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Original Article

Exogenous expression of human SGLT1 exhibits aggregations in sodium dodecyl sulfate polyacrylamide gel electrophoresis

Wei-Chien Huang[1,2,3,4*], Sheng-Chie Hsu[1*], Shyh-Jer Huang[1], Yun-Ju Chen[1,5,6], Yu-Chun Hsiao[2,3], Weihua Zhang[7], Isaiah J Fidler[8,9], Mien-Chie Hung[1,2,9,10]

[1]Center for Molecular Medicine, China Medical University Hospital, Taichung 404, Taiwan; [2]Graduate Institute of Cancer Biology, [3]the Ph.D. program for Cancer Biology and Drug Discovery, China Medical University, Taichung 404, Taiwan; [4]Department of Biotechnology, Asia University, Taichung 413, Taiwan; [5]Department of Medical Research, E-Da Hospital, Kaohsiung 824, Taiwan; [6]Department of Biological Science & Technology, I-Shou University, Kaohsiung 824, Taiwan; [7]Department of Biology and Biochemistry, University of Houston, Houston, TX, 77204, USA; [8]Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA; [9]Graduate School of Biomedical Sciences, The University of Texas Health Science Center, Houston, TX 77030, USA; [10]Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. [*]These authors contributed equally and thus share first-authorship.

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Abstract: Sodium/glucose co-transporter 1 (SGLT1), which actively and energy-dependently uptakes glucose, plays critical roles in the development of various diseases including diabetes mellitus and cancer, and has been viewed as a promising therapeutic target for these diseases. Protein-protein interaction with EGFR has been shown to regulate the expression and activity of SGLT1. Exogenous expression of SGLT1 is one of the essential approaches to characterize its functions; however, exogenously expressed SGLT1 is not firmly detectable by Western blot at its calculated molecular weight, which creates a hurdle for further understanding the molecular events by which SGLT1 is regulated. In this study, we demonstrated that exogenous SGLT1 functions in glucose-uptake normally but is consistently detected near the interface between stacking gel and running gel rather than at the calculated molecular weight in Western blot analysis, suggesting that the overexpressed SGLT1 forms SDS-resistant aggregates, which cannot be denatured and effectively separated on SDS-PAGE. Co-expression of EGFR enhances both the glucose-uptake activity and protein level of the SGLT1. However, fusion with Flag or HA tag at its carboxy- but not its amino-terminus abolished the glucose-uptake activity of exogenous SGLT1 without affecting its protein level. Furthermore, the solubility of SGLT1 aggregates was not affected by other detergents but was partially improved by inhibition of o-link glycosylation. These findings suggested exogenous overexpression of SGLT1 can function normally but may not be consistently detectable at its formula weight due to its gel-shift behavior by forming the SDS-resistant aggregates.

Keywords: Sodium/glucose cotranspoter 1, epidermal growth factor receptor, protein aggregation, glucose uptake, o-link glycosylation

Introduction

Due to rapid growth and proliferation, cancer cells increase their needs for glucose and nutrients. Cancer cells also consume more glucose than normal cells by switching how they produce energy. Unlike the efficient generation of 38 ATP molecules per glucose by oxidative phosphorylation pathway in normal cells, cancer cells predominantly convert one glucose into two ATPs via glycolysis pathway even in the presence of oxygen, a phenomenon called aerobic glycolysis or the Warburg effect [1].

To meet the higher glucose demand, increased ability to engulf glucose is developed in many cancer types as measured by ^{18}fluorodeoxyglucose positron-emission tomography [2]. Increases in glucose uptake in cancer cells can be achieved by the upregulation of non-energy-dependent glucose transporters (GLUTs) [3]. Enhanced expression of GLUT transporters has

been observed in many cancers, including breast, prostate, brain, lung, and cervical cancer and is associated with poor prognosis [4-8]. Relatively higher GLUT1 expression level is correlated with higher grade and proliferative activity [9, 10]. In addition to GLUTs, expression of active energy-dependent sodium/glucose cotransporters (SGLTs) was also found to meet the demand for high glucose in cancer cells [11-19]. SGLT1 is mainly expressed in the microvilli of intestine and kidney and responsible for the absorption of sugars [20, 21], and somatic mutation of SGLT1 causes glucose/galactose malabsorption [22]. In addition to its physiological roles in luminal epithelium of small intestine and kidney, overexpression of SGLT1 has been repeatedly found in various cancer types, including pancreatic [11], prostate [12, 13], colorectal [14, 15], oral [16-18], and lung [19] cancer.

SGLT1 expression in cancer cells has been reported to prevent autophagic cell death by maintaining intracellular glucose level [12] and is significantly correlated with disease-free survival and overall survival rate [11, 15]. SGLT1 but not GLUT1 is abundantly co-expressed with epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, in multiple cancer types [12, 15, 17]. Interestingly, knockdown of EGFR protein expression but not inhibition of its kinase activity decreases SGLT1 expression and intracellular glucose level, unveiling a kinase-independent role of EGFR in maintaining sufficient glucose supply for tumor growth through protein-protein interaction and stabilization of SGLT1 protein [12]. The new kinase-independent role of EGFR in promoting metabolic homeostasis in cancer cells has also been proposed as a potential mechanism conferring resistance to EGFR tyrosine kinase inhibitors [23], although this remains to be confirmed by further investigations. These findings also uncovered important roles of SGLT1 in tumor progression and drug resistance, and further studies of SGLT1 regulations by physical interaction with oncogenic receptors are worth pursuing.

Enforced overexpression of interested genes by transient transfection is a common strategy to investigate protein-protein interaction. Most proteins are separated according to their molecular sizes by SDS-PAGE. However, exceptional proteins, especially glycosylated transmembrane [24, 25] and sulfitoylsed [26] proteins, are known to have a high tendency to form aggregates and exhibit anomalously retarded SDS-PAGE mobility in non-reducing gels. This mobility shift can be explained by the formation of SDS-resistant aggregates [27] and has been used to evaluate the stability and activity of these proteins. From data obtained by freeze-fracture electron microscopy, SGLT1 expressed in *Xenopus laevis oocytes* exists as a asymmetrical monomer containing 15 ± 2 transmembrane helices [28]. However, it remains unclear whether exogenously overexpressed SGLT1 forms SDS-resistant aggregates like other transmembrane proteins. In this study, we found that the protein level and glucose transporter activity of SGLT1 were increased by EGFR. However, we also observed a mobility retardation of SGLT1 on SDS-PAGE and found that the SDS-resistant aggregations of SGLT1 may be, at least in part, attributed to O-link but not N-link glycosylation. These results bring attention to interpretation of Western blot analysis for exogenous SGLT1.

Materials and methods

Cell line and reagents

HEK293 cell line was maintained in DME/F-12 supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin under 5% CO_2 at 37°C. The human SGLT1 expression plasmid, SGLT1-IRES-GFP, was a kind gift from Dr. Wright (Department of Physiology, UCLA). The SGLT1 siRNA and transfection reagent were purchased from Thermo Scientific Dharmacon; rabbit anti-HA-tag antibody (sc-805) from Santa Cruz Biotechnology; mouse anti-HA-tag antibody (H3663), mouse anti-Flag-tag antibody (F1804) and mouse anti-alpha tubulin antibody (T6074) from Sigma-Aldrich; mouse anti-myc-tag antibody (11667149001) from Roche; GST-SGLT1 recombinant protein (H00006523-P01) from Abnova; [^{14}C] a-methyl-D-glucopyranoside (aMDG) (NEC720A050UC) from Perkin Elmer; phloridzin dehydrate (79320) from Sigma-Aldrich.

Generation of anti-SGLT1 antibody

A fragment of human SGLT1 (amino acids: 589-604, IETQVPEKKKGIFRR) was used as an antigen to raise the antibody. The antigen and anti-

body were manufactured by LTK BioLaboratory (Taoyuan, Taiwan).

Construction of N-terminal and C-terminal Flag-tagged SGLT1

To construct the N-terminal Flag-tagged SGLT1, the human SGLT1 full-length cDNA was amplified with the following primers: 5'-ATAGAATTCATGGACAGTAGCACCTGGAG-3' and 5'-CGCGTCGACTCAGGCAAAATATGCATG-3'. The DNA fragments were digested with EcoR I and Sal I and cloned into the pCMV-Tag 2B vector (Stratagene). To construct the C-terminal HA-tagged or Flag-tagged SGLT1, SGLT1 full-length cDNA was digested with Xba I and Age I and then cloned into the pcDNA6-HA and pcDNA6-Flag plasmids, respectively.

Transient transfection

To test the interaction between SGLT1 and EGFR, HEK293 cells were cultured in 10 cm dishes to 80% confluence with DME/F-12 containing 10% FBS and 1% Pen/Strep for 24 hours prior to cotransfection. Cells were cotransfected with SGLT1 and EGFR for 24 hours, and then harvested and subjected to Western blot (WB) and immunoprecipitation was performed with indicated antibodies. For knockdown of SGLT1, cells were cotransfected with or without SGLT1 siRNA by Dharmafect transfection reagent. The cells were harvested after transfection for 48 hours and were subjected to WB analysis.

RNA extraction, reverse transcription (RT), and polymerase chain reaction (PCR)

Total RNA was isolated from transfected HEK293 cells by TriPure Isolation Reagent (Roche) according to the manufacture's protocol. Total RNA (2 μg) was subjected to reverse transcription with M-MLV reverse transcriptase (Invitrogen) and oligo dT to synthesize the first strand cDNA, and 10% of the cDNA was added as template in each PCR reaction. PCR primer sequences for SGLT1 are 5'-TTCCACATCTTCCGAGATCC-3' and 5'-GGACGACACAGGCAATTTTT-3'.

Western blot analysis and competition assay

For WB analysis, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% TritonX-100, 1 mM EDTA, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatine, 1% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, in H_2O). Proteins separated by 8% SDS-PAGE were transferred to PVDF membrane followed by blocking with 5% nonfat dry milk and then incubation with primary antibodies overnight at 4°C. The membranes were washed with 0.1% TBS/T (1XTBS, 0.1% Tween-20) 3 times, each time for 5 min before incubation with secondary antibody. For competition assay, the PVDF membranes were incubated with anti-SGLT1 antibody and/or SGLT1 peptide (50 mg/ml) at 37°C for 30 min followed by incubation with secondary antibody.

Immunoprecipitation

Cells were lysed with RIPA buffer, and lysates containing 1 mg protein were incubated with mouse anti-HA antibody (1 μg) or mouse IgG (1 μg) overnight at 4°C. Then, 40 μl protein A/G beads were added to precipitate the anti-SGLT1 complex. The immunoprecipitates were denatured by sample buffer and subjected to WB analysis.

[^{14}C] a-methyl-D-glucopyranoside (αMDG) uptake assay

The activity of SGLT1 in cells was determined by measuring the uptake of αMDG, a specific substrate for SGLT1. Cells were seeded in a 24-well plate and transfected with the indicated plasmids for 24 hr. Prior to αMDG uptake assays, cells were pre-treated with or without 100 μM phloridzin, an SGLT1-specific inhibitor for 30 min. After treatment of phloridzin, cells were washed with PBS once and incubated with Krebs–Ringer–Henseleit (KRH) solution (120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM HEPES, titrated to pH 7.4 with Tris) containing [^{14}C] αMDG (0.5 μCi/ml) in the presence or absence of 100 μM phloridzin.

Results and discussion

Exogenously expressed SGLT1 forms SDS-resistant aggregates on immunoblot

To examine whether aggregation of overexpressed SGLT1 can be observed on the immunoblot, carboxy-terminal HA-tagged SGLT1 (SGLT1-HA) was transiently co-transfected with

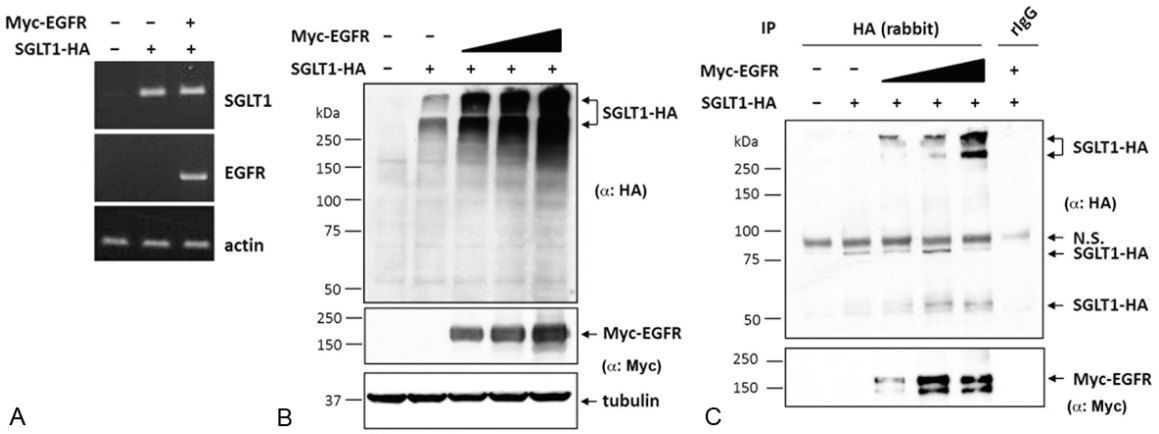

Figure 1. Overexpression of SGLT1 by EGFR shows abnormally retarded mobility on SDS-PAGE. A: Total RNA was extracted from HEK-293 cells transfected with myc-EGFR and/or SGLT1-HA and subjected to reverse transcription followed by PCR reaction with primers against indicated genes. B and C: Total lysate were prepared from HEK-293 cells co-transfected with SGLT1-HA and increasing amount of EGFR and subjected to WB analysis and immunoprecipitation with indicated antibodies. N.S. presents the non-specific signal.

or without myc-tagged EGFR (myc-EGFR) into HEK-293 cells. The transcription of SGLT1-HA driven by the CMV promoter was detectable in HEK-293 cells transfected with SGLT1-HA cDNA alone (**Figure 1A**, lane 2). However, using whole cell lysates, the SGLT1-HA protein detected by WB analysis with anti-HA antibodies did not appear at the formula weight (55 kDa) [29], but is seen as two major bands with high molecular size of over 250 kDa (**Figure 1B**). Moreover, co-transfection with myc-EGFR dose-dependently increased the protein level of SGLT1 (**Figure 1B**) but not its mRNA level (**Figure 1A**, lane 3). These results suggest that exogenously expressed SGLT1 in HEK-293 cells forms SDS-resistant aggregates on the Western blot and that myc-EGFR increases the protein levels of SGLT1. Interestingly, after the SGLT1-HA was enriched by immunoprecipitation using anti-HA antibodies, it appeared at both its formula weight and the high-molecular-weights of the SDS-resistant aggregates. Regardless, both forms of SGLT1 were increased by myc-EGFR (**Figure 1C**). These results indicate that exogenous SGLT1-HA cannot be denatured by SDS and forms aggregates in SDS-PAGE, similar to other multiple transmembrane protein reported previously [24, 25].

To further confirm that the higher-molecular-weight bands correspond to the exogenous SGLT1 protein, we generated an anti-SGLT1 antibody as previously described [30] and used it to detect the SDS-resistant forms. The anti-SGLT1 antibody recognized the recombinant GST-fused SGLT1 but not GST alone at both the high molecular weight of over 250 kDa and the predicted molecular weight around 80 kDa, and these signals were blocked by the antigen peptide of SGLT1 (**Figure 2A**). These results not only validate the specificity of this antibody but also indicate that the purified recombinant GST-SGLT1, similar to the exogenously expressed SGLT1-HA in HEK-293 cells, is present predominantly as the SDS-resistant aggregates on the Western blot. This anti-SGLT1 antibody also recognizes both amino-terminal Flag-tagged and un-tagged SGLT1 aggregates expressed in HEK-293 cells (**Figure 2B**). The anti-Flag antibody only detected Flag-tagged but not the un-tagged SGLT1 (**Figure 2C**). Furthermore, the recognition of Flag-SGLT1 by the anti-SGLT1 antibody can be blocked by the antigen peptides (**Figure 2D**) and siRNA (**Figure 2E**) against SGLT1. All these results suggest that exogenously expressed SGLT1 forms SDS-resistant aggregates with an anomalous gel shift on the SDS-PAGE. Since SGLT1 proteins without tag (**Figure 2B**) or with HA-tag (**Figure 1B**) or Flag-tag (**Figure 2B**) at the carboxy- or amino-terminus all showed the gel-shift behavior on SDS-PAGE, fusion tags likely did not cause the aggregation of SGLT1. Unexpectedly, Flag-tag fusion at both the amino- and the carboxy-termini of SGLT1 (Flag-SGLT1-Flag) greatly prevented the formation of SGLT1 aggregates (**Figure 2F**). GLUT1 has been shown to form oligomers in the plasma membrane of erythro-

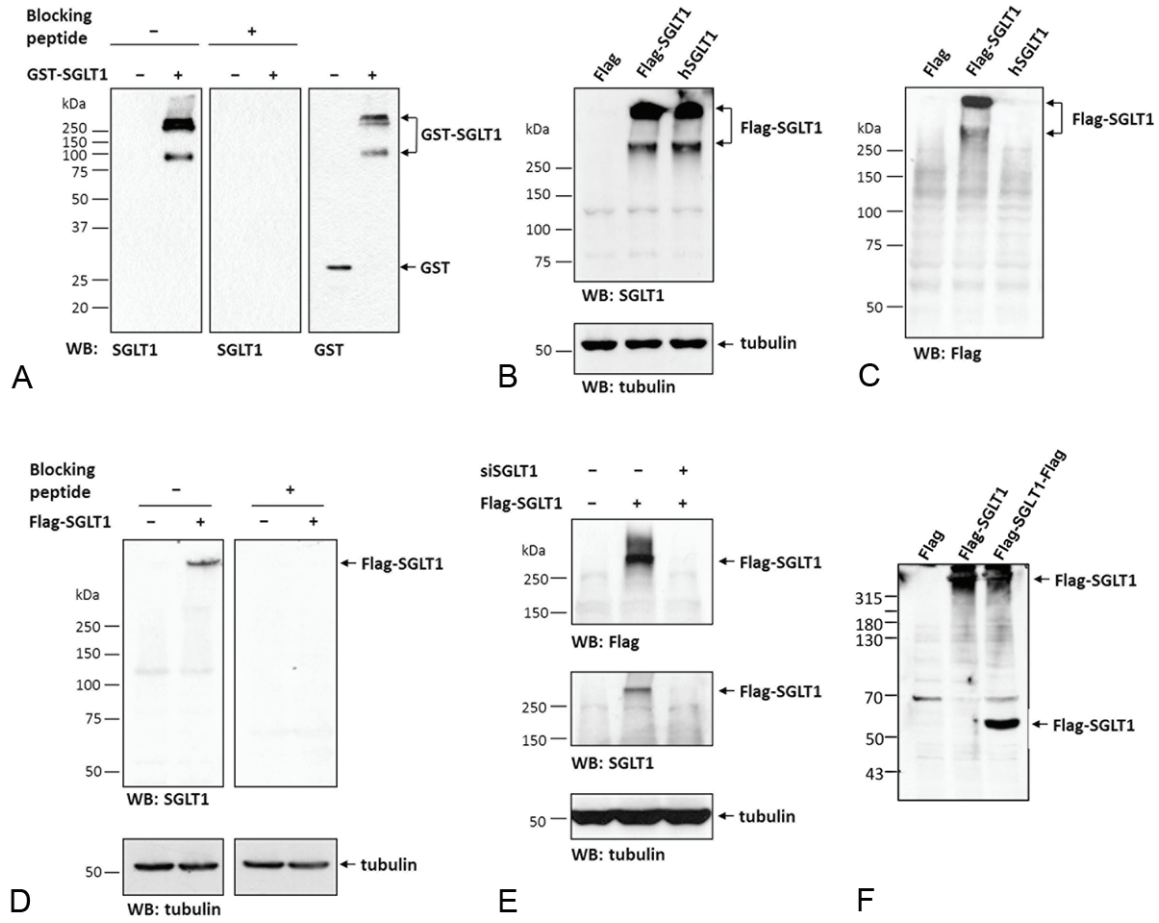

Figure 2. SGLT1 forms SDS-resistant aggregates. A: Recombinant GST-fused SGLT1 and GST were subjected to WB analysis with anti-SGLT1 antibody in the presence or absence of SGLT1 antigen peptide. B and C: Total lysates were prepared from HEK-293 cells transfected with Flag-SGLT1, SGLT1, or Flag-tagged empty vector and subjected to WB analysis with anti-SGLT1 (B) or anti-Flag antibodies. D: Total lysates from HEK-293 cells transfected with or without Flag-SGLT1 were subjected to WB analysis with anti-SGLT1 antibodies in the presence or absence of SGLT1 antigen peptide. E: Total lysates from HEK-293 cells co-transfected with Flag-SGLT1 and SGLT1 siRNA were subjected to WB analysis with anti-SGLT1 or anti-Flag antibodies. F: Total lysates from HEK-293 cells transfected with Flag-SGLT1 or Flag-SGLT1-Flag were subjected to WB analysis with anti-Flag antibodies.

cyte [31], and overexpressed SGLT1 in the plasma membrane has been shown to form asymmetrical monomer in *Xenopus laevis* oocytes using freeze-fracture electron microscopy analysis [28]. These results indicated that SGLT1 may not aggregate in cells; instead, it forms aggregates only after being extracted from cells.

The SGLT1-mediated glucose uptake is enhanced by EGFR

To determine whether exogenously expressed SGLT1 retains its proper biological activity, we analyzed the glucose uptake activity by measuring the uptake of [^{14}C] αMDG, a specific substrate for SGLT1. As shown in **Figure 3A**, untagged SGLT1 exhibited apparent α-MDG-uptake activity, and this activity was blocked by phloridzin, an SGLT1-specific inhibitor. These results indicate that exogenously expressed SGLT1 retains glucose uptake activity even though it is present as SDS-resistant aggregates on SDS-PAGE. The α-MDG uptake activity of SGLT1 was further enhanced by overexpression of EGFR (**Figure 3B**), supporting our previous findings that EGFR stabilizes SGLT1 expression and enhances SGLT1-mediated glucose uptake [12]. Interestingly, the αMDG-uptake activity was observed in HEK-293 cells trans-

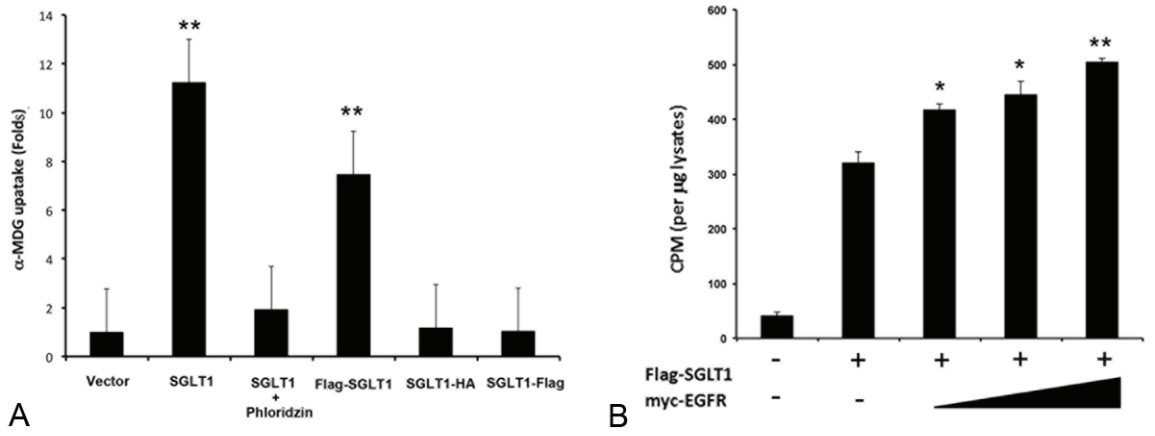

Figure 3. EGFR enhances the glucose transport activity of SGLT1. A: Whole cell lysates from HEK-293 cells transfected with indicated SGLT1 plasmids or treated with or without phloridzin were subjected to αMDG uptake assay. B: Whole cell lysates from HEK-293 cells co-transfected with Flag-SGLT1 and increasing amount of myc-EGFR were subjected to αMDG uptake assay.

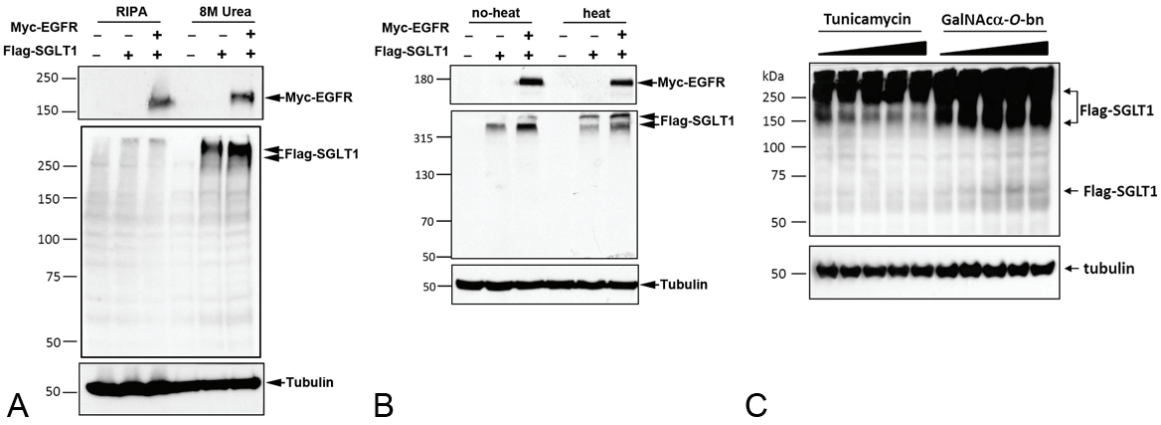

Figure 4. O-glycosylation contributes to SGLT1 aggregation. A: Total lysate of HEK-293 cells, which were transfected with myc-EGFR and/or Flag-SGLT1, were prepared with RIPA buffer or 8 M urea followed by WB analysis with anti-myc or anti-Flag antibodies. B: Total lysate of HEK-293 cells, which were co-transfected with Flag-SGLT1 and Myc-EGFR, were prepared with or without heat denaturation followed by WB analysis with anti-Flag and anti-myc antibodies. C: HEK-293 cells transfected with Flag-SGLT1 were treated with increasing concentration of tunicamycin or GalNAc(α)-O-bn for 24 hr and then subjected to WB analysis with anti-Flag antibody.

fected with N-terminal Flag-tagged SGLT1 (Flag-SGLT1) but not in the cells transfected with the C-terminal HA- or Flag-tagged SGLT1 (SGLT1-HA and SGLT1-Flag), indicating that tag fusion to the C-terminus of SGLT1 abolished its function in transporting glucose (**Figure 3A**).

O-glycosylation contributes to the anomalous SDS-PAGE migration of SGLT1

Next, we asked which factor is involved in the anomalous mobility of SGLT1 on SDS-PAGE. Severe solubility problems frequently hamper WB analysis of multi-transmembrane proteins. It is likely that the transmembrane domains of SGLT1 might be responsible for its poor solubility and the formation of SDS-resistant aggregates. Aiming to disassociate the SDS-resistant aggregates of SGLT1, we included 8M urea and Triton X-100 into the protein extraction buffer however, none of these reagents were able to reduce the aggregation of SGLT1 on SDS-PAGE (**Figure 4A** and data not shown). Heat denaturation in acidic condition and organic solvent

has also been reported to trigger the SDS-resistant aggregation of membrane proteins through polymerization of partially unfolded molecules in the presence of SDS. However, our data indicated that SGLT1 protein freshly prepared from cells without heat denaturation still forms SDS-resistant aggregates (**Figure 4B**). Furthermore, the use of anhydrous trifluoroacetic acid (TFA), a strong acid that has been shown to promote de-aggregation [25], did not affect the aggregation of SGLT1 (data not shown). A study showed that SGLT1 with a single mutation at Arg135 migrates as two aggregated forms at higher molecular weights [32]. The SGLT1 cDNA used in this study does not contain any mutation (data not shown), which rules out the possibility that SDS-resistant SGLT1 aggregation is caused by mutations.

Glycosylation influences protein folding and aggregation of membrane proteins [33]. SGLT1 is known to be N-link glycosylated at Asn248 [34], but its O-link glycosylation has not been identified yet. Sequential removal of N- and O-link glycosylation by treatment with PNGase-F and O-glycosidase, respectively, *in vitro* slightly reduced the apparent molecular size of SGLT1 on SDS-PAGE [35, 36], indicating that glycosylation might cause the retarded mobility of SGLT1. Thus, we next examined whether glycosylation contributes to the formation of SDS-resistant SGLT1 aggregates. Interestingly, treatment with tunicamycin, which inhibits N-linked oligosaccharide chain addition to nascent polypeptides, had no effect on SGLT1 aggregation (**Figure 4C**). However, inhibition of O-link glycosylation by GalNAc(α)-O-bn increased the levels of SGLT1 at lower-molecular-weights including those at 55 kDa, although most of SGLT1 was still present in the aggregated forms (**Figure 4C**). These results suggest that O-glycosylation but not N-glycosylation may, at least in part, contribute to the aggregation of exogenously expressed SGLT1. Other determinants that cause aggregation of SGLT1 remain to be further investigated.

In summary, this study not only supported previous findings that EGFR can enhance the protein expression and glucose transport activity of SGLT1 through protein-protein interaction but also revealed a retarded mobility of SGLT1 on SDS-PAGE. Although the detailed mechanisms await further investigation, the SDS-resistant aggregation of SGLT1 is likely attributed in part to O-link glycosylation. These results also bring our awareness to a more careful data interpretation of WB analysis for SGLT1.

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Address correspondence to: Wei-Chien Huang, Center for Molecular Medicine, China Medical University Hospital, 9F, No.6, Hsueh-Shih Road, Taichung, 404 Taiwan. Phone: 886-4-22052121 ext 7931; Fax: 886-4-22333496; E-mail: whuang@mail.cmu.edu.tw; Mien-Chie Hung, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard Houston, TX 77030. Phone: 713-792-7477; Fax: 713-794-3270; E-mail: mhung@mdanderson.org

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