Trafficking of Kv1.4 potassium channels: interdependence of a pore region determinant and a cytoplasmic C-terminal VXXSL determinant in regulating cell-surface trafficking

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Kv1.4 and Kv1.1 potassium channel homomers have been shown to exhibit different intracellular trafficking programmes and cell-surface expression levels in cell lines: a determinant in the pore region of Kv1.4 and Kv1.1 [Zhu, Watanabe, Gomez and Thornhill (2001) J. Biol. Chem. **276**, 39419–39427] and a cytoplasmic C-terminal VXXSL determinant on Kv1.4 [Li, Takimoto and Levitan (2000) J. Biol. Chem. **275**, 11597–11602] have been described, which affected trafficking and cell-surface expression levels. In the present study, we examined whether trafficking pore determinants influenced any cytoplasmic C-terminal trafficking determinant. We found that removal of VXXSL from a Kv1.4 chimaera that contained the pore of Kv1.1 did not affect cell-surface trafficking. Therefore removal of the C-terminal VXXSL of Kv1.4 inhibited protein surface levels only in the presence of the Kv1.4 pore. In contrast, truncating the cytoplasmic C-terminus of Kv1.1 or truncating a Kv1.1 chimaera with the pore of Kv1.4, had little effect on surface protein levels. Furthermore, the subregion of the Kv1.4 pore trafficking determinant that was required for the inhibitory effect of VXXSL removal was mapped to a threonine residue in the deep pore region. Therefore the Kv1.4 pore determinant affected the trafficking and cell-surface levels directed by the C-terminal VXXSL determinant. Different Kv1 trafficking programmes would affect cell-surface expression levels either positively or negatively and also cell signalling. Cells may use differential trafficking programmes of membrane proteins as a post-translational mechanism to regulate surface protein levels and cell function.

Key words: cell-surface expression, K^+ channel, trafficking.

INTRODUCTION

Excitable tissues express various voltage-gated potassium (K^+) channels that play important roles in shaping action potential waveforms [1]. Numerous voltage-gated K^+ channels have been identified by recombinant DNA methods. Expression studies have shown that channels within a subfamily can be expressed as tetrameric homomers or heteromers and that there are many structural regions involved in channel operation [1–3].

Kv1.4 and Kv1.1 channels are glycosylated proteins [4–8] found in many brain regions and may be associated with cytoplasmic Kvβ subunits [9,10]. Kv1 subunits appear to fold to tetramers in the ER (endoplasmic reticulum). Determinants required for this process include the cytoplasmic N-terminal tetramerization domain and the S1 and S2 transmembrane domains [11,12]. In addition, cell-surface levels of some Kv1 channels in expression systems may be affected by post-translational mechanisms, e.g. by their subunit composition [13], their pore region [8], a cytoplasmic C-terminal VXXSL determinant [14] and, in some cases, association with cytoplasmic Kvβ subunits [15]. Different trafficking programmes of K^+ channels and other membrane proteins appear to be an additional mechanism to control cell-surface levels, which can affect cell signalling and physiology. A number of human disorders are due to ion-channel mutations in which channel misfolding, ER retention and trafficking problems result in an insufficient number of channels on the cell surface [16].

We have used Kv1.1 and Kv1.4 channels as both homotetramers [8] and heterotetramers [17], to study K^+ channel biosynthesis and transport to the cell surface in mammalian cells, since we found striking differences in their trafficking programmes in three different cell lines. As homomers, Kv1.1 exhibited high partial ER retention, low *trans*-Golgi glycosylation and low cell-surface expression, whereas Kv1.4 showed the opposite pattern for all three parameters. A major determinant for high intracellular ER partial retention or high cell-surface expression was mapped to outer pore regions of these channels. The pore of Kv1.1 induced high partial ER retention and low surface expression when transplanted to Kv1.4, whereas the pore of Kv1.4 promoted high cell-surface expression when transplanted to Kv1.1 ([8], see also [18]).

The cytoplasmic C-terminus of Kv1 channels may be grouped into two domains: a constant region of five amino acids towards the end of the S6 transmembrane domain and a variable region after this sequence. The variable C-terminal region (amino acids 574–655) of Kv1.4 promoted high surface expression. Truncation of this region inhibited surface expression levels, partly due to the removal of the VXXSL determinant ([14], see also [8]). In contrast, truncation of the variable C-terminal region (amino acids 420–495) of Kv1.1 did not significantly affect surface expression levels [8]. These findings imply that the variable C-terminal region for Kv1.4 was required for wild-type cell-surface expression levels, but not for Kv1.1. We did not truncate the C-terminus of either Kv1.4 or Kv1.1 into the constant region, because their high identity suggests an important role in function and because a stop mutation in this region in Kv1.1, Arg^{417}, increased intracellular protein retention and was identified as a mutation that caused the human disorder, episodic ataxia type I [16]. Thus the variable C-terminus on two closely related Kv1 channels appeared to play very different roles in protein trafficking to the cell surface.

Abbreviations used: CHO, Chinese-hamster ovary; ER, endoplasmic reticulum; GFP, green fluorescent protein.

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The aims of the present study were to examine the influence of the pore regions of Kv1.4 and Kv1.1 on any C-terminal truncation effect and to investigate which subregion or amino acid in the pore was a major determinant.

EXPERIMENTAL

Cell lines, plasmids and transfections

CHO (Chinese-hamster ovary) pro5 cells and CHO Lec1 cells, from A.T.C.C. (Manassas, VA, U.S.A.), were maintained in Dulbecco's modified Eagle's medium with 0.35 mM proline or α-minimal essential medium with 10 % (v/v) foetal bovine serum at 37 °C in 5 % CO_2 atmosphere. Rat brain Kv1.1 and Kv1.4 cDNAs [19], with a 5′ Kozak signal in front of the start methionine and no 5′- and 3′-untranslated regions, were used to construct various mutant cDNAs by standard PCR mutagenesis [8]. cDNA integrity was confirmed by DNA sequencing. Transient transfections (lipofectAMINE™ Plus, Invitrogen/Gibco BRL) were performed according to the manufacturer's instructions on cells plated in a 35 mm dish. For each transfection, 0.5 μg of plasmid was used. Cells were incubated for 20–24 h post-transfection before they were processed. At 15 h, the cell-surface expression levels for these channel proteins were maximal (results not shown).

Membrane purification and immunoblot analysis of Kv1.1 and Kv1.4

Total crude cell membranes (ER, Golgi and plasma membrane) were isolated in ice-cold hypo-osmotic media with protease inhibitors as described in [8]. Total cell membranes from a 35 mm dish were recovered and approx. 20 μg of membrane protein/gel lane was run on 9 % SDS gels. Proteins were electrotransferred to nitrocellulose (Bio-Rad Laboratories). The filter was blocked in PBS with 5 % (w/v) non-fat milk and then incubated overnight in Kv1.4 mouse monoclonal N-terminal antibody (to amino acids 13–37) in the ratio 1:1000 (Upstate Biotechnology, Lake Placid, NY, U.S.A.) [20] or Kv1.1 polyclonal N-terminal antibody [8,21]. After subjecting to washes, horseradish peroxidase-linked anti-mouse or anti-rabbit secondary antibodies were added, and the bound antibodies were detected by enhanced chemiluminescence (ECL® detection kit; Amersham Biosciences, Piscataway, NJ, U.S.A.) and a pre-flashed Kodak X-AR5 film. Signals were quantified as described in the next section. Membrane preparations of non-transfected CHO cells did not exhibit immunoreactivity to Kv1.4 or Kv1.1 antibodies [8].

Estimation of plasma membrane protein by biotinylation and immunoblot analysis

Cell-surface proteins were biotinylated with either hydrazide-LC-biotin (Pierce, Rockford, IL, U.S.A.) or *N*-hydroxy-succinimidobiotin (Pierce). Procedures for surface biotinylation, streptavidin–agarose bead precipitation of solubilized biotinylated membrane proteins and the specific detection of cell-surface Kv1 proteins by subsequent immunoblotting have been described previously [8]. To control for possible differences in cell density between dishes, actin levels of samples were also observed on immunoblots with actin antibodies (Sigma). Transfection efficiency was estimated by co-transfecting with 0.1 μg of GFP (green fluorescence protein) plasmid and immunoblotting with GFP antibodies (Clontech, Palo Alto, CA, U.S.A.) [8]. Pre-flashed X-ray film was exposed to filters for various lengths of time. Film images were scanned with a MicroTek 8700 scanmaker (dynamic range 0–3.5) and densitometry analysis was performed with the NIH Image 1.6 software that was calibrated with internal and external standards [8]. A value of $100.0 \pm$ S.E.M. was designated for total cell-membrane protein (ER, Golgi and plasma membrane) and cell-surface protein levels of wild-type Kv1.1 or Kv1.4. Truncation mutants were normalized to that value. ECL® detection system (Amersham Biosciences) yielded a linear plot over an approx. 1–20-fold concentration range as assayed by immunoblots on serially diluted membranes from transfected cells (results not shown).

Immunofluorescence microscopy analysis of Kv1.4 and Kv1.1

COS-1 cells (A.T.C.C.) on glass coverslips were transfected with various Kv1.4 or Kv1.1 constructs and incubated for 20–24 h after transfection. Cells were then washed, fixed in 3 % (w/v) paraformaldehyde for 10 min and permeabilized and blocked for 1 h [17]. Kv1 antibody was added overnight in the ratio 1:1000. After a series of washes, the cells were incubated for 1 h with secondary antibody (anti-mouse for Kv1.4 and anti-rabbit for Kv1.1) conjugated with Alexa Flour 568 fluorophore (Molecular Probes, Eugene, OR, U.S.A.). Cells were then washed, mounted on to glass slides and viewed by an Olympus BX50 microscope having a BX-FLA (reflected light fluorescence attachment), using the appropriate filter cube for the fluorophore. More than 200 cells were viewed for each construct and we show a representative cell for each condition. Fluorescent cells were photographed at $\times 1000$ magnification using the automatic exposure option on an Olympus PM-20 exposure control unit. Kodak etachromP1600 film was developed and an image scanned with the MicroTek scanmaker. The photographs should be compared in terms of overall localization pattern and not in terms of signal intensity.

RESULTS AND DISCUSSION

Truncating the variable cytoplasmic C-terminus of Kv1.4 or Kv1.1 affected its cell-surface level by changing the stability and ER export of Kv1.4, but had little effect on the surface levels of Kv1.1

We investigated the mechanism of differential effects of truncation of the cytoplasmic C-terminus of Kv1.4 and Kv1.1 on their cell-surface protein levels. Kv1.4 and Kv1.1 (Figure 1A) mutants, with their variable cytoplasmic C-termini truncated at five different positions (Figure 1B), were used to estimate cell-surface protein levels by surface biotinylation and immunoblotting. As shown in a previous study [8], cell-surface Kv1.1 homomers on immunoblots were detected mostly as immature high-mannose glycoprotein bands termed p60, whereas cell-surface Kv1.4 homomers were mostly a mature glycosylated p110 glycoprotein with lesser amounts of an immature high-mannose p85 glycoprotein (Figures 2A, lane 1 and 2B, lane 1 respectively). All Kv1.1 mutants (T1–T5) that had their C-terminal variable region truncated to different degrees were expressed on the cell surface at approx. 65–90 % of the level of wild-type (Figure 2A). These results suggest that the complete variable C-terminal region (amino acids 420–495) of Kv1.1 played a minor role in governing its surface protein levels, as we have suggested previously [8] using patch-clamping methods on Kv1.1 T1. Partial truncation of the C-terminus produced results somewhat similar to those of complete truncation.

Kv1.4 truncation mutant (T4 and T5) proteins that contained the VXXSL determinant [14] were expressed at similar cell-surface

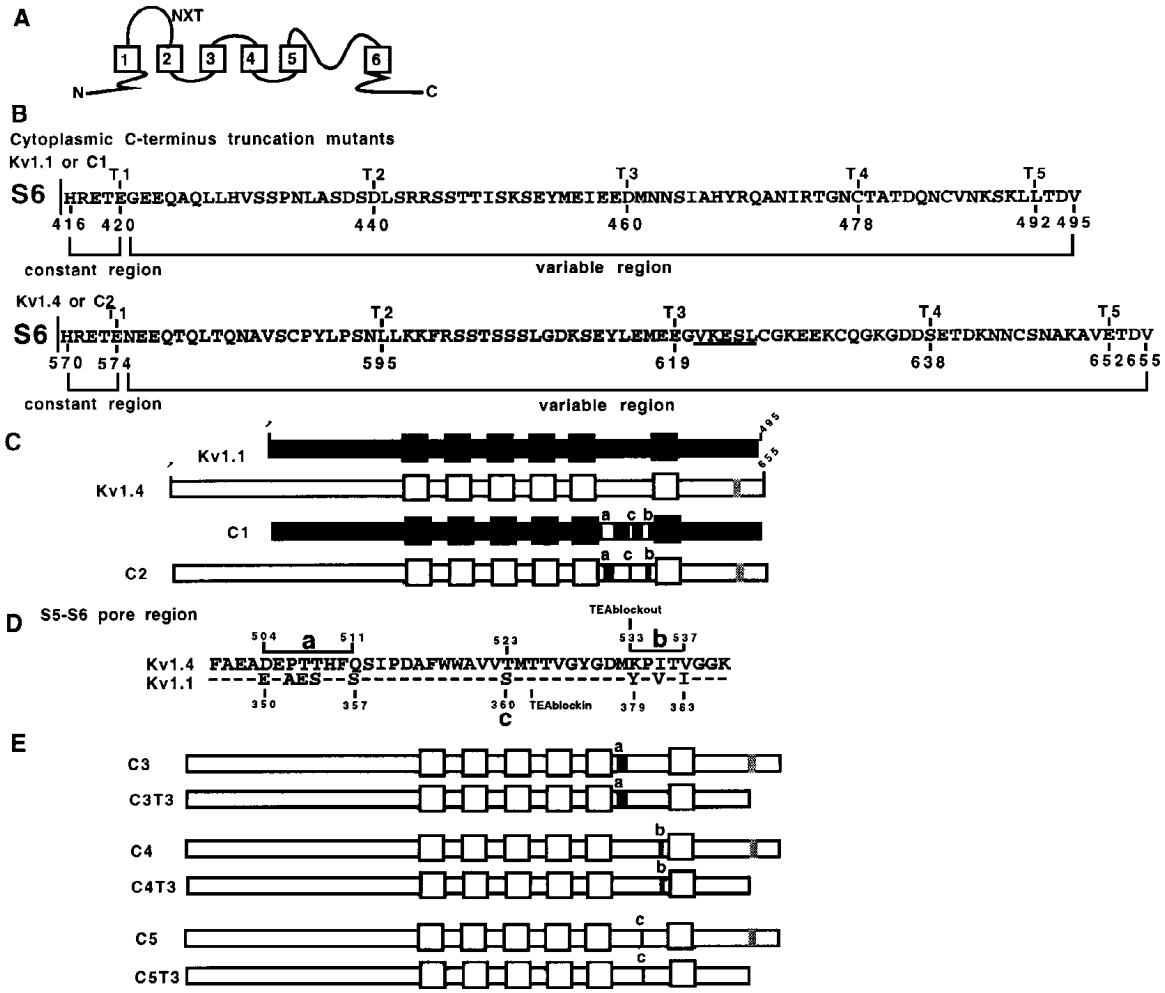

Figure 1 Kv1.1 and Kv1.4 constructs

(**A**) Conventional schematic representation of a Kv1 monomer with its N- and C-termini in the cytoplasm. (**B**) C-terminal amino acid sequences of the constant and variable regions of Kv1.1 and Kv1.4, showing the site of the truncation mutants (T1–T5). (**C**) Schematic representation of Kv1.1 and Kv1.4 monomers and of mutants produced by exchanging regions in the pore. The light grey C-terminal region is the VXXSL motif [14]. (**D**) Amino acid sequences of the S5–S6 pore region of Kv1.4 and Kv1.1. Numbers represent the amino acid numbers in the different constructs and the dashes used for Kv1.1 represent amino acid identity with Kv1.4. (**E**) Schematic representation of Kv1.4 monomers with different Kv1.1 pore regions that have been +/− truncated at the C-terminus to remove the VXXSL motif.

protein levels as wild-type (Figures 2B and 2C). However, the three Kv1.4 truncation mutants lacking the VXXSL determinant (T1, T2 and T3) had only approx. 2–4 % of the surface protein level of wild-type (Figures 2B and 2C), which has been ascribed to high intracellular retention for a construct similar to Kv1.4 T3 [14] or Kv1.4 T1 [8]. In the present study, we also estimated the relative total cell protein levels (from the ER, Golgi and plasma membrane) of Kv1.4 T1–T3 mutants to ascertain if their protein stability had changed. Decreasing Kv1 protein stability could also play a role in decreasing surface protein levels, and this analysis would give insight into mechanisms regulating cell-surface protein levels. We found that Kv1.4 T1–T3 mutants had only approx. 25–50 % of the total protein level of wild-type (Figure 2D), and this decreased level was presumably due to a decrease in protein half-life. Dividing the surface protein by the total protein gave surface/total protein ratios for Kv1.4 T1–T3 constructs and they were much lower than the value of 1.0 for control Kv1.4 (Figure 2E). A ratio much lower than 1.0 suggested that these mutants exhibited different trafficking characteristics and probably had high partial intracellular retention when compared with the Kv1.4 control. Indeed, Kv1.4 T1 exhibited an immunofluorescence localization pattern consistent with high intracellular retention in the ER, which was very different from the pattern for Kv1.4, which was indicative of high cell-surface expression (Figure 2F; cf. Kv1.4 with Kv1.4 T1, which was similar to Kv1.1). The important parameter in our immunofluorescence micrographs is the overall pattern and not the signal intensity. We used COS cells in the present study, because they are large cells with consistent ER and Golgi localization. Kv1.4 or Kv1.1 whole-cell immunofluorescence patterns were not cell type-dependent, because CHO, neuronal-like CAD (Cath-a-differentiated) and COS cells all showed a similar pattern ([17] and results not shown).

These results suggest that the removal of the Kv1.4 VXXSL determinant decreased cell-surface protein levels both by inducing high partial ER retention [14,8] and by decreasing the stability of total protein. We hypothesize that different unknown ER proteins interact with either: (i) Kv1.4 mutants without the VXXSL

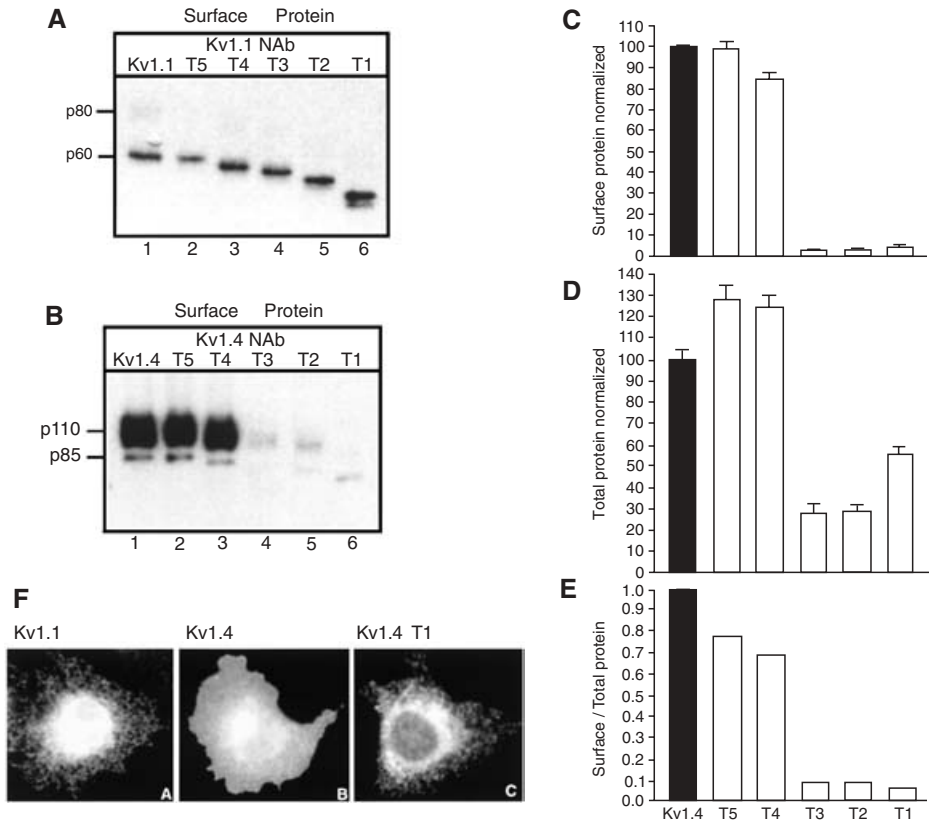

Figure 2 Effects of truncating the Kv1.1 C-terminal variable region on cell-surface levels and removal of the VXXSL determinant on the C-terminus of Kv1.4 on both cell-surface and total protein levels

Immunoblots of cell-surface Kv1.1 (**A**) and Kv1.4 (**B**) proteins isolated by biotinylation and streptavidin purification. (**C**) Kv1.4 surface protein levels from (**B**) were taken as 100.0 ± S.E.M. and the different C-terminal mutants (Kv1.4 T1–T5) were normalized to it ($n = 3$). (**D**) Kv1.4 total protein levels (immunoblot not shown) were taken as 100.0 ± S.E.M. and the different C-terminal mutants (Kv1.4 T1–T5) were normalized to it ($n = 3$). Levels of (**C**, **D**) were also normalized to actin and GFP levels. (**E**) Surface/total protein for Kv1.4 proteins = surface protein (**C**)/total protein (**D**). (**F**) Immunofluorescence localization pattern for Kv1.1, Kv1.4 and Kv1.4 T1 in transfected COS cells.

determinant, thereby inducing their partial high ER retention or (ii) Kv1.4 with VXXSL and this promoted its high ER to Golgi export. In addition, decreased protein stability was evident in Kv1.4 T1–T3 and it probably affected the protein pools from the ER, Golgi and plasma membrane. It is also possible that partial high ER retention led to degradation by the proteasomal pathway and contributed to a decrease in protein stability.

Effect of truncation of the cytoplasmic C-terminus on surface protein was mostly independent of the Kv1.1 pore, whereas the inhibitory effect of Kv1.4 VXXSL removal was dependent on the Kv1.4 pore

We have shown previously that a pore region of some Kv1 channels govern their surface targeting, because exchanging the pore region between Kv1.4 and Kv1.1 conferred on the recipient protein the characteristics of the donor protein for *trans*-Golgi glycosylation and surface expression levels [8]. Does the pore region affect the differential effects of C-terminal truncation for Kv1.4 and Kv1.1 on cell-surface protein levels? Kv1.1 C1 with the pore of Kv1.4 and Kv1.4 C2 with the pore of Kv1.1 were truncated at the same five positions within their C-termini as before to address this question (Figures 1B and 1C). The full-length Kv1.1 C1 chimaera was referred to as C16 [8] or P1 [17] in our previous studies. Note that Kv1.4 and Kv1.1 pore regions have only nine amino acids that are different: five in the **a** region, three in the **b** region and one in the **c** region (Figure 1D). Kv1.4 and Kv1.1 have identical S5 and S6 amino acid sequences.

Kv1.1 C1 exhibited efficient *trans*-Golgi glycosylation (Figure 3A, lane 1), because it contained the pore of Kv1.4, as we have shown previously [8]. When compared with the surface protein level of Kv1.1 C1 control, surface protein levels of Kv1.1 C1T2–T5 were approx. 80–90 %, whereas the Kv1.1 CT1 level was approx. 50 % (Figures 3A and 3C). Total protein levels of Kv1.1 C1T5 and Kv1.1 C1T4 were similar to Kv1.1 C1 control, whereas Kv1.1 C1T1–T3 levels were approx. 60–70 % of control (Figures 3B and 3D). Surface/total protein ratios for these mutants are shown in Figure 3(E). Kv1.1 C1T2 showed a similar immunofluorescence localization pattern as Kv1.1 C1 (Figure 3F), whereas Kv1.1 C1T1 exhibited a pattern that was different and indicative of partial high intracellular retention (Figure 3F). It appeared that cell-surface levels were maintained for all truncation mutants except Kv1.1 T5, in spite of a decreased total protein level in some cases. Thus the Kv1.4 pore did not dramatically change the effect of C-terminal truncation for most of the Kv1.1 C1 mutants. However, Kv1.1 C1T1 exhibited a lower surface protein level than Kv1.1 C1, presumably due to partial intracellular retention and a lower total protein level. All Kv1.1

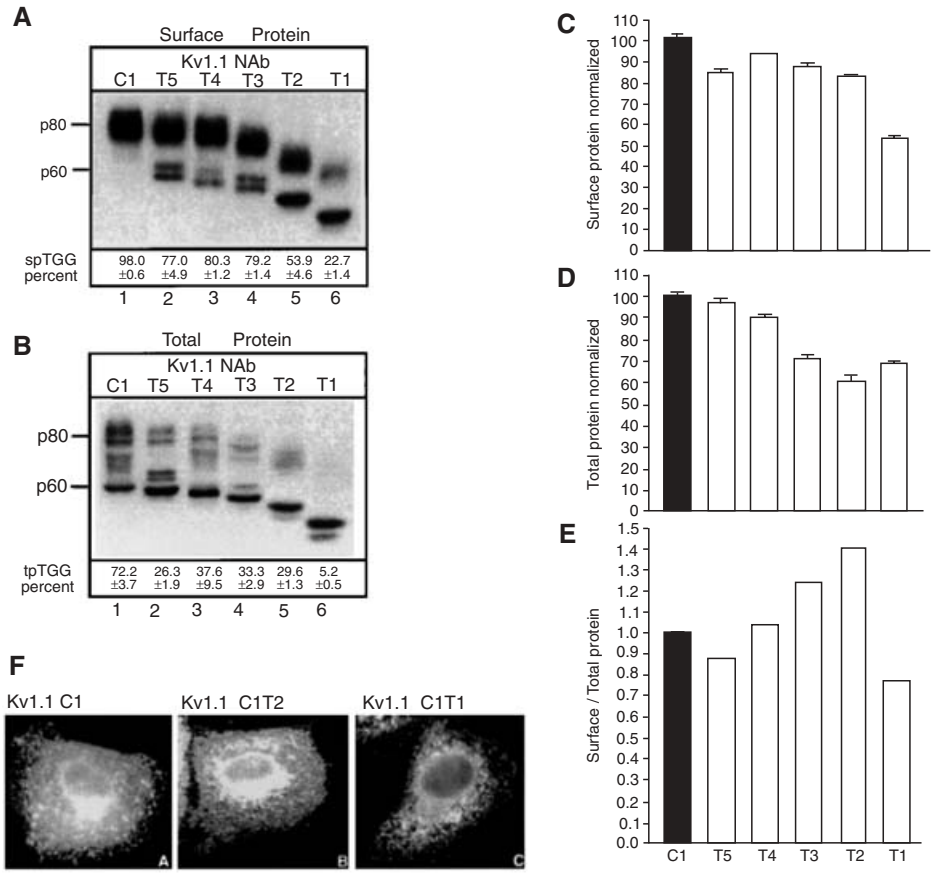

Figure 3 Minor effect of truncating the Kv1.1 C1 (Kv1.1 with the Kv1.4 pore) C-terminal variable region on cell-surface and total protein levels

(**A**, **B**) Kv1.1 mutant cell-surface and total proteins were detected by immunoblots. (**C**) and (**D**) Normalized group data for (**A**) and (**B**) respectively, which were also normalized to actin and GFP levels. Kv1.1 C1 values were taken as 100.0 ± S.E.M. and the different C-terminal truncation mutants (C1T1–T5) were normalized to it ($n = 3$). (**E**) Surface/total protein = surface protein/total protein from (**C**) and (**D**). (**F**) Immunofluorescence localization pattern for Kv1.1 C1, Kv1.1 C1T2 and Kv1.1 CT1 in transfected COS cells.

C1T1–T5 truncation mutants had a decrease in surface protein *trans*-Golgi glycosylation (spTGG; Figure 3A) and total protein *trans*-Golgi glycosylation (tpTGG; Figure 3B) when compared with Kv1.1 C1. Kv1.1 C1T1 exhibited the lowest percentage of conversion for cell-surface and total proteins. Thus the full-length, variable C-terminus was required for the most efficient *trans*-Golgi glycosylation of Kv1.1 C1.

Kv1.4 C2 was expressed as a high mannose-type glycoprotein on the cell surface, because it was *trans*-Golgi-glycosylated inefficiently (Figure 4A, lane 1) [8], and truncating the Kv1.4 C2 variable C-terminus had only a minor effect on surface protein (Figures 4A and 4C) versus Kv1.4 C2. Total protein levels were also not affected by these truncations (Figures 4B and 4D). Thus surface/total protein ratios were also not affected for these mutants when compared with control (Figure 4E). Similar surface/total protein ratios suggested that the immunofluorescence localization pattern for Kv1.4 C2 and Kv1.4 C2 T1 would be similar. Indeed, both exhibited high partial intracellular retention (Figure 4F). In contrast, the equivalent truncations in Kv1.4 had a significant effect on cell-surface and total protein levels as well as immunofluorescence localization (Figure 2). The only difference between Kv1.4 and Kv1.4 C2 was the presence of the Kv1.4 pore in the former construct. Thus the Kv1.4 pore was required for the inhibitory effect on surface protein levels due to VXXSL removal.

Each pore determinant subregion of Kv1.4 affected the degree of inhibition of surface protein levels elicited by removal of the VXXSL determinant, but the deep pore c subregion had the greatest effect

It was observed that the removal of the VXXSL determinant significantly inhibited Kv1.4 surface protein levels if the Kv1.4 pore was present. Are all the three Kv1.4 pore determinant subregions, **a**–**c** in Figures 1(C) and 1(D), required to achieve this level of inhibition? To address this question, we used Kv1.4 constructs that contained only the **a** (termed C3 and referred to as C8 in [8]), **b** (termed C4 and referred to as C9 in [8]) or **c** (termed C5) subregion of the Kv1.1 pore with or without the VXXSL determinant on the C-terminus (Figure 1E). As shown in Figure 2, Kv1.4 T3 was expressed at only approx. 2% of the surface level (Figures 5A, lanes 1 and 2, and 5C) and at approx. 25% of the total protein level (Figure 5B, lanes 1 and 2, and 5D) of Kv1.4. Surface protein levels of C3T3 and C4T3 were approx. 20–25% of that of their respective controls, whereas the surface level of C5T3 was approx. 90% that of its control (Figures 5A and 5C). All exhibited less inhibition of their surface levels when compared with Kv1.4, for all had surface levels greater than approx. 2% that of the respective controls. Replacement of the Kv1.4 **c** pore region almost abolished the VXXSL truncation inhibitory effect on surface levels, whereas replacement of the **a** or **b** region caused partial inhibition of it. Total protein levels were approx. 55–75%

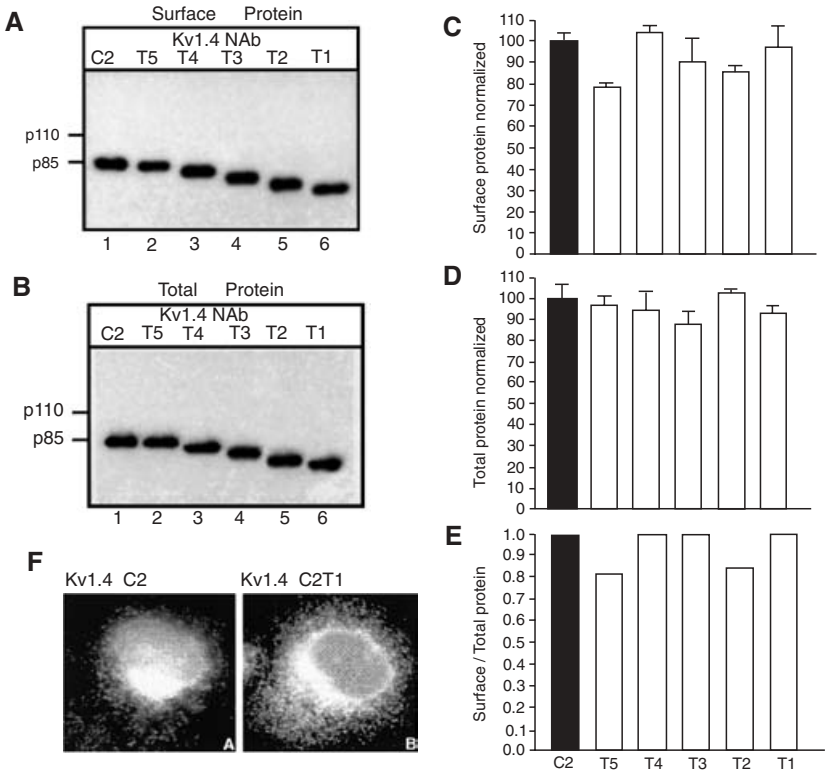

Figure 4 Effect of removal of the VXXSL determinant from Kv1.4 C2 (Kv1.4 with the Kv1.1 pore) on cell-surface and total protein levels

(**A**, **B**) Kv1.4 mutant cell-surface and total membrane proteins were detected by immunoblots. (**C**) and (**D**) Normalized group data for (**A**) and (**B**) respectively, which were also normalized to actin and GFP levels. Kv1.4 C2 values were taken as 100.0 ± S.E.M. and the different C-terminal truncation mutants (Kv1.4 C2T1–T5) were normalized to it ($n = 3$). (**E**) Surface/total protein = surface protein/total protein from (**C**) and (**D**). (**F**) Immunofluorescence localization pattern for Kv1.4 C2 and Kv1.4 C2T1 in transfected COS cells.

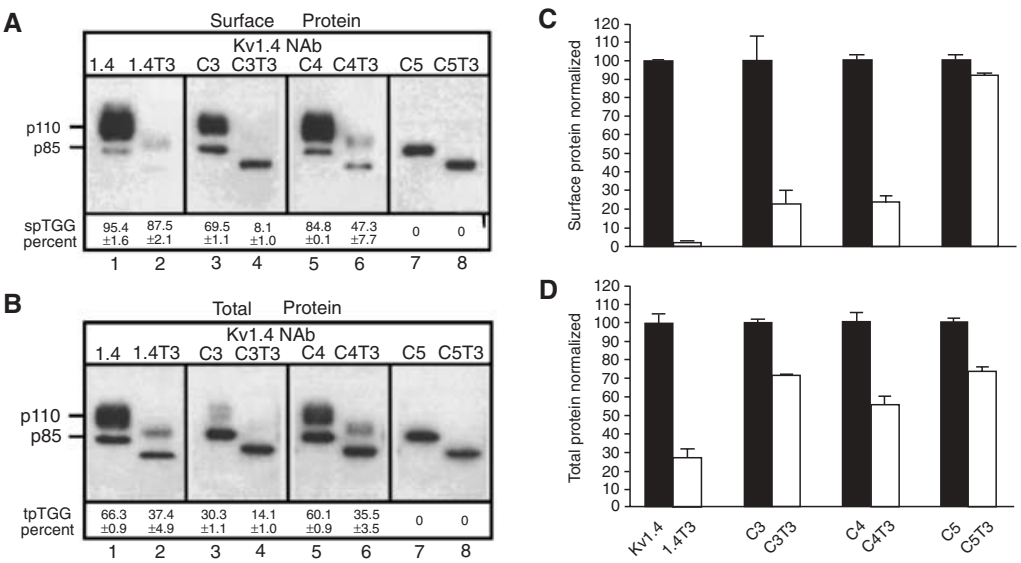

Figure 5 Requirement of Kv1.4 deep pore c region for the inhibitory effect of removal of the VXXSL determinant on cell-surface levels

(**A**, **B**) Kv1.4 construct cell-surface and total membrane proteins were detected by immunoblots. (**C**) and (**D**) Normalized group data for (**A**) and (**B**) respectively, which were also normalized to actin and GFP levels. For each of the panels in (**A**, **B**), the left lane control of each panel was taken as 100.0 ± S.E.M. and the Kv1.4 T3 C-terminal truncation mutant was normalized to it ($n = 3$).

that of their controls for all three mutants (Figures 5B and 5D), which was higher than the approx. 25% level for Kv1.4 T3 when compared with its Kv1.4 control. Thus the **c** region of the pore was required for the highest degree of inhibition from removal of the VXXSL determinant. Kv1.4 T3 surface proteins were still *trans*-Golgi-glycosylated to a high degree and thus

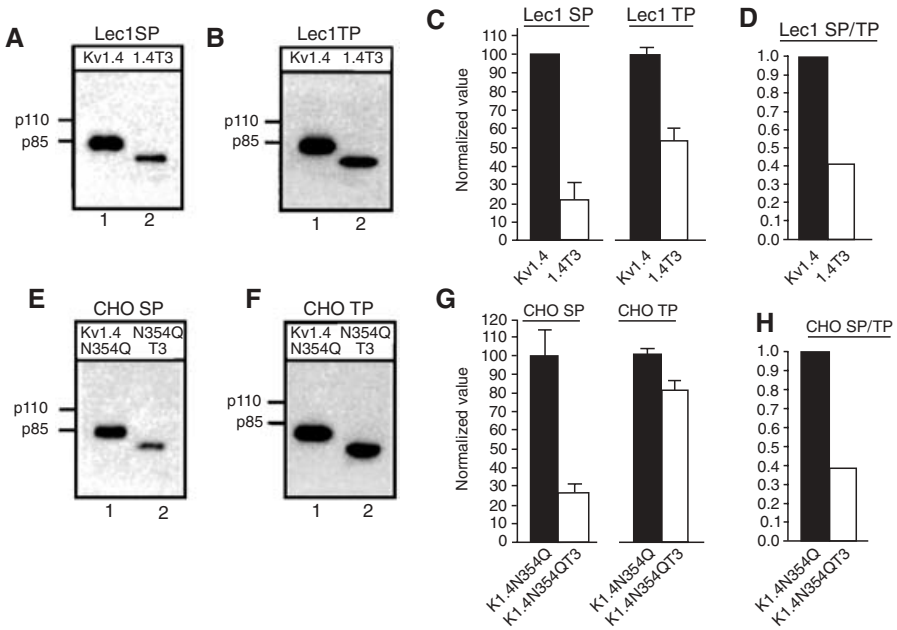

Figure 6 Ineffectiveness of Kv1.4 N-glycosylation in preventing the inhibitory effect of VXXSL removal on surface targeting

(**A**–**D**) Kv1.4 and the Kv1.4 T3 C-terminal truncation mutant expressed in glycosylation-deficient Lec1 CHO cells that express only high-mannose glycoproteins on the cell surface. (**A**) and (**B**) Immunoblots of surface protein (SP) and total membrane protein (TP) respectively. (**C**) Normalized group data for (**A**) and (**B**) respectively, which were also normalized to actin and GFP levels. (**D**) Surface/total protein = surface protein/total protein from (**C**). (**E**–**H**) Kv1.4N354Q and the Kv1.4N354Q T3 C-terminal truncation mutant expressed in wild-type CHO cells. Kv1.4N354Q is not N-glycosylated. (**E**) and (**F**) Immunoblots of SP and TP respectively. (**G**) Normalized group data for (**E**) and (**F**) respectively. (**H**) Surface/total protein = surface protein/total protein from (**G**).

truncation of the VXXSL determinant had only a minor effect on this parameter (Figure 5A, lanes 1 and 2). In contrast, Kv1.4 with different Kv1.1 pore subregions exhibited different degrees of reduction of the percentage of spTGG when the C-terminal VXXSL determinant was truncated (Figures 5A and 5B, lane 3–8). This finding suggests that various Kv1.1 pore subregions affected *trans*-Golgi glycosylation in a dominant negative manner and do so to different degrees.

As shown above, Kv1.4 C-terminal VXXSL removal did not affect surface protein levels of Kv1.4 C2 or Kv1.4 C5 (Kv1.4 with the whole pore or pore **c** region of Kv1.1; Figures 4A and 5A). These Kv1.4 mutants also inhibited TGG in non-truncated Kv1.4. Is it the replacement of the pore region or the lack of high TGG, which occurs because of pore replacement, that is responsible for the lower surface protein levels? Kv1.4 or Kv1.4 T3 was expressed in glycosylation-deficient Lec1 CHO cells [22] only as immature high mannose-type glycoproteins (Figures 6A and 6B). When compared with Kv1.4 Lec1, Kv1.4 T3 Lec1 showed (i) a surface protein level of approx. 20 % (Figures 6A and 6C), but these levels were higher than the approx. 2 % surface level of Kv1.4 T3 versus Kv1.4 in wild-type CHO cells and (ii) a total protein level of approx. 50 % (Figures 6B and 6C), but these levels were higher than the approx. 25 % total level of Kv1.4 T3 versus Kv1.4 in wild-type CHO cells. The surface/total protein ratio for Kv1.4 T3 Lec1 was approx. 0.4 (Figure 6D), which suggested that C-terminal truncation still induced partial high intracellular retention, but not as much as Kv1.4 T3 in wild-type cells. We also used the Kv1.4N354Q mutant (where N354Q stands for $Asn^{354} \rightarrow Gln$), which prevented N-glycosylation at this site, and a C-terminal truncated Kv1.4N354Q mutant in wild-type CHO cells and recorded somewhat similar results as we found using Lec1 cells (Figures 6E–6H). Thus the glycosylation state of Kv1.4 did not prevent the full inhibitory effects of VXXSL removal on surface targeting, although it did affect the extent of the inhibition.

These results suggest that the effect of Kv1.4 VXXSL removal on cell-surface and total protein levels required the Kv1.4 deep pore **c** (threonine) region for the maximal inhibitory effect, and the outer pore **a** and **b** subregions played a lesser role. This effect was disrupted by mutating, in the **c** subregion, the threonine residue to a serine residue, a conservative substitution found in Kv1.1. This threonine residue may form a combined three-dimensional determinant with the outer pore **a** and **b** subregions in Kv1.4 [8], and we speculate that a putative chaperone-like protein interacts with this outer pore determinant, or even possibly an inner pore determinant, to exert its effect. In the Kcsa [23] or the KvAP [24] K^+-channel crystal structures, the equivalent threonine residue is at the C-terminal end of the pore helix in the middle of the lipid bilayer and it is directed towards an aqueous cavity surrounded by the M1 and M2 transmembrane domains [23].

Mechanisms and significance of controlling the levels of K^+ channel surface proteins

In the present study, the following are some of the explanations and possible mechanisms involved in the trafficking of Kv1.4. (i) The Kv1.4 pore determinant and the VXXSL determinant do not influence one another directly, but it is required that both are present to exert their effects, and they may do so by interacting with different unknown trafficking molecules that influence surface expression levels. (ii) There is one Kv1.4 determinant located at either the pore determinant site or the C-terminal site and it is influenced by folding of both sites in a subunit. This site interacts with an unknown trafficking molecule that influences

surface expression levels. It is also possible that the distal Kv1.4 C-terminus, around the VXXSL determinant, directly interacts with the mouth of the inner pore, suggesting a more direct interaction between the C-terminal determinant region and the pore region. (iii) Disrupting the Kv1.4 pore determinant gives maximal inhibition and removing the VXXSL does not contribute to further inhibition.

Altering the cell-surface expression levels of ion channels in excitable cells, as well as other cells, can affect cell signalling characteristics [1]. Cell-surface levels of K^+ channels can be controlled transcriptionally by differential mRNA production. In addition, surface levels could be controlled post-transcriptionally by different inherent channel protein-trafficking programmes. For example, a Kv1 protein surface level may be high in cells expressing the protein as a homomer, whereas in other cells, with a similar mRNA subunit level, the surface protein level may be low if it is heteromerized with another Kv1 subunit that exhibits dominant-negative effects on surface levels. Mapping the different K^+ channel determinants that govern surface levels, as well as understanding which determinants influence one another in homomers and heteromers, will give an insight into the diverse strategies used by cells to control protein expression levels at critical cellular locations. In addition to gene transcriptional control, cells may use post-translational mechanisms to control the surface levels of other membrane proteins and these can alter cell physiology.

This work was supported by grant no. NS29633 of the National Institutes of Health (Bethesda, MD, U.S.A.; to W. B. T.).

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Received 14 June 2003/24 July 2003; accepted 6 August 2003
Published as BJ Immediate Publication 6 August 2003, DOI 10.1042/BJ20030885