THE ROLE OF THE T1 DOMAIN IN THE TRAFFICKING OF Kv1.5

by

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ABSTRACT

The T1 domain of Kv channels is important in the subfamily-specific assembly by clustering individual monomers to increase their chances of interaction into tetramers. Furthermore, T1 domain plays roles in channel gating, stability as well as an interaction site with auxiliary subunits that ultimately modulate the function and expression of the channel.

We identified a 10 amino acid region at the start of the T1 domain that is an important prerequisite for the functional expression of Kv1.5 ion channels. A number of N-terminal and T1 domain deletions were made that changed the ability of the channel to traffick to the cell surface. N-terminal deletions up to the T1 domain (ΔN119 Kv1.5) and larger deletions of at least 60% of the T1 domain (ΔN189 and ΔN209 Kv1.5) yielded functional Kv1.5 channels. However, smaller deletions of 15 (ΔN135 Kv1.5) and 43 (ΔN163 Kv1.5) amino acids of the T1 domain yielded non-trafficking and non-functional Kv1.5 channels. These mutants formed aggregates and were localized in the ER and the degradation pathway. However, re-addition of the first 10 amino acids of the T1 domain to the non-trafficking mutants rescued their trafficking to the plasma membrane. Furthermore, point mutations of residues thought to be involved in the intermolecular interaction during tetramer formation result in subtle alterations of channel trafficking ability. As well, point mutations of previously identified ER retention motifs (RXR) rescue the aggregation of the non-trafficking mutants with a very small rescue in their current density.

We concluded that protein aggregation may be caused by number of states, such as exposure of ER retention motifs, disruption of important β-sheet secondary
structures, disruption of tetramerization contacts, or exposure of large hydrophobic residues. Additionally, the trafficking and function of ΔN119, ΔN189, and ΔN209 Kv1.5 mutants may be affected by deletion of important sites involved in the modulation of Kv1.5 function, such as phosphorylation and interaction with auxiliary subunits. It is also possible that the combination of these individual processes affect the overall Kv1.5 cell surface expression.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>3 Letter Code</th>
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CFTR          Cystic fibrosis transmembrane conductance regulator
COPI          Coat Protein Complex type I
COPII         Coat Protein Complex type II
COS-7         African green monkey kidney cells
ER            Endoplasmic Reticulum
ERAD          Endoplasmic Reticulum Associated Degradation
GABA          γ-aminobutyric acid B
HEK293        Human Embryonic Kidney 293
hERG          human ether-a-go-go channel
K⁺            Potassium
K<sub>ATP</sub> ATP-sensitive potassium channel
Kir           inwardly-rectifying potassium
K<sub>v</sub>  voltage-gated potassium
MAGUK         membrane-associated guanulate kinases
Nav           voltage-gated sodium
NMDA          N-methyl-D-aspartate
PDZ           PSD-95/DLG/ZO-1
PKA           Protein Kinase A
PKC           Protein Kinase C
PSD-95        Postsynaptic density 95
QC            Quality control
SAP-97        Synapse Associated Protein-97
SH3           Src Homology 3
SURI/II  Sulphonylurea I/II
T1 domain  First Tetramerization domain
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CO-AUTHORSHIP STATEMENT

Martina Jochova was responsible for project management, the experimental design (with the help of Dave Steele, Jodene Eldstrom, and Vijay Viswanathan), mutagenesis, transfections, immunoblotting and immunofluorescence experiments, thesis writing, and data analysis including electrophysiological data analysis. Figure preparation was performed with the help of David Fedida, Jodene Eldstrom and Dave Steele. The electrophysiology measurements of the wt and T1 domain deletion mutants were performed by Zuren Wang and of the N-terminal deletion mutants by Harley Kurata. The current density data was provided by Woo Sung Choi (he has also performed the data analysis in figure 3.12). In addition, Jodene Eldstrom has performed and analyzed the immunofluorescent experiments in figure 3-12.
1 INTRODUCTION

Voltage-gated potassium channels in excitable cells play important roles in propagation of action potentials. Delayed rectifier potassium channels open in response to changes in the transmembrane voltage during the repolarization stage of the cardiac action potential, returning the cells of the cardiac muscle to their resting potential (Marban, 2002). By definition, the amplitude of the macroscopic current is dependent on the number of functional channels in the plasma membrane, the single channel conductance, and the open probability of each channel (Delisle et al., 2004). Disturbances of any of these factors can lead to cardiac arrhythmias like torsade de pointes and ventricular fibrillation, and potentially to sudden death. The changes in single channel conductance and probability of opening arise due to factors affecting the biophysical properties of ion channels, more specifically mutations in the "pore" affecting the permeability and mutations affecting the gating properties of the channel (Meacham et al., 2001; Zhou et al., 1998). Changes in the number of functional channels at the plasma membrane arise from changes affecting channel biogenesis and intracellular trafficking. For example, truncation mutation lead to missfolding or misassembly and subsequently to early protein degradation, and mutations that cause a decrease in transcription efficiency reduce channel expression levels (Curran et al., 1995; Delisle et al., 2004; Tristani-Firouzi et al., 2001). Therefore, an understanding of the structure and biogenesis of ion channels is of great importance and interest.
1.1 Structure and function of voltage-gated potassium channels

In our lab, the focus is on Kv1.5 voltage-gated potassium channels. Kv1.5 is a delayed-rectifier and a mammalian homologue of Shaker. It is expressed ubiquitously, but most significantly in the mammalian cardiac ventricle and atrium (Feng et al., 1997; Ordog et al., 2005; Mays et al., 1995), in the sciatic nerve and other parts of nervous system (Attali et al., 1997; Jou et al., 1998; Peretz et al., 1999; Mi et al., 1995), in pulmonary artery smooth muscle (Yuan et al., 1998; Archer et al., 1998), and in both vascular and visceral smooth muscle (Overturf et al., 1994; Clement-Chomienne et al., 1999). Voltage-gated K⁺ channels, like Kv1.5, consist of four identical monomer subunits assembled in four fold symmetry around a gated central potassium conducting pore, as seen in Figure 1.1B (Jan and Jan, 1997). Each monomer contains six transmembrane domains, S1 – S6, as well as cytoplasmic NH₂ and COOH termini (Figure 1.1A). The transmembrane domains contain mainly hydrophobic residues and are connected by alternating intracellular and extracellular linkers. The S1-S4 domains contain the voltage sensor, largely consisting of the S4 domain which senses changes in the potential across the membrane (Hille, 1992). S4 contains several Arg’s (arginines), spaced at every 3rd position, that cause the S4 to move across the membrane electric field subsequently forcing the channel into open and closed conformations (Bezanilla et al., 1991; Liman et al., 1991; McCormack et al., 1991; Papazian et al., 1991). The S5 and S6 domains line the pore of the tetrameric channel. The pore is structurally adapted for ion selectivity and gating, and regulates the ion conduction through the channel.
Figure 1-1 Schematic Structure of Kv channel

A. Schematic structure of Kv channel monomer, consisting of six transmembrane domains and cytoplasmic NH$_2$ and COOH termini. The TM domains are mainly hydrophobic and are connected by alternating intracellular and extracellular linkers. TM domains S1 – S4 make up the voltage sensor. S4 is made up of a number of positively charged Arg residues. The S5, P-loop (selectivity filter), and S6 form the pore of the channel that regulates the ion conduction through the channel.

B. Tetrameric structure of Kv channel. Four monomers, each consisting of six transmembrane domains, assemble together in a four-fold symmetry around a central gated pore. (from Sigma RBI)
The P-loop, connecting S5 and S6, contains the selectivity filter in the ion gateway between the extracellular and intracellular environments (Hartmann et al., 1991; Heginbotham et al., 1992; Pongs, 1992; Yool and Schwarz, 1991). More specifically, in Shaker and mammalian Kv1 channels, the selectivity filter signature sequence, TXXTXGYGD, allows efficient permeation of K$^+$ ions but not other cations like Na$^+$ (Heginbotham et al., 1992; Heginbotham et al., 1994). The carbonyl oxygens line the selectivity filter facing the pore and mimic hydration shells as dehydrated K$^+$ ions are directed through the pore (MacKinnon, 2003; Valiyaveetil et al., 2004). The structure of the selectivity filter is maintained by number of interactions involving the residues of the signature sequence and the rest of the Kv channel; these include adjacent tryptophan residues (W67 and W68 in KcsA, W434 and W435 in Shaker) that form an 'aromatic cuff' and interact with tyrosine (Y78 in KcsA, Y445 in Shaker) near the extracellular entrance of the selectivity filter (Doyle et al., 1998; Kurata and Fedida, 2005). These interactions are critical for the function of the selectivity filter since their disruption causes changes in pore selectivity and gating (Heginbotham et al., 1994; Perozo et al., 1993; Kirsch et al., 1995; Yang et al., 1997).

The gating of voltage-gated potassium channels involves opening and closing of the channel according to the voltage across the membrane, so as to permit or prevent the passage of ions. The KcsA and MthK channels crystal structures are consistent with the intracellular part of the pore functioning as a 'gate' and their structural differences have been used to model the gating mechanisms of Kv channels (Jiang et al., 2002b). The crystal structure of KcsA shows the intracellular side of the M2 domains (same as S6 in Kv channels) crossing over and forming a
bundle at the intracellular side of the channel, known as the inner helix bundle or the bundle crossing (Doyle et al., 1998; Jiang et al., 2002b). The crystal structure of MthK, on the other hand, shows that the intracellular side of the M2 domains are bent so that the pore is wide open, exposing the pore cavity to the cytoplasm (Doyle et al., 1998; Jiang et al., 2002a; Jiang et al., 2002b). It is believed that the difference between the two crystal structures accounts for the difference between a 'closed' (KcsA crystal structure) channel and an 'open' (MthK crystal structure) channel. In addition, the crystal structure of Kv1.2 shows that the extracellular side of the pore is highly conserved in all prokaryotic and eukaryotic channels, whereas the inner side of the pore of Kv1.2 falls between the 'open' conformation (MthK and KvAP crystal structure) and the 'closed' conformation (KcsA crystal structure) (Long et al., 2005). Therefore, it is suggested that the S6 domain undergoes a conformational change during gating (the process by which the pore opens and closes), forcing the α helices to swing open, exposing the pore cavity to the intracellular environment (Jiang et al., 2002b; Kurata and Fedida, 2005). The gating mechanism is thought to involve a "gating hinge" made up of highly conserved glycine residue (Gly 83 in MthK, Gly 99 in KcsA, Gly 386 in Shaker, Gly 504 in Kv1.5) in the 'inner' helices of the S6 region (Jiang et al., 2002b), or the Pro-X-Pro (X is any amino acid) motif in Shaker channels (absent in prokaryotic channels) thought to kink the S6 inner helices (Long et al., 2005) to achieve the different conformations.
1.1.1 Structure and function of T1 domain

Members of the *Shaker* (Kv1) family contain a highly conserved region in their cytosolic NH$_2$ termini, known as the first tetramerization (T1) domain (Tempel et al., 1987; Tempel et al., 1988; Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988; Yokoyama et al., 1989). Initially, sequence alignment of the NH$_2$ terminus in Kv1 ion channel family members showed three highly conserved regions (≥70% homology) interspersed by variable regions (Shen and Pfaffinger, 1995; Xu et al., 1995). By deletion mutagenesis, the authors identified the first two conserved regions, or the T1 domain, to be important in subfamily-specific monomer interactions. Later studies showed the importance of the T1 domain in maintaining overall stability of the channel where channels without the T1 domain are easily degraded into monomers (Strang et al., 2001). Furthermore, the T1 domain has been shown important in gating of Kv channels (Cushman et al., 2000; Minor et al., 2000; Kurata et al., 2002) and as an attachment site of the auxiliary β subunit proteins (Gulbis et al., 2000).

The crystal structures of the isolated *Shaker* T1 domain (Kobertz et al., 2000) and of the Kv1.2 T1 domain – β subunit complex (Long et al., 2005), show the tetrameric T1 domain suspended in the cytoplasm below the pore of the channel by linkers between the T1 and S1 domains, just like a “hanging gondola” (see figure 1.2 and figure 1.4A). The T1 – S1 linkers project outward from the channel’s central axis maintaining a separation between the pore and the intracellular regions (Long et al., 2005). Ions reach the pore through the “windows” between the linkers or “cables” of the gondola-shaped T1 domain (Kobertz et al., 2000). In addition, the side ‘windows’
are large enough to allow entry of the N-terminal inactivation "ball" which functions as an inactivation domain during the N-type inactivation process in *Shaker* Kv channels (Hoshi et al., 1990; Zagotta et al., 1990). This inactivation ball domain may be a part of the Kvα channel or a part of the interacting Kvβ subunit (see below text and figure 1.4A). The T1-S1 linkers may be involved in establishing electrostatic interactions with the hydrophobic region of the N-terminal ball during inactivation (Gulbis et al., 2000; Zhou et al., 2001; Long et al., 2005).

![Figure 1-2 Crystal structure of T1 domain and β subunit](image)

**Figure 1-2 Crystal structure of T1 domain and β subunit**

Ribbon representation of T1 tetramer, shown from the N-terminal side (bottom), C-terminal side (top) and side view. The T1 monomers assemble in a four-fold symmetry just like the TM domains of the Kv channels. The monomer interaction takes place via antiparallel β-sheet structure at the N-terminal part of T1 domain (Minor et al., 2000).
Four T1 domains come together, one from each monomer, to form a tetrameric structure. Using the NH$_2$ terminus of the Shaker B channel as a polypeptide, Min Li and colleagues, were among the first to show that a distal portion of the NH$_2$ terminus (aa83-196 in Shaker B channel) is involved in intersubunit interactions among monomers belonging to the same family of ion channels (Li et al., 1992). Conversely, studies of chimeras consisting of NH$_2$ terminus of an unrelated family of ion channels (DRK1) added to the rest of the Shaker B channel, showed a lack of interaction between the chimeric channel and the wt Shaker B channel. These findings were confirmed by yeast-two-hybrid studies using chimeric constructs consisting of a synthetic peptide, homologous in sequence to the T1 domain, replacing the NH$_2$ terminus of either Kv1.1 or Kv3.1 channels (Shen and Pfaffinger, 1995; Xu et al., 1995). Attaching the T1 domain to both Kv1.1 and Kv3.1 channels resulted in homotetramer formation among Kv1.1 or Kv3.1 monomers as well as heterotetramerization among Kv1.1 and Kv3.1 monomers/dimers. Furthermore, the authors hypothesized that in order to allow for the subfamily interactions to take place, the cytoplasmic NH$_2$ termini must be folded in such a way as to expose the particular amino acids involved in the interactions. Disruption of the proper folding by affecting the orientation of the interacting residues will lead to lack of tetramer formation. Therefore, one function for the T1 domain was identified: to bring together monomers into structural and functional homotetramers (monomers of the same family member, i.e. Kv1.1) and heterotetramers (monomers of different members of the same subfamily, i.e. Kv1.1 and Kv1.4), and to prevent interaction between different families of ion channels. In later studies, it was shown that Shaker
B channels can tetramerize even in the absence of the T1 domain, however the tetramerization efficiency was quite low (Zerangue et al., 2000). Therefore, the T1 domain plays an important first step in the assembly, where it clusters monomers in order to increase their chances of interaction, thus promoting the formation of functional channels.

During the process of gating, in response to membrane depolarization, the S4 transmembrane domain moves across the membrane electric field forcing the channel into open and closed conformations (Bezanilla et al., 1991; Liman et al., 1991; McCormack et al., 1991; Papazian et al., 1991). It is hypothesized that the T1 domain is physically linked to the conformational changes that take place in the transmembrane pore (Cushman et al., 2000; Minor et al., 2000). The exact mechanism is not understood but it is thought that changes in the conformation of the pore, force a global conformational change on the T1 domain, affecting the positions and interactions of a number of polar residues within the T1 domain.
1.2 The Biogenesis of ion channels

In order to avoid insertion of incorrectly folded or oligomerized ion channels into the plasma membrane, the biogenesis and intracellular trafficking of ion channels from the endoplasmic reticulum (ER) to the plasma membrane are highly regulated. Therefore, to ensure that only structurally and functionally correct channels are expressed, the cell is equipped with various quality control (QC) checkpoints along the secretory pathway. The ER is the organelle with stringent QC, since this is the start of the protein biogenesis where newly synthesized proteins are folded and assembled into functional moieties. As a result, ER has primary and secondary QC mechanisms to ensure that only functional proteins enter their final destinations. The primary QC mechanism involves association of newly synthesized proteins with ER chaperone proteins to ensure correct folding and assembly before exit out of ER. Secondary QC involves modulatory mechanisms responsible for protein export out of the ER, including masking and unmasking of motifs encoded in the primary amino acid sequence of the nascent protein (see below). Missfolded proteins are tagged by covalent binding of ubiquitinating protein(s) for degradation (Haas and Siepmann, 1997). Once the proteins pass the ER QC machinery they can move into the Golgi network for further maturation processes, including more complex glycosylation and directional sorting to the plasma membrane.
Proteins are synthesized, folded and assembled in the ER, where membrane-associated ribosomes are shown in yellow. If the proteins are missfolded, they are shunted into the cytosol and degraded by the proteasome. If the proteins are correctly folded, they leave the ER through the ER exit sites, where the COPII coat proteins (yellow triangles) bind the nascent proteins. Inclusion of proteins into COPII vesicles is selective. Once the COPII vesicles have detached from the ER, the cargo protein is released into the Golgi and the COPII coat is replaced by the COPI coat (yellow rectangles). COPI vesicles are involved in the formation of return vesicles that bring ER resident proteins and any proteins that escaped ER QC, back to the ER. The Golgi network, from which cargo moves to the plasma membrane, serves also as a location for the rerouting of misfolded proteins into degradation pathway (Ellgaard et al., 1999).
The transportation of the newly synthesized proteins throughout the secretory pathway (between ER, Golgi and plasma membrane) is made possible via secretory vesicles (Figure 1.3). The main components of the transport vesicles are coat proteins that interact with specific amino acid motifs encoded in the primary amino acid sequence of the cargo protein (Balch et al., 1994; Aridor and Balch, 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996). To date, three types of coat proteins and their functions have been identified; clathrin, COPI and COPII coat proteins. The clathrin coat is involved in the late secretory pathway between Golgi and plasma membrane (Kirchhausen, 2000; Robinson, 1994). COPI and COPII coat proteins are involved in the early secretory pathway between ER and Golgi in retrograde and anterograde directions, respectively (Antonny and Schekman, 2001; Klumperman, 2000; Orci et al., 1997). The COOH-terminal tyrosine-based (YXXΦ; X is any amino acid and Φ is a bulky/hydrophobic amino acid) and di-leucine (LL) motifs are recognized by clathrin-mediated endocytosis (Kirchhausen, 2000; Kirchhausen, 2002). The C-terminal di-lysine motifs are recognized by COPI coat protein and position-independent di-acidic motifs, identified in fast ER export, are recognized by COPII coat protein (Bonifacino and Traub, 2003). Once the newly synthesized proteins reach the plasma membrane, they are then endocytosed into the cytoplasm and either recycled back to plasma membrane or shunted to the degradation pathway (Abriel and Staub, 2005).
1.2.1 Regulation of ion channel expression via transcription

The QC mechanism of the expression of ion channels begins with the regulation of transcription by differential splicing of introns from the heteronuclear RNA to produce mRNAs of different nucleotide content (Levitan et al., 1991; Takimoto et al., 1993; Mori et al., 1995). Gene transcription can be upregulated by the glucocorticoid receptor agonist dexamethasone as well as natural glucocorticoids (i.e. corticosterone) that act by directly stimulating glucocorticoid-response element found upstream of the translation start site in Kv1.5 (Levitan et al., 1991; Takimoto et al., 1993; Mori et al., 1995). In vivo, injection of dexamethasone glucocorticoid was seen to up-regulate Kv1.5 protein expression 20-fold in the heart (Takimoto and Levitan, 1994). Additional regulations on transcription and translation of Kv1.5 and other voltage-gated ion channels in neurons and heart have been also shown; i.e., Kv1.5 and Kv2.1 expression is downregulated by neuropeptide TRH (Takimoto et al., 1995), Kv1.5 and Kv1.6 expression in striatal neurons is reduced by opiates (pharmacological stimulus) (Mackler and Eberwine, 1994), and ventricular Kv4.2 and Kv4.3 expression in the heart is enhanced by thyroid hormone (Shimoni et al., 1997). This type of regulation is slow, occurring over a period of hours, and therefore relevant mainly to long-term effects.

1.2.2 Regulation of ion channel expression in the ER

Mammalian membrane proteins, like ion channels, are thought to enter the ER co-translationally and through a ribosome that is attached to a translocon (Ellgaard and Helenius, 2003). A translocon is a membrane protein, located in the
ER membrane which has a tunnel-like structure that serves as a passageway for the nascent protein as it is being translated in the ER lumen (White and von Heijne, 2004). While the nascent protein is still attached to the ribosomes, co-translational folding takes place followed by post-translational folding, post-translational modifications, and oligomerization of monomers (Ellgaard and Helenius, 2003). The post-translational modifications, like disulphide-bond formation, signal-peptide cleavage, and N-linked glycosylation, are important for the correct folding and assembly of the new protein. These modifications are performed with the help of ER chaperones, like calnexin, calreticulin, BiP, GRP94, ERP57, ERP72 and protein disulfide isomerase (PDI), which are also part of the QC machinery (Ellgaard et al., 1999). During normal biogenesis these chaperone proteins recognize, even if only transiently, exposed features like hydrophobic patches and exposed mobile loops.

Kv1 channels undergo core glycosylation in the ER, followed by further processing of the oligosaccharide tree in the Golgi (Kornfeld and Kornfeld, 1985). The Glu\(_3\)Man\(_9\)GlcNac\(_2\) core sugar is added co-translationally (Nilsson and von Heijne, 1993) to the N-glycosylation site, in multi-transmembrane glycoproteins, by oligosaccharyl transferase (Silberstein and Gilmore, 1996; Helenius and Aebi, 2001). The consensus N-glycosylation site in Kv1.1-1.5 is Asn-X-Thr/Ser (NXT/S) located in the S1-S2 linker, where X can be any amino acid except proline (P) (Zhu et al., 2003; Stuhmer et al., 1989). The core glycosylation site must be extracellular, usually at the first linker, and must have at least 10 amino acid residues from either S1 or S2 transmembrane domains in order to be accessible to ER glycotransferase (Landolt-Marticorena and Reithmeier, 1994; Zhu et al., 2003). After the core sugar is
added, the terminal glucose residues are cleaved by ER glucosidases I and II, mediating a binding of the new glycoprotein to the calnexin or calreticulin chaperones (Hammond et al., 1994; Ware et al., 1995; Spiro et al., 1996). If the glycoprotein cannot be correctly folded during its glycosylation cycles, it is recognized by GT (glucosyltransferase) which can reglucosylate the nascent protein and allow it to reassociate with calnexin causing it to re-enter the glycosylation cycle. The new glycoprotein can only leave the cycle once it is no longer reglycosylated by GT. If, however, the nascent glycoprotein is constantly misfolded, it is marked for degradation by mannosidase I and shunted to the ER degradation pathway (Jakob et al., 1998; Liu et al., 1999; Cabral et al., 2000; Su et al., 1993). Calnexin and calreticulin chaperones are, therefore, not only assisting in the core glycosylation but are also believed to bind the nascent proteins in order to promote their proper folding (Hammond et al., 1994; Hebert et al., 1995; Parodi, 2000; Trombetta and Parodi, 1992). As a result, the trimming of the N-linked oligosaccharide is linked to the QC mechanism in ER. Once the glycoproteins are correctly folded, they exit the cycle and are exported to the Golgi for further trimming and modification of the sugar tree.

The functional consequence of core and complex glycosylation is not well understood. A number of studies have shown that it is somehow important for protein stability and trafficking, but not necessary for proper folding, oligomerization, or channel function (Conti et al., 2002; de Souza and Simon, 2002; Khanna et al., 2001; Watanabe et al., 2004; Much et al., 2003; Petrecca et al., 1999). Other studies have proposed that complex glycosylation is necessary for correct protein folding and has an effect on the channel gating because the negatively charged sialic acids
in the sugar tree affect the surface potential (Thornhill et al., 1996; Watanabe et al., 2003).

Initially, it was believed that once the proteins are folded and oligomerized, they leave the ER and enter the Golgi by a default pathway. Subsequently, a mechanism for QC system was found encoded in the primary protein sequence of the cytoplasmic domains of the new membrane proteins (see below). To date, a number of QC motifs have been identified and they fall into 2 categories; the retention/retrieval motifs and the ER export motifs.

1.2.2.1 Retention and/or retrieval of proteins in endoplasmic reticulum

The first retention/retrieval signals were identified in ER-resident lumenal and transmembrane proteins; a KDEL (Lysine – Aspartic acid – Glutamic acid – Leucine) sequence located in the COOH terminus of lumenal ER-resident proteins and a KKXX (di-lysine motif followed by any amino acids) sequence found at the COOH terminus of transmembrane ER-resident proteins (Ellgaard et al., 1999; Murshid and Presley, 2004). The main function of these motifs is the retrieval of ER-resident proteins, after they exit the ER accompanying the new glycoproteins to ER-Golgi intermediate compartment or to Golgi network. ER-resident lumenal proteins with KDEL amino acid sequence are believed to bind a transmembrane receptor, that cycles between ER and Golgi (Hardwick et al., 1990; Semenza et al., 1990), by a pH-dependent mechanism (Wilson et al., 1993). Therefore, it is believed that if the KDEL proteins are transported into the Golgi's acidic environment by mistake, they bind with high affinity to KDEL receptors and are returned to ER, which has a neutral
pH, forcing the receptor-protein complex to dissociate (Pelham, 1996). In addition, some KDEL ER-resident proteins (calreticulin) are retained, rather than retrieved, in the ER by a KDEL-independent mechanism (Sonnichsen et al., 1994). The KKXX motif, found in the transmembrane ER-resident proteins and proteins that cycle between ER and Golgi (Cosson and Letourneur, 1994; Letourneur et al., 1994), is similar to the COPI binding motif except the last two amino acids in cycling proteins are phenylalanines, which is similar to COPII binding motif (Dominguez et al., 1998; Fiedler et al., 1996; Kappeler et al., 1997). Mostly, it is believed that KKXX proteins reside in the ER by retrieval and not by retention, just like KDEL ER-resident proteins. However, there are some studies that show retention-type of ER residence in vitro (Andersson et al., 1999).

An ER retention/retrieval signal important in newly synthesized protein has been first identified in Kir6.2/6.3 (K\textsubscript{A TP}) and its accessory subunit SUR1/2 (Zerangue et al., 1999). It has been shown to consist of RXR (arginine - X - arginine; X represents any amino acid but usually neutral or basic amino acid) amino acid residues. Initially, the authors observed that in order to be expressed at the plasma membrane, Kir6.2 and Kir6.3 (\(\alpha\) subunit) must co-assemble with SUR1 and SUR2 \(\beta\) subunits, respectively. If the \(\alpha\) and \(\beta\) subunits are only partially assembled or transfected alone, they will end up localized in the ER and will not express at the plasma membrane. Zerangue et al., found that deletion mutations and point mutations of the RXR motifs can rescue the surface expression of individually transfected Kir6.2/6.3 and SUR1/2 proteins. Therefore, the authors postulated that the RXR motif must be "masked" during correct oligomerization between Kir6.2/6.3
and SUR1/2 β subunits. Incorrect stoichiometric assembly, due to either incorrect monomer folding or a mutation in the interacting residue(s), results in an inability of the subunits to assemble. Furthermore, incorrect protein assembly leads to the exposure and recognition of the RXR motif(s) by ER resident protein(s) which retain the incorrectly folded proteins in the ER. The authors confirmed the function of the ER retention motif by adding the RKR residues to Kir2.1 subfamily member, the β2 adrenergic receptor (β2-AR), or an unrelated CD4 protein. All three proteins normally express easily at the plasma membrane, but attaching the RXR peptide, results in ER retention and a lack of cell surface expression. Alternatively, conversion of the RXR motif to AAA in these peptides, allows efficient surface expression of Kir2.1, β2-AR, and CD4 proteins.

Subsequent groups have assessed the roles of di-arginine motifs in the expression of additional ion channels. The GABA (γ-amino butyric acid B) receptors, NMDA (N-methyl-D-aspartate) receptors, CFTR (cystic fibrosis transmembrane conductance regulator), hERG (human ether-a-go-go), and NaV (sodium voltage-gated) channels have all been investigated. In GABA_B receptors, Margeta-Mitrovic et al. have found a C-terminal RSRR motif to be important in heteromeric assembly between GABA_B1 and GABA_B2 subunits lack of which leads to RXR exposure and ER retention (Margeta-Mitrovic et al., 2000). Point mutations have identified the arginines (Arg) in the 1st and 3rd positions to be functionally important in ER retention, and the Arg in the 4th position to only strengthen the ER retention. Similar to K_ATP channels, the amino acid in the 2nd position can tolerate any point mutations without affecting its ER retention function. Therefore, the two subunits, GABA_B1 and
GABA\textsubscript{B2}, must interact and mask each other’s RXR motifs, in order to be trafficked to the cell surface. This masking and interaction was further found to take place through the coiled-coil interaction of their \( \alpha \) helices.

Similarly, an RRR amino acid motif responsible for ER retention, has been found in the C1 cassette of NMDA receptors (Standley et al., 2000). The authors found the ER retention motif by examining different splice variants of NMDA receptors that showed ER localization when expressed alone. Deletion and point mutations showed that the ER localization is indeed due to exposure of the RXR motifs. These results were confirmed by another study, where the authors fused a C-terminal region of the NMDA receptor, containing the RXR motif, to CD8 protein and found that the C-terminal RXR motif was capable of forcing the localization of the fusion protein in the ER (Xia et al., 2001).

The CFTR channel has a total of four RXR motifs, but only two (located in the NBD1 domain) were found to play a role in ER retention/retrieval (Owsianik et al., 2003). Co-expression of misfolded \( \Delta F508 \) CFTR with truncated CFTR constructs containing RXR motif(s) resulted in a rescue of non-trafficking \( \Delta F508 \) CFTR to the cell surface. In another study, point mutations of arginine to a structurally similar lysine amino acid in \( \Delta F508 \) CFTR, also resulted in rescue of its surface expression (Chang et al., 1999). Therefore, these studies confirmed that the exposed RXR motifs lead to protein retention in the ER but proper masking of these motifs, due to correct folding, lead to cell surface expression.

Similarly, Kupershmidt et al. have identified an RGR motif located in the COOH terminus of HERG (human ether-a-go-go) ion channels and shown that, for
expression, the motif must be masked, again, by a proper folding of the long COOH terminus. Exposing the RGR motif through truncation mutations localizes the HERGΔ147 channel in the ER (Kupershmidt et al., 2002).

Overall, the above results show the involvement of the RXR(R) amino acid motif in the correct stoichiometric assembly of α subunits (GABA<sub>B</sub> receptors and NMDA receptors), heteroassembly of α and β subunits (K<sub>ATP</sub> channels) and protein folding (CFTR). Later studies have also shown that exposed RXR motifs bind COPI coat proteins in retrograde transport between Golgi and ER (Yuan et al., 2003). In addition, the neighboring phophoserine, of the masked RXR motifs, may interact with ER chaperone proteins (i.e. 14-3-3) and may function in suppression of ER retention perhaps by displacing the COPI complex. However, the precise mechanism by which this occurs is not yet known.

1.2.2.2 ER export motif

Studies on trafficking of inwardly rectifying family of potassium channels revealed a second type of QC in the ER, the forward/export trafficking signals. Like the retention/retrieval signals, these signals are encoded in the primary sequence of the cargo proteins and are location independent. Unlike the retention/retrieval signals, these motifs must be exposed on the surface of the protein, in order to be rapidly exported out of the ER. The ER export motifs were first discovered in Kir channels, by noticing that different subfamilies express to different degrees at the plasma membrane (Nichols and Lopatin, 1997). Ma et al., set out to investigate the cause of this behavior by constructing deletion mutations, point mutations, and C-
terminal chimeras between the different subfamilies of Kir channels (Ma et al., 2001). They have identified a five amino acid motif (FCY)ENE or EXD that, when mutated or deleted, results in a lack of plasma membrane expression in Kir2.1 and Kir1.1, respectively. Assuming, that these results are due to failure of folding and/or assembly, the group examined the biophysical properties: ion permeation, rectification, and the single-channel properties of the Kir2.1 wt and deletion mutant (C-terminal deletion after residue 374 which contains the diacidic residues). The authors found that the lack of cell surface expression is not due to a failure of protein assembly, but rather to lack of trafficking to the plasma membrane. In addition, to confirm the function of the FCYENE motif as an ER export signal, they tested its ability to allow surface expression of non-trafficking proteins. Fusion of the di-acidic motif to Kir 6.2Δ36 (deletion of 36 amino acids removes RXR motif so that it does not interfere with ER export) and GABAβ1-ASRR (point mutation of the first R removes the ER retention motif, so that it does not interfere with the ER export) proteins, resulted in surface expression. Similarly, fusing the FCYENE motif to an unrelated ion channel family (Kv1.2) or Kir4.1, which normally require the help of either Kvβ2 subunit or other auxiliary proteins to traffic to the plasma membrane, allowed the Kv1.2 and Kir4.1 surface expression without the auxiliary subunits (Ma et al., 2001; Stockklausner et al., 2001).

In another study, Ma et al., identified the location and function of a di-acidic ER export motif in G protein-activated inwardly rectifying potassium channels, Kir3 (GIRK) (Ma et al., 2002). GIRK is made up of heterotetramers between Kir3.1, Kir3.2, Kir3.3, and Kir3.4 α-subunits, depending on the tissue in which they are
expressed. The authors identified an ER export motif, (D/N)QD(V/M)E(S/I)(P/G)V, in the NH$_2$-terminus of Kir3.2 and Kir3.4 that is not present in Kir3.1 or Kir3.3. Therefore, Kir3.1, when expressed alone, localizes to the ER. However, coassembly of Kir3.2/Kir3.1 and Kir3.4/Kir3.1 causes the heterotetramers to express at the cell surface. Again, fusing the NH$_2$-terminal 35 amino acids of Kir3.4 or Kir3.2 to a member of a different subfamily, Kir2.1 with Ala substitutions of its ER export motif, as well as to an unrelated protein GABA$_B_1$-ASRR, results in surface expression. Alternatively, fusion of Kir2.1's C-terminal di-acidic motif to Kir3.4 (36-419 deletion to remove its di-acidic motif) and Kir3.2 (1-44 deletion to remove its di-acidic motif) results in surface expression. Therefore, the above chimera studies confirm the functions of the di-acidic motifs in Kir1.1, Kir2.1, Kir3.2, and Kir3.4 in regulating ER export and promoting cell surface expression. Interestingly, Ma et al., found that fusing the di-acidic motif to full-length Kir6.2 and wt GABA$_B_1$ proteins, does not increase the surface expression of these proteins, indicating that the ER export motif is not able to override the ER retention motif (Ma et al., 2001).

As in Kir channels, an ER export motif has been identified in the COOH terminus of the Kv1 channels (Li et al., 2000). However, unlike Kir channels, Kv1 channels do not employ a di-acidic motif for this purpose. Instead, the authors have identified VXXSL (valine-X-X-serine-leucine) and LXXSL (leucine-X-X-serine-leucine) motifs as ER export signals in Kv1.4 and Kv1.5, respectively. Initially, the authors noticed differences in the levels of surface expression of Kv1.4 as compared to Kv1.5 ion channels. Interchanging the ER export motifs between Kv1.4 and Kv1.5 reversed this relationship and deletion of the motif caused a failure of surface
expression, as assayed by glycosylation experiments. By sequence alignment, a similar motif, VXXSN (valine-X-X-serine-asparagine), has been identified in Kv1.2 (Li et al., 2000). Normally, Kv1.2 requires the help of β subunit in order to be properly trafficked to the plasma membrane. Interchanging the leucine residue (the last residue of VXXSL motif) and asparagine residue (the last residue of VXXSN motif) between Kv1.2 and Kv1.4, caused Kv1.4 to be retained in the ER and Kv1.2 to be efficiently trafficked to cell surface. Furthermore, the low levels of cell surface expression of Kv1.4L623N were rescued by addition of β subunit and the Kv1.2N469L expressed well at the plasma membrane without the help of the β subunit. These results show that the (V/L)XXS(L/N) motif seems to be involved in a step between oligomerization and Golgi processing, but acts independently of β subunit proteins. Furthermore, the ER export motif is proposed to be a recognition signal for the COPII transport vesicles in the anterograde direction between ER and Golgi network (Klumperman, 2000).

Unlike the di-acidic motif, however, the (V/L)XXS(L/N) motif is not important in subunit multimerization (Li et al., 2000). Using chimera studies, Manganas et al., have identified a pore region important to heteromerization (Manganas et al., 2001). Normally, Kv1.1 is retained in the ER and only trafficks to the plasma membrane when heterotetramerized with Kv1.4. The authors have identified, using immunofluorescence and Proteinase K experiments, the P-loop domain (linker between S5 and S6 domains) of Kv1.1 as a dominant regulator of the Kv1.1/Kv1.4 heterotetramer trafficking. They postulate that a P-loop motif is in fact an ER-
retention signal, acting on folding and assembly, and the C-terminal VXXSL is the ER export signal. However, the mechanism behind this retention is unknown.

### 1.2.3 Regulation of ion channel processing in Golgi network

Provided that the newly synthesized proteins have passed the ER QC machinery, they can leave the ER and enter Golgi where they are subjected to further modifications along with QC checkpoints. Golgi network consists of stacks of flattened cisternae with each stack being polarized with three regions: cis-Golgi network, medial-Golgi network, and trans-Golgi network (Rambourg and Clermont, 1990). Newly synthesized proteins from ER or ER-Golgi intermediate compartment enter the cis-Golgi network for the initial complex glycosylation. In the medial-Golgi network, the protein undergoes cycles of adding and trimming of the carbohydrate tree. Consequently, the newly synthesized protein enters the trans-Golgi network for completion of glycosylation and plasma membrane sorting or lysosomal degradation. Although a number of studies have shown the molecular mechanisms involved in early secretory trafficking pathways, the QC mechanisms of trafficking from the Golgi to the plasma membrane are much less understood. It is known that the chaperone proteins that are found in the ER are not found in the Golgi network (Castro-Fernandez et al., 2005). In addition, the chemical environment of the Golgi, which is more acidic and contains less number of chaperone proteins, differs from ER environment. However, a few studies of Kir2 homo- and heterotetrameric channels have shown an N-terminal cluster of basic residues and C-terminal tyrosine-dependent (YXXΦ) motifs, both important for Golgi exit/sorting (Stockklausner and...
Klocker, 2003; Hofherr et al., 2005). In one study, Stockklausner et al., postulated that the positively charged N-terminal residues function in Golgi QC by electrostatic interactions with the negatively charged head groups of sphingolipids making up the transport vesicles (Stockklausner and Klocker, 2003). The C-terminal YXXΦ motif has been shown to interact with clathrin coat proteins and may therefore be involved in transport between plasma membrane and Golgi (Kirchhausen, 2000).
1.3 Modulation of Kv channel function

1.3.1 Modulation of Kv channels by β subunits

Four Kvβ genes have been identified in the mammalian genome. Their products are >85% conserved in their amino acid “core” region although they differ in their NH₂ termini (England et al., 1995a; Leicher et al., 1996). Kvβ1.1, Kvβ1.2 and Kvβ1.3 are splice variants of the same gene whereas Kvβ2.1, Kvβ3.1, and Kvβ4.1 are products of three different genes. The crystal structure of Kvβ2 has revealed structural similarities to aldo-keto reductases exemplified by a symmetrical tetrameric barrel structure with bound NADP⁺ co-factor (Gulbis et al., 1999). The crystal structure of co-expressed rat Kvβ2 with the rat Kv1.1 T1 domain, is shown in Figure 1.4A, and shows Kvβ2 hanging under the cytoplasmic T1 domain of Kv channel in a T1₄ – β₄ hetero-octamer conformation around a central axis running along its entire length (Gulbis et al., 2000). More specifically, the highly conserved FYQLGDEAM residues of NH₂ terminus in Kv1.1 – Kv1.6 (residues 193 – 201 of NH₂ terminus in Kv1.5) are involved in the interaction with Kvβ1 (figure 1.4B) (Sewing et al., 1996).

The main functions of Kvβ subunits involve modulation of gating, stability, and trafficking of Kv1α channels. Additional proposed functions of Kvβ subunits include tethering of Kvα channels to other cellular proteins such as cytoskeletal proteins and protein kinases (Levin et al., 1996; Nakahira et al., 1998; Gong et al., 1999; Kwak et al., 1999; Williams et al., 2002). Kvβ1 and Kvβ3.1 contain inactivating “β ball”
Figure 1-4 Crystal structure of T1 domain in complex with β subunit

A. A model of the T1 domain located under the TM part of the channel and the β subunit (blue). The model is based on the crystal structure of KcsA potassium channel. The NH₂ terminus is shown to enter the TM pore through the openings between T1-S1 linkers.

B. A close-up of the residues involved in the interaction between the T1 domain loop (red) and β subunit (blue) (Gulbis et al., 2000).
domains in their NH$_2$ termini. Therefore, Kv$\beta$1 and Kv$\beta$3 isoforms function mainly in an increase of the rate of inactivation in otherwise non-inactivating Kv1$\alpha$ channels (Rettig et al., 1994; Heinemann et al., 1995; Heinemann et al., 1996), or speed up the inactivation of a slowly inactivating channels (Uebele et al., 1996; Uebele et al., 1998). Other functions of Kv$\beta$1 and Kv$\beta$3 include, a shift in the voltage dependence of activation towards the hyperpolarized direction (England et al., 1995a; Heinemann et al., 1996; Uebele et al., 1996), and slowing of deactivation in Kv1.5 channels (England et al., 1995a; England et al., 1995b; De Biasi et al., 1997). Furthermore, point mutations within the NADP$^+$ binding pocket of Kv$\beta$1.1, were seen to attenuate its inactivating function in Xenopus oocytes and therefore, couple the $\beta$ subunit function and the gating of Kv1 channels to the oxidation state of the cell (Bahring et al., 2001). Alternatively, Kv$\beta$3 was seen to increase current density of Kv2 channels, probably by assisting in Kv2 trafficking (Shi et al., 1996; Martens et al., 2000).

Kv$\beta$2 and Kv$\beta$4, both of which lack an inactivating domain, are mainly involved in maturation and trafficking of Kv$\alpha$ channels. Nagaya et al. have shown that the Kv$\beta$2 subunit attaches co-translationally to the hydrophobic residues of the nascent Kv1.2$\alpha$ NH$_2$ terminus, while Kv$\alpha$ is still attached to the ribosome, and appears to assist channel folding in the endoplasmic reticulum (Shi et al., 1996; Nagaya and Papazian, 1997). In a number of Kv1$\alpha$ homo- and hetero-tetramers, the co-translational binding of Kv$\beta$2 was shown to increase their stability, their degree of N-linked glycosylation, and their cell surface expression (Shi et al., 1996; Manganas and Trimmer, 2000). In addition, Kv$\beta$2 has been shown to increase the current density of Kv4.3 channels without affecting its gating properties (Yang et al., 2001;
Deschenes and Tomaselli, 2002). The Kvβ4 subunit interacts with the COOH terminus (rather than NH₂ terminus, as seen with other Kvα channels) of Kv2.2 channel only, and no other family of potassium channels (Kv1, Kv3 or Kv4) or even the closely related Kv2.1 channel, increasing Kv2.2 channel's surface expression (Fink et al., 1996). Furthermore, point mutations within the NADP⁺ binding pocket of Kvβ2 residues, revealed an inability of Kv1.2α trafficking to the plasma membrane in COS-1 cells and an inability of axonal targeting in cultured hippocampal neurons (Campomanes et al., 2002). Therefore, NADP⁺ binding and the oxidoreductase activity of β subunit may be crucial for its function.

1.3.2 Modulation of Kv channel function by phosphorylation

The α subunits of voltage-gated K⁺ channels can be regulated by both serine/threonine and tyrosine phosphorylation. Phosphorylation acts as a fast regulator of ion channel trafficking and, therefore, adds to the complexity of channel expression profiles. Protein kinase A (PKA) activation was shown to enhance Kv1.1 current density due to increase in protein synthesis (Levin et al., 1996; Winklhofer et al., 2003). Protein kinase C (PKC) activation, despite the presence of a canonical PKC phosphorylation site, does not lead to direct phosphorylation of Kv1.1 channel but indirectly increases its protein synthesis (Winklhofer et al., 2003). Kv1.2 current density was found to be down-regulated by G-protein/PKC phosphorylation while up-regulated by PKA phosphorylation (Huang et al., 1993; Huang et al., 1994) and Kv1.3 current density was decreased by both PKA and PKC activation (Payet and Dupuis, 1992). Kv1.4 and Kv1.5 currents were found to be modulated by tyrosine
phosphorylation via Src-dependent and Src-independent binding to Src homology 3 (SH3) domain (Holmes et al., 1996; Nitabach et al., 2002). Upon tyrosine phosphorylation, Kv1.4 and Kv1.5 currents were suppressed via Src-binding to SH3 domain. Conversely, activation of the tyrosine-phosphorylation pathway without Src-binding leads to slowed inactivation kinetics in Kv1.4 as well suppression of Kv1.5 currents.

1.3.2.1 Modulation of Kv channel trafficking by phosphorylation

A mechanism of phosphorylation affecting ion channel trafficking has been discovered in Kir1.1 channels. Kir1.1 ion channels, must be phosphorylated at S44 for high surface expression (O’Connell et al., 2005; Yoo et al., 2005). Point mutations of S44 to Alanine eliminates the possibility of phosphorylation and results in ER/Golgi retention; mimicking phosphorylation by mutagenizing S44 to Aspartic acid (D44), rescues the Kir1.1 surface expression. Even though, the phosphorylation site (S44) is located in the COOH terminus and the ER/Golgi retention motifs are located in the NH2 terminus, the crystal structure of prokaryotic Kir channel (KirBac1.1) shows that the cytoplasmic NH2 and COOH termini are in close proximity interacting by hydrogen bonding. The authors, therefore, hypothesized that S44 phosphorylation ‘masks’ either the di-arginine ER retention motif or the basic residue cluster found in the primary sequence of Kir1.1. Similarly, other phosphorylation-induced ER export has been found in NMDA receptors (Scott et al., 2001; Scott et al., 2003), glutamate receptors (Ren et al., 2003), the KDEL receptor (Cabrera et al., 2003), and human cardiac Na channels (Zhou et al., 2002). In these proteins,
however, the phosphorylation site and the ER/Golgi retention motifs are located in close proximity. These results imply that both the ER and the Golgi may accumulate a pool of properly folded channels and a phosphorylation switch can modulate a fast delivery to the cell surface.

1.3.3 Modulation of Kv channel function by PDZ domains

PDZ domains are modular protein-protein interaction domains of proteins that bind specific C-terminal peptide sequences of membrane proteins (Doyle et al., 1996; Ponting et al., 1997; Sheng and Kim, 1996; Songyang et al., 1997) or other PDZ containing proteins (Brenman et al., 1996). Many proteins contain a number of PDZ domains, allowing for multimerization or scaffolding of protein complexes (Kim et al., 1998; Niethammer et al., 1998; Pawson and Scott, 1997; Tsunoda et al., 1997). MAGUK (membrane-associated guanulate kinases) proteins are part of a major PDZ containing class of proteins involved in the clustering of protein complexes at specialized membrane domains like cell junctions in epithelial cells and synaptic junctions in neurons (Anderson, 1996). In terms of clustering of ion channels, Kim et al., have shown that the PSD-95 family of membrane associated MAGUKs are involved in clustering of Shaker-related ion channels (Kim et al., 1995). The direct interaction between Shaker channel and PSD-95 proteins takes place at the cytoplasmic COOH terminus of the potassium channel. This clustering requires a simultaneous binding of cysteine residues located on the NH₂ terminus of PSD-95 (Hsueh and Sheng, 1999). In Kv1.5, PSD-95 was shown to interact directly, resulting in downregulation of Kv1.5 currents (Eldstrom et al., 2002). In contrast, SAP-97
(another member of MAGUK family of proteins) was shown to interact with Kv1.5 channels indirectly, resulting in upregulation of the channel's current density (Eldstrom et al., 2003). Direct interaction between PSD-95 and Kv1.2 ion channels does not affect the channel's trafficking but instead increases the stability of the channels at the plasma membrane (Tiffany et al., 2000). Furthermore, the function of PDZ binding domain proteins, like SAP 97, may be modulated by PKC phosphorylation (Mathur et al., 2006).

In our study, we have identified a 10 amino acid region in the N-terminal end of the T1 domain that is an important prerequisite for functional expression of Kv1.5 ion channels. A number of N-terminal and T1 domain deletions have been made that resulted in variations in Kv1.5 cell surface trafficking. N-terminal deletions up to the T1 domain and larger deletions in which at least 60% of the T1 domain was deleted yielded functional channels. However, smaller deletions of 15 and 43 amino acids of the T1 domain yielded non-trafficking Kv1.5 channels. Interestingly, addition of first 10 amino acids of the T1 domain to the non-trafficking mutants is able to rescue their trafficking to the plasma membrane.
2 METHODS

2.1 Channel Mutagenesis

The mammalian expression vector pcDNA3 was used for expression of all Kv1.5 constructs in this study. All primers used were synthesized by Sigma Genosys (Oakville, Ontario, Canada). All constructs were sequenced to check for errors during PCR amplification and to ensure correct reading frame.

ΔN119 Kv1.5, ΔN163 Kv1.5, ΔN189 Kv1.5 and ΔN209 Kv1.5 mutants were generated as previously described (Kurata et al., 2002).

Kv1.5 ΔN135 was generated by PCR amplification of hKv1.5 pcDNA3 encoding residue 135 to the COOH-terminus of hKv1.5 using a primer of sequence CCCAAGCTTATGGCACCCTGGCGCAGTTC at the 5'end, which includes a HindIII site (introduced restriction site is underlined and introduced start codon is italicized) and CCGCGCGGCCACCCAGAGCTC primer at the 3' end. The resultant product was cloned into wtKv1.5 on pcDNA3 as a HindIII – BspEI fragment, replacing a similarly-bounded fragment from the wild-type channel.

The T1120-140+ΔN163 Kv1.5 mutant was constructed in two parts that were PCR amplified and then ligated. For preparation of Kv1.5 pcDNA3 encoding amino acids 163 to COOH terminus, wtKv1.5 pcDNA3 digested with Scal at the 5' end and NotI at the 3' end was PCR amplified using primer of sequence CAGCTGGGCACCCTGGCGTACTTCTTTGCGCGAAGCCGCAACCC (start of aa 163 is underlined) at the 5' end and AAGACCGAGACGATGGCGATG at the 3'end to yield a ~300bp product. For the preparation of the “T1120-140” part, wtKv1.5 pcDNA3 was
digested with *HindIII* at the 5’end and *ScaI* at the 3’ end yielding a ~500bp product that was again digested with *Drall* to yield a ~450pb product. The ~450bp product was PCR amplified using a primer of sequence CCCAAGCTTATGCAGCGCGTCCACATCAACATC at the 5’ end and CGCCAGGGTGCCCAGCTG at the 3’ end to yield a ~60bp product. This ~60bp product was ligated with the ~300bp product and then subcloned back into analogous location of wtKv1.5 pcDNA3 digested with *HindIII* at the 5’end and *BspEI* at the 3’end.

**T1(1)+ΔN163 Kv1.5** and **T1(2)+ΔN163 Kv1.5** were generated by PCR amplification of T1120-140+ΔN163 Kv1.5 pcDNA3 construct using CCCAAGCTTATGCAGCGCGTCCACATCAACATCTCCGGGTACTTCTTCGACCGCAACGGGC and CCCAAGCTTATGCTGCACGTTCAGACGCAGCTG primers at the 5’ end, respectively, introducing *HindIII* restriction sites. At the 3’ end, a AAGACCGAGACGATGGCGATG primer was used in both constructs for PCR amplification. The PCR products were digested with *HindIII* at the 5’end and *BspEI* at the 3’end, followed by subcloning into similarly digested hKv1.5 pcDNA3 to replace the analogous region.

**T1(1)+ΔN140 Kv1.5** and **T1(2)+ΔN140 Kv1.5** were generated by PCR amplification of hKv1.5 in pcDNA3 using CCCAAGCTTATGCAGCGCGTCCACATCAACATCTCCGGGCAGTTCCCCAACACACTCCTGG and CCCAAGCTTATGCTGCACGTTCAGACGCAGCTG primers in the forward direction, respectively, introducing a *HindIII* restriction site. The primer CCTCATCCTCGCGGAAGC was used in the 3’ direction for both constructs. The
PCR products were digested with *HindIII* at the 5’end and *BspEI* at the 3’end, and subcloned into similarly digested hKv1.5 pcDNA3 to replace the analogous region.

Site-directed mutagenesis of Kv1.5 was performed using QuickChange method by Stratagene. For preparation of A_{131-133}ΔN140 and A_{131-133}ΔN163 mutants, the primers used were GCTTATGGCTTGGCAGGGCACCCTGGCG and GCTTATGCTGGCTTTGcGgCGgcGCTGGGCACCCTGGCGC, respectively, and their complements (small case codons represent changes to create the point mutations). For preparation of A_{166}ΔN140 and A_{166}ΔN163 mutants, the primers used were GGAACGAGTACTTCTTCGcCCGCAACCGGCCCAGCTTCG and CACCCTGGCGTACTTCTTCGcCCGCAACCGGCCCAGCTTCG, respectively, and their complements. For RXA^1 (R^{167}N^{168}A^{169}), RXA^2 (R^{184}L^{185}A^{186}), and RXA^3 (R^{212}F^{213}A^{214}) point mutations, the primers used were GTACTTCTTCGACGCCAACCGGCCCAGCTTCG, CTACCAGTCCGGGCGGCCCTTCGAGGAGGGGGTC, GACGAGGCCATGGAGGCGCTTCCGCGAGGATGAG, respectively, and their complements.

### 2.2 Cell culture and transfection

Human embryonic kidney (HEK)293 or COS-7 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in an air, 5%CO₂ incubator. Cultured cells were transiently transfected using Lipofectamine 2000 (Invitrogen) reagent and method.
For electrophysiological experiments, one day before the transfection, the cells were plated on a 1μm wide sterile glass coverslip in 35-mm Petri dishes with 10-20% confluence. 1-2μg of pGFP expressing vector was co-transfected with 1.5 – 2.5μg of channel DNA in 100μL serum-free medium and 3μl of Lipofectamine 2000 added to dishes of HEK293 cells containing a total of 1ml of MEM +10% FBS. GFP was used to allow for the identification of transfected cells during electrophysiological experiments which were performed 24hr after transfection.

For immunocytochemical applications, 2 or 3 days before transfection, the cells were plated on a 1.5μm wide sterile glass coverslip 35-mm Petri dishes with 40-50% confluence. 2.5 – 3μg of channel DNA in 100 μl serum-free medium and 5 – 6μl of Lipofectamine 2000 was added to dishes of HEK293 cells containing a total of 1ml of MEM + 10% FBS. COS-7 cells, used for immunocytochemical experiments, were also co-transfected with pGFP vector in for easy visual identification of the cytoplasm. For the glycerol treated cells, 2.5-3μg of channel DNA in 100μl serum-free medium and 5 - 6μl of Lipofectamine 2000 were added to dishes of HEK293 cells containing a total of 1ml of MEM +10% FBS + 10% Glycerol. ~20 hours after transfection, the glycerol was slowly diluted out, over a period of 1 or 2 hours, by addition of MEM+10% FBS media. The cells were allowed to recover from the glycerol treatment for at least 1hr prior to immunocytochemistry. The immunocytochemical experiments were performed 24hr after transfection.

For immunoblotting applications, the cells were plated in a sterile T-25 flask 5-7 days before transfection and transfected once grown to 90-100% confluence.
μg of channel DNA in 500 μl serum-free medium and 12 or 18μl of Lipofectamine 2000 were added to dishes of HEK293 cells containing a total of 3ml of MEM + 10% FBS. The immunoblotting experiments were performed 24hr after transfection.

2.3 Electrophysiology

Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume 250 μl) containing the bath solution at ambient temperature (22–23°C), and perfused with bathing solution throughout the experiments. Transfected cells were selected for using fluorescence microscopy. Whole-cell current recording and data analysis were performed using an Axopatch 200B amplifier and pClamp 9.2 software (Axon Instruments, Foster City, CA, USA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL, USA). Electrodes had resistances of 1–3 MΩ when filled with the filling solution. Capacitance compensation and 80% series resistance compensation were routinely used in all whole cell recordings. Measured series resistance was between 1 and 4 MΩ for all recordings. When the series resistance changed during the course of an experiment, data were discarded. Data were sampled at 10–20 kHz and filtered at 5–10 kHz. Membrane potentials were not corrected for small junctional potentials between bath and pipette solutions. No leak subtraction was used when recording currents. Data for K⁺ current analysis and presentation were leak subtracted off-line if required. For recording K⁺ current, patch pipettes contained (in mM): NaCl, 5; KCl, 135; Na₂ATP, 4; GTP, 0.1; MgCl₂, 1; EGTA, 5; HEPES, 10; and was adjusted to pH 7.2 with KOH. The bath solution
contained (in mM): NaCl, 135; KCl, 5; HEPES, 10; sodium acetate, 2.8; MgCl₂, 1; CaCl₂, 1; and was adjusted to pH 7.4 with NaOH. All chemicals were from Sigma Aldrich Chemical (Mississauga, ON, Canada). Statistical significance between groups of data was determined using one-way ANOVA, with pairwise multiple comparisons. A value of \( p<0.05 \) was deemed statistically significant. Data are presented as mean±S.E.M.

### 2.4 Immunocytochemistry

24hr post-transfection, HEK293 cells or COS-7 cells were washed 3 times with ice-cold 1X Phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄). Cells were then fixed at room temperature for 12 min using 4% formaldehyde and followed by incubation in Quenching buffer (50mM Glycine, 1X PBS) for 10 min to remove autofluorescence. After three 5 min washes with 1X PBS at room temperature, the cells were blocked and permeabilized using Blocking solution (2% BSA and 0.2% Triton X-100 in 1X PBS) for 30min at room temperature. Cells were then incubated with mouse monoclonal antibody to the T7 tag (1:1000; Novagen) or a rabbit anti-Kv1.5 polyclonal antibody to the C-terminal region of Kv1.5 (1:500; generated in our laboratory, aa 537-553 EQGTQSQGPGLDRGVQR) in blocking solution and incubated for 2 hr at room temperature or overnight at 4°C. In the Calnexin labeling colocalization experiments, the cells were incubated with rabbit anti-calnexin polyclonal antibody (1:200; Stressgen) and mouse anti-T7 monoclonal antibody (1:1000; Novagen) in blocking solution for 2 hr at room temperature. Following three 5 min washes with 1X PBS at
room temperature, cells were incubated with Alexa 594-conjugated goat anti-mouse IgG antibody and/or Alexa 488-conjugated goat anti-rabbit IgG antibody (1:1000; Molecular Probes) for 1 hr at room temperature. After incubation with 300nM 4',6-Diamidino-2-phenylindole (DAPI; Molecular Probes) to stain the nuclei, the cells were washed three times for 5 min with 1X PBS at room temperature. Cells were then mounted with 10 μl of a 90% glycerol + 2.5% w/v DABCO-PBS solution. Images of labeled cells were observed under Nikon C1 laser scanning confocal microscope (Nikon DEclipse C1, Melville, NY) at 60X oil immersion objective and processed using the operation EZ-C1 software for Nikon C1 confocal microscope (Nikon). Images were later viewed and prepared using Adobe Photoshop software packages.

2.5 Proteinase K treatment and cell lysis

24 hrs post-transfection, HEK293 cells were washed 3 times with ice-cold 1X PBS and then treated with Proteinase K buffer (10 mM Hepes, 150 mM NaCl, 2mM CaCl2; pH 7.4) without (control) or with 200 μg/ml Proteinase K for 20 – 30 min at 37°C in an air, 5%CO2 incubator (Manganas et al., 2001; Zhou et al., 1998). The reaction was stopped using 1.3 ml of Quenching Buffer (25 mM EDTA, 6mM PMSF), in which the cells were harvested by centrifugation at 1000Xg at 4°C. The cells were then washed three times for 5 min in 1X PBS and again centrifuged at 1000xg at 4°C. Then, the cells were lysed in Lysis buffer (LB; 25mM Phosphate Buffer at pH7.4, 150mM NaCl, 2.5mM EDTA, 10% Glycerol) with 1% IGEPAL or 1% Triton detergent for 1 hr at 4°C or on ice with occasional mixing. Additional protease
inhibitors (1 mM iodoacetamide, Aprotinin, 1 mM PMSF) were added to the LB to prevent degradation of the protein. The lysates were then centrifuged at 10 000xg for 25 min at 4°C to separate supernatant and pellet fractions.

2.6 Western Blotting

Cell extracts were run on 8% or 10% SDS – PAGE in Running buffer and transferred for 2hr at 60V or overnight at 20V in Transfer buffer according to standard protocol. PVDF membranes were blocked for either 30min at 37°C or 1.5hr at room temperature in 10% Blocking solution (10% w/v non-fat milk powder in Rinse buffer; 100mM NaCl, 10mM Tris-Cl pH8.0, 1mM EDTA, 0.1% Tween 20). The membranes were then incubated with rabbit anti-Kv1.5 polyclonal antibodies directed against C-terminal Kv1.5 protein (1:5000; generated in our laboratory, aa 537-553 EQGTQSQGPGGLDRGVQR) in 5% Blocking Solution (5% w/v non-fat milk powder in Rinse buffer) for either 1hr at 37°C or overnight at 4°C. After four 15min washes at 37°C or at room temperature in Rinse buffer, the membranes were incubated with secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase (HRP; Jackson Immunoresearch, West Grove, Pa. USA) and 5% Blocking solution for 30min at 37°C. Following four 15min washes at 37°C in Rinse buffer, the antibody – protein interactions were visualized by using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences) and detected on X-ray film (Kodak).
3 RESULTS

3.1 Small deletions of T1 domain affect the trafficking of Kv1.5

Previously it was shown in our lab that a naturally occurring truncated version of Kv1.5, (missing 209 amino acids from the NH₂ terminus; ΔN209), was able to produce measurable macroscopic currents with few changes in the inactivation properties (Kurata et al., 2001). This channel lacks the bulk of the Kv1.5 NH₂ terminus including ~80% of the channel's T1 domain. Smaller deletions of T1 domain, namely ΔN135 (missing 15 amino acids from the T1 domain), ΔN140 (missing 20 amino acids from the T1 domain), and ΔN163 (missing 43 amino acids from the T1 domain), produced channels that failed to functionally express as evidenced by an inability to measure ionic currents (Kurata et al., 2002). Therefore, we set out to investigate whether this lack of macroscopic currents is due to the channels' inability to traffic to the plasma membrane or due to an inability of the channels to function after insertion into the plasma membrane. We have constructed a number of NH₂-terminal and T1 deletion mutants (Figure 3.1), namely, ΔN119 (the whole T1 domain is intact), ΔN135, ΔN163, ΔN189 (deletes ~70 amino acids of the T1 domain), and ΔN209 (deletes >80% of the T1 domain). Figure 3.1 shows schematic diagrams of Kv1.5 deletion mutants. The diagrams represent the cytoplasmic NH₂ terminus where black fill shows the boundaries of T1 domain and grey fill represents the rest of the NH₂ terminus.
In order to determine each channel’s ability to traffic to the plasma membrane, the localization of the constructs were assayed by immunofluorescence/confocal microscopy and by testing the channel’s sensitivity to Proteinase K enzyme (Figure 3.2). Immunofluorescence assay of transiently transfected ΔN189 Kv1.5 mutant in HEK293 cells, shows plasma membrane as well as some intracellular staining (red staining) (Figure 3.2A). Wt Kv1.5, used as a positive control, shows clear plasma membrane staining, as do ΔN119 Kv1.5 and ΔN209 Kv1.5 (not shown). On the other hand, confocal images of transiently transfected ΔN135 Kv1.5 and ΔN163 Kv1.5 mutants in HEK293 cells, show aggregated and diffuse intracellular staining without any plasma membrane labelling. Therefore, these results show that deletion of the first half of the T1 domain impairs trafficking of the channel perhaps caused by some type of missfolding. Deletion of the NH$_2$-terminus (ΔN119) excluding T1 domain and deletion of >60% of T1 domain (ΔN189 and ΔN209) yields properly assembled channels that are able to reach and insert into the plasma membrane.
Figure 3-2 Small T1 domain deletions abolish Kv1.5 surface expression.

A. Confocal images of transiently transfected HEK293 cells expressing ΔN135, ΔN163, ΔN189, and Wt Kv1.5. Each image is a single optical slice of fixed cells taken through the middle of the cell. Red staining represents Kv1.5 protein and blue staining represents nuclear staining (DAPI). The size bars are 10μm.

B. Western blot of post-nuclear lysates from control and Proteinase K treated HEK293 cells transiently transfected with ΔN135, ΔN163, ΔN189 and Wt Kv1.5 (control). Where applicable, cells were treated with 20μg/μl Proteinase K for 30min, followed by regular lysis and immunoblotting protocol. Left lane on the western blots represents untreated protein under regular lysis and immunoblotting protocol.
The results obtained by confocal microscopy were confirmed by subjecting the mutants to Proteinase K (PK) digestion (Figure 3.2B). PK is a non-specific serine protease that is unable to penetrate cellular membrane and is, therefore, ineffective in clearing intracellular proteins. However, PK will efficiently digest proteins or parts of proteins that are exposed to the extracellular environment and thus, combined with subsequent immunoblotting assay, PK digestion can be used as an assay for detecting the presence of transmembrane proteins at the cell surface (Manganas et al., 2001). In Kv1.5 expressed at the plasma membrane, PK enzyme cleaves the extracellular loops connecting the transmembrane domains of the channel and the mature pool of proteins will be detected at a much smaller size on the western blot. If the protein is not inserted in the cellular membrane, the PK enzyme will not cleave the intracellular Kv1.5 proteins and there will be no difference between the untreated and treated samples in the western blot. Figure 3.2B shows an immunoblot of Wt Kv1.5, ΔN135 Kv1.5, ΔN163 Kv1.5, and ΔN189 Kv1.5. Untreated Wt Kv1.5 protein runs as a doublet at around 83kDa, where the upper band represents mature, fully glycosylated pool of proteins present at the plasma membrane and the lower band represents immature, core-glycosylated pool of proteins mostly likely present in the ER (Eldstrom et al., 2002; Li et al., 2000). As seen in Figure 3.2B, treatment of wt Kv1.5 with PK enzyme results in digestion of mature pool of proteins which are then detected at a much smaller size of about 32 kDa. This band represents the S6 transmembrane domain with the cytoplasmic COOH-terminal fragment of wt Kv1.5 because the antibody we used detects the COOH-terminus of Kv1.5. The immature pool of proteins, as expected, is not affected by PK treatment. Similarly, the bands in
the untreated ΔN189 Kv1.5 lane run as doublet at ~ 47kDa. PK treatment of ΔN189 Kv1.5 eliminates the upper band and has no effect on the lower band. The sizes of ΔN135 Kv1.5 and ΔN163 Kv1.5 proteins have been calculated to be 53kDa and 49.5kDa, respectively. Untreated ΔN163 Kv1.5 runs as a single band at around 50kDa on the western blot. However, PK digestion of ΔN163 Kv1.5 has no effect on this band. Therefore, it would appear that ΔN163 Kv1.5 is not present at the plasma membrane. Similarly, ΔN135 Kv1.5 is resistant to PK digestion even though it runs as a doublet at around 50kDa on a western blot. Overall, these results confirm that T1 deletions of at least 15 residues but less than 43 residues prevent the channel from trafficking and insertion to the cell membrane.

To determine whether the T1 and NH₂-terminal deletion mutants present at the cell surface are functional, we tested for currents using whole cell patch clamping methods (Figure 3.3). Figure 3.3A shows representative macroscopic current traces of wt Kv1.5, pGFP, ΔN119 Kv1.5, ΔN135 Kv1.5, ΔN163 Kv1.5 and ΔN209 Kv1.5. GFP (green fluorescent protein) current traces show the endogenous current since these cells were transfected with GFP DNA carrying vector only, without any Kv1.5 protein, and therefore serve as a negative control. Similar to the magnitude of the endogenous macroscopic current in HEK293 cells, previously shown to be less than 1nA (Yu and Kerchner, 1998; Zhu et al., 1998), cells transfected with pGFP alone exhibited currents of <1nA. The macroscopic currents of ΔN135 Kv1.5 and ΔN163 Kv1.5 were also measured to be less than 1nA, consistent with failure to express at the cell surface (figure 3.3A).
Figure 3-3 Small T1 deletions cannot produce whole-cell macroscopic currents whereas other deletion mutants can.

A. Representative current traces from HEK293 cells transiently transfected with Wt, ΔN119, ΔN135, ΔN163, ΔN189, and ΔN209 Kv1.5 or pGFP. The cells were held at -80 mV and stepped to voltages ranging between -70 mV to +80 mV in 5 or 10 mV steps for at least 100 ms. GFP was co-transfected to enable identification of transfected cells.

B. Summary of averaged current density of the above mutants at +60 mV depolarization normalized to cell capacitance. The results are statistically significant (ΔN119 and ΔN209 vs Wt and vs GFP, p<0.05)
Alternatively, whole cell current traces of ΔN119 Kv1.5, ΔN189 Kv1.5 (not shown) and ΔN209 Kv1.5 deletion mutants show large macroscopic currents of about 20nA; and wt Kv1.5 peak currents can reach as much as 100nA. Therefore, these channels are functional at the plasma membrane. Figure 3.3B shows a summary plot of current density values, which represents averaged macroscopic current measured at +60mV depolarization and normalized to cell capacitance (nA/pF). The opening threshold of Wt Kv1.5 channel is around -40mV to -50mV, which means that at +60mV majority of the ion channels are open. The average current densities of ΔN135 Kv1.5 and ΔN163 Kv1.5 mutants are the same as average current density of pGFP transfected cells, and therefore these mutants are not functional. The average current densities of ΔN119 Kv1.5 and ΔN209 Kv1.5 was calculated to be about 1nA/pF at +60mV, which is about one-third of the current density of transiently transfected Wt Kv1.5 (calculated to be 3nA/pF). Therefore, these results confirm that ΔN119 Kv1.5 and ΔN209 Kv1.5 are not only present in the plasma membrane but are also functional at the plasma membrane.

3.2 ΔN163 Kv1.5, but not ΔN135 Kv1.5, is localized to the ER

Immunofluorescence studies showed that ΔN135 Kv1.5 and ΔN163 Kv1.5 aggregate in the cellular cytoplasm (figure 3.2A). Perhaps, if we can identify their exact location in the cytoplasm, we can better determine the reason for their aggregation and their lack of trafficking. Calnexin is a resident ER membrane protein thought to be involved in the processing of proteins during their biogenesis in the ER
Therefore, we used this protein to mark the location of ER in HEK293 cells and to determine whether or not Kv1.5 non-trafficking mutants colocalize with it. Figure 3.4 shows confocal images of cells expressing channel mutants stained with anti-Kv1.5 and anti-calnexin. The top three images show that ΔN135 KV1.5 (red staining) and calnexin (green staining) do not co-localize. Thus, the ΔN135 KV1.5 mutant is not retained in the ER. However, the lower three images show that ΔN163 Kv1.5 (red staining) and calnexin (green staining) do co-localize (yellowish staining), indicating that ΔN163 Kv1.5 is retained in the ER.

3.3 Glycerol rescues the expression of ΔN163 Kv1.5

Several studies have successfully employed chemical and pharmacological chaperones to rescue the trafficking of non-trafficking mutants (Sato et al., 1996; Brown et al., 1996; Zhou et al., 1999; Ficker et al., 2000; Kagan et al., 2000; Gong et al., 2004). One example of a chemical chaperone is glycerol which has been shown to rescue the expression of missfolded proteins like CFTR ΔF508 (Sato et al., 1996; Brown et al., 1996) and hERG (Ficker et al., 2000; Kagan et al., 2000; Zhou et al., 1999) mutants. Although the exact mechanism of its action is unknown, glycerol is thought to facilitate correct folding by acting as a protective agent by physico-chemical interactions with the missfolded protein and perhaps preventing ER chaperones from binding to the missfolded protein (Qu et al., 1997). Other proposed mechanisms include slowing of protein synthesis in order to help the folding of the
protein and altering the energy barrier for the formation of a properly folded protein conformation (Gekko and Timasheff, 1981; Welch and Brown, 1996). We set out to investigate whether glycerol could rescue the trafficking of our non-trafficking mutants. If the channel mutants are missfolded or misassembled, glycerol treatment might rescue their trafficking or at least eliminate their aggregates. Figure 3.5 shows the confocal images of ΔN135 Kv1.5 and ΔN163 Kv1.5 treated with 10% glycerol. The last image in figure 3.5 represents wt Kv1.5 treated with 10% glycerol, which was used as a positive control of channel capable of trafficking. The staining pattern of glycerol treated ΔN163 Kv1.5 (red staining) has been changed from aggregated staining to what looks like Golgi and plasma membrane staining with a notable absence of any aggregation. Therefore, it appears that the surface expression of this mutant has been rescued by the treatment, and implies that the 163 residue NH₂-terminal deletion causes the channel to misfold or misassemble. Similarly, the immunostaining pattern of ΔN135 Kv1.5 was also changed from an aggregated pattern to more diffuse intracellular staining, however, there does not seem to be clear plasma membrane or even Golgi staining.
Figure 3-4 ΔN163 Kv1.5 but not ΔN135 colocalizes with calnexin

Confocal images of transiently transfected HEK293 cells expressing ΔN135 and ΔN163 Kv1.5. Each image is a single optical slice of fixed cells taken through the middle of the cells. Red staining represents Kv1.5 protein, blue staining represents nuclear staining (DAPI), and green staining represents ER marker calnexin. Colocalization between Kv1.5 mutants and calnexin is represented in the "Merge" image as orange to yellow color. The size bars are 10 μm.

Figure 3-5 10% Glycerol rescues the expression of ΔN163 Kv1.5

Confocal images of transiently transfected HEK293 cells expressing ΔN135, ΔN163 and Wt Kv1.5 (positive control). Each image is a single optical slice of fixed cells taken through the middle of the cells. The cells were incubated at 37°C with 10% Glycerol, during transfection. Red staining represents Kv1.5 protein and blue staining represents nuclear staining (DAPI). The size bars are 10 μm.
3.4 First 10 amino acids of T1 domain rescue surface expression of non-trafficking mutants

So far we showed that the ΔN119 Kv1.5 deletion mutant characterized by deletion of all NH$_2$-terminal amino acids up to T1 domain, is able to assemble, traffic, and express at the cell surface. Deletion of less than 50% of the T1 domain of Kv1.5 (deletion of 15 T1 domain residues in ΔN135 Kv1.5 and deletion of 43 T1 domain residues in ΔN163 Kv1.5) yields likely misfolded proteins that are retained in the ER and/or are unable to insert into the plasma membrane. Since adding the first 20 amino acids of T1 domain (T1$_{120-140}$) to ΔN135 Kv1.5 yields functional channels (T1$_{120-140}$+ΔN140 or ΔN119 Kv1.5), we decided to add the same region to the ΔN163 Kv1.5 mutant (T1$_{120-140}$+ΔN163). In order to find out whether this new mutant T1$_{120-140}$+ΔN163 Kv1.5 is able to rescue the trafficking of ΔN163 Kv1.5, we subjected T1$_{120-140}$+ΔN163 Kv1.5 to immunofluorescence and whole cell patch clamping studies. Figure 3.6 shows a schematic diagram of the new T1 mutants.
Figure 3-6 Schematic representation of Kv1.5 T1 mutations

The transmembrane domains are shown for the first mutant only and left out, thereafter, for clarity. The mutants were prepared as described in "Methods". Black fill represents the T1 domain and grey fill represents the rest of the NH$_2$-terminus. T1(1) (amino acids 120-128) and T1(2) (amino acids 129-140) were inserted into both $\Delta$N163 and $\Delta$N140. SE stands for Surface Expression.
Figures 3.7 and 3.8 show the immunofluorescence of T1 mutants in HEK293 and COS-7 cells, respectively. The COS-7 cell line was used to confirm the observations seen in HEK293 cells as well as eliminate any "cell-specific" occurrences from HEK293 cells. In addition, COS-7 cells have a large cytoplasm and a smaller nucleus, compared to HEK293 cells, and are thus a better choice for following intracellular localization and trafficking of proteins. Immunostaining results from both HEK293 cells (Figure 3.7) and COS-7 cells (Figure 3.8) show that the T1_{120-140}+ΔN163 Kv1.5 mutant is distributed throughout the cytoplasm, ER and Golgi, as well as the surface of the cell. The staining of T1_{120-140}+ΔN163 Kv1.5 mutant looks similar to immunofluorescence of T1_{120-140}+ΔN140 Kv1.5 (ΔN119 Kv1.5) mutant in these cells, which means that addition of the first 20 residues of T1 domain appears to rescue the trafficking of ΔN163 Kv1.5 to the plasma membrane.

From an examination of the Kv1.2 T1 domain crystal structure, it is apparent that the first 20 amino acids of T1 domain (T1_{120-140}) form an antiparallel β-sheet structure. More specifically, the first 10 amino acids of the T1 domain (N-terminal residues 120-128; T1(1)) form the first part of the antiparallel β-sheet and amino acids 11-20 of T1 domain (N-terminal residues 129-140; T1(2)) form the second part (or antiparallel part) of the β-sheet structure (represented in Figure 3.6). These two parts of the β-sheet are connected by a random coil. Therefore, in light of the fact that T1_{129-140} can be split into two β-sheet halves, we constructed two additional mutants adding either the first or the second part of the β-sheet structure to both ΔN135 Kv1.5 and ΔN163 Kv1.5 mutants.
Figure 3-7 Addition of first 10 amino acids of T1 domain rescues non-trafficking mutants in HEK293 cells.

Confocal imaging of transiently transfected HEK293 cells expressing T1_{120-140} + ΔN163, T1(1)+ΔN163, and T1(2)+ΔN163 Kv1.5 mutants as well as T1_{120-140} + ΔN140 (same as ΔN119), T1(1)+ΔN140, and T1(2)+ΔN140 Kv1.5 mutants. The arrows point to membrane staining. Each image represents a single optical slice through the middle of fixed cells. Red staining represents Kv1.5 protein and blue staining represents nuclear staining (DAPI). The size bars are 10 μm.
Figure 3-8 Addition of first 10 amino acids of T1 domain rescues non-trafficking mutants in COS-7 cells.

Confocal imaging of transiently transfected COS-7 cells expressing T1_{120-140}+ΔN163, T1(1)+ΔN163, and T1(2)+ΔN163 Kv1.5 mutants as well as T1_{120-140}+ΔN140 (same as ΔN119), T1(1)+ΔN140, and T1(2)+ΔN140 Kv1.5 mutants. The arrows point to membrane staining. Each image represents a single optical slice through the middle of fixed cells. Red staining represents Kv1.5 protein, blue staining represents nuclear staining (DAPI), and green staining represents GFP used for identification of the size of cytoplasm. The size bars are 10 μm.
As illustrated in Figure 3.6, adding the first 10 residues of T1 domain to AN135 Kv1.5 and AN163 Kv1.5 mutants, yields T1(1)+ΔN140 Kv1.5 and T1(1)+ΔN163 Kv1.5 mutants, respectively. Adding only the second part of the β-sheet structure (residues 11-20 of the T1 domain) to AN135 Kv1.5 and AN163 Kv1.5 mutants, yields T1(2)+ΔN140 Kv1.5 and T1(2)+ΔN163 Kv1.5, respectively. Immunofluorescence images of all transiently transfected T1(1) and T1(2) mutants in COS-7 and HEK293 cells, show substantially more intracellular staining resembling the staining pattern of T1120-140+ΔN163 Kv1.5 (red staining) and eliminating aggregation (Figs. 3.7, 3.8). In addition, T1(1)+ΔN140 Kv1.5 and T1(1)+ΔN163 Kv1.5 mutants show Golgi and some plasma membrane staining whereas T1(2)+ΔN140 Kv1.5 and T1(2)+ΔN163 Kv1.5 mutants do not. T1(2)+ΔN140 Kv1.5 and T1(2)+ΔN163 Kv1.5 mutants show limited intracellular staining, as seen better in COS-7 cells, and lack plasma membrane staining. Therefore, adding the first 10 amino acids of T1 domain to non-trafficking mutants seems to rescue the trafficking of both ΔN135 Kv1.5 and ΔN163 Kv1.5, whereas adding the second 10 amino acid region does not.

In parallel experiments the macroscopic currents from these mutants were measured using whole-cell patch clamping. Current traces of Wt Kv1.5, T1120-140+ΔN163 Kv1.5, T1(1)+ΔN163 Kv1.5 and T1(2)+ΔN163 Kv1.5 are shown in figure 3.9A. All three mutants produced relatively small current compared to wt Kv1.5. Figure 3.9A shows that T1(2)+ΔN163 Kv1.5 mutants produced, mostly, currents of less than 1nA (similar to GFP transfected controls) with about 3/15 cells producing currents slightly above 1nA, whereas T1(1)+ΔN163 Kv1.5 produced current of about 15nA.
A. Representative current traces from wt, T1_{120-140}+ΔN163, T1(1)+ΔN163, T1(2)+ΔN163 Kv1.5 transiently transfected in HEK293 cells. The cells were held at -80 mV and stepped to voltages ranging between -70 mV to +80 mV in 10 mV steps for at least 100 ms. GFP was co-transfected with pcDNA3 in order to identify transfected cells.

B. Summary of averaged current density at +60 mV depolarization normalized to cell capacitance. The results are statistically significant (T1_{120-140}+ΔN163, T1(1)+ΔN163, T1(2)+ΔN163 vs. wt Kv1.5; p<0.05).

Figure 3-9 Addition of first 10 amino acids of T1 domain results in functional channels.
Interestingly, T1\textsubscript{120-140}+\Delta N163 Kv1.5 produced medium-size currents, falling between T1(1)+\Delta N163 Kv1.5 and T1(2)+\Delta N163 Kv1.5 peak currents, with 1 cell producing currents of 6nA and 5 cells producing currents of 1.5nA – 2.5nA. Figure 3.9B shows the summary plot of current density values, calculated from averaged macroscopic current measured at +60mV depolarization and normalized to cell capacitance (nA/pF). The current density of T1\textsubscript{120-140}+\Delta N163 Kv1.5 mutant is about 0.2nA/pF, meaning that even though T1\textsubscript{120-140}+\Delta N163 Kv1.5 channels seem to traffic towards the plasma membrane, the channel can not insert into or is not functional at the plasma membrane. The current density of T1(2)+\Delta N163 Kv1.5 is less than 0.1nA/pF, and therefore, T1(2)+\Delta N163 channels are not functional. On the other hand, the current density of T1(1)+\Delta N163 Kv1.5 was measured and calculated to be 0.8nA/pF, showing that this mutant is able to both express and be functional at the plasma membrane. Therefore, the first 10 amino acids of T1 domain rescue the surface expression of \Delta N163 Kv1.5. However, compared to wt Kv1.5, the current density of T1(1)+\Delta N163 Kv1.5 is significantly lower (>2.5 nA/pF, Figure 3.9B, inset). Whole-cell patch clamp data from T1\textsubscript{120-140}+\Delta N140 Kv1.5 expressing cells is shown in Figure 3.3 (same as \Delta N119 Kv1.5) and its average current density is close to 1nA/pF. We did not obtain whole-cell patch clamp data of the T1(1)+\Delta N140 Kv1.5 and T1(2)+\Delta N140 Kv1.5 mutants, since the fluorescence images of these mutants resemble the ones of T1(1)\textsubscript{120-128}+\Delta N163 Kv1.5 and T1(2)\textsubscript{129-140}+\Delta N163 Kv1.5, respectively.
3.5 Point mutations within the T1 domain have an effect on surface expression

Since we have found that the first 10 residues of the Kv1.5 T1 domain rescue the surface expression of ΔN135 Kv1.5 and ΔN163 Kv1.5, we decided to attempt to determine whether a specific amino acid motif or region was responsible for the rescue of the non-trafficking mutants. Recently, a study was published where the authors used computer homology modeling of the Kv1.5 T1 domain (and other Kv1 channels) based on the crystal structure of the Shaker channel T1 domain (Liu et al., 2005). From this model, it would appear that important residues involved in intra-domain T1 interactions reside in the first 20 amino acids of the T1 domain (T1_{120-140}) (figure 3.10). In particular, E_{132}T_{133}Q_{134} residues are predicted to form side chain interactions with D_{166} on adjacent monomers during assembly of the Kv1.5 tetramer. Therefore, we performed point mutations on these residues to Ala in T1(2)+ΔN163 (AAA132-134ΔN163 and D166AΔN163) and T1(2)+ΔN140 (AAA132-134ΔN140 and D166AΔN140) backgrounds. Figure 3.11 shows the immunofluorescence of the D166AΔN163 Kv1.5 and D166AAΔN140 Kv1.5 in HEK293 cells demonstrate an aggregated staining pattern compared to T1 mutants without point mutations and wt Kv1.5. AAA132-134ΔN163 Kv1.5 and AAA132-134ΔN140 Kv1.5 showed more cytoplasmic and some plasma membrane staining. Therefore, it seems that mutating D_{166} to Alanine (D166AΔN163 and D166AΔN140) results in aggregation of the T1 mutants and mutating E_{132}T_{133}Q_{134} to Alanine (AAA132-134ΔN163 and AAA132-134ΔN140) results in thorough intracellular and some plasma membrane staining.
Figure 3-10 Representation of intersubunit interaction in tetramer formation

A. Crystal structure of Shaker T1 domain forming a tetramer. The blue labelling shows the location of E_{132}T_{133}Q_{134} and D_{166} residues contributing to the tetramer structure.

B. Hypothetical intersubunit interaction of highly polar residues in Kv1.5, based on homology modeling of Shaker T1 crystal structure. Only side chain residues and their interactions are shown (Liu et al., 2005).
Figure 3-11 Effects of key residues point mutations in T1 domain on trafficking.

Confocal images of transiently transfected HEK293 cells expressing D166A\N163, AAA132-134\N163, D166A\N140, and AAA132-134\N140 Kv1.5 mutants and wt Kv1.5 (positive control). Each image represents a single optical slice through the middle of fixed cells. Red staining represents Kv1.5 protein and blue staining represents nuclear staining (DAPI). The size bars are 10 µm.
However, we did not confirm these observations by testing the functionality of these mutants at the plasma membrane by electrophysiology.

3.6 Point mutations in the NH$_2$-terminal di-Arginine ER retention motif cause a slight rescue of $\Delta$N163 Kv1.5 trafficking

Thus far, we have found that $\Delta$N135 Kv1.5 and $\Delta$N163 Kv1.5 are unable to traffic to the plasma membrane. In addition, we have shown that $\Delta$N163 Kv1.5 is retained in the ER. A number of amino acid sequences have been identified to be involved in ER retrieval/retention in transmembrane proteins including ion channels. If the protein is missfolded such that its retention/retrieval motif(s) are exposed, these motifs are thought to be recognized by ER resident chaperone proteins and the newly synthesized protein is retained in the ER. In a number of different protein families, including $K_{ATP}$ (Zerangue et al., 1999); CFTR (Owsianik et al., 2003); GABA (Margeta-Mitrovic et al., 2000); hERG (Kupershmidt et al., 2002); and Na$_V$ channels (Zhou et al., 2002), RXR (arginine-X-arginine) motifs serve this function. Therefore, we decided to look for the di-arginine motif(s) in our non-trafficking mutants. $\Delta$N135 Kv1.5 does not contain any RXR motifs in the region between amino acid 135 and 163. $\Delta$N163 Kv1.5 has three RXR motifs, located at positions 167 – 169 ($R^{167}N^{168}R^{169}$; aka RXR$^1$), 184 – 186 ($R^{184}L^{185}R^{186}$; aka RXR$^2$), and 212 – 214 ($R^{212}F^{213}R^{214}$; aka RXR$^3$). Therefore, we constructed point mutations converting one, two, or three RXR motifs in the $\Delta$N163 Kv1.5 mutant to RXA (arginine-X-alanine).
Figure 3.12A shows immunofluorescent confocal imaging in HEK293 cells and figure 3.12B shows the confirming whole-cell patch clamping data. From confocal imaging we can see that mutating the di-arginine motif changes the Kv1.5 staining (red) from aggregated pattern, as seen in ΔN163 Kv1.5 and RXA\(^1\)⁺(R\(^{167}\)N\(^{168}\)A\(^{169}\)), to more diffuse intracellular pattern as well as some cell membrane staining, as seen in RXA\(^2\) (R\(^{167}\)N\(^{168}\)A\(^{169}\) and R\(^{184}\)L\(^{185}\)A\(^{186}\)) and RXA\(^3\) (R\(^{167}\)N\(^{168}\)A\(^{169}\), R\(^{184}\)L\(^{185}\)A\(^{186}\) and R\(^{212}\)F\(^{213}\)A\(^{214}\)). Similarly, the whole-cell current density (figure 3.12B) at +60mV shows a slight increase from 40pA/pF in ΔN163 Kv1.5 and RXA\(^1\)+ΔN163 Kv1.5, to ~100pA/pF in RXA\(^2\)+ΔN163 Kv1.5 and RXA\(^3\)+ΔN163 Kv1.5. These results show, therefore, that a sequential removal of the di-arginine motifs show a very small increase in ΔN163 Kv1.5 surface expression associated with a more diffuse distribution of the channels in the cell.
**Figure 3-12** NH$_2$-terminal di-arginine ER retention motif point mutations in Kv1.5 show a small rescue of ΔN163 Kv1.5 trafficking.

A. Confocal images of transiently transfected HEK293 cells expressing single, double, and triple arginine to alanine mutations, encompassing three potential NH$_2$-terminal di-arginine ER retention signals of ΔN163. Each image represents a single optical slice through the middle of fixed cells. Red staining represents channel protein and blue staining represents nuclear staining (DAPI). The size bars are 10 μm.

B. Summary of averaged current density of the above mutants at +60 mV depolarization normalized to cell capacitance. The results are statistically significant (p < 0.05).
4 DISCUSSION AND CONCLUSIONS

4.1 Discussion

We have found that deletion of N-terminal ~120 amino acids of Kv1.5 has a significant effect on the cell surface protein expression, as measured using the patch clamp technique (Figure 3.3). Similarly, deletion of the whole NH₂ terminus, including more than 60% of the T1 domain (Kv1.5 ΔN209) causes problems for the normal surface expression of Kv1.5. However, smaller deletions of 15 to ~40 amino acids at the start of the T1 domain impair the normal trafficking of Kv1.5 far more seriously, as determined by immunofluorescence as well as PK experiments and whole cell patch clamp. ΔN135 and ΔN163 Kv1.5 mutants form aggregates in the cytoplasm and are not able to traffic to the plasma membrane (figure 3.2A). Co-localization studies have shown that ΔN163 is most likely retained in the ER as evidenced by co-localization with an ER marker calnexin, whereas ΔN135 did not colocalize with calnexin suggesting lysosomal or other organelle localization. Addition of the first 10 amino acids of the T1 domain (T1(1)+ΔN140 and T1(1)+ΔN163) to non-trafficking Kv1.5 mutants ΔN135 and ΔN163, eliminated their aggregation and allowed trafficking to the cell surface.

4.1.1 ΔN163 Kv1.5 can not traffic to the plasma membrane

Interestingly, we have found that ΔN163 is retained in the ER and its surface trafficking can be rescued by the help of a chemical chaperone, glycerol. Previous
studies have shown that newly synthesized proteins are retained in the ER if they are missfolded, misassembled, or can not properly tetramerize with their accessory subunits (Kowalski et al., 1998; Kopito, 1997). Glycerol as well as other chemical chaperones have been shown to repair missfolded proteins or help missfolded proteins to escape the ER QC machinery (Sato et al., 1996; Zhou et al., 1999; Gong et al., 2004). Although the exact mechanism of its action is unknown, glycerol is thought to facilitate correct folding by acting as a protective agent by physico-chemical interactions with the missfolded protein and perhaps preventing ER chaperones from binding to the missfolded protein (Qu et al., 1997). Other proposed mechanisms include slowing of protein synthesis in order to help the folding of the protein and altering the energy barrier for the formation of a properly folded protein conformation (Gekko and Timasheff, 1981; Welch and Brown, 1996). In our study, addition of glycerol changed the distribution of ΔN135 and ΔN163 Kv1.5 mutants from intracellular aggregates to more diffuse intracellular localization and possibly allowed cell surface expression (figure 3.4). Unfortunately, we were not able to measure whole cell currents of the glycerol treated, otherwise non-trafficking mutants, because glycerol quenches the GFP signal needed for identification of transiently transfected cells during patch clamping experiments. Attempts at biochemical assays, like Proteinase K digestion, were unsuccessful because a great majority of cells detached during glycerol treatment. Further experiments need to be done to determine whether these channels are able to insert into the plasma membrane and, if they do, whether they are functional.
As stated above, Zerangue et al. have shown that during missfolding and incorrect assembly of a new protein a particular motif, RXR, is exposed and recognized by the ER QC machinery (Zerangue et al., 1999). As a result, the missfolded proteins are retained in the ER until the mistake is corrected or are shunted to the degradation pathway. These and other studies have shown that during correct folding (Owsianik et al., 2003; Kupershmidt et al., 2002) and correct assembly with accessory subunits (Zerangue et al., 1999; Margeta-Mitrovic et al., 2000), the di-arginine motif(s) is/are masked. In our study, ΔN163 Kv1.5 has been found to localize to the ER, as determined by calnexin co-localization and immunofluorescence studies (Figure 3.4). The primary sequence of ΔN163 Kv1.5 has three RXR motifs located at the N-terminal-most position of the mutant, at positions 167 – 169 (R\textsuperscript{167}N\textsuperscript{168}R\textsuperscript{169}), 184 – 186 (R\textsuperscript{184}L\textsuperscript{185}R\textsuperscript{186}), and 212 – 214 (R\textsuperscript{212}F\textsuperscript{213}R\textsuperscript{214}). Therefore, it is possible that some or all of the ER retention motifs are exposed in ΔN163 Kv1.5 mutant and that these may be responsible for the lack of plasma membrane expression of ΔN163 Kv1.5. In fact, we showed that mutating the last Arg of each di-arginine motif(s) results in a small increase in current density and sequential recovery from aggregation from RXA\textsuperscript{1} to RXA\textsuperscript{2} to RXA\textsuperscript{3} (A = Alanine) (Figure 3.11). The reason for only a small increase in current density could be the fact that we did not mutate both arginines of the RXR motif since it has been shown before that both arginines play a role in the ER retention and so both arginines must be masked (Zerangue et al., 1999; Margeta-Mitrovic et al., 2000; Standley et al., 2000; Kupershmidt et al., 2002; Zhou et al., 2002; Owsianik et al., 2003). Alternatively, it is possible that the RXR motifs are suppressed by phosphorylation.
and/or binding with PDZ proteins, as shown previously in cardiac sodium channel and NMDA receptors (Standley et al., 2000; Scott et al., 2001; Zhou et al., 2002). It is also possible that these ER retention motifs do not play any functional role in Kv1.5 channel trafficking in HEK293 cells.

4.1.2 First 10 amino acids of T1 domain may be involved in masking ER retention motif(s)

Addition of the first 10 amino acids of the T1 domain (amino acids 120 – 128 in the wt Kv1.5) to ΔN163 Kv1.5 (T1(1)+ΔN163 Kv1.5) rescues cell surface expression of the mutant, as shown by electrophysiology and confocal imaging experiments. However, we have not been able to determine the exact mechanism by which the rescue is affected. It is possible that this 10 amino acid region of T1 domain (this region forms the antiparallel β-sheet structure) contains a trafficking motif necessary for ER export, but examining the primary amino acid sequence reveals no known trafficking motifs. It is also possible that this region is involved in “masking” of one or all RXR motifs during correct folding of the T1 domain, as seen when observing the crystal structure of Kv1.2 ion channel (Minor et al., 2000). Kv1.2 and Kv1.5 amino acid sequences are about 60% homologous, but the sequence similarity between their T1 domains is more than 90%. Therefore, it is safe to assume that the secondary folding of the Kv1.5 and Kv1.2 ion channel T1 domains are very similar. Yet another possibility is that the 10 amino acid region, that forms part of the antiparallel β-sheet structure, is involved in intermolecular interactions contributing to the tetramer formation and/or stability of the tetramer T1.
Region of N-terminal residues 129 – 140 (residues 9 – 20 of T1 domain; T1(2)+ΔN163 Kv1.5) was not able to rescue the surface expression of non-trafficking ΔN163 Kv1.5, as shown by electrophysiology and confocal imaging experiments. Even though these residues appear to mask the RXR motifs, deletion of the first 10 amino acids of T1 domain may disrupts the β-sheet structure. Disruption of the β-sheet structure may contribute to missfolding of a part or the whole NH2 terminus, leading to exposure of the ER retention motifs.

4.1.3 β-sheet structure may play a role in protein trafficking

Recently, it has been shown that a coiled-coil structure is necessary for correct trafficking of cardiac potassium channel KCNQ1 (Kanki et al., 2004). Coiled coil structures consist of two α helices, oriented antiparallel to each other (Monera et al., 1994; Monera et al., 1996; Myszka and Chaiken, 1994). Disruptions or mutations in the coiled coil structure in COOH terminus of KCNQ1 channel, result in LQT phenotype (Kanki et al., 2004). This region does not contain any trafficking motifs except a leucine zipper, which when disrupted without affecting the secondary structure, resulted in normal surface expression. Coiled coil structures have also been shown to be necessary for protein interaction in GABA receptors where GABA$_B_1$ and GABA$_B_2$ interact via coiled-coil to form a mature functional channel and, at the same time, "mask" the RXR motif(s) (Kammerer et al., 1999; Kuner et al., 1999). Disruption of this interaction and subsequent exposure of the di-arginine motif(s) causes retention of the receptor subunits in the ER (Margeta-Mitrovic et al., 2000; Pagano et al., 2001).
Similarly, exposed strands of β-sheets are important for protein-protein interaction and disruption of these secondary structures can result in protein aggregation. Such a mechanism has been implicated in a number of neurodegenerative disorders (Richardson and Richardson, 2002; Siepen et al., 2003). As an example, defensin HNP-3 protein, involved in the mammalian immune response, consists of three β-sheet strands in each monomer that hydrogen bond with the neighboring monomer to form a six-stranded β-sheet (Hill et al., 1991). This β-sheet conformation contributes to a stable and compact structure, preventing the small protein from being digested by proteases. In our study, we have seen that the disruption of the first 20 amino acids of the T1 domain, which forms an antiparallel β-sheet structure, by either deletion or point mutations, results in aggregation of the Kv1.5 mutant channels. The crystal structures of Shaker and the Kv1.2 T1 domain, as well as the homology models for T1 domain of Kv1 family of channels, indicate that several interactions between monomers take place via the β-sheet structures (Kobertz et al., 2000; Cushman et al., 2000; Minor et al., 2000; Liu et al., 2005). Therefore, it is possible that the aggregation observed in immunofluorescent studies is due to the inability of the monomer to form a tetrameric structure. Just like defensin protein, disruption of the two stranded β-sheet structure may threaten the stability of the tetrameric T1 domain and/or the whole channel leading to collapse and misfolding.

In addition, NH2-terminal residues 155-165 form another small antiparallel β-sheet structure. Therefore, it is possible that the reason for lack of trafficking of ΔN163 Kv1.5 is the disruption of this β-sheet. Incidentally, this small antiparallel β-
sheet structure has been determined to be involved in the β–subunit interaction. Perhaps, disruption of this antiparallel β-sheet structure may have an effect on the ion channel folding and/or assembly in the ER and, as a result, is caught by the QC machinery. Further experiments need to be done in order to determine whether any of the above hypotheses are relevant in the trafficking of the ΔN163 Kv1.5 mutant.

4.1.4 Importance of specific residues in T1(2) for trafficking of Kv1.5

A study, using computer homology modeling of the Kv1.5 T1 domain based on the crystal structure of Shaker channel T1 domain, showed that certain residues are important in intermolecular interactions contributing to the tetrameric structure (Liu et al., 2005). In their model, the authors propose that Kv1.5 N-terminal residues 132 – 134 (E132T133Q134 in Kv1.5) on one monomer are involved in side chain interactions with residue 166 (D166 in Kv1.5) on adjacent monomer (Figure 3.10B). Interestingly, E132T133Q134 are located in the second half of the 20 amino acid region of T1 domain, i.e., T1(2)+ΔN140 and T1(2)+ΔN163. Therefore, we have performed point mutations of these residues to Ala (A132A133A134 and A166) in both T1(2)+ΔN140 and T1(2)+ΔN163 Kv1.5 mutants. Our immunofluorescence studies of D166AΔN140, D166AΔN163 and AAA132-134ΔN163 identified an aggregated staining pattern unlike the immunofluorescence of T1(2)+ΔN140 and T1(2)+ΔN163 mutants which showed mostly diffuse intracellular staining (compare Figure 3.7 and Figure 3.10). AAA132-134ΔN140 also showed increased cytoplasmic as well as some plasma membrane staining (Figure 3.11). Therefore, residues including E132T133Q134 and
D_{166} may play a role in the tetramerization of the channel. In addition, the A_{166} point mutation, similarly, causes significant aggregation of both T1(2)+ΔN140 and T1(2)+ΔN163 Kv1.5 as would be expected from its hypothesized interaction with E_{132}T_{133}Q_{134}.

### 4.1.5 Mutation of residues in T1 domain that are involved in gating may abolish protein trafficking

The T1 domain has been implicated in the gating mechanics of Kv1 ion channels, previously (Cushman et al., 2000; Minor et al., 2000). The exact mechanism is not well understood but the authors hypothesize that the gating process forces a global conformational change on T1, affecting the positions and interactions of a number of polar residues within the T1 domain; i.e., the T1 domain may be physically linked to the changes that take place in the transmembrane pore. The Kv1.5 NH₂-terminal residues thought to be involved include N_{124} F_{131} E_{132} T_{133} T_{137} F_{164} D_{166} D_{173} V_{189} D_{194} and D_{198} (Minor et al., 2000). It is possible that the interactions of these residues are not only important in their gating function but also in the proper folding and assembly of the T1 domain while the channel is synthesized in the ER. Therefore, a deletion or mutation of these residues may result in missfolding that may be detected by the QC machinery preventing their exit from the ER. Alternatively, the trafficking of these Kv1.5 mutants might not be compromised or slowed down, but their gating mechanics may be altered, resulting in low macroscopic current. These hypotheses require further testing.
4.1.6 $\Delta N135$ Kv1.5

Just like $\Delta N163$, the other Kv1.5 non-trafficking mutant in our study, $\Delta N135$ also forms aggregates in the cytoplasm of HEK293 cells (see figure 3.2A). We were unable to determine why the $\Delta N135$ Kv1.5 mutant is unable to traffic to the plasma membrane or why it aggregates in the cytoplasm. The primary amino acid sequence of Kv1.5 between residues 135 and 163 contains no known ER retention motifs or any other previously identified trafficking motif(s). Our experiments do show, however, that $\Delta N135$ Kv1.5 is not retained in the ER, as shown by the lack of co-localization with calnexin in immunofluorescence experiments. In addition, its aggregation staining seems to be attenuated by the help of glycerol to a much smaller extent than the $\Delta N163$ mutant. Since this mutant is not retained in the ER, it may be folded and assembled properly. In addition, it may be able to leave the ER and enter the Golgi network. In this scenario, the QC machinery in the Golgi does not allow the sorting and the exit of the protein to the plasma membrane and instead the mutant may be shunted into the degradation pathway. Since the Golgi QC checkpoints involved in protein trafficking are not well understood, it is difficult to say how a protein that enters the Golgi would be retained there and subsequently enter the degradation pathway. Alternatively, since we could not detect the $\Delta N135$ Kv1.5 localization in the ER, it is more likely that the mutant is shunted into the degradation pathway directly from the ER. During this process, called the ER-associated degradation (ERAD), misfolded and/or unassembled proteins are recognized by the ERAD machinery and are retrotranslocated into the cytoplasm where they are degraded by the proteasome (Abriel and Staub, 2005).
Proteinase K experiments provided useful information. Whereas ΔN163 Kv1.5 runs as a single band in the western blot before and after the treatment with the enzyme (Figure 3.2B), consistent with its retention in the ER, ΔN135 Kv1.5 runs as a double band in the Western blots. Interestingly, both bands of ΔN135 are resistant to the Proteinase K treatment (Figure 3.2B). While it is conceivable that the upper band is a result of complex glycosylation associated with entrance into Golgi network it is much more likely that the two bands represent degraded pool(s) of ΔN135 proteins. In addition, both bands of ΔN135 mutant run in the Western blot at a smaller molecular weight than ΔN163 Kv1.5 (at about 47kDa, whereas the theoretical molecular weight of un-glycosylated ΔN135 Kv1.5 is 53kDa) and the fact that this mutant was rapidly cleared out of most cells, confirming the possibility that this mutant is in the degradation pathway.

Addition of the first 10 amino acids of the T1 domain, T1(1)+ΔN140 Kv1.5, rescues the trafficking and surface expression of ΔN135, just as was the case for ΔN163 Kv1.5 (Figure 3.7 and 3.8). Observing the crystal structure of the Kv1.2 T1 domain, the 20 amino acid region that is deleted in the ΔN163 does not form any particular secondary structure. However, this region consists of a number of large hydrophobic residues and contains a small antiparallel β-sheet at positions ~155 – 165 (Minor et al., 2000). Therefore, it is possible that the first 10 amino acids of the T1 domain that form the antiparallel β-sheet structure may act as a “stabilizer” of the overall T1 domain, as discussed above. When the “stabilizer” β-sheet is deleted, the T1 domain cannot fold properly due to its exposed large hydrophobic residues (residues 135 – 163) and, as a result, forms aggregates. In addition, if the T1
domain can not fold correctly, it may also be possible that this leads to exposure of the ER retention RXR motifs.

Adding the second part of the 20 amino acid region of T1 domain to the δN135 mutant (T1(2)+δN140; N-terminal residues 129-140) does not seem to rescue the trafficking of the δN135 mutant to the cellular membrane (Figure 3.7 and 8), most likely for the same reasons as listed above for T1(2)+δN163 Kv1.5. Further experiments must be done to determine whether there are any new trafficking motifs involved or whether it is the incorrect folding of the T1 domain that is responsible for the lack of trafficking and aggregation of δN135 Kv1.5.

4.1.7 δN119, δN189, and δN209 deletions yield functional Kv1.5 channels with low current density

We have found that even though δN189 and δN209 Kv1.5 constructs are missing more than 60% of T1 domain, they are still able to traffic, insert, and be functional in the plasma membrane. Perhaps these results are not surprising since we have deleted two of the di-arginine ER retention motifs and therefore, the ER QC chaperone proteins that are involved in recognition of these motifs may not be able to bind to the missfolded protein. Furthermore, it has been shown previously that ion channels without a T1 domain can still fold correctly, tetramerize, and function at the plasma membrane (Zerangue et al., 2000), but the tetramerization efficiency of these channels is compromised. Therefore, it would appear that a significant fraction of δN189 and δN209 Kv1.5 constructs are correctly assembled and trafficked to the
plasma membrane, but at much slower rates than wt Kv1.5. Also, it has been shown that a channel without the T1 domain is less stable than a wt channel (Strang et al., 2001). This means that these proteins may easily be degraded along their secretory pathway and therefore, smaller numbers of ion channels are able to make it to the plasma membrane. As a result, the measured macroscopic current densities are lower than those of channels with intact T1 domains (wt). We have not measured the kinetic properties of most of these constructs but we have shown (Kurata et al., 2001) a small shift in the half-activation voltage of ΔN209 Kv1.5 in the hyperpolarized direction compared to wt Kv1.5. All of the theories above may contribute to the low macroscopic current density of ΔN189 and ΔN209 Kv1.5 mutants compared to that of wt Kv1.5. Further studies will be necessary to properly evaluate these possibilities.

Deletion of the N-terminal 189 or 209 amino acids removes the attachment site for β subunits and most of the T1 residues thought to play roles in gating of voltage-gated potassium channels (Minor et al., 2000; Cushman et al., 2000). The β subunit interaction loop is located in the T1 domain of Kv1 channels (Gulbis et al., 2000). In Kv1.5 ion channels, the residues thought to be involved in this interaction include amino acids 156 – 163 (FDPLRNEY) most of which, as inferred from the crystal structure of the Kv1.2 T1 domain, are part of the antiparallel β-sheet discussed earlier. Even though Kv1.5 is able to traffic to the plasma membrane without the help of a β subunit, it has been shown that β subunits alter Kv1.5 kinetics (England et al., 1995a; Uebele et al., 1996; De Biasi et al., 1997; Uebele et al., 1998). In addition, studies of Kv1.2 ion channel synthesis found that β subunits
assist in the folding of the newly synthesized protein, as soon as its NH$_2$ terminus is translated and enters the ER from the translocon (Shi et al., 1996; Nagaya and Papazian, 1997). Therefore, it is possible that there are ER-resident QC proteins that somehow check for the $\beta$ subunit interaction. If this is true, a lack of $\beta$ subunit interaction site might slow down the QC processes of the nascent $\Delta$N189 and $\Delta$N209 Kv1.5 protein and result in reduced ion channel number at the cell surface.

Interestingly, the $\Delta$N119 Kv1.5 has an intact T1 domain but its whole-cell current density is 1/3 that of wt Kv1.5 ion channel. This means that the long NH$_2$ terminus, preceding the T1 domain, may be important in the biogenesis and trafficking of Kv1.5 ion channels. In fact, it is interesting that Kv1.1 and Kv1.2 ion channels which have very short NH$_2$ termini preceding their T1 domains are only able to express at the cell surface as either a heterotetramer, in the case of Kv1.1, or with the help of a $\beta$ subunit, in the case of Kv1.2. The long NH$_2$ terminus of Kv1.5 contains two SH3-binding domains as well as additional proline-rich domains. SH3 domains have been implicated in recruitment of tyrosine kinases to a protein. Recently, our lab has identified the importance of the first SH3 domain in Kv1.5 endocytosis via a dynein-dependent pathway (Choi et al., 2005). The authors showed that deletion of N-terminal amino acids 65 to 93, which include both SH3 domains, has no effect on the currents upon treatment with p50, compared to control (no p50 treatment) and wt Kv1.5 (increase in current density with p50 treatment). Although it is not known whether an SH3 domain is involved, trafficking from the ER to the Golgi also requires dynein (Presley et al., 1997; Hamm-Alvarez et al., 1993; Valetti et al., 1999; Presley et al., 2002). Perhaps deleting the SH3 domain(s), which
are located between residues 65 – 93, may have a small impact on the forward trafficking between ER and Golgi. Similarly, the ΔN119 mutant is missing the two SH3 domains and therefore, its trafficking to the plasma membrane may be slowed down for the same reasons.

Phosphorylation has been further implicated in the regulation of Kv1.5. Kv1.5 phosphorylation by src tyrosine kinases results in a decrease of Kv1.5 current density (Holmes et al., 1996), and inhibition of Protein Kinase A (PKA) results in a decrease of Kv1.5 current density (Mason et al., 2002). The latter occurs despite the fact that PKA activation has no effect on Kv1.5 current density (Mason et al., 2002). Therefore, the trafficking of Kv1.5 channel is directly and indirectly modulated by different phosphorylation kinases and removal of their sites of action from the target protein will likely have an effect on current density and trafficking of the ion channel.

Another reason for the downregulation of ΔN119 Kv1.5 relative to wild-type may involve α-actinin. N-terminal residues 63 – 150 of Kv1.5 have been shown to interact with α-actinin (Maruoka et al., 2000; Cukovic et al., 2001). This interaction very probably ties Kv1.5 channels to the actin cytoskeleton, linking them to regulatory kinases and phosphatases which have been shown to up-regulate KV1.5 current in X. laevis oocytes (Mason et al., 2002). ΔN119 Kv1.5 mutant lacks more than 60% of the α-actinin interaction domain in Kv1.5. Therefore, disruption of this cluster of ion channels/actin and associated regulatory enzymes and phosphatases may affect the trafficking of ΔN119 Kv1.5 to the plasma membrane or its retention there and, therefore, its current density level.
A novel PDZ-binding domain has been found in the NH$_2$ terminus of Kv1.5, comprising roughly N-terminal amino acid residues 17 – 65 (Eldstrom et al., 2002). Members of the PDZ-binding family of proteins interact directly or indirectly with Kv1 ion channels exerting different effects on the ion channel function. For example, PSD95 was shown to interact with Kv1.5 ion channel directly resulting in downregulation of Kv1.5 whole-cell current density (Eldstrom et al., 2002). Alternatively, SAP97 was shown to interact with Kv1.5 ion channel indirectly, resulting in upregulation of Kv1.5 whole-cell current density (Eldstrom et al., 2003). Direct interaction between PSD95 and Kv1.2 ion channels does not affect the channel's trafficking, but instead has been shown to increase the stability of the ion channel at the plasma membrane (Tiffany et al., 2000). Therefore it is possible that the deletion of the NH$_2$ terminus with PDZ-binding and actin-binding domains weakens the channel's stability or its ability to cluster at the plasma membrane, ultimately resulting in lower whole cell current density.

4.2 Conclusions

Our studies have uncovered a number of interesting phenomena. We have identified a 10 amino acid region at the start of the T1 domain that is an important prerequisite for functional Kv1.5 ion channels. N-terminal deletions up to the T1 domain (ΔN119 Kv1.5) and large deletions of the T1 domain (ΔN189 and ΔN209 Kv1.5) have no effect on the surface expression of Kv1.5 but minor effects on the function of Kv1.5. Small deletions at the start of the T1 domain (ΔN135 and ΔN163 Kv1.5) yield mutants unable to traffic to the plasma membrane. We found that the
non-trafficking mutants aggregate in the ER, are probably degraded by the proteosome of the ERAD, and are rescued from aggregation by treatment with the chemical chaperone glycerol. Furthermore, addition of the first 10 amino acids of the T1 domain, N-terminal residues 120 – 128 (or residues 1-8 of T1 domain), rescues the non-trafficking mutants from aggregation and renders them functional at the cell surface. This T1 region does not contain any trafficking motifs, but it forms an antiparallel β-sheet structure and contains key residues involved in channel tetramerization.

Our results are inconclusive in terms of the exact mechanisms that determine the lack of trafficking and low macroscopic current density of these particular mutants. Instead a number of hypothetical mechanisms have been listed throughout the discussion. To summarize, it is possible that the trafficking of ΔN135 and ΔN163 Kv1.5 mutants is abolished because of missfolding of the T1 domain and/or the NH₂ terminus that can lead to protein aggregation. The protein aggregation may be caused by number of states, such as exposure of ER retention motifs, disruption of important β-sheet secondary structures, disruption of tetramerization contacts, or exposure of large hydrophobic residues. Additionally, the trafficking and function of the ΔN119, ΔN189, and ΔN209 Kv1.5 mutants may be affected by deletion of important sites involved in the modulation of Kv1.5 channel function, such as phosphorylation and interaction with auxiliary subunits. It is also possible that a combination of these individual processes affects the Kv1.5 cell surface expression. Further studies will be necessary in order to determine the relative roles of these activities in the biogenesis of the Kv1 family of ion channels.
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