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The Degree of N-glycosylation Affects the Trafficking and Cell Surface Expression Levels of Kv1.4 Potassium Channels

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Abstract Kv1.4 potassium channels are heavily glycosylated proteins involved in shaping action potentials and in neuronal excitability and plasticity. Kv1.4 N354Q, without an N-glycan, exhibited decreased protein stability and trafficking to the cell surface (Watanabe et al. in J Biol Chem 279:8879–8885, 2004). Here we investigated whether the composition of the N-glycan affected Kv1.4 cell surface expression. Kv1.4 proteins carrying N-glycans with different compositions were generated by adding glycosidase inhibitors or using N-glycosylation-deficient mutant cell lines. We found that oligomannose-type, hybrid-type, or incomplete complex-type N-glycans had a negative effect on surface protein expression of Kv1.4 compared with complex-type N-glycans. The decrease in surface protein level of Kv1.4 was mainly due to a reduction in total protein level, induced by altered N-glycan composition. Kv1.4 in CSTP-treated cells carried a unique oligomannose-type N-glycan that contains three glucose residues. This N-glycan had the most negative effect on cell surface expression of Kv1.4. It decreased Kv1.4 surface protein level by a combined mechanism of reducing total protein level and increasing ER-retention. Our data suggest that composition of the N-glycan plays an important role in protein stability and trafficking, and a sialylated complex-type N-glycan promoted high cell surface expression of Kv1.4.

Keywords Potassium channel · N-glycosylation · Trafficking · Cell surface expression

Abbreviations

CSTP	Castanospermine
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
MMN	1-Deoxymannojirimycin
Kv	Voltage-gated potassium channel
RSE	Relative surface expression
SWSN	Swainsonine

Introduction

Membrane glycoproteins are synthesized in the rough endoplasmic reticulum (ER) and undergo glycosylation modifications that occur either co-translationally or post-translationally. N-linked glycans are attached to the asparagine residue in the consensus sequence of NXT(S), where X is any amino acid except proline. N-glycans are processed further in the Golgi apparatus to form an oligomannose, hybrid, or complex structure. N-glycosylation plays an important role in numerous membrane proteins for folding, stability, trafficking, and interaction, as well as signaling (Molinari 2007; Napp et al. 2005; Roy et al. 2010; Zhao et al. 2008) and is involved in the normal cellular function and

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contributes to some diseases. Abnormality in N-glycosylation has been associated with congenital disorders (Eklund and Freeze, 2006), immune diseases (Rudd et al. 2001), and cancer (Varki et al. 2009).

Potassium channels are a diverse group of membrane proteins that conduct K^+ ions through the cell membrane. Voltage-gated K^+ (Kv) channels open and close upon changes of the transmembrane potential and are important in the physiology of both excitable and non-excitable cells. The roles of Kv channels in excitable cells are well established, including modulating resting membrane potential, action potential shape and duration, and neurotransmitter release. Kv channels function as either homotetramers or heterotetramers, and some of them are modulated by auxiliary proteins. A Kv channel α subunit consists of six transmembrane segments (S1–S6), which connected by linkers and flanked by intracellular N- and C-termini. S1–S4 forms the voltage sensor domain, whereas S5–S6 contributes to the central pore. The Kv1 subfamily comprises at least eight members, Kv1.1–Kv1.8. Each of them contains one or two N-glycosylation consensus sites on the extracellular S1–S2 linker, except for Kv1.6. Kv1.1–Kv1.5 have been shown to be *N*-glycosylated at the corresponding site (Zhu et al. 2001, 2003, 2012, Schwetz et al. 2010), whereas Kv1.7 and Kv1.8 have not been examined. N-glycosylation had differential effects on cell surface expression and function of Kv1 subfamily members. Removal of N-glycosylation decreased cell surface expression level of Kv1.2, Kv1.3, and Kv1.4, but had no effect on Kv1.1 in transfected cell lines (Watanabe et al. 2004, 2007; Zhu et al. 2012). Removal of N-glycosylation altered gating properties of Kv1.1, Kv1.2, and Kv1.5, but had no effect on Kv1.4 in transfected cell lines (Thornhill et al. 1996; Watanabe et al. 2003, 2004; Schwetz et al. 2010). Furthermore, the location and composition of an N-glycan also affected Kv1 channel cell surface expression level (Zhu et al. 2009, 2012). These findings indicate the importance of N-glycosylation in modulating Kv1 expression and function and the complexity of the mechanisms involved.

Kv1.4 is a unique member of the Kv1 subfamily due to its fast inactivation properties (Rasmusson et al. 1995). It is the sole Kv1 subtype expressed in smaller diameter dorsal root ganglion neurons (Rasband et al. 2001), indicating the potential clinical importance. Kv1.4 contains one N-glycosylation consensus site (NDT) on its S1-S2 linker and is heavily *N*-glycosylated. Mutation of the NDT site (Kv1.4N354Q) resulted in a significant reduction in protein stability, trafficking efficiency, and cell surface expression of Kv1.4 (Watanabe et al. 2004). However, the effects of N-glycan composition in ion channel trafficking and cell surface expression is still not well known.

In this study, we used two different methods, blocking N-glycosylation pathway by adding specific glycosidase inhibitors or using N-glycosylation-deficient mutant cell lines, to modify N-glycan composition. We aimed to investigate whether altered N-glycan composition affects Kv1.4 trafficking and cell surface expression to gain additional insights into the role of N-glycans in ion channel biology.

Materials and Methods

Cell Culturing

Chinese hamster ovary (CHO) pro5 cells are the parentals of the Lec mutants. CHOpro5 cells will be referred to in the text as CHO cells. N-glycosylation-deficient CHOpro5 mutant cells, Lec1, Lec8, and Lec2, and COS7 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in α-MEM medium, supplemented with 0.35 mM proline, 2 mM glutamine, 100 I.U. penicillin/streptomycin, and 10 % (v/v) fetal bovine serum) at 37 °C under 5 % CO_2.

cDNA and Transfection

Kv1.4 has one extracellular NDT at N354DT. Kv1.4N354Q mutation was generated using replication mutagenesis, as described previously (Zhu et al. 2001). Integrity of constructs was confirmed by DNA sequencing. Transient transfection was performed according to manufacturer's protocol. For biotinylation and immunoblotting, CHO or Lec cells were seeded directly onto wells and transfected the next day at ~80 % confluence. Cells were rinsed with DMEM alone and transfected for 3 h with 0.5 μg cDNA per well using Lipofectamine Plus (Life Technologies, Gaithersburg, MD). Cell transfection efficiency was routinely checked by cotransfection with 0.1 μg cDNA for green fluorescent protein (GFP). For immunofluorescence, COS7 or CHO cells were seeded onto UV-sterilized glass coverslips in 35 mm wells and transfected the next day at ~50 % confluence.

Total Membrane Isolation and Glycosidase Treatment

Total membrane proteins were isolated from cells as described previously (Zhu et al. 2001). Glycosidase treatment was performed according to manufacturer's protocol. Total membranes were digested with Endo H (0.16 μ/ml) or PNGase F (13 μ/ml) at 37 °C for 20 h before testing by immunoblotting.

Immunoblotting

Protein samples (~20 μg/gel lane) were fractionated on 9 % SDS-PAGE and transferred to PVDF membrane. The

membrane was blocked with 5 % nonfat milk in PBS and probed with Kv1.4 mouse monoclonal antibodies (1:1000 dilution) (UC Davis/NINDS/NIMHNeuroMab Facility, Davis, CA) at 4 °C overnight. For total protein immunoblots, actin monoclonal antibodies (Sigma-Aldrich, St. Louis, MO) were routinely used as a loading control. The membrane was washed with PBS/0.1 % Tween and then incubated for 1.5 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10000 dilution) (GE Healthcare Life Sciences, Pittsburgh, PA) and washed again. An enhanced chemiluminescent (ECL) substrate was added to detect HRP activity from antibodies. The membrane was exposed to Kodak XAR5 film and developed.

Cell Surface Biotinylation

After 15–20 h transfection, cells were washed with ice-cold PBS contained 0.7 mM $CaCl_2$/0.4 mM $MgCl_2$ and biotinylated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (Thermo Scientific, Waltham, MA) at 4 °C for 15 min. Cells were then washed with PBS containing 10 mM Tris (pH7.4) and solubilized with RIPA lysis buffer (120 mM NaCl, 50 mM Tris, 0.4 % DOC, 1.2 % Triton X-100, 400 μM PMSF, 10 μM leupeptin/pepstatin). Lysates were centrifuged at 14,000×*g* for 30 min at 4 °C to remove the nuclei. The supernatant was incubated with 50 μl streptavidin-agarose beads (Thermo Scientific, Waltham, MA) at 4 °C overnight. The beads were washed and eluted with 6x SDS sample buffer. Biotinylated proteins were separated on 9 % SDS-PAGE and immunobloted with anti-Kv1.4 antibodies.

Glycosylation Inhibitor Treatment

Glycosidase inhibitors castanospermine (CSTP), 1-deoxymannojirimycin (MMN), and swainsonine (SWSN) were purchased from EMD Millipore (Billerica, MA) and dissolved in ddH_2O as instructed. CHO cells were pre-incubated with CSTP (200 μg/ml), MMN (200 μg/ml), or SWSN (2 μg/ml) for overnight before transfection. During the 3 h transfection process, the inhibitors were omitted from the medium. After transfection, cells were cultured in fresh medium supplemented with inhibitors for 20–24 h. No apparent changes in cell morphology and density were observed under this condition. To eliminate non-specific effects of glycosidase inhibitors on protein synthesis, Kv1.4N354Q, a Kv1.4 mutant with NDT mutated, was used as a control and tested with all inhibitors in the same way (data not shown).

Immunocytochemistry

Transfected COS7 or CHO cells were fixed with 3 % paraformaldehyde in PBS for 10 min at room temperature and washed three times with PBS. Cells were then permeabilized by incubating with PGBA buffer (PBS plus 0.1 % gelatin, 1 % BSA, 0.05 % sodium azide) containing 2 % normal goat serum and 0.1 % Triton X-100 on a rocker for 30 min. The solution was replaced with PGBA buffer containing Kv1.4 monoclonal antibodies (1:1000 dilution) overnight on a rocker at 4 °C. The cells were washed three times with 1 % Triton X-100 in PBS and incubated with AlexaFluor-conjugated secondary goat anti-mouse antibody (1:500 dilution) (Nova Biologicals, Littleton, CO) for 1 h on a rocker at room temperature. After rinsing with 1 % Triton X-100 in PBS, the coverslips were mounted onto glass slides using Slow Fade Antifade Reagent (Molecular Probes, Eugene, OR) and sealed. Fluorescent cells were analyzed at 1000X magnification using an Olympus BX-50 microscope equipped for epi-fluorescence illumination.

Detergent Solubility Assay

Transfected cells were lysed in 500 μL of hypotonic buffer containing 50 mM Tris-buffered saline, pH 8.0, 5 mM EDTA, 1 mM iodoacetamide, and a protease inhibitor mixture (2 μg/mL aprotonin, 1 μg/mL leupeptin, 2 μg/mL antipain, 10 μg/mL benzamidine, and 0.2 mM PMSF) and either 0 % or 0.1 % Triton X-100. Lysates were centrifuged at 4 °C for 5 min at 14,000×*g*. The soluble fractions were diluted with an equal volume of 2X SDS sample buffer. Pellet fractions were washed three times with ice-cold PBS and mixed with 1X SDS sample buffer to match the volume of the diluted soluble fraction. Samples were boiled and separated on 9 % SDS-PAGE and immunoblotted with anti-Kv1.4 antibodies.

Electrophysiology

Whole cell patch clamping of cultured cells has been described in details previously (Zhu et al. 2001). Cells were cotransfected with 0.5 μg Kv1.4 cDNA and 0.1 μg GFP cDNA to allow visualization of transfected cells by fluorescence microscopy. Patch pipettes had tip resistances of 1.5–2.5 mΩ when filled with internal solution. The bath solution contained 150.0 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 5.0 mM glucose, and 10.0 mM HEPES (pH 7.3; NaOH). The intracellular solution contained 70.0 mM KCl, 65.0 mM KF, 5.0 mM Na, 1.0 mM $MgCl_2$,10.0 mM EGTA, 5.0 mM glucose, and 10.0 mM HEPES (pH 7.3; KOH). Kv1.4 currents were recorded by Axopatch 200B amplifier. Currents were elicited by depolarizing steps from −80 to +50 mV in 10 mV increments every 80 ms from a holding potential of -80 mV. The voltage induced currents were filtered at 5 kHz. Linear leak currents and residual capacity transients were subtracted

using P/4 protocol. Data were analyzed using Pclamp8 software. Maximum conductance values were obtained from the peak leak-subtracted current using Ohm's law. A predicted Nernst K^+ equilibrium potential is −83 mV. Conductance density was calculated by maximum conductance divided by capacitance values.

Results

Kv1.4 in Wild Type CHO Cells has Both Oligomannose-Type and Complex-Type N-glycans

To investigate the N-glycosylation state of Kv1.4 proteins (Fig. 1a), rat Kv1.4 cDNA was transiently transfected into CHO cells, and total membranes, including ER, Golgi, and plasma membranes, were isolated for immunoblotting. Kv1.4 proteins were detected as two bands with molecular masses of ~110 and ~80 kDa, respectively (Fig. 1b). Peptide-N-glycosidase F (PNGase F) and endoglycosidase H (Endo H) were used to analyze N-glycans. PNGase F removes the entire N-glycan from the protein, whereas Endo H removes oligomannose- and hybrid-type glycans, but not complex-type glycans. Both ~110 and ~80 kDa bands were sensitive to PNGase F, suggesting that both of them contained N-glycans (Fig. 1b). Furthermore, the ~110 kDa band was insensitive to Endo H, whereas the ~80 kDa band was sensitive to Endo H, indicating that the former contained complex-type N-glycans and the latter contained oligomannose- or hybrid-type N-glycans (Fig. 1b). Since CHO cells synthesize a broad range of complex- and oligomannose-type N-glycans and very few hybrid-type N-glycans (North et al. 2010), we speculate that the ~80 kDa band contains only oligomannose-type N-glycans. Thus, it appears that Kv1.4 in CHO cells consists of two populations, one with oligomannose-type and one with complex-type N-glycans.

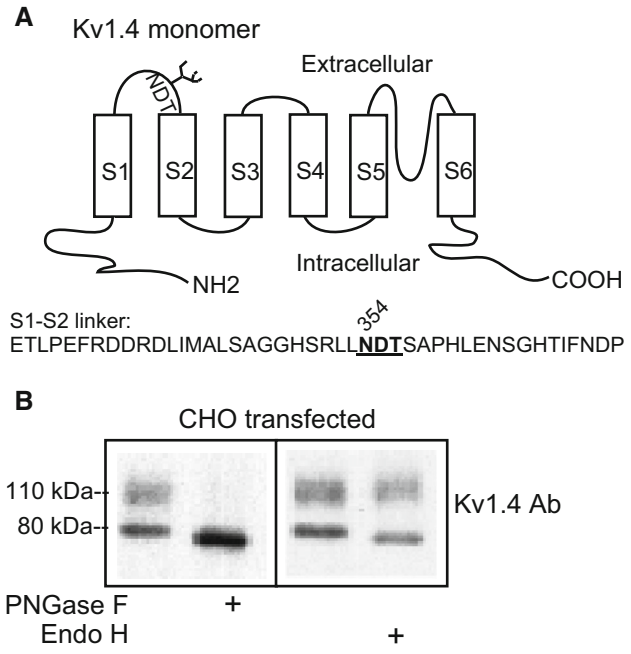

Fig. 1 Glycosidase digestion of Kv1.4 in CHO cells. **a** Schematic of a Kv1.4 monomer with six putative transmembrane domains (S1–S6). An N-glycan was attached to the N-glycosylation consensus site (NDT) at the extracellular S1–S2 linker. Amino acid sequence of the S1–S2 linker with the consensus site at 354 highlighted in bold. **b** Glycosidase digestion of Kv1.4 proteins in CHO cells. Endo H cleaves oligomannose- and hybrid-type N-glycans, whereas PNGase F cleaves oligomannose-, hybrid- and complex-type N-glycans. +, indicates glycosidase digestion

Kv1.4 N-glycan Composition is Altered by Specific Glycosidase Inhibitors

N-glycosylation begins when a multisubunit protein complex transfers a preassembled 14-sugar glycan, $Glc_3Man_9GlcNAc_2$, from dolichol pyrophosphate (Dol-P–P) precursor to NXT(S) sequons in newly synthesized regions of proteins. The protein-bound N-glycan is subsequently trimmed, extended, and modified by a series of processing reactions in the ER and the Golgi apparatus. The α-glucosidase I initiates the processing by cleaving the terminal α1-2Glc residue from the 14-sugar glycan. CSTP inhibits α-glucosidase I activity (Fig. 2 (1)). In the presence of CSTP, N-glycans keep glucose residues but may trim away one or two mannose residues in the pathway. The predicted N-glycan structure is $Glc_3Man_{7-9}GlcNAc_2$ (Stanley et al. 2009). Kv1.4 in CSTP-treated cells showed one major band on immunoblots with a molecular mass of ~ 80 kDa. The ~ 80 kDa band was sensitive to both PNGase F and Endo H, indicating that it contains oligomannose- or hybrid-type glycans (Fig. 3a, lane 1-3). Since this band had a molecular mass similar to that of the lower band of wild type Kv1.4 (~80 kDa), we speculate that this band contains only oligomannose-type N-glycans. Thus, in the presence of CSTP, Kv1.4 had oligomannose-type N-glycans, probably with the structure $Glc_3Man_{7-9}GlcNAc_2$.

The majority of glycoproteins exiting the ER to the Golgi carry N-glycans with the structure $Man_8GlcNAc_2$. Cis-Golgi α1-2 mannosidase I removes α1-2 Man residues from the 10-sugar glycan to produce $Man_5GlcNAc_2$, a key intermediate in the pathway to hybrid- or complex-type N-glycans (Stanley et al. 2009). 1-Deoxymannojirimycin (MMN) inhibits cis-Golgi α1-2 mannosidase I (Fig. 2 (2)). In the presence of MMN, $Man_8GlcNAc_2$, the oligomannose-type 10-sugar glycan would accumulate. Kv1.4 in MMN-treated cells showed one major band on an immunoblot with a molecular mass of ~80 kDa. This band was sensitive to both PNGase F and Endo H, indicating that it contains oligomannose- or hybrid-type glycans (Fig. 3a, lane 4-6). We speculate that this band contains oligomannose-type

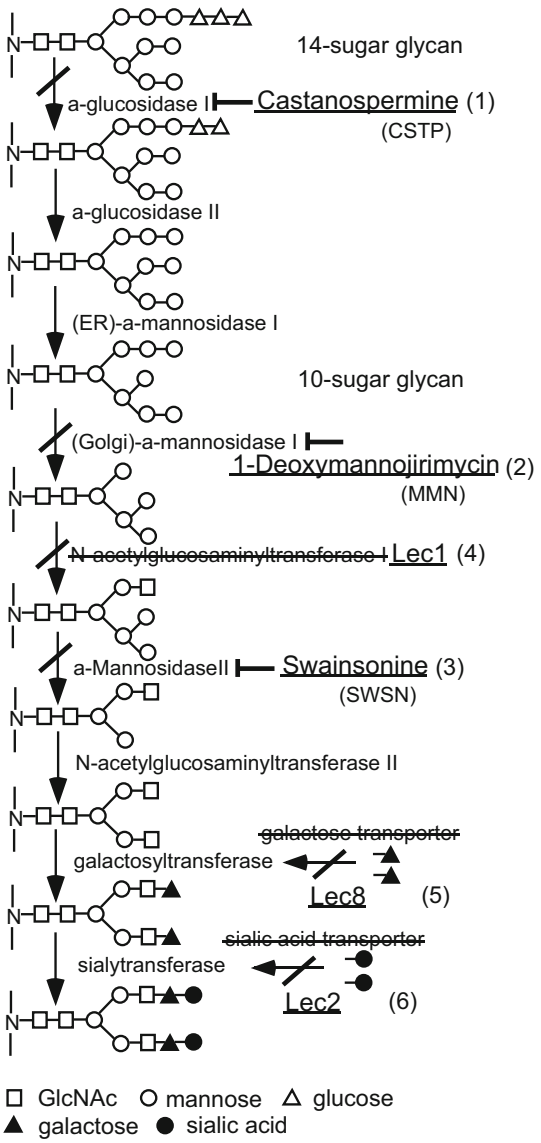

Fig. 2 Diagram of N-glycosylation pathway. The blocking sites of glycosidase inhibitors, CSTP, 1-deoxymannojirimycin (MMN), and SWSN, labeled (1), (2), and (3), respectively. The glycosylation defect sites of CHO mutant cells, Lec1, Lec8, and Lec2, labeled (4), (5), and (6), respectively

N-glycans, since it had a molecular mass similar to that of the lower band of wild type Kv1.4. Thus, in the presence of MMN, Kv1.4 carried oligomannose-type N-glycans, probably with structure of $Man_8GlcNAc_2$.

Medial-Golgi α mannosidase II removes the terminal α1-3Man and α1-6Man residues from $GlcNAcMan_5GlcNAc_2$ to form $GlcNAcMan_3GlcNAc_2$, an essential step in the pathway to generate complex-type N-glycans (Stanley et al. 2009). Inhibition of α mannosidase II would induce synthesis of hybrid-type N-glycans. SWSN is an inhibitor of medial-Golgi α mannosidase II (Fig. 2 (3)). Kv1.4 in SWSN-treated cells showed two bands on immunoblots with molecular masses of ~95 and ~80 kDa, respectively. Both bands were sensitive to PNGase F and Endo H, suggesting that they contain oligomannose- or hybrid-type N-glycans (Fig. 3a, lane 7–9). Because the lower band had a molecular mass similar to that of the lower band of wild type Kv1.4, it is speculated to contain oligomannose-type N-glycans. As for the upper band, it is speculated to contain hybrid-type N-glycans. Therefore, in the presence of SWSN, Kv1.4 consists of two populations, one with oligomannose-type N-glycans and one with hybrid-type N-glycans.

Kv1.4 N-glycan Composition Affects its Surface Protein Level

We examined next whether altered N-glycan composition affects Kv1.4 cell surface protein level. Kv1.4 proteins were expressed in CHO cells in the presence or absence of glycosidase inhibitors. Surface protein level was estimated by cell surface biotinylation and immunoblotting. Surface conductance density was estimated by whole cell patch clamping. The surface protein level of Kv1.4 in CSTP-treated cells was decreased significantly compared with Kv1.4 in untreated cells, or Kv1.4 control. Kv1.4 in treated cells was only ~24 % of control level (Fig. 3b, c). The surface conductance density of Kv1.4 in CSTP-treated cells was also decreased significantly, ~26 % of control level (Fig. 3f). Thus, cell surface biotinylation and patch clamping gave similar estimates for cell surface expression of Kv1.4 proteins. Furthermore, the surface protein level of Kv1.4 in MMN- or SWSN-treated cells was also decreased, ~55 % of control level (Fig. 3b, c). Therefore, for surface expression level: Kv1.4 (100) > SWSN-treated (55) ~ MMN-treated (55) > CSTP-treated (24). As we have described, wild type Kv1.4 on the cell surface contains mainly complex-type N-glycans, surface Kv1.4 in SWSN-treated cells contains mainly hybrid-type N-glycans, whereas surface Kv1.4 in MMN- or CSTP-treated cells contains oligomannose-type N-glycans but with different compositions. These data suggest that N-glycan composition had a significant impact on cell surface protein levels of Kv1.4.

A decrease in surface protein level could be due to a decrease in total protein level and/or a decrease in protein trafficking efficiency. Total protein level was estimated by immunoblotting. The total protein level of Kv1.4 in CSTP-, MMN-, or SWSN-treated cells was ~50, ~60 or ~75 % of Kv1.4 control level, respectively (Fig. 3d, e). Therefore, alteration of N-glycan composition by SWSN, MMN, or CSTP significantly decreased Kv1.4 total protein level, although to different degrees.

Dividing surface protein level by total protein level gives a relative surface expression (RSE) level, which is considered an index for protein trafficking efficiency. The

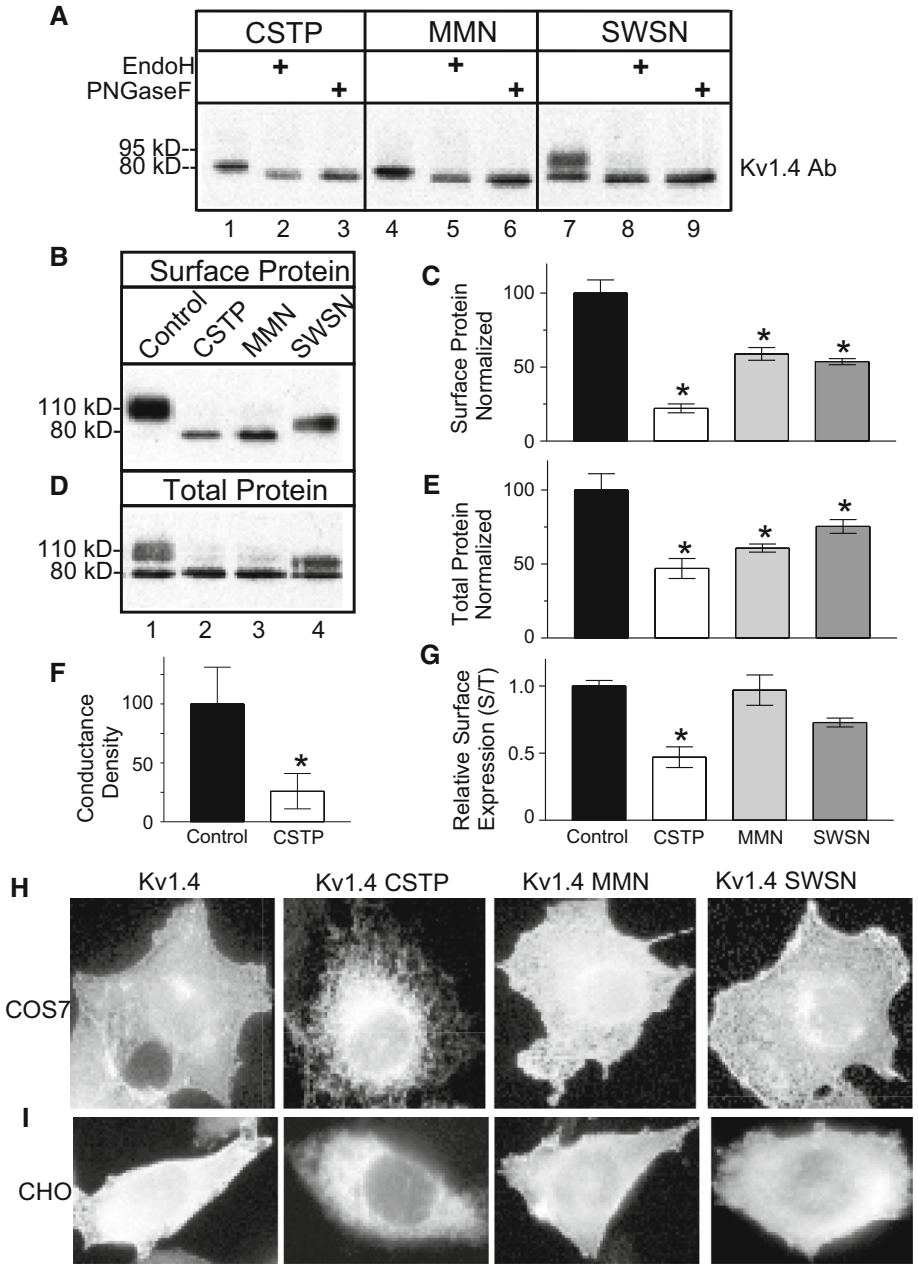

Fig. 3 Kv1.4 in glycosidase inhibitor-treated CHO cells has decreased surface protein levels compared with Kv1.4 in untreated cells. Kv1.4 proteins were expressed in CHO cells in the presence or absence of CSTP, MMN, or SWSN. Surface protein level was estimated using biotinylation and immunoblotting. Surface conductance density was estimated using whole cell patch clamping. Total protein level was estimated by immunoblotting. **a** Glycosidase digestion of Kv1.4 proteins in CSTP, MMN, or SWSN-treated cells. +, indicates glycosidase digestion. **b** and **c** Normalized surface protein level of Kv1.4 in CSTP, MMN, or SWSN-treated cells. The Kv1.4 value in untreated cells, or Kv1.4 control, was designated 100 ± SEM (standard error of the mean, $n = 3$), the mean value of others were normalized to it. Representative immunoblot shown. **d** and **e** Normalized total protein level of Kv1.4 in CSTP-, MMN-, or SWSN-treated cells. The control value was designated 100 ± SEM ($n = 3$), the mean value of others were normalized to it. Representative immunoblot shown. **f** Normalized conductance density of Kv1.4 in CSTP-treated cells. The control value was designated 100 ± SEM (n = 10), and the mean value of Kv1.4 in CSTP-treated cells was normalized to it. **g** Relative surface expression (RSE) of Kv1.4 in CSTP-, MMN-, or SWSN-treated cells. The RSE level was calculated by dividing surface protein level by total protein level (S/T). *, indicates significant difference from Kv1.4 control, analyzed by one-way ANOVA and Tukey's post-test ($p < 0.05$). **h** Immunofluorescence staining of Kv1.4 in glycosidase inhibitor-treated COS7 cells. **i** Immunofluorescence staining of Kv1.4 in glycosidase inhibitor-treated CHO cells. The important parameter to observe is localization pattern and not intensity

RSE level of Kv1.4 in CSTP-treated cells was reduced significantly compared with Kv1.4 control level, ~0.5 versus 1.0 for control, whereas the RSE level of Kv1.4 in MMN- or SWSN-treated cells was similar to control level (Fig. 3g). Thus, for trafficking efficiency: Kv1.4 (1) ~ MMN-treated (1) ~ SWSN-treated (0.8) > CSTP-treated (0.5). Therefore, Kv1.4 in CSTP-treated cells is expected to have higher partial intracellular retention compared with Kv1.4 control. In contrast, Kv1.4 in MMN- or SWSN-treated cells would be predicted to have a distribution pattern similar to that of Kv1.4 control.

Immunofluorescence imaging showed that Kv1.4 in CSTP-treated cells had a perinuclear and web-like staining indicative of high ER-retention, whereas wild type Kv1.4 and Kv1.4 in MMN- or SWSN-treated cells had a diffuse staining with cell perimeter clearly visible, indicative of higher cell surface localization in both transfected COS7 and CHO cells (Fig. 3h, i). The parameter of interest in these images is the Kv1.4 protein overall localization and not signal intensity.

The above results indicate that a change in composition of N-glycans decreased Kv1.4 cell surface protein level. The decrease observed in MMN- or SWSN-treated cells was mostly the result of a decrease in total protein level, whereas the decrease observed in CSTP-treated cells was due to a combination of decreased total protein level and higher partial ER-retention versus control.

Kv1.4 has Different N-glycan Compositions in N-glycosylation-deficient Mutant CHO Cells

We next transfected Kv1.4 cDNAs into N-glycosylation-deficient mutant CHO cells (Lec1, Lec2, or Lec8) to obtain Kv1.4 proteins carrying N-glycans with various compositions. Lec1 mutant cells lack *N*-acetylglucosaminyltransferase I activity, synthesize only $Man_5GlcNAc_2$, oligomannose-type N-glycans, and do not synthesize complex- or hybrid-type N-glycans (Fig. 2 (4)) (North et al. 2010). Kv1.4 proteins in Lec1 showed only one band on an immunoblot with molecular mass of ~80 kDa. This band was sensitive to both Endo H and PNGase F, indicating it contained oligomannose-type glycans (Fig. 4a, lane 1–3), consistent with the prediction.

Lec8 mutant cells lack UDP-galactose Golgi transporter activity and synthesize complex-type N-glycans without both galactoses and sialic acids (Fig. 2 (5)) (Esko and Stanley, 2009). The predicted N-glycan structure is $GlcNAc_2Man_3GlcNAc_2$. Kv1.4 proteins in Lec8 showed only one band on an immunoblot with molecular mass of ~80 kDa. This band was sensitive to PNGase F but resistant to Endo H, indicating it contains complex-type N-glycans (Fig. 4a, lane 4–6), consistent with the prediction.

Lec2 mutant cells lack CMP-sialic acid Golgi transporter activity and synthesize complex-type N-glycans without sialic acids, $Gal_2GlcNAc_2Man_3GlcNAc_2$ (Fig. 2 (6)) (North et al. 2010). Kv1.4 proteins in Lec2 showed two bands on immunoblots with molecular masses of ~95 and ~80 kDa, respectively. The ~95 kDa band was sensitive to PNGase F but resistant to Endo H, indicating it contains complex-type N-glycans. The ~80 kDa band was sensitive to both PNGase F and Endo H, indicating it contains oligomannose-type glycans (Fig. 4a, lane 7–9). Thus, Kv1.4 in Lec2 cells consists of two populations, one with oligomannose-type N-glycans and one with complex-type asialo-N-glycans.

Kv1.4 in Lec Mutant Cells has Decreased Cell Surface Expression Compared with Expression in CHO Parental Cells

Surface protein level of Kv1.4 in Lec mutants was estimated and compared with Kv1.4 level in CHO cells, or wild type Kv1.4. The surface protein level of Kv1.4 in Lec1, Lec8, and Lec2 was ~50, ~35, and ~65 %, of wild type Kv1.4 level, respectively (Fig. 4b, c). Also, the surface Kv1.4 in CHO and Lec2 cells contained mainly complex-type N-glycans. Thus, for Kv1.4 surface protein level, wild type > Lec2 > Lec1 > Lec8. The total protein level of Kv1.4 in Lec1, Lec8, and Lec2 was ~50, ~40, and ~70 % of wild type Kv1.4 level, respectively (Fig. 4d, e). Thus, for Kv1.4 total protein level, wild type > Lec2 > Lec1 > Lec8. The RSE level of Kv1.4 in Lec1, Lec8, and Lec2 was similar to wild type Kv1.4 level, suggesting that mutant and wild type Kv1.4 had similar trafficking efficiency (Fig. 4f). The above results suggest that Kv1.4 in Lec1, lec8, and Lec2 mutant cells exhibited decreased surface protein level compared with Kv1.4 in wild type CHO cells. The decrease observed was mostly the result of a decrease in total protein level, presumably due to altered N-glycan composition.

The High Partial ER-retention of Kv1.4 in CSTP-Treated Cells Does Not Appear to be Due to Gross Protein Misfolding

Only Kv1.4 in CSTP-treated cells exhibited high partial ER-retention. We used detergent solubility assay to examine whether the increased ER-retention of Kv1.4 in the presence of CSTP is due to protein misfolding. Triton X-100, a nonionic detergent, was utilized to solubilize Kv1.4 proteins. Misfolded transmembrane proteins often require a higher Triton X-100 concentration in order to be solubilized from the membrane (Manganas et al. 2001). Kv1.4 was expressed in CHO cells in the presence or absence of CSTP. Cells were lysed in buffers containing 0 and 0.1 % Triton X-100. The critical micelle concentration of Triton X-100 is 0.015 %. The Triton-soluble

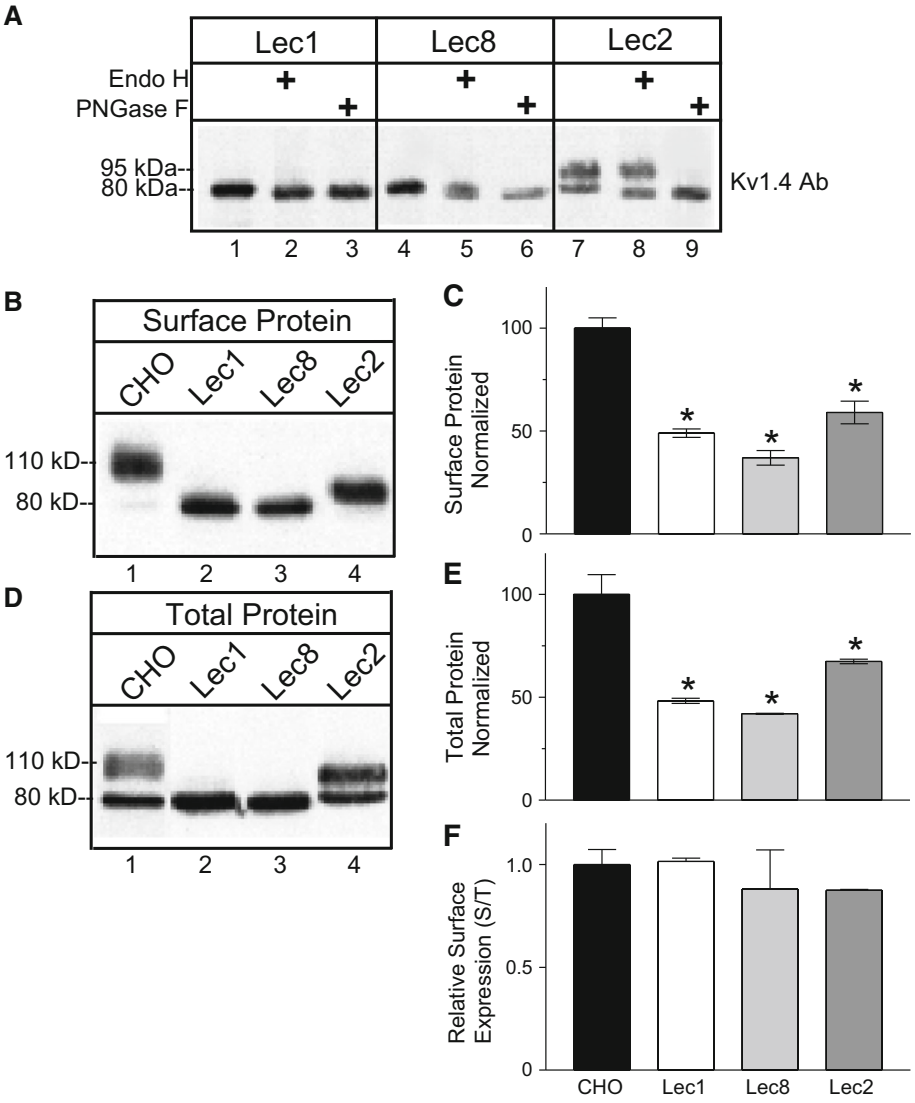

Fig. 4 Kv1.4 in N-glycosylation-deficient mutant CHO cells has decreased surface protein level compared with Kv1.4 in wild type CHO cells. Kv1.4 cDNAs were transfected into N-glycosylation-deficient mutant CHO cells, Lec1, Lec8, or Lec2. **a** Glycosidase digestion of Kv1.4 proteins in Lec mutant cells. +, indicates glycosidase digestion. **b** and **c** Normalized surface protein level of Kv1.4 in Lec mutant cells. The wild type Kv1.4 value was designated 100 ± SEM ($n = 3$), the mean value of others were normalized to it. Representative immunoblot shown. **d** and **e** Normalized total protein level of Kv1.4 in Lec mutant cells. The wild type value was designated 100 ± SEM ($n = 3$), the mean value of others were normalized to it. Representative immunoblot shown. **f** RSE of Kv1.4 in Lec mutant cells. *, indicates significant difference from wild type Kv1.4, analyzed by one-way ANOVA and Tukey's post-test ($p < 0.05$)

and -insoluble fractions in the lysates were separated by centrifugation and examined by immunoblotting (Fig. 5). As expected, Kv1.4 control and Kv1.4 in CSTP-treated cells were insoluble in 0 % Triton X-100. At 0.1 % Triton X-100, Kv1.4 control and Kv1.4 in CSTP-treated cells showed similarity in solubility and were 100 % soluble. Therefore, the high partial ER-retention of Kv1.4 in CSTP-treated cells does not appear to be due to gross protein misfolding.

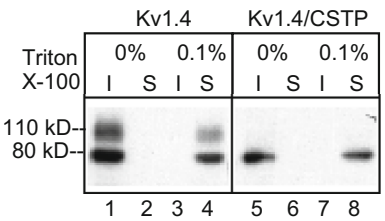

Fig. 5 Altered N-glycan composition has little or no effect on nonionic detergent solubility of Kv1.4. Kv1.4 proteins were expressed in CHO cells in the presence or absence of CSTP. Cells were lysed in different concentrations of nonionic detergent Triton X-100. Soluble fractions (*S*) and insoluble fractions (*I*) separated by centrifugation, fractionated by SDS-PAGE, and immunoblotted

Discussion

In this study, we produced Kv1.4 proteins carrying N-glycans with six different apparent compositions using two methods (Fig. 2). These N-glycans include three oligomannose-types (CSTP- or MMN-treated, or Lec1), one hybrid-type (SWSN-treated), and two complex-types (Lec8 or Lec2). The complex-type N-glycans in Lec2 lack terminal sialic acids, whereas the complex-type N-glycans in Lec8 lack both sialic acids and galactoses, compared with complex-type N-glycans in wild type Kv1.4. Our data showed

that all Kv1.4 N-glycosylation mutants had decreased surface protein levels compared with wild type Kv1.4, although differing in degree, suggesting that oligomanose-type, hybrid-type, or galactose- and/or sialic acid-deficient complex-type N-glycans have a negative effect on surface protein expression of Kv1.4 compared with complex-type N-glycans. It also suggests that the sialic acid content in complex-type N-glycans plays a critical role in surface protein levels of Kv1.4, because other glycans tested do not have or have a reduced amount of sialic acids (i.e., hybrid-type). Furthermore, all Kv1.4 N-glycan mutants had decreased total protein levels compared with wild type, suggesting that their protein stability was significantly reduced. It appears that the decrease in surface protein level of Kv1.4 was mainly due to a reduction in protein stability, which was induced by altered N-glycan composition (mainly by a reduction in sialic acid content). We have shown previously that Kv1.4N354Q, without its N-glycan, did have a significantly reduced protein half-life versus Kv1.4 (Watanabe et al. 2004).

Sialic acids are located at the terminal end of the N-glycan structure. Influence of sialic acids on cell surface expression varies among glycoproteins. CD133, a transmembrane glycoprotein, has been used as a biomarker for normal and cancer stem cells. Sialylation stabilized the protein conformation of CD133, and desialylation accelerated its degradation (Zhou et al. 2010). Increased sialylation was also known to improve cell surface expression of human thyrotropin receptors (Frenzel et al. 2005). On the other hand, desialylation was found to restrict endocytosis and therefore enhance surface protein level of TRPV5 channels (Cha et al. 2008). Supplementation of N-acetylglucosamine reduced sialic acid content and increased the degree of branching in N-glycans, which in turns promoted cell surface expression of Kv1.3 channels (Zhu et al. 2012). Thus, the effect of sialylation on surface expression of glycoproteins might be a combined result of stabilizing protein conformation and increasing endocytosis.

The N-glycan of Kv1.4 in CSTP-treated cells had unique properties compared with other N-glycans tested. The predicted structure of this N-glycan is $Glc_3Man_{7-9}GlcNAc_2$, an oligomannose-type N-glycan with terminal glucose residues attached. This glycan structure had the most inhibitory effect on Kv1.4 surface protein expression- the Kv1.4 level in the presence of CSTP was only ~24 % of Kv1.4 control level. Furthermore, it affected Kv1.4 surface level by a combined mechanism of decreasing total protein level (presumably by reducing protein stability) and increasing ER-retention. Stem cell surface glycosylation is dominated by oligomannose-type glycans, ~75 % of total N-glycan species. Moreover, they are rich in $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ and have ~7 % of $Glc_3Man_9GlcNAc_2$ (An et al. 2012). Oligomannose-type N-glycans are elevated during breast cancer progression. Man9-containing glycans are enriched in both mouse and human sera in the presence of breast cancer (de Leoz et al. 2011), whereas Man8-containing glycans are prevalent on the cell surface of various tumor cells (Liu et al. 2013). This is in contrast with fully differentiated normal mammalian cells that are rich in complex-type N-glycans. It is speculated that oligomannose-type N-glycans on stem cells and tumor cells might play a role in cellular binding and recognition (An et al. 2012; de Leoz et al. 2011).

Our results suggest that oligomannose-type N-glycans, especially glucose-containing N-glycans, negatively impacted protein stability and trafficking of Kv1.4. Understanding the role of oligomannose-type N-glycans on surface expression of glycoproteins could be applicable to stem cell biology and cancer biology.

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