Ile-177 and Ser-180 in the S1 Segment Are Critically Important in 
Kv1.1 Channel Function*

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Ile-177 and Ser-180 are conserved residues in the first transmembrane 
segment (S1) of the Shaker, Shab, Shaw, and Shal subfamilies of voltage-gated K+ channels. Here we report that the mutation of these residues in Kv1.1 to 
leucine, proline, or arginine abolished the expression of outward potassium currents in Xenopus oocytes. Co-injection of these mutant cRNAs and wild type Kv1.1 cRNA into Xenopus oocytes exerted a potent dominant negative effect resulting in the suppression of Kv1.1-encoded currents. Transient transfection experiments of COS-7 cells revealed that the S1 mutants directed the synthesis of 
Kv1.1 polypeptides. Quantitative co-immunoprecipitation assays revealed that most of the S1 mutants co-assembled and formed both homo- and heterotetrameric complexes. Furthermore, the mutated polypeptides could reach the plasma membranes of transfected Sol8 cells. We conclude that mutations of Ile-177 and Ser-180 do not interfere with either the assembly of multimeric channel complexes or the targeting of these complexes to the plasma membrane. It is likely that these residues are involved in helix-helix interactions that are critical to the proper functioning of voltage-gated potassium channels.

Voltage-gated potassium channels, the largest and the most diverse group of ion channels, play a central role in the propagation of signals and the determination of cellular excitability (1). At least four subfamilies of voltage-gated K+ channels have been identified. These subfamilies encode the Shaker (Kv1), 
Shab (Kv2), Shaw (Kv3), and Shal (Kv4) channel polypeptides and their mammalian homologues, which are highly conserved across species. Each channel is synthesized as a monomeric α subunit, which assembles into a pore-forming tetrameric channel. These channels share several common architectural designs, such as a conserved core domain which is comprised of six transmembrane segments, and a teteramerization domain (T1 or NAB) at the amino terminus (2).

During the past several years, we have learned a great deal about the rules governing the assembly and multimerization of voltage-gated potassium channels. It is now established that the co-assembly of monomeric subunits occurs primarily within the same subfamily of Kv channels, resulting in the formation of either homo- or heterotetrameric complexes (3–7).

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The total amount of cRNA injected at 1:1 and 1:3 ratios of wild type to mutants is increased 2- and 4-fold, respectively; therefore, more channel complexes are expected to form. The amplitudes of the resultant currents would be expected to be greater than that of the wild type.
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alone, if the subunits do not exert dominant negative suppression. The percentage of the current will be the sum of the conducting complexes and can be predicted if one, two, or three subunits are required to block the channels (15, 16).

Construction of FL-Kv1.1, FL-Kv1.1Ile177, FL-Kv1.1Ser180 Mutants, and TR-Kv1.1HA—Kv1.1 cDNA cloned from rat soleus muscle (17) was the starting template for creating the constructs. It was amplified by polymerase chain reaction using the T7 primer and a redundant reverse primer carrying Arg, Leu, and Pro codons at either Ile-177 or Ser-180 in the S1 segment and a silent mutation to introduce an XhoI site. The polymerase chain reaction product was subcloned and sequenced. An XhoI/KpnI fragment from Kv1.1 cDNA was added to each mutant to generate the full-length channel construct in Bluescript II vector (Stratagene) (FL-Kv1.1). Each of these Kv1.1Ile177 and Kv1.1Ser180 mutants was digested with KpnI enzyme, blunt-ended with T4 DNA polymerase, and then digested with BstEII enzyme. These fragments were then used to generate the FLAG epitope-tagged Kv1.1 (FL-Kv1.1) and the FLAG epitope-tagged mutants (FL-Kv1.1Ile177 or FL-Kv1.1Ser180) in pCDNA3 (11). We used the gene SOEing method to construct the truncated Kv1.1 containing the NH2-terminal 205 amino acid residues tagged with three copies of the HA epitope (TR-Kv1.1HA) (18).

Transient Transfections in COS-7 Cells—COS-7 cells were grown to about 70% confluency in Petri dishes (100 mm). LipofectAMINE reagent (30 μg/10 μg of DNA) was used in Opti-MEM medium (Life Technologies, Inc.) for 24 h and then changed to the complete medium for a total period of 66–72 h (11). The cells were washed with 2 × 10 ml of PBS* and scrapped in a total 1.5 ml of chilled PBS on ice. For co-transfections, 1 μg of each cDNA was used. The cells were washed (3 × 1.5 ml of PBS with Ca2+ and Mg2+), fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and reacted with the anti-FLAG antibody (M2) (1:1000) (20). The secondary antibody used for staining was the fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100) (Jackson ImmunoResearch). The cells were visualized by laser-scanning confocal microscopy (MRC1024, Bio-Rad).

RESULTS

Ile-177 and Ser-180 Mutants Did Not Express Outward Potassium Currents in Oocytes—Fig. 1A shows the primary structure of the first transmembrane segment (S1) of Kv1.1 and the mammalian homologues of four voltage-gated K+ channels, Shaker, Shaw, Shab, and Shal. The wild type Kv1.1 polypeptide used in this study was tagged with a FLAG epitope at the amino-terminal end did not produce any noticeable change in the properties of this current (10, 17). In contrast, oocytes injected with each of the mutant cRNA showed no detectable outward potassium currents even after injection of higher doses of cRNAs or recording after longer post-injection periods (Fig. 2, B and C). Thus, the mutation of either Ile-177 or Ser-180 to Arg, Leu, or Pro abolished the Kv1.1-encoded out-
ward potassium currents.

**Dominant Negative Suppression of Kv1.1-encoded Currents by Ile-177 and Ser-180 Mutants**—To address the question of whether the mutations affected the ability of these subunits to co-assemble with the Kv1.1 polypeptide, we carried out cRNA co-injection experiments. In these experiments, the wild type and mutant subunits were expected to co-assemble into both homo- and heterotetrameric complexes, since they all have an intact T1 domain. The probability of each type of homo- and heterotetrameric complexes that would form is given by a binomial distribution (see “Materials and Methods”). The total current observed would be the sum of the currents carried by each of the conducting complexes. An accurate prediction by this equation rests upon several critical parameters. 1) The amplitudes of the expressed currents must be linear as a function of the amount of cRNA injected and the protein it yielded. 2) Both the wild type and the mutant subunits must be able to co-assemble with an equal probability. 3) Co-assembly with one or more mutant subunits may or may not completely suppress the assembled channel’s conductance, but should not alter it.

In order to ensure the validity of the first consideration, we first measured the amplitude of outward potassium currents expressed in oocytes injected with increasing amounts of FL-Kv1.1 cRNA, using the same pulse protocol as above (Fig. 2D). As clearly shown in Fig. 2, E and F, the linearity of the current amplitudes was maintained fairly well at different voltages with the amounts of cRNA tested. Based on these results, we chose to use 0.69 ng of FL-Kv1.1 cRNA (expressing 10 to 30 μA current at 0 mV) in all our co-injection experiments. Co-injection of FL-Kv1.1 with mutant cRNAs at a 1:1 ratio resulted in a significant suppression of Kv1.1-encoded currents (Fig. 3, A and C-H, p < 0.01). The extent of current suppression was greatly enhanced at a 1:3 ratio of wild type to mutant cRNA (Fig. 3, C-H), except for the FL-Kv1.1Ile177P mutation, which had no noticeable effect on the amplitude of FL-Kv1.1 encoded currents (Fig. 3G). By contrast, we did not observe any suppression of these currents by co-injection of a 3-fold excess of control cRNA (pTRI-Xef1) (Fig. 3B). Co-injection of the S1 mutant cRNAs did not alter the slope conductance, voltage-dependence or kinetics of activation of the Kv1.1-encoded currents (data not shown). Thus, suppression of the Kv1.1-encoded currents was specifically related to the presence of the mutant cRNAs. Using the binomial equation, the percentages of the predicted currents at both 1:1 and 1:3 ratios of co-injections are given if one or more mutant subunits are sufficient to block the formation of functional channels (Table I). The observed values were very close to the predicted values at the 1:3 ratio, assuming that co-assembly with one mutant subunit was sufficient to block the formation of functional channels. At a lower ratio of 1:1, a somewhat weaker suppression than predicted was observed. This phenomenon may have resulted from the lesser amounts of protein expressed by the mutant cRNAs as compared with the amount expressed by the wild type, thereby shifting the actual ratios of the subunits available for co-assembly. Indeed, we have consistently observed variations in the level of expression of mutant proteins in transfected COS-7 cells (see protein results below). The protein analysis also revealed that the level of the expressed FL-Kv1.1Ile177P protein was dramatically reduced, thus explaining its inability to suppress the currents in oocytes.

**FL-Kv1.1Ile177 and KL-Kv1.1Ser180 Mutants Form Both Homo- and Heteromultimeric Complexes**—To obtain direct evidence for co-assembly at the protein level, we carried out co-transfection experiments in COS-7 cells, followed by co-immunoprecipitation analyses. The FL-Kv1.1, FL-Kv1.1Ile177, FL-Kv1.1Ser180 mutants, and an HA epitope-tagged truncated Kv1.1 construct (TR-Kv1.1HA) were transfected (Fig. 1B). As previously established by us, an HA-tagged deletion fragment of similar size was necessary and sufficient to retain its ability to co-assemble with Kv1.1, Kv1.4, and Kv1.5 channels in vitro, in GH3 cell lines and mouse heart (10, 11, 21). The principle of this method is based on a quantitative depletion of the pool of one of the interacting proteins (the full-length FL-Kv1.1, the FL-Kv1.1Ile177, or FL-Kv1.1Ser180 mu-

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**Fig. 2.** Two-electrode voltage clamp analysis of macroscopic Kv1.1 currents expressed in Xenopus oocytes. A–C show representative current traces recorded from oocytes injected with 0.92 ng of cRNA encoding FL-Kv1.1, FL-Kv1.1S180P, and FL-Kv1.1I177L, respectively. D, pulse protocol used. E, steady-state current-voltage (I-V) relationships of the FL-Kv1.1 current. F, current amplitudes at 0 mV were plotted as a function of the amount of the FL-Kv1.1 cRNA injected. Data were fitted by a linear equation: $f(x) = 42.4x - 1.5$ ($r = 0.994$). Data are shown as mean ± S.E. (n = 3–4).
tants), which is in direct co-assembly with TR-Kv1.1HA. The heteromultimeric complexes were precipitated from an oocyte injected with 0.69 ng of FL-Kv1.1 (top), co-injected with FL-Kv1.1 and FL-Kv1.1S180R (1:1) (middle), or co-injected with FL-Kv1.1 and FL-Kv1.1I177L (1:1) (bottom). B-H, I-V curves after co-injecting the FL-Kv1.1 cRNA (●) with a control cRNA (B), FL-Kv1.1S180L (C), FL-Kv1.1S180P (D), FL-Kv1.1S180R (E), FL-Kv1.1I177L (F), FL-Kv1.1I177P (G), and FL-Kv1.1I177R (H) at 1:1(●) and 1:3(▲) ratios. Data are shown as mean ± S.E. (n = 4–9).

| Mutants cRNA ratio | % current predicted if i subunits are required to block channel | % of WT current recorded at 0 mV |
|--------------------|---------------------------------------------------------------|---------------------------------|
| Mutants WT:mutant  | i = 1 | i = 2 | i = 3 |                                  |
| S180L 1:1          | 12.5 | 63 | 138 | 67 |
| S180R 1:1          | 12.5 | 63 | 138 | 34 |
| S180P 1:1          | 12.5 | 63 | 138 | 36 |
| I177L 1:1          | 12.5 | 63 | 138 | 45 |
| I177R 1:1          | 12.5 | 63 | 138 | 23 |
| I177P 1:3          | 12.5 | 63 | 138 | 94 |

In Fig. 4, A and B. When FL-Kv1.1 was transfected alone, anti-FLAG antibody could immunoprecipitate multiple polypeptides with apparent molecular masses of 56 to 59 kDa (Fig. 4A, lane 3). These bands most likely correspond to the differentially glycosylated and phosphorylated forms of Kv1.1 a subunit (17, 22, 23). In contrast, two successive rounds of immunoprecipitation with anti-HA antibody failed to precipitate any Kv1.1 polypeptide (Fig. 4A, lanes 1 and 2). The multiple, closely moving bands of FL-Kv1.1 and FL-Kv1.1Ile or FL-Kv1.1Ser mutants that we have observed here have similar molecular masses and therefore reflect different maturation stages. The transfection of TR-Kv1.1HA alone followed by its precipitation by either anti-HA antibody or anti-FLAG antibody showed that it could only be brought down by anti-HA antibody (Fig. 4B, lanes 1 and 2). The multiple, closely moving bands of FL-Kv1.1 and FL-Kv1.1Ile or FL-Kv1.1Ser mutants that we have observed here have similar molecular masses and therefore reflect different maturation stages. The transfection of TR-Kv1.1HA alone followed by its precipitation by either anti-HA antibody or anti-FLAG antibody showed that it could only be brought down by anti-HA antibody (Fig. 4B, lanes 1 and 2).
tation with anti-FLAG antibody did not precipitate any TR-Kv1.1HA polypeptides (lane 9), validating the usefulness of the scheme. In contrast, in the mixed lysates of separately transfected COS-7 cells, the FL-Kv1.1 protein could be brought down only by anti-FLAG antibody and not by anti-HA antibody (Fig. 4, lanes 10–12). This observation confirms that the co-assembly of FL-Kv1.1 and TR-Kv1.1HA proteins depended upon their co-translation. Mock transfection using the vector alone did not yield any specific protein bands (Fig. 4, A and B, lanes 13–15).

All the FL-Kv1.1Ile177 and FL-Kv1.1Ser180 mutants were then tested for their ability to co-assemble by this sequential method. Fig. 4A shows the positions and the intensities of the polypeptides encoded by FL-Kv1.1Ile177 and FL-Kv1.1Ser180 mutants. Co-transfection of each of the FL-Kv1.1Ile177 or FL-Kv1.1Ser180 mutants with TR-Kv1.1HA resulted in the co-precipitation of multiple protein bands with apparent molecular masses similar to those of the FL-Kv1.1 polypeptides. However, we consistently observed variations in their expression levels (Fig. 4A, lanes 16–33). Co-transfection of the FL-Kv1.1Ile177L followed by co-precipitation yielded somewhat elevated levels of this protein. In contrast, co-transfection of FL-Kv1.1Ile177P resulted in the co-precipitation of dramatically reduced levels of protein (Fig. 4A, lanes 31–33). Other mutants displayed lesser variations, and their co-expression levels were comparable to those achieved with co-transfected wild-type constructs (Fig. 4A, lanes 7–9). Despite these variations, the mutants were efficiently co-immunoprecipitated with the anti-HA antibody (Fig. 4A, lanes 16–33). A corresponding depletion of the TR-Kv1.1HA with anti-HA antibody is not shown for the mutants, since it was exactly as obtained in the controls (Fig. 4B). Fig. 4C shows the ratios of the FL-Kv1.1 and mutant polypeptides that were immunoprecipitated with either the anti-HA or anti-FLAG antibody. Mean ± S.D. from three independent experiments shown.

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**FL-Kv1.1Ile177 and FL-Kv1.1Ser180 Mutants Are Targeted to the Plasma Membrane of Sol8 Cells**—The failure of all of our mutants to form functional channels in oocytes led us to speculate that they were “folding mutants” which were trapped in the endoplasmic reticulum compartment and were therefore unable to reach the plasma membranes (11, 21, 24). Hence, we decided to study their subcellular localization using immunofluorescence imaging of transfected cells. This study could also shed some light on the hitherto unknown subcellular distribution of defective K+ channels in episodic ataxia (25) and in several types of long QT syndrome (26).

Since the FL-Kv1.1 cDNA was cloned from a rat soleus cDNA library (17), we decided to examine its subcellular expression in Sol8, a myogenic cell line derived from the mouse slow-twitch soleus muscle. Indeed, transiently transfected FL-Kv1.1 could be detected on the membranes of Sol8 cells as well as in the cytoplasm (Fig. 5B). Interestingly, we consistently observed
that plasma membrane staining was more commonly found in regions of high confluency where some of the cells appeared to fuse, reminiscent of myotube formation. Untransfected Sol8 cells displayed some background fluorescence, but did not reveal any membrane staining (Fig. 5A). Thus, these results demonstrated that FL-Kv1.1 could be targeted to the plasma membrane of Sol8 cells. When we tested for membrane expression of FL-Kv1.1I177L, FL-Kv1.1I177R, and FL-Kv1.1SER180 mutants, they could all be detected on the plasma membranes of transfected Sol8 cells. Two representative examples, FL-Kv1.1I177L and FL-Kv1.1SER180, are shown in Fig. 5, C and D. Thus, the mutation of either Ile-177 or Ser-180 to leucine, arginine, or proline did not prevent the targeting of the Kv1.1 polypeptides to the plasma membrane. Transient transfection experiments of FL-Kv1.1 and FL-Kv1.1 mutants into other cell lines (including COS-7, HEK293, Chinese hamster ovary, and Madin-Darby canine kidney) did not yield sufficient plasma membrane expression detectable by anti-FLAG antibodies. In contrast, the transfection of a control membrane protein, the cationic amino acid transporter (27), revealed clear membrane staining in these cells (data not shown). Taken together, these results indicate that Sol8 cells can express detectable levels of FL-Kv1.1 polypeptides on the plasma membrane, with or without mutations in the S1 region.

**DISCUSSION**

*Significance of the Results of Ile-177 and Ser-180 Mutations in the S1 Segment of Kv1.1—*A significantly high level of conservation of amino acids in the core region of voltage-gated channels is indicative of functionally important sites and forms an important basis for their three-dimensional structural and functional modeling (28). The mutations of either Ile-177 or Ser-180, the two completely conserved amino acid residues in the S1 segments of the Kv1–4 voltage-gated K⁺ channels, abolished the expression of outward potassium currents in oocytes (Fig. 2, B and C). In transfected COS-7 cells, all mutant cDNA constructs (except FL-Kv1.1I177P) directed the synthesis of proteins, which were matured normally and formed products of the expected molecular mass, albeit with some variation in their steady-state levels (Fig. 4). It is conceivable that the presence of the potentially helix-disrupting proline residue at position 177 resulted in decreased protein stability.

Our analysis indicated that a block in the biosynthesis or maturation of Kv1.1 polypeptides could not explain the generation of non-functional channels. Indeed, the mutations did not alter the consensus sites for the processing and maturation of Kv1.1 protein in transfected cells (22, 23, 29, 30). Moreover, most of the wild type or mutated Kv1.1 polypeptides formed heteromultimeric complexes with TR-Kv1.1HA (Fig. 4C). The smaller fraction, which was immunoprecipitated with anti-FLAG antibody in the last step, most likely contains both homomultimeric channel complexes and unassembled subunits. These results were in agreement with those from the electrophysiological measurements, in which co-injection of the mutants with the wild type cRNA in *Xenopus* oocytes suppressed most of the Kv1.1 encoded currents (Fig. 3). Collectively, we conclude that none of the Ile-177 or Ser-180 mutations obliterated the co-assembly of homo- or heteromultimeric complexes with either the wild type or the mutated subunits. Furthermore, the incorporation of a single mutant subunit was most likely sufficient to suppress Kv1.1-encoded currents.

The first transmembrane segment plays a critical role in initiating the insertion of newly translated polypeptides into the endoplasmic reticulum membrane and in promoting the stability and clustering of the other transmembrane segments of membrane proteins (31). Indeed, several studies (9, 32), including ours (10, 11), have shown that the deletion of this segment abolished the assembly of Kv1 subunits. The distinct staining of each of the mutant FL-Kv1.1 channel protein on the plasma membranes of the Sol8 cells is an intriguing phenomenon (Fig. 5). We speculate that differentiated Sol8 myocytes express high levels of membrane-associated proteins that are important for the trafficking and membrane expression of Kv1.1 polypeptides. Assuming a co-translational co-assembly for all subunits (shown for the wild type in Fig. 4A, lanes 10–12), the staining probably represents homomultimeric complexes and not a single subunit. These complexes could reach
the plasma membrane and form non-functional channel complexes. In this context, it is worth noting that the W434F, a mutation in the pore region of the Shaker B K⁺ channel which rendered the channel non-conducting, apparently reached the membranes and “expressed” gating currents (33).

A Proposed Role for Ile-177 and Ser-180 in Critical Subunit Interactions—The S1 segment contains both the hydrophobic and hydrophilic residues (Fig. 1A). Current three-dimensional structural models of voltage-gated K⁺ channels predict that the S1 segment forms a transmembrane amphipathic α helix (34). This model depicts the S1 segment closely packed with the S2 and S4 segments in a cylindrical bundle of α helices in the outer half of the channel’s “open” conformation. Direct experimental evidence for functionally critical interactions among the charged residues in the S2, S3, and S4 segments has emerged from recent elegant biochemical studies on the native Shaker K⁺ channel (35) and from studies investigating the interactions of synthetic transmembrane segments in phospholipid membranes (36). Our evidence for the existence of non-functional channels in the membranes suggests that both the Ile-177 and Ser-180 residues in the S1 segment of Kv1.1 might be critically important in helix-helix interactions, rather than in the assembly process per se, as we had previously hypothesized (10). It is likely that inclusion of one “bad” subunit in the tetrameric channel here could hinder critically important intra- or inter-subunit interactions, which could in turn impede the sequential steps as the channel proceeds from the closed to the final open state. This is perhaps the best interpretation of all our results, which showed that all of the mutant proteins (except the I177P) were well expressed, could co-assemble to form multimeric complexes, exerted a dominant negative effect on the wild-type K⁺ currents, and could also reach the plasma membranes. In summary, our results highlight the important structural models of voltage-gated K⁺ channels predict that the membranes (36). Our evidence for the existence of non-functional intermediates in the transition from the closed to the final open state and that the Ile-177 and Ser-180 residues in the S1 segment of Kv1.1 might be critically important in helix-helix interactions, rather than in the assembly process per se, as we had previously hypothesized (10). It is likely that inclusion of one “bad” subunit in the tetrameric channel here could hinder critically important intra- or inter-subunit interactions, which could in turn impede the sequential steps as the channel proceeds from the closed to the final open state. This is perhaps the best interpretation of all our results, which showed that all of the mutant proteins (except the I177P) were well expressed, could co-assemble to form multimeric complexes, exerted a dominant negative effect on the wild-type K⁺ currents, and could also reach the plasma membranes. In summary, our results highlight the important role that the Ile-177 and Ser-180 residues play in the function of Kv1.1 channels. These observations will help increase our understanding of the cellular mechanisms in long QT-syndrome and ataxia/myokymia, which have been envisaged to arise by dominant negative mechanisms of defective voltage-gated K⁺ channels, including Kv1.1 (37).

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