Up-regulation of Sodium-Dependent Glucose Transporter by Interaction with Heat Shock Protein 70

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Running title: Heat shock protein 70 increases glucose uptake

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ABSTRACT

Heat shock stress induces some heat shock proteins including Hsp70, and activates sodium-dependent glucose transport in porcine renal LLC-PK1 cells. But its mechanisms are not clarified in detail. We investigated whether sodium-dependent glucose transporter (SGLT1) interacts with Hsp70 to increase SGLT1 activity. Heat shock stress increased SGLT1 activity without changing of SGLT1 expression. The increase of SGLT1 activity was completely inhibited by an anti-TGF-β1 antibody. Instead of heat shock stress, TGF-β1 increased SGLT1 activity in dose- and time-dependent manners without changing of SGLT1 expression. We found that the amount of Hsp70 immunoprecipitated from TGF-β1-treated cells with an anti-SGLT1 antibody was higher than that of the control cells. Transfection of an anti-Hsp70 antibody into the cells inhibited the increase of SGLT1 activity. With confocal laser microscopy, both SGLT1 and Hsp70 was localized near to the apical membrane in the TGF-β1-treated cells and an anti-Hsp70 antibody disturbed these localization. Furthermore, we clarified that an anti-Hsp70 antibody inhibited interaction of SGLT1 with Hsp70 in vitro. These results suggest that Hsp70 forms a complex with SGLT1 and increase the expression level of SGLT1 in the apical membrane, resulting in up-regulation of glucose uptake.

Key words: heat shock stress, immunoprecipitation, LLC-PK1 cells, sodium-dependent glucose transport, transforming growth factor-β1
INTRODUCTION

Regulation of glucose absorption plays an essential role in maintaining cellular and organic functions. In mammalian, glucose uptake across epithelial cells is mediated via two distinct glucose transporters: Na⁺-dependent glucose transporter (SGLT) located in the apical membrane and facilitate glucose transporter (GLUT) located in the basolateral membrane of kidney (1). SGLT family includes three homologous: a high-affinity transporter (SGLT1) and low-affinity transporters (SGLT2 and SGLT3). LLC-PK₁ cells derived from porcine kidney is useful model system for investigation of glucose transport because they selectively express SGLT1 and SGLT3 on apical membrane same as in vivo (2). So far, Rabito and Ausiello (3) reported that over 85 % of total Na⁺-dependent glucose uptake in LLC-PK₁ cells is mediated via SGLT1.

SGLT1 contains a number of potential protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites (4). The expression level of SGLT1 protein on plasma membrane was mainly regulated by these two kinases: PKA increased the number of SGLT1 in the plasma membrane while PKC decreased it in SGLT1-expressed xenopus oocytes (5). Furthermore, it has been reported that PKC lowered turnover rate in SGLT1-expressed COS-7 cells (6). The stabilization of SGLT1 in the plasma membrane is an important step to increase of glucose uptake.

Stress- or injury-induced protection and functional enhancement are often associated with increased synthesis and accumulation of heat shock proteins, particularly Hsp70 (for review see, 7-9). The Hsp70 is ascribed to need for preventing aggregation and misfolding of proteins. However, it plays an essential role under normal condition, including folding of assisting of newly synthesized proteins, translocating proteins to the appropriate organs, and dissociating protein aggregates. In addition, Hsp70 interacts with specific native proteins expressed on plasma membrane such as A₁ adenosine receptor (10), Na⁺/H⁺-exchanger (11), and Na⁺,K⁺-ATPase (12).

Hsp70 is ubiquitously presence in all renal tubular epithelial cells (13). During cellular recovery from renal ischemia, Hsp70 interacts with cytoskeletal elements (12). In LLC-PK₁ cells, it has been reported that heat shock stress increased Hsp70 and SGLT1 activity, and produce protection of cell function (14). It is, however, unclear what mechanism is concerned with the increase of SGLT1 activity. In the present study, we show that heat shock stress increase SGLT1 activity mediated via production of transforming growth factor-β1 (TGF-β1). Furthermore, we found that TGF-β1 increases the interaction of SGLT1 with Hsp70, resulting in the increase of SGLT1 activity.
EXPERIMENTAL PROCEDURES

Materials --- A mouse monoclonal antibody raised Hsp70 (SPA-810) was purchased from StressGen Biotechnologies (Victoria, Canada). A rabbit polyclonal antibody raised actin (C-11) and a goat polyclonal antibody raised aminopeptidase N were from Santa Cruz Biotechnology (CA, U.S.A.). A rabbit polyclonal antibody raised Hsp70 was from Upstate Biotechnology (NY, U.S.A.). This antibody was used in the experiments of transfection and interaction of SGLT1 with Hsp70 in vitro. A rabbit polyclonal antibody raised porcine SGLT1 was kindly provided from Prof. Julie, E., Lever (University of Texas Medical School, Houston, U.S.A.). Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG was from American Qualex (CA, U.S.A.). Texas red-labeled anti-rabbit IgG was from EY Laboratories (CA, U.S.A.). AMCA-labeled anti-goat IgG was from Jackson Immuno Research Laboratories (PA, U.S.A.). A porcine TGF-β1 was from Wako Pure chemical (Osaka, Japan). Chariot, a transfection reagent capable of delivering antibodies was from Active Motif (CA, U.S.A.). Protein G-sepharose bead was from Amersham Pharmacia Biotech (NJ, U.S.A.). [14C]-α-methyl-glucopyranoside (AMG) was from NEN Life Science Products (Boston, MA, U.S.A.). All other reagents were of the highest grade of purity available.

Cell culture --- The porcine renal epithelial LLC-PK₁ cells were obtained from JCRB (Tokyo, Japan). Cells were maintained in a Medium199 (Sigma, MO, U.S.A.) supplemented with 10% fetal calf serum (FCS), 100 µg/ml penicillin and 100 µg/ml streptomycin in an atmosphere of 5% CO₂ in air at 37°C.

Measurement of SGLT1 activity --- Cells were grown to sub-confluence or confluence conditions on 24-well plate, and then treated with heat shock stress or TGF-β1 in FCS-free Medium199. Heat shock stress was performed at 42°C for 3 h, then 37°C for 12 h. TGF-β1 was added in FCS-free Medium199 at indicated time and concentrations. The SGLT1 activity was assayed by incubating in a Hanks balanced salt solution (HBSS) containing [14C]-AMG (0.4 µCi/ml), 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES, pH 7.4 in the presence and absence of phloridzin (0.5 mM), a potent SGLT1 inhibitor. After incubation at 37°C for 30 min, the
solution was aspirated quickly and washed by ice-cold HBSS without $^{14}$C-AMG for 4 times. The cells were solubilized with 0.5 N NaOH and the aliquots were taken for determination of radioactivity and protein concentration. Protein concentration was measured using the protein assay kit (Bio-Rad Laboratories, CA, U.S.A.) with bovine serum albumin as the standard.

**Preparation of membrane fraction from LLC-PK$_1$ cells** --- Whole membrane fraction was prepared from the cells cultured in 10-cm Petri dishes by the procedure of Peng and Lever (15). In brief, cells were washed three times with HBSS, scraped, and suspended in PBS containing 5 mM EDTA. After centrifugation at 80 x g for 5 min, the pellet was solubilized in 20 mM Tris-HCl, sonicated, and centrifuged at 1,000 x g for 5 min. The supernatant was centrifuged at 100,000 x g for 60 min, and the pellet was suspended in 20 mM Tris-HCl (membrane fraction).

**SDS-polyacrylamide gel electrophoresis and Western blotting** --- SDS-polyacrylamide gel electrophoresis was carried out as described previously (16). In brief, membrane preparations (20 µg) were applied to the SDS-polyacrylamide gel. Proteins were blotted onto a PVDF membrane and incubated for 1.5 h with each primary antibody following by peroxidase-conjugated anti-rabbit IgG or mouse IgG. Finally, the blots were stained with the ECL Western blotting kit from Amersham Pharmacia Biotec.

**Immunoprecipitation** --- The apical membrane fraction prepared as described elsewhere (17). The samples solubilized in a lysis buffer containing 1 % Triton X-100, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 8.0 were incubated with Protein G-sepharose bead and an antibody specific for the SGLT1 at 4 °C for 1 h with gentle rocking. After centrifugation at 6,000 x g for 1 min, the pellet was washed four times with a lysis buffer. The pellet was solubilized in a sample buffer for SDS-polyacrylamide gel electrophoresis. The Western blotting was carried out as described above.

**Transfection of antibodies** --- Cells were grown to confluence on 24-well plate. A polyclonal antibody raised Hsp70 or an anti-rabbit IgG were transfected into the cells using chariot kit according to the appendant protocol. After 3 h of transfection, the cells were treated with TGF-β1 followed by examining SGLT1 activity and localization of both SGLT1 and Hsp70.
Immunocytochemistry --- The cells grown on cover glass were incubated with FCS-free medium 199 in the presence and absence of TGF-β1, and washed twice with PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ prior to fixation with 3 % paraformaldehyde for 7 min at room temperature. The cells were then permeabilized with 0.3 % Triton X-100 for 15 min, and 5 % goat serum in PBS (blocking solution) for 30 min. Incubation with anti-SGLT1, anti-Hsp70, and aminopeptidase N antibodies (final dilution 1/120) for 90 min at room temperature was followed by washes with PBS, and then incubating for 90 min with Texas red-labeled anti-rabbit IgG that combine with anti-SGLT1 antibody, FITC-labeled anti-mouse IgG that combine with anti-Hsp70 antibody, and AMCA-labeled anti-goat IgG that combine with anti-aminopeptidase N antibody in a blocking solution (dilution 1/20). Immunolabeled cells were visualized on a LSM 510 confocal microscope (Carl Zeiss, Germany) that set up with the appropriate filter for FITC (488 nm excitation, 530 nm emission filter), Texas red (543 nm excitation, 585-615 nm emission filter), and AMCA detection (351 nm excitation, 450 nm emission filter). Images were collected at 1.0-μm increments (vertical direction) beginning at the apical membrane and ending at the basal membrane. Images were further processed using Adobe Photoshop (Adobe System, Inc).

Complex formation of SGLT1 with Hsp70 in vitro --- The apical membrane fraction was prepared from TGF-β1 (2 ng/ml, 2 h)-treated cells. The sample was pre-incubated with 5 mM ATP/10 mM Mg²⁺ at 30 °C for 10 min, and then incubated with the lysis buffer containing hexokinase (50 units/ml) and 15 mM glucose at 30 °C for 10 min to remove ATP from the incubation solution (18). The aliquot was incubated with Protein G-sepharose bead at 4 °C for 1 h with gentle rocking. After centrifugation at 6,000 x g for 1 min, the supernatant was incubated with the mixture of new bead and an antibody for SGLT1 at 4 °C for 12 h to immunoprecipitate proteins specifically interacting with SGLT1. After centrifugation, the pellet was solubilized in a sample buffer, and then SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as described above.

Statistics --- Results are presented as the means ± S.E. Differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparison was made by using Tukey’s multiple comparison test. Statistically significant differences were assumed at P < 0.05.
RESULTS

Expression of SGLT1 and Hsp70 in LLC-PK1 cells --- LLC-PK1 cells were utilized to examine the expression of SGLT1 and glucose absorption in renal proximal tubule. In this cell line, SGLT1 activity has been observed to develop after cell confluence (2, 19). At first, we checked the expression of SGLT1 and Hsp70 in the different growing stages (Fig. 1A). Hsp70 was detected in both the sub-confluent and the confluent conditions, but SGLT1 was not detected in the sub-confluent condition. As a sample loading control, we observed that actin exists as a same amount between the confluent and the sub-confluent conditions (data not shown). To confirm the expression pattern of SGLT1, we measured the SGLT1 activity using $^{14}$C-AMG. SGLT1 activity was observed in the confluent condition, but not in the sub-confluent condition (Fig. 1B). This result was coincident with the expression pattern of SGLT1 (Fig. 1A).

Neutralization of heat shock response by an anti-TGF-β1 antibody --- At the confluent condition, heat shock stress increased the SGLT1 activity that is neutralized by anti-TGF-β1 antibody (Fig. 2A). Interestingly, Hsp70 expression was potently increased by heat shock stress, but SGLT1 was unchanged (Fig. 2B). An anti-TGF-β1 antibody scarcely affected the expression of SGLT1 and Hsp70. These results indicate that heat shock stress increased the SGLT1 activity mediated via production of TGF-β1 and the inhibition of SGLT1 activity by an anti-TGF-β1 antibody was not caused by decrease of SGLT1 expression. Next, we examined the regulatory mechanism of SGLT1 activity by TGF-β1.

Effects of TGF-β1 on SGLT1 activity and expression --- TGF-β1 increased SGLT1 activity in a time-dependent manner, and the maximal effect was observed at 2 h (Fig. 3A). The effect of TGF-β1 (0.05 - 20 ng/ml) was a dose-dependent, and the EC₅₀ was 2 ng/ml (Fig. 3B). It has been reported that TGF-β1 increased glucose uptake by enhancing GLUT1 expression in mesangial cells (20, 21). So we checked expression level of SGLT1. TGF-β1 unchanged SGLT1 expression compared with control (Fig. 4A). Furthermore, heat shock stress increased Hsp70 expression, but TGF-β1 did not change it. Taken together, increase of Hsp70 expression was not involved in the up-regulation of SGLT1 activity. So far, it has been reported that Hsp70 and related proteins interacted with plasma
membrane proteins such as Na+/H+-exchanger (11), Na+/K+-ATPase (12), and cystic fibrosis transmembrane conductance regulator (22). Next, we examined interaction level of SGLT1 with Hsp70. Membrane fractions prepared from control and TGF-β1-treated cells were immunoprecipitated with an anti-Hsp70 or an anti-SGLT1 antibody. Then, each sample was reacted with an anti-SGLT1 antibody or an anti-Hsp70 antibody, respectively. As shown in Fig. 4B, TGF-β1 increased interaction of SGLT1 with Hsp70.

**Localization of SGLT1 and Hsp70** --- We determined the localization of SGLT1 and Hsp70 by immunocytochemistry (Fig. 5A). Aminopeptidase N, an apical membrane marker protein, was appeared as blue fluorescence only in apical membrane site. Hsp70, appeared as green fluorescence, was localized in entire plasma membrane and cytosol fraction. SGLT1 was appeared as red fluorescence. The image of SGLT1 merged with that of aminopeptidase N showed that intermediate color of purple in the TGF-β1-treated cells, indicating that SGLT1 and aminopeptidase N were co-localized near the apical membrane site (Fig. 5A, right). Furthermore, co-localization of SGLT1 and Hsp70, appeared as yellow, was moved from cytosol fraction to apical membrane site by TGF-β1 (Fig. 5A, left). Next, we examined the effect of an anti-Hsp70 antibody on localization of SGLT1 and Hsp70. Transfection of an anti-Hsp70 antibody into the cells using a chariot kit inhibited the TGF-β1-induced movement of SGLT1 and Hsp70 to apical membrane site (Fig. 5B, right). In control cells, an anti-rabbit IgG did not affect co-localization of SGLT1 and Hsp70 (Fig. 5B, left).

**Inhibition of SGLT1 activity by an anti-Hsp70 antibody** --- In the rat-1 fibroblasts overexpressing human insulin receptors, microinjection of an anti-Hsp70 antibody into the cells partially inhibited insulin stimulated mitogenesis (23). To examine the necessity of the interaction of SGLT1 with Hsp70 in the elevation of SGLT1 activity, we examined the effect of an anti-Hsp70 antibody on SGLT1 activity. This antibody (1.8 μg/ml) inhibited TGF-β1-elicited SGLT1 activation and slightly inhibited the basal SGLT1 activity (Fig. 6). This inhibitory effect on SGLT1 activity consisted with that of localization of SGLT1 and Hsp70. In control cells, an anti-rabbit IgG (1.8 μg/ml) did not inhibit TGF-β1-elicited SGLT1 activation. These results indicate that interaction of SGLT1 with Hsp70 induced elevation of SGLT1 activity.

**Interaction of SGLT1 with Hsp70 in vitro** --- Hsp70 and its related protein bind specifically to
hydrophobic peptide segments in an ATP-dependent manner (24). We examined whether SGLT1 interacts with Hsp70 \textit{in vitro} (Fig. 7). Hsp70 was immunoprecipitated with an anti-SGLT1 antibody in the absence of ATP. After incubation of the apical membrane fraction with ATP (5 mM), Hsp70 dissociated from SGLT1. Interestingly, the removal of ATP by hexokinase and glucose induced re-interaction of Hsp70 with SGLT1. This interaction was inhibited by an anti-Hsp70 antibody, but not by an anti-rabbit IgG. We detected no band in the membrane that was not incubated with an anti-Hsp70 antibody (data not shown), indicating an anti-Hsp70 antibody did not contaminate in the samples. The interaction of Hsp70 with SGLT1 consisted with the data of co-localization as shown in Figure 6.

**DISCUSSION**

The mRNA and transport activity of SGLT1 are not detectable in sub-confluent LLC-PK\textsubscript{1} cells as shown by northern blotting and glucose transport assay (2, 19, 24). We also showed that SGLT1 protein did not express in the sub-confluent condition, and SGLT1 protein and activity appeared only in the confluent condition (Fig. 1). On the contrary, Hsp70 expressed in both the sub-confluent and the confluent conditions. Hsp70 and related proteins play an essential role under normal physiological condition, including folding of assisting of newly synthesized proteins, translocating proteins to the appropriative organs, and dissociating protein aggregates (7-9). So far, mammalian Hsp70 was reported to interact with some transporters expressed in epithelial plasma membrane to maintain their functions (10-12). However, there is no report whether Hsp70 interacts with SGLT1, and involves in the regulation of SGLT1 activity.

Heat shock, oxidants, tissue trauma, and hormonal stimulation increased expression of Hsp70 and related proteins. Some stresses induce production of TGF-\(\beta\)\textsubscript{1}, a multifunctional cytokine, which transmits various cellular responses such as cell proliferation and formation of the extracellular matrix (25-30). Interestingly, the release of TGF-\(\beta\)\textsubscript{1} increased in LLC-PK\textsubscript{1} cells developing after cell confluence (29). Our results indicated that heat shock stress increased SGLT1 activity mediated via production of TGF-\(\beta\)\textsubscript{1} (Fig. 2A). TGF-\(\beta\) receptors are divided into three types; type I (53 kDa), type II (70-85 kDa), and type III (250-350 kDa) (30). The signal primarily through TGF-\(\beta\) type II receptor, then phosphorylation of type I receptor activates protein kinases. TGF-\(\beta\)\textsubscript{1}
was reported to quickly stimulate adenylate cyclase activity in LLC-PK₁ cells (31). Activation of PKA up-regulates SGLT1 mRNA level after 2-4 days lag period, accompanied by pronounced stabilization of the message (15). In the present study, heat shock stress unchanged the expression level of SGLT1 protein within 12 h (Fig. 2B). We suggest that heat shock stress induced SGLT1 activation without increase of SGLT1 expression in short term period.

TGF-β1 increased SGLT1 activity, but did not change expression levels of SGLT1 and Hsp70 (Figs. 3 and 4). An anti-TGF-β1 antibody inhibited SGLT1 activation induced by heat shock stress, but has no effect on the Hsp70 expression (Fig. 2). These results suggest that the increase of Hsp70 is independent from the regulation of SGLT1 activity, and not an important phenomenon to up-regulate SGLT1 activity. Recently, Bidmon et al. (12) reported that the interaction of Hsp70 with Na⁺/K⁺-ATPase was increased followed by stabilizing of Na⁺/K⁺-ATPase within cytoskeletal fraction during the restoration of the renal cells after ischemia. We found that SGLT1 interacted with Hsp70 in normal condition, and TGF-β1 increased interaction level between them (Fig. 4B).

We hypothesized that the interaction of SGLT1 with Hsp70 and localization of these proteins in the apical membrane site are important to increase SGLT1 activity. In immunocytochemistry, TGF-β1 made move both SGLT1 and Hsp70 near the apical membrane site (Fig. 5A). Furthermore, an anti-Hsp70 antibody inhibited the co-localization of SGLT1 and Hsp70 in TGF-β1-treated cells (Fig. 5B). Transfection of an anti-Hsp70 antibody inhibited the elevation of SGLT1 activity elicited by TGF-β1 (Fig. 6). These results suggest that translocation of Hsp70 from cytosol to apical membrane site was important to stabilize SGLT1 expression on the membrane and up-regulate glucose uptake.

To confirm the interaction of SGLT1 with Hsp70, we performed immunoprecipitation assay in vitro. Hsp70 and its related protein bind specifically to hydrophobic peptide segments, that regions are not conserved, in an ATP-dependent manner (32). The ADP-bound form of Hsp70 has a high affinity for peptides, whereas the ATP form has a low affinity. ATP dissociated Hsp70 from SGLT1 in apical membrane fraction (Fig. 7). Removal of ATP induced re-interaction of these proteins. Furthermore, an anti-Hsp70 antibody inhibited the interaction in vitro similar as shown in Figure 5B. These results indicate that Hsp70 interacts with SGLT1 in an ATP-dependent manner and an anti-Hsp70 antibody blocked interaction of there proteins, lead to inhibition of SGLT1 activity.

In conclusion, we found that heat shock stress increased SGLT1 activity mediated via TGF-β1 production. However, the treatment of the cells with heat shock stress or TGF-β1 for short period
unchanged SGLT1 expression. TGF-β1 increased the interaction of SGLT1 with Hsp70 and translocated them near the apical membrane. These results suggest that Hsp70 supports the apical localization and function of SGLT1 during normal and restoring conditions after injury with heat shock stress.

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FIGURE LEGENDS

Fig. 1  Expression and transport activity of SGLT1 in sub-confluence and confluence LLC-PK<sub>1</sub> cells. A, membrane fractions were prepared from sub-confluence and confluence cells. Samples were run on SDS-PAGE gels and immunoblotted with an anti-SGLT1 antibody (SGLT1) or an anti-Hsp70 antibody (Hsp70). B, SGLT1 activity was determined by [14C]-AMG uptake carried out at 37°C for 30 min in sub-confluence (open column) and confluence cells (closed column). **, significantly different from the sub-confluent condition (P < 0.01). n = 4.

Fig. 2  Effects of an anti-TGF-β1 antibody on the heat shock response. A, Heat shock stress was performed at 42°C for 3 h (closed columns), followed by incubation at 37°C for 12 h. During these incubations, the media contained an anti-TGF-β1 antibody (0.1, 1, and 10) or uncontained the antibody (N). Instead of heat shock, control cells were incubated continuously at 37°C (open column). Then, SGLT1 activity was determined at 37°C for 30 min (n = 5-6). B, heat shock stress (HS) was performed in the absence (-) and the presence (+) of an anti-TGF-β1 antibody (10 µg/ml). Control cells were incubated continuously at 37°C (left lane). Then, each membrane fraction was collected, run on SDS-PAGE gels, and immunoblotted with an anti-SGLT1 antibody (SGLT1) or an anti-Hsp70 antibody (Hsp70).

Fig. 3  Increase of SGLT1 activity by TGF-β1. A, LLC-PK<sub>1</sub> cells were incubated with 2 ng/ml TGF-β1 for indicated time, then SGLT1 activity was determined (n = 3-4). B, the cells were incubated with TGF-β1 at indicated concentration for 2 h, then SGLT1 activity was determined (n = 3-4).
**Fig. 4  Comparison of SGLT1 and Hsp70 expression levels.** A, the membrane fractions were prepared from control (-) and 2 ng/ml TGF-β1-treated (+) cells. Samples were run on SDS-PAGE gels and immunoblotted with an anti-SGLT1 (SGLT1) or an anti-Hsp70 antibody (Hsp70). B, the membrane fractions prepared from control (-) and TGF-β1-treated (+) cells were immunoprecipitated with an anti-Hsp70 (left) or an anti-SGLT1 antibody (right), then immunoblotted with an anti-SGLT1 or an anti-Hsp70 antibody, respectively.

**Fig. 5  Effects of an anti-Hsp70 antibody on SGLT1 and Hsp70 localization.** A, the cells were treated with an anti-SGLT1, an anti-Hsp70, and an anti-aminopeptidase N antibodies. Images of confocal microscope (x-z axis) showed localization of SGLT1 (red), Hsp70 (green), and aminopeptidase N (blue) in control and 2 ng/ml TGF-β1-treated cells. AP, apical membrane site; BL, basal membrane site. B, the cells were transfected with 1.8 µg/ml anti-mouse IgG (IgG) or 1.8 µg/ml anti-Hsp70 antibody (Hsp70) using chariot kit followed by incubation with 2 ng/ml TGF-β1. The merging colors showed the co-localization of aminopeptidase N with SGLT1 (purple) and Hsp70 with SGLT1 (yellow). Scale bar is 10 µm.

**Fig. 6  Effects of an anti-Hsp70 antibody on SGLT1 activity.** LLC-PK₁ cells were transfected with an anti-Hsp70 antibody (1.8 µg/ml) using chariot. Then, the cells were incubated without (open column) and with 2 ng/ml TGF-β1 at 37°C for 2 h (closed columns). An anti-rabbit IgG (1.8 µg/ml) was transfected into the control cells (N) instead of an anti-Hsp70 antibody. n = 3-4. ***, significantly different from the value in the absence of an anti-Hsp70 antibody (P < 0.01).
Fig. 7  Effects of ATP on interaction between SGLT1 and Hsp70 in vitro. A, the membrane fractions were prepared from 2 ng/ml TGF-β1-treated cells, and then pre-incubated in the absence (-) and presence (+) of 5 mM ATP/10 mM Mg^{2+} at 30°C for 10 min. As indicated, the samples were incubated with the lysis buffer containing hexokinase (50 units/ml) and 15 mM glucose in the presence of an anti-Hsp70 antibody (1.8 µg/ml) or an anti-rabbit IgG (1.8 µg/ml) at 30°C for 10 min. Finally, the samples were incubated with the mixture of Protein G-sepharose and an anti-SGLT1 antibody at 4°C for 12 h to collect proteins interacted with SGLT1. The immunoprecipitated protein was detected with anti-Hsp70 antibody.
Fig. 2 Ikari et al.

A

SGLT1 activity
(nmol AMG/mg protein/30 min)

N 0.1 1 10

anti-TGF-β1 antibody (μg/ml)

**

B

SGLT1

Hsp70

HS

- + -
Fig. 3 Ikari et al.
Fig. 4 Ikari et al.

A

| TGF-β1 |   | + |
|--------|---|---|
| SGLT1  |   |   |
| Hsp70  |   |   |

B

| TGF-β1 |   | + |
|--------|---|---|
| SGLT1  |   |   |
| Hsp70  |   |   |
Fig. 5  Ikari et al.

A

| Control     | TGF-β1   |
|-------------|----------|
| SGLT1 + amino N |          |
| SGLT1 + Hsp70  |          |

B

| IgG         | Hsp70    |
|-------------|----------|
| SGLT1 + amino N |          |
| SGLT1 + Hsp70  |          |
SGLT1 activity
(nmol AMG/mg protein/30 min)

Fig. 6 Ikari et al.
Fig. 7 Ikari et al.

Hsp70

|                | ATP | + | + | + | + |
|----------------|-----|---|---|---|---|
| hexokinase + glucose | -   | - | + | + | + |
| anti-Hsp70 antibody   | -   | - | - | - | + |
| rabbit IgG            | -   | - | - | + | - |
Up-regulation of sodium-dependent glucose transporter by interaction with heat shock protein 70
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