for SGLT-GFP gradually increased, and SGLT-GFP was localized at whole plasma membrane by 4 hr (Figs. 3C, 4C). During the course of transition of SGLT-GFP localization from apical to whole plasma membrane, the staining pattern of the tight junction framework was unaffected (Fig. 4A–C). When the colcemid was washed out with the conventional medium after 4-hr colcemid treatment, microtubule networks were reconstructed and reverted to the normal shape by 2 hr (Fig. 3D). Apical localization of SGLT-GFP was also restored accompanied with the reversion of the microtubule networks. These results suggest that the apical localization of SGLT-GFP is maintained by the microtubule cytoskeleton.



**Fig. 1.** (A) Immunofluorescence laser confocal images for SGLT-GFP (left frames) and ZO-1 (right frames) of confluent MDCK cells. Projection images of consecutive x-y optical sections are shown in the upper large panels, and an x-z vertical scanning image of each upper panel is shown in the lower small panel. (B–G) Differential interference-contrast images (B and C) and fluorescence images of SGLT-GFP (D–G) in MDCK cells in the  $\text{Ca}^{2+}$ -switch. (B) Sufficient numbers of the cells for confluent culture were cultured in the  $\text{Ca}^{2+}$ -free medium for 24 hr. Cells show fibroblast-like shape and epithelial junctions are not seen. (C) Two hours after the medium change, cell-to-cell contact is restored, and polarized epithelial cell sheets are formed. (D) Twenty-four hours in  $\text{Ca}^{2+}$ -free medium, SGLT-GFP is primarily localized at the perinuclear cytoplasmic region. (E) One hour after the medium change, SGLT-GFP at the plasma membrane is evident. (F) Two hours after the medium change, SGLT-GFP is gradually accumulating in the apical membrane. (G) Eighteen hours after the medium change, SGLT-GFP is preferentially localized at the apical membrane. Bars=10  $\mu\text{m}$ .



**Fig. 2.** Immunofluorescence laser confocal images of M $\beta$ CD-treated cells. Projection images of consecutive x-y optical sections are shown in the upper large panels. An x-z vertical scanning image of each upper panel is shown in the lower small panel. Green and red signals indicate the SGLT-GFP and ZO-1, respectively. Arrowheads indicate the basolateral SGLT-GFP signals. Bar=10  $\mu$ m. Confluent MDCK cells (A) were treated with 15 mM M $\beta$ CD for 1 hr (B), 2 hr (C), or treated together with M $\beta$ CD and 30  $\mu$ g/ml cholesterol for 2 hr (D). (A) SGLT-GFP is localized at apical plasma membrane in the confluent cells. (B) One hour after the M $\beta$ CD treatment, gradual transition of SGLT-GFP from apical to whole plasma membrane is seen. (C) Two hours after the treatment, SGLT-GFP is localized along the whole plasma membrane. The staining pattern of ZO-1 is not affected during the course of the transition of SGLT-GFP. (D) Addition of cholesterol completely counterbalances the effect of M $\beta$ CD on the localization of SGLT-GFP.

#### *Effects of M $\beta$ CD and colcemid on tight junction function*

To examine whether the function of the tight junctions is influenced by M $\beta$ CD or colcemid treatment, we examined TER and the localization of other apical and basolateral marker proteins. The mean value of TER of the confluent MDCK cells was  $1618 \pm 23 \Omega\text{cm}^2$ . Colcemid treatment did not affect TER, whereas the M $\beta$ CD treatment rapidly reduced the TER of MDCK cells (Fig. 5). Interestingly, occludin and ZO-1 were localized at the apico-lateral aspect of the cells and exhibited the typical tight junction frame-

work as seen in control cells either after treatment with M $\beta$ CD for 2 hr or colcemid for 4 hr (Fig. 2B–C and Fig. 4B–C). Moreover, the polarized localization of apical glycoprotein gp135 was not affected by either treatment (Fig. 6). These results suggested that the function for prevention of lateral diffusion is retained normally during the M $\beta$ CD treatment for 2 hr or colcemid treatment for 4 hr, and that the M $\beta$ CD treatment may only affect the barrier function of tight junctions.

#### *Basolateral SGLT-GFP is transported from apical aspect of plasma membrane*

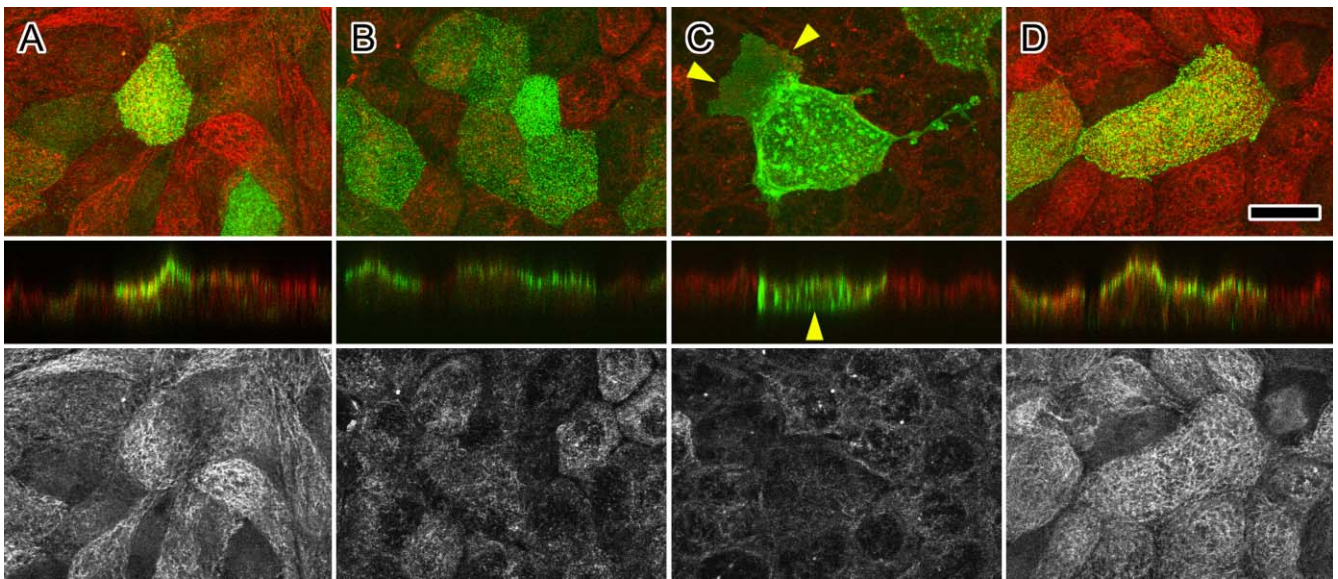
The above results raise a question as to the source of the basolateral SGLT-GFP, for which there are two possible answers: one is that the basolateral SGLT-GFP is transported from the apical plasma membrane domain, and the other is that the newly synthesized SGLT-GFP protein is nonspecifically transported to the whole plasma membrane. To assess these possibilities, *de novo* protein synthesis was inhibited by cycloheximide administration during the colcemid or M $\beta$ CD treatment. Administration of cycloheximide (2  $\mu$ g/ml) did not affect the transition of apical SGLT-GFP to whole plasma membrane in colcemid-treated cells (Fig. 4D). Cycloheximide also did not affect the transition of apical SGLT-GFP to whole plasma membrane by M $\beta$ CD treatment (data not shown). These results indicate that the basolateral SGLT-GFP arises from apical plasma membrane possibly by a transcytosis-like mechanism accompanied with the destruction of a system for their apical localization.

## IV. Discussion

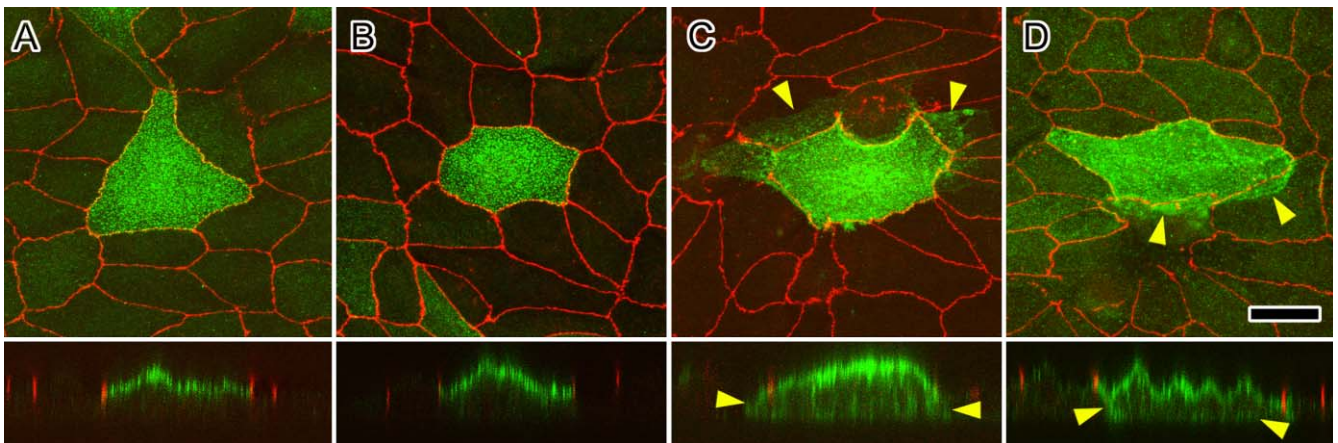
SGLT-GFP localized at the apical plasma membrane is dependent on the formation of the tight junction framework as seen in wild type SGLT1 [13]. This observation indicates that SGLT-GFP is useful as a fluorescent probe for apical membrane molecules in living MDCK cells. When the cells were cultured in Ca $^{2+}$ -free medium for 24 hr, cells could not form intercellular junctions and most of SGLT-GFP signals were localized in cytoplasmic vesicles in the perinuclear region. These SGLT-GFP-positive vesicles started to transfer to the whole plasma membrane 1 hr after the Ca $^{2+}$ -switch. SGLT-GFP vesicles may be launched to the whole plasma membrane by a vesicular transport system that is stimulated by a signal transduction following the construction of adherence junctions by the Ca $^{2+}$ -switch. SGLT-GFP signals then gradually accumulated in the apical membrane domain accompanying the formation of the tight junctions, and preferentially localized at the apical membrane after subsequent overnight culture. These results indicate that localization of SGLT-GFP to the apical membrane is dependent on the formation of the tight junction framework, and that the localization pattern of SGLT-GFP during the formation of cell polarity is similar to that in wild type SGLT1.

It has been reported that SGLT1 associates with detergent-resistant membrane microdomains, and disruption





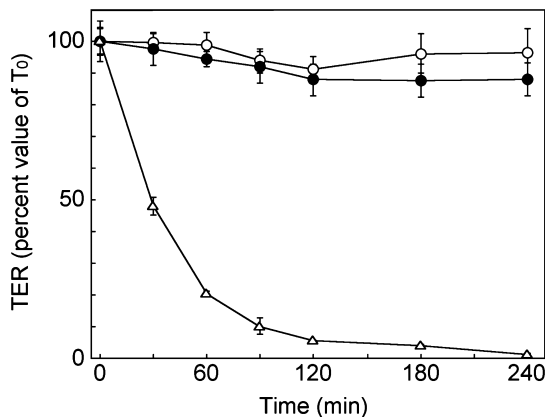
**Fig. 3.** Immunofluorescence laser confocal images of colcemid-treated cells. Projection images of consecutive x-y optical sections are shown in the upper large panels. An x-z vertical scanning image of each upper panel is shown in the lower small panel. Green signal indicates SGLT-GFP, and red signal indicates  $\alpha$ -tubulin. Monochrome images show the channel of  $\alpha$ -tubulin. Arrowheads indicate the basolateral SGLT-GFP signals. Bar=10  $\mu$ m. Confluent MDCK cells (**A**) were treated with 200 ng/ml colcemid for 1 hr (**B**), 4 hr (**C**), treated with colcemid for 4 hr and cultured in normal medium for 2 hr (**D**). (**A**) SGLT-GFP is localized at apical plasma membrane in the confluent cells. (**B**) One hour after the colcemid treatment, whereas the microtubule networks are completely disrupted, SGLT-GFP is still localized at apical plasma membrane. (**C**) Four hours after the colcemid treatment, SGLT-GFP localizes along the whole plasma membrane. (**D**) Two hours after the removal of colcemid, microtubule networks are reconstructed and reverted to normal shape. Apical localization of SGLT-GFP is also restored.



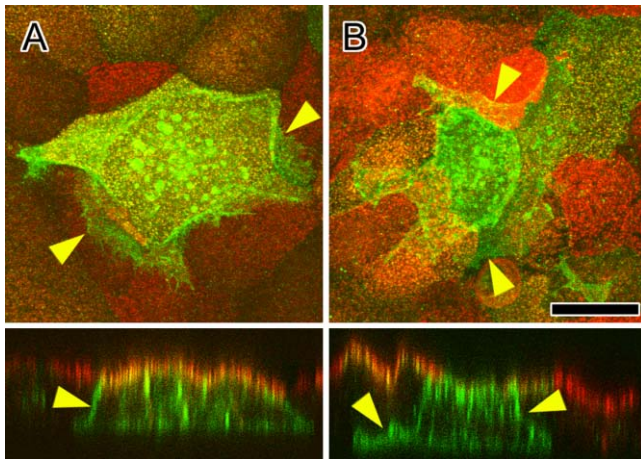
**Fig. 4.** Immunofluorescence laser confocal images of colcemid-treated cells. Projection images of consecutive x-y optical sections are shown in the upper large panels. An x-z vertical scanning image of each upper panel is shown in the lower small panel. Green signal indicates SGLT-GFP, and red signal indicates ZO-1. Arrowheads indicate the basolateral SGLT-GFP signals. Bar=10  $\mu$ m. Confluent MDCK cells (**A**) were treated with 200 ng/ml colcemid for 1 hr (**B**), 4 hr (**C**), treated together with colcemid and 2  $\mu$ g/ml cycloheximide for 4 hr (**D**). (**A**–**C**) The staining pattern of ZO-1 does not change during the course of transition of SGLT-GFP by colcemid treatment. (**D**) Four hours after the treatment with colcemid and cycloheximide, SGLT-GFP is localized along the whole plasma membrane as seen in cells treated with colcemid alone in panels C.

of these microdomains by M $\beta$ CD decreased the amount of SGLT1 in detergent-resistant membrane microdomains, a change that was paralleled by a decrease of sodium-dependent glucose transport activity [10]. These results suggested that cellular cholesterol may be important for the localiza-

tion of SGLT1. It has been reported that cellular cholesterol was rapidly depleted from 60% to 50% within 30 min to 2 hr after treatment with M $\beta$ CD [2], and that apical targeting of membrane protein HA associated with lipid raft was perturbed [4, 12]. When confluent MDCK cells were



**Fig. 5.** Changes of TER in control (closed circle), M $\beta$ CD (open triangle)-, and colcemid (open circle)-treated MDCK cells. TER values are shown as percentage of values at time 0. The mean value of TER of the confluent MDCK cells was  $1618 \pm 23 \Omega\text{cm}^2$ . Values are mean  $\pm$  S.E.M.



**Fig. 6.** Immunofluorescence laser confocal images of M $\beta$ CD- and colcemid-treated cells. Projection images of consecutive x-y optical sections are shown in the upper large panels. An x-z vertical scanning image of each upper panel is shown in the lower small panel. Green signal indicates SGLT-GFP. Red signal indicates gp135. Arrowheads indicate the basolateral SGLT-GFP signals. Bar=10  $\mu\text{m}$ . Confluent MDCK cells were treated with M $\beta$ CD for 2 hr (A) or colcemid for 4 hr (B), and immunostained for apical glycoprotein gp135. Whereas the SGLT-GFP signals are localized at whole plasma membrane, the gp135 is localized at apical membrane in either M $\beta$ CD (A) or colcemid (B) treatment.

treated with M $\beta$ CD, the localization of SGLT-GFP was perturbed and switched from apical to whole plasma membrane. This response appeared within 30 min, and the transition of SGLT-GFP to whole plasma membrane was completed by 2 hr. Since the immunofluorescence staining patterns of tight junction protein ZO-1 and apical marker protein gp135 did not change during the transition of SGLT-GFP by M $\beta$ CD treatment, the change of localization is not due to lateral diffusion by the destruction of the tight junction borders.

The effect of M $\beta$ CD for the transition of SGLT-GFP was completely counterbalanced by the addition of cholesterol. This result indicated that cellular cholesterol is essential for apical localization of SGLT-GFP.

Cytoskeletal tracks are essential for the vesicular transport and selective targeting of membrane proteins. Apical targeting of influenza HA is disrupted accompanied with destruction of microtubule filaments by nocodazole treatment [4, 12]. We examined possible involvement of microtubules in the localization machinery of SGLT-GFP. By 1 hr after the treatment with colcemid, microtubule networks were completely disrupted, whereas apical localization of SGLT-GFP was maintained. Apical localization of SGLT-GFP was gradually perturbed and SGLT-GFP localized to the whole plasma membrane by 4 hr. Microtubule networks were reconstructed and reverted to normal shape within 1 hr after the removal of colcemid. The apical localization of SGLT-GFP was concomitantly restored accompanied with the reversion of the microtubule filaments. Since the immunofluorescence staining patterns of ZO-1 and gp135 did not change during the transition of SGLT-GFP by colcemid treatment, it is considered that the transition is not due to lateral diffusion by the loss of fence function of the tight junctions. When the cells were treated with colcemid and cycloheximide, transition of SGLT-GFP to whole plasma membrane was observed as well. This result indicates that the basolateral SGLT-GFP originates from that at apical plasma membrane rather than from a newly synthesized source. Destruction of microtubules by colcemid treatment may perturb the apical retention system for SGLT-GFP and the subsequent transcytosis-like transport of apical SGLT-GFP to basolateral membrane, resulting in the whole membrane distribution of it.

From these results, we conclude that the apical localization of SGLT-GFP is maintained by cellular cholesterol and microtubules. Localization of SGLT-GFP to the apical membrane may be maintained by recycling with continuous endocytosis and exocytosis, and cholesterol microdomains and microtubules play important roles in the recycling machinery.

## V. Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Kato Memorial Bioscience Foundation. We thank Dr. M. Kasahara (Teikyo University, Tokyo, Japan) for providing the rat SGLT1 cDNA, Dr. G. K. Ojakian (SUNY Downstate Medical Center, Brooklyn, NY) for the anti-gp135 antibody.

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