Allowed N-glycosylation sites on the Kv1.2 potassium channel S1–S2 linker: implications for linker secondary structure and the glycosylation effect on channel function

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N-glycosylation is a post-translational modification that plays a role in the trafficking and/or function of some membrane proteins. We have shown previously that N-glycosylation affected the function of some Kv1 voltage-gated potassium (K^+) channels [Watanabe, Wang, Sutachan, Zhu, Recio-Pinto and Thornhill (2003) J. Physiol. (Cambridge, U.K.) **550**, 51–66]. Kv1 channel S1–S2 linkers vary in length but their N-glycosylation sites are at similar relative positions from the S1 or S2 membrane domains. In the present study, by a scanning mutagenesis approach, we determined the allowed N-glycosylation sites on the Kv1.2 S1–S2 linker, which has 39 amino acids, by engineering N-glycosylation sites and assaying for glycosylation, using their sensitivity to glycosidases. The middle section of the linker (54 % of linker) was glycosylated at every position, whereas both end sections (46 % of linker) near the S1 or S2 membrane domains were not. These findings suggested that the middle section of the S1–S2 linker was accessible to the endoplasmic reticulum glycotransferase at every position and was in the extracellular aqueous phase, and presumably in a flexible conformation. We speculate that the S1–S2 linker is mostly a coiled-loop structure and that the strict relative position of native glycosylation sites on these linkers may be involved in the mechanism underlying the functional effects of glycosylation on some Kv1 K^+ channels. The S3–S4 linker, with 16 amino acids and no N-glycosylation site, was not glycosylated when an N-glycosylation site was added. However, an extended linker, with an added N-linked site, was glycosylated, which suggested that the native linker was not glycosylated due to its short length. Thus other ion channels or membrane proteins may also have a high glycosylation potential on a linker but yet have similarly positioned native N-glycosylation sites among isoforms. This may imply that the native position of the N-glycosylation site may be important if the carbohydrate tree plays a role in the folding, stability, trafficking and/or function of the protein.

Key words: extracellular linker, N-glycosylation, endoplasmic reticulum, voltage-gated potassium channel.

INTRODUCTION

Potassium (K^+) channels are responsible for re-polarizing and shaping action potentials (reviewed in [1]). Voltage-gated Kv K^+ channels, from the same subfamily, functionally express themselves as homotetramers or heterotetramers, and numerous amino acid regions are involved in the channel function (reviewed in [1–3]).

Kv1.2 voltage-gated K^+ channels [4] are glycosylated proteins [5–8] which are found throughout the brain [9–11] and are a major component of the dendrotoxin-binding protein complex [5–7]. It has been proposed that Kv1 subunits form tetramers in the ER (endoplasmic reticulum), and determinants involved in proper folding include the cytoplasmic N-terminus and the S1 and S2 segments [1–3]. Conventional models of the membrane topology of Kv K^+ channel have hypothesized that there are six α-helical transmembrane segments (S1–S6) in each monomer [1–3]. In contrast, the crystal structure of a bacterial KvAP voltage-gated K^+ channel suggested that its S1–S4 α-helices are not full transmembrane segments [12]. We have shown that N-glycosylation of Kv1.1 voltage-gated K^+ channels on their S1–S2 extracellular linkers affected their functional properties [13,14], and we found a similar functional effect for glycosylation of Kv1.2 channels (preliminary results). We have proposed that glycosylation of Kv1 channels affected gating both by a surface potential and a co-operative subunit interaction mechanism [13,14]. That is, the negatively charged sialic acids on the tree appeared to form part of the effective surface charge density. Hence, the surface potential affected gating, and the sugar tree also affected co-operative subunit interactions that affected channel function. Kv1.2 has one extracellular N-glycosylation consensus site (NST) on its S1–S2 linker (N-glycosylation consensus sites are NXT/S, with X being any amino acid except proline [15]).

N-glycosylation of plasma membrane proteins takes place in the ER. It may be a co- or post-translational modification, and processing of the sugar tree continues in the ER and Golgi before delivery of the glycoprotein to the cell surface [15]. Analysis of numerous plasma membrane proteins has suggested a number of general rules for native N-glycosylation (reviewed in [16]): (a) the consensus site was on an extracellular linker, (b) the consensus site was usually on the first extracellular linker from the N-terminus, (c) the consensus site was >10 amino acids from a putative transmembrane domain and (d) consensus sites that fulfilled (a–c) still may not be glycosylated. These observations suggested that a consensus site must also be accessible or in a flexible conformation for glycosylation by the membrane-bound ER glycotransferase complex. Kv1.2, similar to Kv1.1, has only

Abbreviations used: CHO, Chinese-hamster ovary; endo H, endoglycosidase H; ER, endoplasmic reticulum; PNGase F, peptide N-glycosidase F.

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one N-glycosylation consensus site on its S1–S2 linker which fulfils the above requirements and is glycosylated. In contrast with Kv1.2, the Kv2.1 S1–S2 linker, which has only 18 amino acids and no native N-glycosylation consensus site, has been proposed to have a rigid α-helical structure with one face of the helix interacting with the protein embedded in the membrane [17].

The aim of the present study was to determine the allowed N-glycosylation sites on the S1–S2 linker of Kv1.2 K^+ channels by a scanning mutagenesis approach. The accessibility of amino acid residues for glycosylation would give insight into the rigidity or flexibility of a linker, the environment (aqueous versus membrane), i.e. the regions where the S1–S2 linkers reside, and the mechanism of the glycosylation effect on channel function. We also determined whether the S3–S4 linker could be glycosylated.

EXPERIMENTAL

Cell lines, plasmids and transfections

CHO (Chinese-hamster ovary) pro5 cells, from A.T.C.C. (Rockville, MD, U.S.A.), were maintained in Dulbecco's modified Eagle's medium with 0.35 mM proline or α-modification of Eagle's medium with 10 % (v/v) foetal bovine serum at 37 °C under 5 % CO_2. Mutagenesis used standard PCR or replication mutagenesis [18]. The Kv1.2N207Q cDNA was used as a template to construct different cDNAs with one added N-glycosylation site (NST) at various positions along the S1–S2 extracellular linker. Kv1.1 and Kv1.6 [4] cDNAs were also used. cDNAs were subcloned into the eukaryotic expression vector pcDNA3 and sequencing confirmed the integrity of the constructs. LIPOFECTAMINE™ Plus (Gibco BRL) reagent was used for transient transfections on cells plated in a 35 mm dish using 0.5 μg of plasmid/dish following the manufacturer's instructions, and cells were incubated for 20–24 h post-transfection before processing. Cell-surface expression levels for the Kv1.2 protein were maximal at approx. 15 h (results not shown).

Membrane isolation and enzyme treatment/immunoblot analysis

Total cell membranes (ER, Golgi and plasma membrane) were isolated in ice-cold hypo-osmotic media with protease inhibitors and stored at −85 °C [18]. Total cell membranes recovered from a 35 mm dish were used to run on SDS [9 % (w/v) gel] with approx. 25 μg of membrane protein/gel lane. Proteins were electrotransferred to nitrocellulose (Bio-Rad); the filter was blocked in PBS with 5 % (w/v) non-fat milk, and then incubated overnight in Kv1.2 mouse monoclonal C-terminal antibodies (to amino acids 428–499), at 1:2000 (Upstate Biotechnology, Lake Placid, NY, U.S.A.). The solubilized total membranes were treated according to the manufacturer's instructions for 20–24 h at 37 °C, with the final concentration of glycosidases (Boehringer Mannheim, Germany) at 0.16 unit/ml for endo H (endoglycosidase H), 13 units/ml for PNGase F (peptide N-glycosidase F) or 0.01 unit/ml for sialidase or 60 units/ml for calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, U.S.A.). The specificity of the Kv1.2 monoclonal antibody has been described in [19]. After washes, Kv1.2 proteins were then visualized with horseradish peroxidase-linked anti-mouse secondary antibodies with enhanced chemiluminescence (ECL® detection kit; Amersham Biosciences) using preflashed Kodak X-AR5 film. A Microtek 8700 scanmaker was used to scan a film image for display and/or densitometry analysis [18]. Non-transfected CHO cells showed no immunoblot signals when antibodies to Kv1.2 were used (results not shown).

Kv1.2 cell-surface protein analysis

Cell-surface biotinylation used hydrazide-LC-biotin (Pierce), which is specific for carbohydrates or (sulphosuccinimidyl-6-(biotinamido)ethyl-1,3-dithiopropionate)biotin (Pierce), which is specific for primary amines. Transfected CHO cell 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 [18]. In certain cases, densitometry analyses were performed as described previously [18].

Protein secondary-structure prediction programs

Protein secondary-structure prediction programs were used to predict the α-helical, β-strand and coiled-loop content of different amino acid sequences of Kv1 channels. Both the total amino acids of the protein or just the amino acids of a subregion were used. The protein secondary prediction program PSIPRED was used to generate the data in Figures 3(A) and 3(B) [20,21]. The PSIPRED web site is http://bioinf.cs.ucl.ac.uk/psipred/psiform.html. Other prediction programs gave similar results, including PHDsec [22,23], PROF [24], APSSP2 [25], SSpro and SSpro8 [26] and JPred [27].

RESULTS AND DISCUSSION

Kv1.2 proteins in brain and in transfected cell lines exhibited different banding patterns

A Kv1.2 [4] monomer is represented in a conventional topology model with its six transmembrane segments and its N-glycosylation site at N207 (NST) (Figure 1A). The NST site is on the Kv1.2 S1–S2 linker and has 25 and 15 amino acids respectively from the S1 and S2 segments (Figure 1B). Immunoblot analysis indicated that the denatured Kv1.2 monomer in brain was a diffuse 80 kDa (p80) band (Figure 1C, lane 1) that was insensitive to endo H (Figure 1C, lane 2), which cleaves only high mannose trees, but sensitive to (a) PNGase F (Figure 1C, lane 3), which cleaves all N-linked trees, (b) sialidase (Figure 1C, lane 4), which cleaves terminal sialic acids and (c) calf intestinal alkaline phosphatase (Figure 1C, lane 5), which cleaves phosphates from proteins. Thus brain Kv1.2 protein was a mature glycoprotein that was glycosylated in the *trans*-Golgi and was basally phosphorylated.

In contrast, transiently expressed Kv1.2 in CHO cell total membranes (ER, Golgi and plasma membrane) was detected on immunoblots predominantly as a 60 kDa (p60) band with a lesser amount of an 80 kDa (p80) band (Figure 1D, lane 2). This p80 band was similar to the one in brain and was sensitive to PNGase F but insensitive to endo H. This suggested that it was a mature glycoprotein, whereas the p60 band was sensitive to both glycosidases, which implied that it was a high mannose-type glycoprotein (Figure 1E). Cell-surface biotinylation/immunoblotting showed that the Kv1.2 p80 band was enriched on the cell surface (Figure 1D, lane 4) as against a whole-cell immunoblot (ER, Golgi and plasma membranes) (Figure 1D, lane 2). This finding suggested that most of the p60 in total cell membranes was partially ER-retained, but these glycoproteins were eventually glycosylated to mature glycoproteins in the Golgi. Kv1.2N207Q exhibited bands (Figure 1D, lane 3) that were insensitive to both glycosidases, which showed that there was only one N-linked sugar tree at N207 (Figure 1E, lanes 4–6). The predominant p57 band was not N-glycosylated and the upper bands were also not N-glycosylated but phosphorylated (results not shown).

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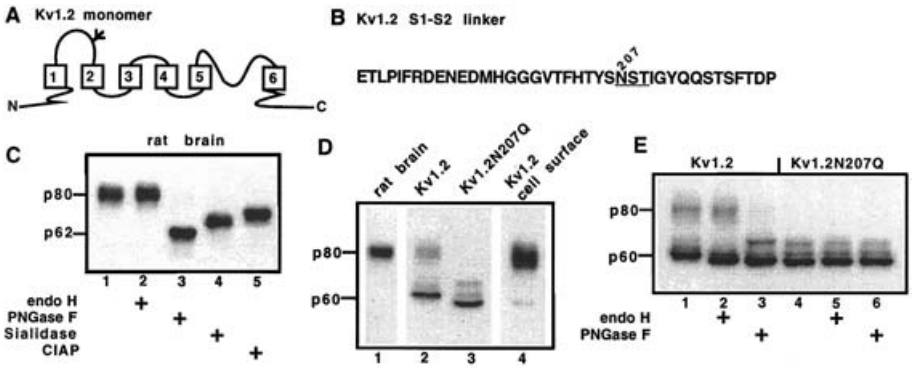

Figure 1 The Kv1.2 channel, a heavily post-translationally processed protein

(**A**) Conventional membrane topology model of a Kv1.2 monomer with the S1–S2 extracellular linker containing an N-glycosylation tree. (**B**) Amino acid sequence of the Kv1.2 S1–S2 linker with the NST consensus site underlined. (**C**) Rat brain total membranes were submitted to different enzymic treatments (endo H, PNGase F and sialidase are glycosidases and CIAP is calf intestinal alkaline phosphatase), run on SDS gels and immunoblotted with Kv1.2 antibody. (**D**) Kv1.2 immunoblot of total membranes (ER, Golgi and plasma membranes) and cell-surface biotinylated Kv1.2 proteins from CHO cells transfected with Kv1.2 cDNA. (**E**) Endo H and PNGase F treatment of Kv1.2 and Kv1.2N207Q.

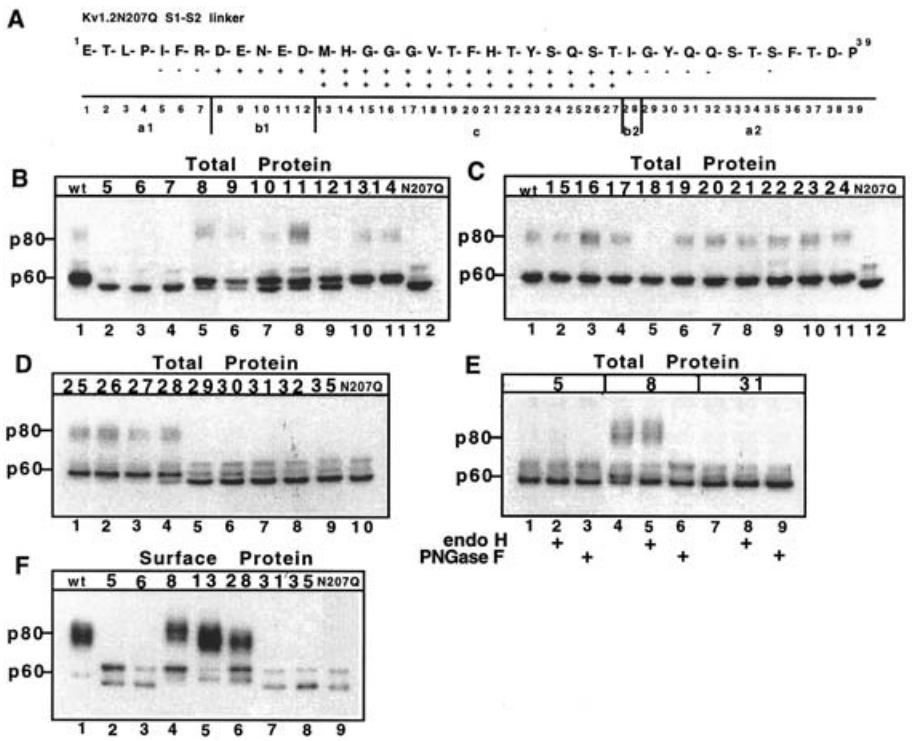

Figure 2 Possibility of N-glycosylation at every position in the middle region of the Kv1.2 channel S1–S2 linker

(**A**) Kv1.2 S1–S2 linker amino acid sequence and a summary of the results from (**B–D**). NST sites were engineered on the Kv1.2N207Q construct at various positions. For example, position 5 represents that an NST site replaced the IFR sequence and position 6 represents that an NST site replaced the FRD sequence. −, site was not glycosylated; +, both glycosylated and unglycosylated proteins were detected; ++, all protein bands were glycosylated. (**B–D**) Immunoblot of total membranes from CHO cells transfected with different Kv1.2 cDNAs. wt = Kv1.2 and N207Q = Kv1.2N207Q, which are control cDNAs and 5–32 and 35 cDNAs are Kv1.2N207Q constructs with one engineered NST site at various positions along the S1–S2 linker as shown in (**A**). (**E**) Endo H and PNGase F treatment of selected Kv1.2 constructs. (**F**) Selected Kv1.2 construct cell-surface protein pattern by surface biotinylation and immunoblotting.

The Kv1.2 S1–S2 linker can be N-glycosylated at every position along its middle section and this linker is predicted to be mostly a coiled-loop structure

An N-glycosylation site-scanning mutagenesis approach, with the Kv1.2N207Q template, was used to introduce an NST site at various positions along the S1–S2 linker (Figure 2A). For example, the number 5 position in the S1–S2 linker in Figure 2(A) shows that an NST site replaced the Ile-Phe-Arg amino acid sequence. The NST sequence was used since it is the wild-type consensus sequence. These Kv1.2 mutants were transiently transfected into CHO cells, and Kv1.2 proteins were assayed for N-glycosylation by immunoblotting total cell-membrane proteins along with Kv1.2 and Kv1.2N207Q controls (Figures 2B–2D). Glycosidase

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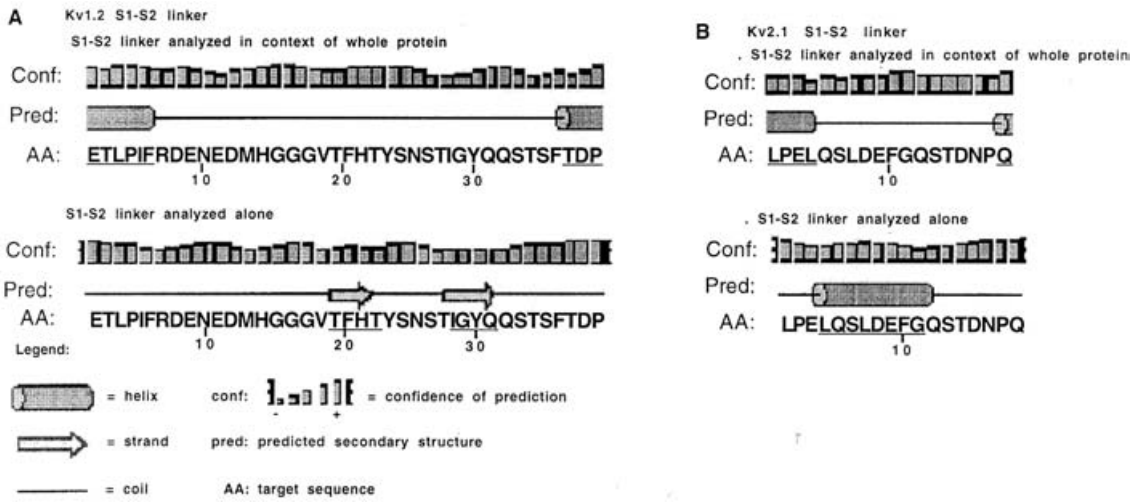

Figure 3 Prediction of a coiled-loop structure for the Kv1.2 S1–S2 linker

(**A**, **B**) Kv1.2 and Kv2.1 S1–S2 linker predicted protein secondary structure, using the total amino acids of the protein or just the S1–S2 linker amino acids by the PSIPRED method, see the Experimental section [20,21].

sensitivity was used to examine whether Kv1.2 bands were glycosylated or unglycosylated core proteins, and selected examples are shown in Figure 2(E). Results starting from the S1–S2 linker N-terminal region are discussed below and a summary is shown in Figure 2(A). We found that (a) positions 5–7 showed no N-glycosylation (Figure 2B, lanes 2–4), which we include in the a1 linker section (Figure 2A), (b) positions 8–12 showed incomplete N-glycosylation with both glycosylated and non-glycosylated bands (Figure 2B, lanes 5–9), which we term as the b1 section (Figure 2A), (c) positions 13–27 exhibited complete N-glycosylation (Figure 2B, lanes 10 and 11; Figure 2C, lanes 2–11; Figure 2D, lanes 1–3), which we term the c section (Figure 2A), (d) position 28 showed incomplete N-glycosylation (Figure 2D, lane 4), which we term the b2 section (Figure 2A), and (e) positions 29–32 and 35 were not N-glycosylated (Figure 2D, lanes 5–9), which we include in the a2 section (Figure 2A).

These results suggested the following for the Kv1.2 S1–S2 linker. Every position in the middle sections (b1, c and b2) or 54% (21/39) of the linker was partially or completely glycosylated, whereas positions on both end sections (a1 and a2) or 46% (18/39) of the linker were not glycosylated (Figure 2A). This finding suggested that the structure of the middle section was accessible to the ER glycotransferase at every position. We speculate that the linker here is flexible and that it resides away from the outer membrane surface. This secondary structure is probably a coiled loop without α-helical content, since we detected no periodicity with allowed glycosylation sites. Consistent with this notion, protein secondary structure programs (e.g. the PSIPRED method [20,21]) predicted that the S1–S2 linker of Kv1.2 (or the S1–S2 linker of Kv1.1) was a coiled loop in the bulk of the linker with only short-end regions that showed some α-helical content using total amino acids of the protein, and only short middle regions that showed some β-strand content when using only linker amino acids (Figure 3A).

In contrast, the Kv2.1 S1–S2 linker, with 18 amino acids and no N-glycosylation consensus sites, has been proposed to be a partial α-helix on the basis of an alanine scanning mutagenesis study [17]. It was hypothesized further that an α-helical face was in close proximity to the channel's protein surface in the membrane and implied that this linker had a very rigid structure. Protein secondary structure programs predicted different α-helical content for the Kv2.1 S1–S2 linker: low α-helical content when the total amino acid sequence of the protein was analysed and significant α-helical content if only linker amino acids were analysed (Figure 3B). Thus it appears that two voltage-gated K^+ channels in different subfamilies may have quite different S1–S2 linker structures.

The Kv1.2 a1 and a2 sections were not accessible to the ER glycotransferase, presumably because of their close proximity to the S1 and S2 membrane domains. The a1 section was shorter (seven amino acids) when compared with the a2 section (11 amino acids), which implied that the N-terminal region of the linker was more accessible to the ER glycotransferase. This differential accessibility may be related to how the membrane segments are inserted into the ER membrane and how the S1–S2 and S3–S4 linkers are folded. The b1 and b2 sections were accessible to the ER glycotransferase but incomplete glycosylation occurred in these sections. The b1 section was longer (five amino acids) when compared with the b2 section (one amino acid), which suggested again that the N-terminal linker region was more accessible to the ER glycotransferase. The native b1 section has four charged amino acids and some of these charges, along with the positively charged arginine in a1, probably contributed to the aqueous exposure and accessibility of this section for glycosylation.

We next determined that unglycosylated mutant proteins at sections a1 and a2 were expressed on the cell surface by cell-surface biotinylation/immunoblotting (Figure 2F, lanes 2, 3, 7 and 8). In addition, mutant proteins at sections b1 and b2 were expressed only as glycosylated proteins on the cell surface (Figure 2F, lanes 4 and 6) as was a mutant protein at section c (Figure 2F, lane 5). These results suggested the following: (a) N-glycosylation sites placed near the S1 and S2 membrane domains of Kv1.2 did not interfere with its proper ER translocon insertion and these proteins were trafficked to the cell surface. (b) Non-glycosylated proteins at sections b1 and b2 in the ER in total membranes were all N-glycosylated eventually before trafficking to the cell surface. Thus ER glycotransferase activity appeared inefficient here, as some full-length protein was synthesized before it was glycosylated; however, given enough time, these proteins were glycosylated post-translationally.

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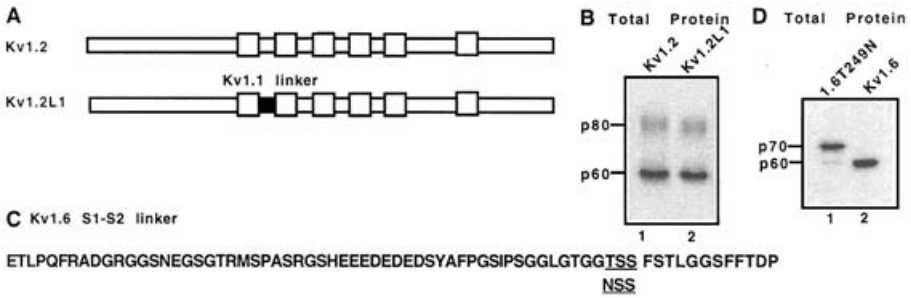

Figure 4 Further investigation of N-glycosylation sites on Kv1 proteins

(**A**) Schematic representation of a Kv1.2L1 construct that had the Kv1.1 S1–S2 linker. (**B**) Immunoblot of total membranes from transfected CHO cells with Kv1.2 or Kv1.2L1. (**C**) The Kv1.6 S1–S2 linker, which does not contain a native N-glycosylation site, with an engineered NSS site. (**D**) Immunoblot of total membranes from transfected CHO cells with Kv1.6 or Kv1.6T249N. Both constructs had an engineered haemagglutinin epitope tag on the C-terminus and specific haemagglutinin antibodies were used for the immunoblot.

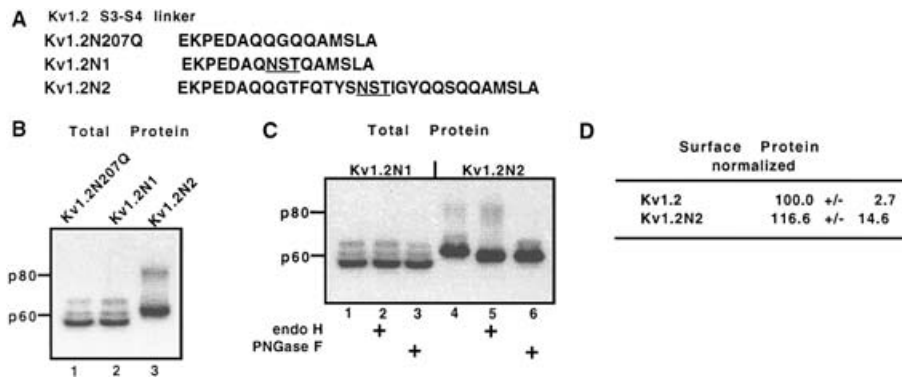

Figure 5 Possibility of N-glycosylation of the Kv1.2 channel S3–S4 linker on placing an additional N-glycosylation site on an extended linker

(**A**) Amino acid sequence of the S3–S4 linker in Kv1.2, in Kv1.2N1 with an added NST site and in Kv1.2N2 with an added NST site on an extended linker. Kv1.2N2 S3–S4 linker was extended with the amino acid sequence TFQTYNSTIGYQQS, which is essentially identical with an equivalent sequence on the Kv1.2 S1–S2 linker, and we used different codons to construct the mutant cDNA. (**B**) Immunoblot analysis of total proteins of CHO cells transfected with Kv1.2, Kv1.2N1 or Kv1.2N2. (**C**) Glycosidase (endo H or PNGase F) treatment of Kv1.2N1 and Kv1.2N2. (**D**) Kv1.2 and Kv1.2N2 normalized surface protein levels by cell-surface biotinylation and immunoblotting. The Kv1.2 level was taken as 100.0 $\pm$ S.E.M. and the Kv1.2N2 level was normalized to it.

Kv1.2 with the Kv1.1 S1–S2 linker was glycosylated similarly to wild-type, and Kv1.6 was glycosylated on its S1–S2 linker if an N-glycosylation site was engineered on it

The Kv1.2L1 mutant (Figure 4A), with the Kv1.1 S1–S2 linker having 34 amino acids instead of the 39 amino acids of Kv1.2, showed a similar glycosylation pattern in total membranes as did wild-type Kv1.2 (Figure 4B, lanes 1 and 2). Kv1.6 does not have a native N-glycosylation site on its S1–S2 linker. However, Kv1.6 with an engineered N-glycosylation site on its S1–S2 linker (Figure 4C) was glycosylated (Figure 4D, lanes 1 and 2). These results suggested that (a) Kv1 subfamily S1–S2 linkers, with different amino acid sequences and different lengths, may be glycosylated similarly when swapped among members, and (b) adding an N-glycosylation site to the Kv1.6 S1–S2 linker may lead to normal N-glycosylation, which is consistent with the notion that this native linker is exposed to the extracellular aqueous environment. Thus Kv1 member extracellular S1–S2 linkers are probably interchangeable with regard to N-glycosylation potential and they may not negatively affect intra-subunit folding and tetramerization of chimaeric channels. However, it is possible that a non-native linker may affect the channel function.

The Kv1.2 S3–S4 linker was only glycosylated if an N-glycosylation site was placed on an engineered extended linker

The Kv1.2 S3–S4 linker has 16 amino acids and no native N-glycosylation consensus site (Figure 5A). A Kv1.2N207Q construct with an engineered N-linked site in the middle of the S3–S4 linker, termed Kv1.2N1 (Figure 5A), was not glycosylated (Figure 5B, lane 2; Figure 5C, lanes 1–3), whereas if this linker was extended to 31 amino acids with an N-glycosylation site, termed Kv1.2N2 (Figure 5A), it was glycosylated (Figure 5B, lane 3; Figure 5C, lanes 4–6). Kv1.2 and Kv1.2N2 were expressed on the cell surface at similar levels when assayed for their surface protein levels by biotinylation/immunoblotting (Figure 5D). These findings are consistent with the notion that the artificially extended S3–S4 linker did not interfere with ER translocon insertion or proper protein folding. The results thus suggested the following. (a) The Kv1.2 S3–S4 linker with an engineered NST site in the context of 16 amino acids was not glycosylated because the site was <10 amino acids from the S3 and S4 segments; the site was probably not accessible to the ER glycotransferase. (b) The S3–S4 linker was glycosylated when the linker, with an added N-glycosylation site, was extended to 31 amino acids; the N-linked

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A

Kv1 S1-S2 linkers

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Kv1.1 ETLPELKDDKDFTGTIHRID.....................NTTVIYTSNIFTDP
Kv1.2 ETLPIFRDENEDMHGGGVTFHTYS..............NSTIGYQQSTSFTDP
Kv1.3 ETLPEFRDEKDYPASPSQDVFEAAN.....,.....NSTSGASSGASSFSDP
Kv1.4 ETLPEFRDDRDLIMALSAGGHSRLL..........NDTSAPHLENSGHTIFNDP
Kv1.5 ETLPEFRDERELLRHPPVPPQPPAPAPGINGSVSGALSSGPTVAPLLPRTLADP
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B

Kv1 channel	S1-S2 linker (aa)	From S1 TM N-site (aa)	From S1 TM RD	From S2 TM N-site (aa)	From S2 TM RD
Kv1.1	34	21	0.62	14	0.41
Kv1.2	39	25	0.64	15	0.38
Kv1.3	41	26	0.63	16	0.39
Kv1.4	44	26	0.59	19	0.43
Kv1.5	54	30	0.55	25	0.46

Figure 6 Location of Kv1 K^+ channel S1–S2 linker N-glycosylation sites at similar relative distances on the linker

(**A**) Kv1 S1–S2 linker amino acid sequences with the underlined NXS/T N-glycosylation consensus sites. Dots represent spaces in the sequences. (**B**) Characteristics of Kv1 S1–S2 linkers; aa, amino acid number; TM, transmembrane domain; RD, relative distance of the N-glycosylation site from the S1 or S2 segment.

site in this context was accessible to the ER glycotransferase and must be in the extracellular aqueous environment.

Native Kv1 N-glycosylation sites are at a similar relative distance from their S1 and S2 segments and this position may be involved in the glycosylation effect on channel function

In the present study, we showed that N-glycosylation on the S1–S2 linker occurred on both sides of Kv1.2 N207, at 17 positions on the N-terminal side and at 3 positions on the C-terminal side (Figure 2A). In the Kv1 subfamily, Kv1.1–Kv1.5 have S1–S2 linkers that have different lengths and different amino acid sequences with one N-glycosylation site (Figure 6A). However, their N-glycosylation consensus sites are at similar relative distances from the S1 and S2 segments (Figure 6B). Approximately 54 % of the Kv1.2 linker can be glycosylated, but the N-glycosylation site is at a similar relative position in Kv1 channels. Therefore we speculate that the position of the sugar tree on this linker may play a role in the underlying mechanism of glycosylation on Kv1.1 (or Kv1.2) channel function [13,14]. Our previous studies provided evidence for some Kv1 channels being affected by N-glycosylation on their S1–S2 linker by a combined surface-potential mechanism, owing to negatively charged sialic acids on the tree affecting the local electric field sensed by the activating-gating machinery, and a co-operative subunit interaction mechanism, due to the bulky sugar tree affecting conformational transitions. Probably, the similar relative N-glycosylation tree positions on Kv1 channels may affect both the surface potential and the co-operative subunit interactions that have been proposed to affect gating.

Kv K^+ channel membrane topology model and a KvAP K^+ channel structure

Modelling and mutational studies for Kv K^+ channels have suggested that the S1–S6 segments are transmembrane helices with the S1–S3 helices at the perimeter lipid boundary, the S4 helix is packed in the middle of the other helices and, at some regions, the S1–S2 and the S3–S4 linkers are in the extracellular aqueous phase [1–3, 28–30]. In contrast, the three-dimensional structure of a bacterial KvAP voltage-gated K^+ channel has each protein monomer with only the S5 and S6 as complete transmembrane helices, whereas the S1–S2 and the S3–S4 helices, and their complete linkers, are positioned mostly horizontally in the middle or on the inner surface of the lipid bilayer respectively [12]. It was also proposed that the KvAP structure is probably similar for eukaryotic Kv K^+ channels. Although our results do not readily distinguish between these two proposed structures/models, it does constrain regions of the mammalian Kv1.2 S1–S2 linker to the extracellular aqueous phase. Our results suggest that at least 54 % of the S1–S2 linker (21/39 amino acids), as well as a region of the non-native extended S3–S4 linker, resides in the extracellular aqueous environment, since these regions could be glycosylated with a large hydrophilic carbohydrate tree. If the new structural model for bacterial KvAP K^+ channels is applicable to eukaryotic Kv K^+ channels, then one would have to envision that the C- and N-terminal regions of the Kv1.2 S1–S2 linker reside in the membrane phase, whereas the middle linker region is extracellular.

Conclusion

Our N-glycosylation scanning mutagenesis study of Kv1.2 suggested that (a) the Kv1.2 S1–S2 linker secondary structure is probably mostly a coiled loop, (b) the similar relative positions of native N-glycosylation sites on Kv1.2 and Kv1.1 channel S1–S2 linkers may be important in the underlying mechanism of the glycosylation effect on channel function and (c) regions of the S1–S2 linker, as well as an extended S3–S4 linker, must reside in the extracellular aqueous environment, which limits us to models of Kv K^+ channels that have these linker regions in the membrane.

Other ion channels, or other membrane proteins, may also have a high glycosylation potential on a linker (i.e. many engineered positions can be glycosylated) but yet have relatively positioned native glycosylation sites among isoforms. This may imply that the position of the native N-glycosylation site may be important if the carbohydrate tree plays a role in the folding, stability, trafficking and/or function of the membrane protein.

This research was supported by National Institutes of Health grant no. NS29633 (W. B. T.) and a Glycoscience award from the Mizutani Foundation (W. B. T.).

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Received 4 April 2003/6 August 2003; accepted 11 August 2003
Published as BJ Immediate Publication 11 August 2003, DOI 10.1042/ BJ20030517

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