Molecular Compatibility of the Channel Gate and the N Terminus of S5 Segment for Voltage-gated Channel Activity*

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Marco Caprinig, Marianna Fava‡, Pierluigi Valente‡, Gregorio Fernandez-Ballester‡, Carmela Rapisarda‡, Stefano Ferroniti, and Antonio Ferrer-Montiels‡

From the ‡Department of Human and General Physiology, University of Bologna, Via San Donato 19/2, 40127 Bologna, Italy and the ‡Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Alicante, Spain

Voltage-gated ion channels are modular proteins designed by the structural linkage of a voltage sensor and a pore domain. The functional coupling of these two protein modules is a subject of intense research. A major focus has been directed to decipher the role of the S4–S5 linker and the C-end of the inner pore helix in channel gating. However, the contribution of the cytosolic N terminus of S5 remains elusive. To address this issue, we used a chimeric subunit that linked the voltage sensor of the Shaker channel to the prokaryotic KcsA pore domain (denoted as Shaker-KcsA). This chimera preserved the Shaker sequences at both the N terminus of S5 and the C-end of S6. Chimeric Shaker-KcsA subunits did not form functional homomeric channels but were synthesized, folded, and trafficked to the cell surface, as evidenced by their co-assembly with Shaker wild type subunits. Sequential substitution of Shaker amino acids at the C-end of S6 and the N terminus of S5 by the corresponding KcsA created voltage-sensitive channels with voltage-dependent properties that asymptotically approached those of the wild type Shaker channel. Noteworthy, substitution of the region encompassing Phe401–Phe404 at the N-end of Shaker S5 by KcsA residues resulted in a significant gain in voltage sensitivity of the chimeras. Furthermore, analysis of channel function at high [K+]o, revealed that the Phe401-Phe404 region is an important molecular determinant for competent coupling of voltage sensing and pore opening. Taken together, these findings indicate that complete replacement of Shaker S5 and S6 by KcsA M1 and M2 is required for voltage-dependent gating of the prokaryotic channel. In addition, our results imply that the region encompassing Phe401-Phe404 in Shaker is involved in protein-protein interactions with the voltage sensor, and signal to the Phe401 in the S5 segment as a key molecular determinant to pair the voltage sensor and the pore domain.

Channel proteins constitute a functionally important class of membrane proteins that mediate the transmembrane passage of ions and other small molecules in their thermodynamically favorable direction. The voltage-gated ion channel superfamily, which includes Na+, Ca2+, and K+ channels, shows a very high sequence similarity suggesting a similar molecular architecture. Among this family of channels, voltage-gated K+ channels (Kv) are involved in a host of cellular processes from setting the resting membrane potential and shaping the action potential waveform and frequency to controlling synaptic strength (1). A canonical voltage-gated K+ channel consists of four α-subunits that are assembled to form the ion-permeation pathway across the membrane. In this context, a prototypical K+ channel α-subunit is formed by an N- and C-terminal domains and six transmembrane helices (S1–S6). The Kv channel family shows a subunit modular organization, constituted by a tetramerization domain, a voltage sensor, and a permeation pathway domain (2).

The structural basis for the channel ionic selectivity has been established in greatest detail for K+ channels because of the high resolution structure of the prokaryotic K+ selective KcsA channel (3). The crystalization of other prokaryotic K+ channels such as KirBac (4), MthK (5), and KvAP (6) have been useful to recognize regions in the protein structure that are important to gate the channel in response to activating stimuli. In particular, the crystal structure of the KvAP channel from Aeropyrum pernix has provided the first high resolution view of a full-length, voltage-gated K+ channel (6). Unexpectedly, the crystal structure of KvAP revealed a structural arrangement for the voltage sensor dramatically different from the conventional models. In the crystal, the voltage sensor is located at the channel cytosolic perimeter, and adopts a “paddle-like” conformation (6, 7). In this model, S4 and part of S3 constitute the so-called paddle, which crosses the whole cellular membrane in response to membrane depolarization, thus providing a novel gating mechanism that notably departs from the conventional voltage-sensing models proposed for eukaryotic K+ channels (7, 8). Nonetheless, the paddle model does not appear to account for functional data on the voltage gating of eukaryotic Kv channels (9–15).

Although the high resolution structures of prokaryotic K+ channels have supported many general features of the pore domains, the functional coupling of the voltage sensor and pore module remains poorly understood. Recently, it was shown as a critical role of the S4–S5 linker and the C-terminal end of the inner pore helix in channel gating (16–20). Our and others groups (21–23) have used a chimeric-based approach to identify amino acid motifs involved in the transduction of the electrical stimuli by the sensor module into a conformational change of
the pore module. A chimical strategy that combined the voltage sensor module of the eukaryotic Shaker channel and the pore domain of the prokaryotic KcsA protein, showed that the chimeric channels are synthesized, folded, and trafficked to the membrane (24). Furthermore, voltage sensitivity and K+ selectivity may be provided to the chimeric channels when the C terminus of the eukaryotic protein is present in the chimera, demonstrating that the ion conduction pore is conserved among K+ channels (22). Additional studies on the functional coupling of the pore and voltage sensor domains suggested the critical importance of the structural compatibility of the S4–S5 linker and the C-end of the inner pore helix in channel gating (23).

Here, we have further addressed this issue to gain molecular and mechanistic information on the cross-talk between the voltage sensor and pore domains. We constructed a chimera where the S5-P-S6 region of the Shaker channel was replaced by the KcsA M1–M2 counterpart. The prokaryotic channel was inserted between the junction of exons 8 and 9 at the N-end of the S5 domain of the eukaryotic channel, and the junction of the proline-valine-proline (PVP) motif (Fig. 1). Unexpectedly, this chimera, which preserved the Shaker sequences at the N-end of S5 and C-end of S6, was not functional, although channel activity could be rescued when co-expressed with wild type Shaker subunits. Replacement of the eukaryotic amino acids at the S6 by their prokaryotic counterparts produced active channels gated by voltage and/or [K+]o. Additional swapping of the S5 sequence gave rise to chimeric channels with voltage-dependent gating that approach that characteristic of the Shaker channel, and identify the Phe401–Phe404 region in Shaker as a key molecular determinant for coupling the voltage sensor and the pore domain.

EXPERIMENTAL PROCEDURES
Molecular Biology of Chimeras Design and Mutations—Standard molecular biological techniques were applied as described (25). Shaker inactivating removed (Sh4IR) and Shaker inactivating (Sh4IR) were a gift of L. Toro (UCLA) (26), and KcsA was provided by S. Chee (Salk Institute). The Shaker-KcsA was constructed by replacing the region of Sh4IR encompassing from the junction of exons 8 and 9 at the S5 segment to the PVP domain at the S6 segment (amino acids 405 to 472), with that corresponding to KcsA (amino acids 39 to 105). PCR was used to introduce NdeI and KpnI sites in Shaker, respectively, at amino acids 363 and 431–432. Two oligonucleotide primers were utilized to amplify KcsA from amino acid 39 through the stop codon while simultaneously introducing a blunt end at amino acid 39, and a KpnI at amino acid 105, which was subsequently used to subclone KcsA into the Shaker/pBluescript construct. Thereafter, the regions S6 (Thr172–PVPVIS177) and S5 (992LIFVLE404) containing the amino acid sequences of the Shaker channel were stepwise replaced by their KcsA counterparts (200VTAALAT112 and 311AVTLVV137) by site-specific mutagenesis (QuickChange Site-directed Mutagenesis Kit, Stratagene). The ile14 amino acid is conserved in the two sequences. The sequences of the transferred and mutated segments were verified by both restriction analysis and automated DNA sequencing. For in vitro transcription, chimeric and wild type channel clones were linearized and used as a template using the mMESSAGE mMACHINE kit (Ambion, Austin, TX).

Electrophysiological Recordings—Xenopus oocytes were defolliculated using the calcium-free Barth’s solution, collagenase (2 mg/ml) and slow agitation (50–60 rpm) for 1–2 h. Oocytes were stored at 18 °C for 12 h before the injection. In vitro transcribed RNA was injected into Xenopus oocytes (5–10 ng/oocyte) as described (27). Electrophysiological recordings were made 2–4 days after injection. Two electrode voltage clamp was performed using an Electrode Voltage Clamp amplifier (TEC 10CD, NPI Electronic, Tamm, Germany). Throughout the experiments oocytes were continuously perfused with an external solution contain-

ing (in mM): 1 MgCl2, 0.3 CaCl2, 10 Na-HEPES, pH 7.5. According to the type of experiments carried out, 3, 10, 30, and 100 mM KCl was also used, and N-methyl-d-glucamine was added to keep constant the ion strength. All recordings were obtained at room temperature ~22 °C. Electrodes were made with hematocrite glass capillaries and pulled with a P-97 puller (Sutter Ins. Co., Novato, CA); they were filled with 1 M KCl buffered with 10 mM TES and typically had resistance of 300–500 KΩ. The currents were sampled at 4–5 kHz after filtering at 1 kHz. Leak subtraction was accomplished with two inverted quarter amplitude pre-pulses that were scaled and subtracted from the test pulse (24). The standard voltage protocol consisted of a family of 800-ms long depolarizing pulses from −120 mV to +120 mV with 20 mV steps, from a holding potential (Vh) of −80 mV. Shaker data were usually obtained in a similar manner by using a family of 200-ms long depolarizing pulses from −120 to +120 mV, ΔV = 20 mV, and Vh = −80 mV. The G-V curves were obtained by converting the maximal current values from the family step stimuli to conductance by using the relation G = IV + V2, where G is the conductance (gS), I (μA) is the peak current recording, V is the command pulse potential, and EK is the theoretical K+ reversal potential obtained with the Nernst equation considering the [K+]o, of 120 mM (T = 295 K) (28). Conductance values were normalized and fitted to a two-state Boltzmann distribution of the form,

\[ G_{\text{max}} = \frac{C}{1 + \left(\frac{V - V_{\text{rev}}}{\Delta V}\right)^n} \]

where \( G_{\text{max}} \) is the maximal conductance, \( V_{\text{rev}} \) is the value of the membrane potential at which 50% of the maximal conductance is reached, and \( n \) is the slope of the G/V curve. C denotes the fraction of the voltage-independent conductance. Data were acquired and analyzed using Pulse/PulseFit 8.11 (HEKA Elektronik, Lambrecht Germany), Origin 7.0 SM0 (OriginLab Corp., Southampton, MA), and ANA (Pusch M., IBF, CNR, Genoa, Italy). Data are shown as mean ± S.D., with \( n \) (number of oocytes) ≥ 5.

Molecular Modeling—The crystallographic structure of the full KvAP potassium channel (PDB code 1ORQ) was used as a starting structure for simulations. The structure was edited with Swiss Protein Data Bank viewer 3.7 (29) and Insight II ( Biosym/MSI). The structural model was constructed by the regularization of the S3 and S4 α-helices and the reorganization of the S1 to S4 helices to form a compact crystal structure. The external ends of S1 and S3 are ~31 Å from the external opening of the pore, whereas the S3–S4 linker is located ~19 Å from the pore, as indicated by directed cysteine mutation findings and blockade with a series of compounds varying in length (30). Localization of the S4 relative to S5 was tested in terms of energy with FOLDX (foldx.embbl.de), and taking into account functional data suggesting that S4 is at the interface between adjacent subunits interacting with S5 (31), the analysis showed better energy values when S4 was located at 40–45 degrees with respect to the main axis of the pore. This is consistent with the notion that S4 is slightly tilted with respect to the pore axis (31). For this reason, a ≤40 degree tilting angle for S4 produces a strong clash with the C-terminal of the adjacent S5 and/or with the N terminal of the S5 within its subunit. Conversely, a >45 degree angle for S4 results in a loss of the interaction with adjacent S5. Thus, we favor a location of the S4 segment at ~30–40 degrees from the pore axis, thus establishing a direct interaction with the S5 helix of the contiguous monomer. In support to this, a recent model reports that the S3–S4 voltage sensor paddle is closed to the C-terminal of S5 of the adjacent subunit in the resting state, which is fully consistent with this conformation (32).

The orientation and optimization of the side chains corresponding to this interaction were carried out in two steps: first, those residues making van der Waals clashes were selected and fitted with “Quick and Dirty” algorithms; second, the model was energy minimized (100 steps). The resulting structure was subjected to a Ramachandran plot with 91.3% of the residues in most favored regions, the accessibility of its atoms and residues, the backbone dihedral angle distribution, and the properties of the structure, such as its atomic contact map, the accessibility of its atoms and residues, the backbone dihedral angles, and to the H-bond and electrostatic networks of the protein. In addition, the model was evaluated with PROCHECK (34) showing a Ramachandran plot with 91.3% of the residues in most favored regions, and 8.6% in additional allowed regions. Molecular graphics were created with the program Pymol (www.pymol.org).

RESULTS
Design and Heterologous Expression of a Chimeric Shaker-KcsA Channel—Our previous chimeric channels containing the full-length KcsA into the background of the voltage-sensing module of mKv1.1 or Sh4IR did not produce functional voltage-dependent channels, although they were normally trafficked to the cell surface (24). In contrast, functional channels could be
rescued when the C terminus of the Shaker channel was included into the chimera (22). To investigate the protein domain important for enduring voltage-sensing activity to the Shaker-KcsA channels, we designed a chimera by replacing the Ile405–Pro473 region of Shaker with the Ile38–Leu105 of KcsA. The chimera conserved the S4–S5 loop, the cytoplasmic part of the outer helix (S5), the C-end of the inner helix (S6), and the cytoplasmic C-terminal end of Sh4IR. B, sequential substitution of Shaker residues at both the N terminus of S5 and C-end of S6 by those corresponding to KcsA, to transfer the complete M1 and M2 segments of the prokaryotic channel to the chimera.

To investigate this notion, we co-injected the Sh4In wild type and chimera subunit at a ratio of 1:3 and measured the voltage-gated responses. As illustrated in Fig. 2C, in the co-injected oocytes voltage-gated currents were characterized by a voltage-dependent fast-inactivating phase followed by a non-inactivating current. The voltage-dependent increase of the peak-to-peak steady state current ratio shown by homomeric Sh4In was drastically reduced in the heteromer Sh4In:Shaker-KcsA assemblies, primarily because of the increase in the magnitude of the non-inactivating component (Fig. 2D). Because the chimeric subunit was obtained in the Sh4IR background, this result suggests that the non-inactivating, voltage-dependent component corresponds to heteromeric channels that contain the chimera. This notion is further substantiated by the ~50 mV shift toward depolarizing potentials of the conductance to voltage curve (G-V), and the 50% decrease in gating valence of the Sh4IR:Shaker-KcsA heteromers, as compared with homomeric Sh4IR channels (Fig. 2E). Taken together, these findings indicate that chimeric Shaker-KcsA subunits co-assemble with wild type monomers. Thus, the chimera is efficiently synthesized, folded, and trafficked to the plasma membrane. Consequently, the lack of function on homomeric Shaker-KcsA assemblies may arise from uncoupling the voltage sensor and channel gate.

Mutations at the C-end of the S6 Segment in the Shaker-KcsA Subunit Lead to Functional Channel—Comparison of the amino acid sequence of our Shaker-KcsA chimera with that designed by Lu et al. (22) reveals differences at the N terminus of segment S5 and the C-end of segment S6 (Fig. 1). The main difference is that our Shaker-KcsA chimera preserves the sequences of the Shaker channel instead of those of KcsA, suggesting that functional coupling of both modules depends on the molecular compatibility of these subunit regions. Hence, to investigate the molecular requirements of functional coupling, we replaced the Shaker amino acids at both the C-end of S6 and the N-end of S5 of the Shaker-KcsA chimera by those corresponding to KcsA.

First, we focused on the channel intracellular gate, and sequentially mutated the Pro573–Ser479 sequence to Val106–Thr112 in the background of the Shaker-KcsA chimera. Chimeric mutant channels were heterologously expressed in Xenopus oocytes and the voltage-gated activity was recorded at 3 mM [K\(^+\)]. Low [K\(^+\)], was chosen to monitor voltage-dependent gating in the absence of [K\(^+\)]-dependent activation. Mutation of the PVP motif to the corresponding VTA sequence of KcsA did not result in voltage-gated function at either of the
conditions tested (data not shown). The lack of channel activity of these mutants was not because of abrogation of protein biosynthesis, as demonstrated by Western immunoblot analysis of whole cell extracts (data not shown). However, additional mutation of Val476 to Ala and Val478 to Ala gave rise to voltage-gated responses that were larger than those obtained from

**FIG. 2.** Co-expression of the Shaker-KcsA chimera with wild type Shaker subunits gives rise to functional heteromeric channels. Reduction of the voltage-dependent channel activity of Sh4In (A) and Sh4IR (B) as function of the relative amount of chimera Shaker-KcsA. Insets in panels A and B depict representative current responses of each construct alone and co-injected with Shaker-KcsA at a ratio 1:3 (w/w). Bars are representative of the peak current amplitudes normalized with respect to those obtained from homomeric Shaker channels (n ≥ 4). Whole oocyte currents were measured 2 days after injection with a voltage protocol consisting of a square pulse to +50 mV of 300-ms duration from a holding potential (V_h) of −80 mV. C, co-expression of Sh4In with Shaker-KcsA at a ratio of 1:3 (w/w) generates functional channels that mediate a voltage-dependent, non-inactivating current. Channel activity was elicited from −80 to +80 mV, with 20 mV potential increments. D, Changes of the peak current (I_p) and steady state current (I_ss) as a function of the applied potentials of mutants shown in panel C. E, G-V relationships for Sh4IR and Sh4IR with Shaker-KcsA at a ratio of 1:3 (w/w). Conductance changes were obtained from the respective I-V relationships using $G = I/V_m - E_K^*$, where $V_m$ is the stimulation potential value and $E_K$ is the theoretical reversal potential for K⁺, calculated with the Nernst relation considering $[K^+]_{out} = 120$ mM. $[K^+]_{in}$ was 100 mM. Solid lines depict the best fit to a Boltzmann distribution. $V_{0.5}$ values were −20 ± 2 mV for Sh4IR and +31 ± 4 mV for Sh4IR:Shaker-KcsA chimera. The slope values ($a_n$ (mV)) of the G-V curve were 14 ± 2 mV for Sh4IR and 29 ± 3 mV for Sh4IR:Shaker-KcsA chimera. All values are mean ± S.D. with n ≥ 5.
Oocytes were held at standard Ringer solutions containing 3 mM [K+] outside and depolarized from -80 mV to +120 mV with voltage step increments of 20 mV. Ionic currents were recorded in two-state Boltzmann distribution, with a $V_{0.5}$ of 82 ± 11 mV, a slope ($a_n$) of 21 ± 8 mV, and a modest voltage-independent component $C$ of 0.06 (Fig. 3C). Note that the $V_{0.5}$ of the chimera is remarkably ≥85 mV more depolarized than that of wild-type Shaker channels ($V_{0.5} = -6.5 ± 0.3$ mV, Fig. 3C). Taken together, these results imply that the C-terminus of the inner pore helix is primarily involved in defining the energetics of the channel gate.

Mutations in this domain create functional K$^+$-selective channels that either gate in a voltage-independent manner or require high voltages to be activated.

**Mutations at the N Terminus of S5 Segment in the Shaker-KcsA Chimera Creates Voltage-dependent Homomeric Channels**—Because restoring the KcsA gate region in the chimera background gave rise to channels with low voltage sensitivity, we questioned whether the amino acid sequence at the N terminus of S5 contributes to couple the voltage sensor to the channel gate. To address this issue, we sequentially mutated residues encompassing Leu$^{398}$–Phe$^{404}$ to the corresponding amino acids of KcsA (Ala$^{31}$–Val$^{37}$) in the background of the Shaker-KcsA chimera that contains the KcsA gate (Fig. 1, bottom). Replacement of Leu$^{398}$ and Leu$^{399}$ by Ala and Ile$^{400}$ to Thr produced a chimera that required high voltage for activation ($V_{0.5} = 101 ± 20$ mV, $a_n = 26 ± 8$ mV, $C = 0.02$) (Fig. 4, A and C). In marked contrast, subsequent mutation of Phe$^{401}$ to Val yielded voltage-activated chimeric subunits whose voltage-dependent conductance curve was shifted by ~85 mV toward hyperpolarizing potentials ($V_{0.5} = 14 ± 8$ mV) without affecting the apparent gating valence ($a_n = 25 ± 6$) and with a modest voltage-independent component ($C = 0.02$) (Fig. 4, A and C).

Thus, this result strongly implies an improved coupling between the voltage sensor and the channel gate. Additional mutation of Phe$^{402}$ to Leu did not further hyperpolarize the G-V curve ($V_{0.5}$ of 24 ± 3 mV, $a_n$ of 25 ± 3 mV and $C = 0.02$) (Fig. 4, B and C). However, replacement of Phe$^{404}$ by Val, a change that completely transfers the KcsA M1 sequence, created channels with further hyperpolarized $V_{0.5}$ to 6 ± 1 mV, and exhibited an $a_n = 18 ± 1$ mV with a modest voltage-independent component $C$ of 0.10 (Fig. 4, A and C). Taken together, stepwise transference of KcsA M1 residues at the N-terminus of the chimera S5 segment created voltage-dependent channels with a $V_{0.5}$ that remarkably approaches that of the Shaker channel (Fig. 5A), without virtually affecting the gating valence (Fig. 5B). Therefore, these findings illustrate that the complete replacement of Shaker S5 by KcsA M1 is required for efficient voltage-dependent coupling, and indicates that residue Phe$^{401}$ is a molecular determinant of competent voltage-dependent gating of chimeric channels.

To further examine the relationship between substitutions at the N terminus of S5 and the C-terminus of S6 and the gating properties, we calculated the free energy difference between the closed and open states at 0 mV ($\Delta G_e$) from $V_{0.5}$ and the apparent gating valence ($\varepsilon$) of the G-V relationship. Notice that $\Delta G_e$ quantity does not account for the voltage-independent conductance because it relies only on the voltage dependence of the channel (20). Fig. 5C depicts the values of $\Delta G_e$ obtained for the chimera and Sh4IR channel at 3 mM [K$^+$]$_o$. Inspection of the plot reveals two

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**Fig. 3. Replacement of Shaker residues at the C-End of S6 in the Shaker-KcsA chimera produces functional channels.** A and B, representative voltage-dependent ionic currents of non-injected oocytes, Sh4IR, and chimeras in which the Shaker amino acids (bold) at the S6 have been replaced by the corresponding of KcsA (gray). C, G-V relationships. Solid lines depict the best fit to a Boltzmann distribution. Oocytes were held at -80 mV and depolarized from -120 to +120 mV with voltage step increments of 20 mV. Ionic currents were recorded in standard Ringer solutions containing 3 mM [K$^+$]$_o$. All values are mean ± S.D. with n ≥ 5.

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non-injected oocytes, although much smaller than currents from homomeric wild type channels (Fig. 3, A and B (left)). The G-V was obtained to study the voltage-dependent gating of this chimera (Fig. 3C). Noteworthy, the channel exhibits a significant conductance at negative potentials, indicating the presence of a strong voltage-independent component. In accordance, the conductance change as a function of the applied voltage can barely be described as a two-state model ($V_{0.5} = 23 ± 10$ mV and $a_n = 24 ± 9$ mV), with a voltage-independent component C of 0.60. Thus, partial restoration of the KcsA M2 segment in the background of the Shaker-KcsA chimeras produces channels that exhibit a rather voltage-independent channel gating.

We next mutated residues Ile$^{477}$ to Leu and Ser$^{479}$ to Thr to recapitulate the entire M2 segment of KcsA into the chimera background (Fig. 1). These mutations produced a chimeric subunit that also assembled into functional channels (Fig. 3B, right). The G-V relationship depicts a voltage-dependent change in conductance that can readily be described with a two-state Boltzmann distribution, with a $V_{0.5}$ of 82 ± 11 mV, a slope ($a_n$) of 21 ± 8 mV, and a modest voltage-independent component C of 0.06 (Fig. 3C). Note that the $V_{0.5}$ of the chimera is remarkably ≥85 mV more depolarized than that of wild-type Shaker channels ($V_{0.5} = -6.5 ± 0.3$ mV, Fig. 3C). Taken together, these results imply that the C-terminus of the inner pore helix is primarily involved in defining the energetics of the channel gate.
features: (i) at variance with Sh4IR, all chimeras exhibit $\Delta G > 0$ kcal/mol; (ii) mutation of Phe$^{401}$ to Val in the N terminus of S5 reduces the $\Delta G \approx 1.5$ kcal/mol, with no significant energetic gain for the additional substitutions in the S5 N terminus of the chimera. Accordingly, this analysis shows that in the Shaker N terminus of S5 and, in particular, residue Phe$^{401}$ are critical molecular determinants for coupling voltage sensing to channel opening.

Extracellular $K^+$ Modulates the Channel Activity of Shaker-KcsA Chimeras—Extracellular $K^+$ modulates the activity of Kv channels primarily by stabilizing the open state of the channel, as evidenced by the remarkable prolongation of tail current kinetics at high $[K^+]_o$ (35). In addition, high $[K^+]_o$ favors channel gating of Kv and chimeric channels (22, 23, 36). Thus, to further understand the functionality of our chimeras we recorded the voltage-dependent ionic currents at 100 mM $[K^+]_o$ (Fig. 6). At variance with non-injected oocytes and Shaker wild type, a raise in the $[K^+]_o$ augmented the ionic currents of most of the chimeras. Noteworthy, current to voltage ($I-V$) relationships at high $[K^+]_o$ show that the chimeras did not exhibit the strong outward rectification characteristic of Sh4IR channels (Fig. 6, B and D), consistent with an altered coupling between both protein modules in the chimera. Two of the chimeras S6(VTAAIAS) and S5(AATVFLF) displayed a virtually linear $I-V$, indicating that these channels equally conduct at negative and positive potentials. Indeed, the ratio of ionic currents elicited at $-80$ and $80$ mV ($I_{-80}/I_{80}$) was $>0.45$. In contrast, the currents of wild type Shaker channels modestly decrease and the ratio $I_{-80}/I_{80}$ was unchanged when the $[K^+]_o$ was increased to 100 mM. Analysis of the $G-V$ relationships for these chimeras also shows a significant increment in the fraction of the voltage-independent conductance at high $[K^+]_o$ (Fig. 7A). Furthermore, conduction through these chimeras could not be abrogated at strong negative potentials suggesting that the energy released by $K^+$ binding to the protein suffices to open the channel. Note, however, that $K^+$ selectivity was virtually
unaffected, as shown by the change in the reversal potential upon increasing the [K\textsuperscript{+}]\textsubscript{o} to 100 mM (Fig. 6, B and D).

Comparison of the voltage-dependent properties of the chimeras at 3 and 100 mM [K\textsuperscript{+}]\textsubscript{o} reveal interesting features (Fig. 7B). (i) Stepwise recapitulation of the KcsA amino acid sequence at both the C-end of S6 and N terminus of S5 create channels with V\textsubscript{0.5} that progressively approach to the value of Shaker channel at both [K\textsuperscript{+}]\textsubscript{o}. (ii) Mutation up to F401V produces channels whose V\textsubscript{0.5} is depolarized at high [K\textsuperscript{+}]\textsubscript{o}. (iii) Subsequent replacement of Phe402 by Leu give rise to channels whose V\textsubscript{0.5} does not change upon raising the [K\textsuperscript{+}]\textsubscript{o}. (iv) Additional mutation Phe404 to Val produces channels whose V\textsubscript{0.5} is hyperpolarized at high [K\textsuperscript{+}]\textsubscript{o}, similar to the behavior seen in the Shaker wild type. (v) The gating valence is not significantly changed by raising the [K\textsuperscript{+}]\textsubscript{o} (Table I). (vi) Mutation of Phe402 and Phe404 reverted the voltage-independent gating at high [K\textsuperscript{+}]\textsubscript{o} that resulted from mutation of Phe401 to Val (Fig. 7A). Collectively, these results suggest that Phe402 and Phe404 also play important roles in pairing the voltage sensor and the pore domain, thus complementing the role of Phe401.

To further unmask the roles of Phe402 and Phe404 in voltage sensing, we examined the variation in the free energy difference between the closed and open states at 3 and 100 mM [K\textsuperscript{+}]\textsubscript{o} (\(\Delta\Delta G = \Delta G_{100K} - \Delta G_{3K}\)).

![Fig. 6. Voltage-dependent responses of Shaker-KcsA chimeras at high [K\textsuperscript{+}]\textsubscript{o}](image)
gain of ≥0.5 kcal/mol was obtained when Phe⁴⁰² was replaced by Leu, which was further enhanced by 0.3 kcal/mol after substitution of Phe⁴⁰⁴ by Val. Thus, these two positions in S5 are involved in the competent coupling of the voltage sensor and the channel gate.

**Shaker-KcsA Chimeric Mutants May Be Gated by [K⁺]ₒ—** We found that some of the chimeras studied show a remarkable sensitivity to changes in the [K⁺]ₒ, suggesting that [K⁺]ₒ may directly gate the channel. To further investigate this property, we evaluated the [K⁺]ₒ-dependent activation of all chimeras

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**TABLE I**

**Functional properties of chimaeric channels**

|       | V dependence | [K⁺] dependence |
|-------|--------------|-----------------|
|       | Vₜ₅ (mV)    | αₜ (mV)         | C               | EC₅₀ | nₗ | I₀K | Iₘ₅ |
| Sh₄IR | -24 ± 2      | 13 ± 1          | 0.01 ± 0.001    | 41   | 0.039 | 0.01 | 0.04 |
| S₆(VTAAI) | 88 ± 9  | 20 ± 6          | 0.06 ± 0.03     | 48   | 0.026 | -6.1 | 0.3  |
| S₅(AAIFFLF) | 149 ± 20 | 29 ± 8          | 0.07 ± 0.02     | 39   | 0.030 | -47  | 0.6  |
| S₅(AATTFFLF) | 94 ± 9   | 23 ± 5          | 0.05 ± 0.02     | 39   | 0.106 | 10   | 0.09 |
| S₅(AATVFLF)  | 31 ± 2    | 14 ± 2          | 0.02 ± 0.04     | 45   | 0.024 | 232  | 3.6  |
| S₅(AATVLLF)  | 25 ± 3    | 23 ± 2          | 0.1 ± 0.03      | 39   | 0.041 | 55   | 3.5  |
| S₅(AATVLLV)  | 1.0 ± 0.5  | 19 ± 1          | 0.1 ± 0.01      | 46   | 0.031 | -47  | 1.4  |

a Voltage-dependent properties of chimeric channels at 100 mM [K⁺]ₒ. Vₜ₅ (mV), αₜ (mV), and C were obtained from the fitting of G-V curves to a Boltzmann distribution. C denotes the fraction of voltage-independent conductance. Data are given as mean ± S.E., with n ≥ 6.

b Characteristics of the [K⁺]ₒ-activated ionic currents. Dose-response curves were fitted to a Michaelis-Menten binding isotherm: I = I₀K + (Iₘ₅/1 + ([K⁺]ₒ/EC₅₀)), nₗ, where Iₘ₅ is the maximum current, I₀K is the [K⁺]ₒ-independent current component, EC₅₀ is the [K⁺]ₒ concentration, which elicits the half-maximal response, and nₗ is the Hill coefficient. Data are given as mean ± S.E., with n ≥ 4.
expressed in Xenopus oocytes. To this end, oocytes were held at −80 mV and bathed with Ringers solution containing 3 mM $K^+$. As indicated, the solution was changed to Ringers containing 10, 30, and 100 mM $K^+$ (Fig. 8A). Exposure to high $K^+$ was interspersed with washes with 3 mM $K^+$. Fig. 8A illustrates that oocytes expressing the Shaker channel did not respond to increments in the $K^+$, while raises in $K^+$ elicited, in a concentration dependent manner, large non-inactivating, inward ionic currents from oocytes expressing chimeras S6(VTAAIAS), S5(AATVFLF), and S5(AATVLLF). Analysis of the $K^+$-activated currents of all chimeras revealed that these three species exhibited the largest $K^+$-activated ionic currents (Fig. 8B). Dose-response relationships for $K^+$-evoked currents were best described with a sigmoidal curve, which could be fitted to a Michaelis-Menten binding isotherm (Fig. 8C). This analysis depicts the best fit to a Michaelis-Menten binding isotherm. The $I_{\text{max}}$ was 3.5 µA, the value of the current at $[K^+]_o = 0$ mM ($I_{0K}$) was −0.23 µA, the slope of the curve was 0.031, and the concentration of $[K^+]_o$ needed to activate half-maximal response was 41 mM. Values are given as mean ± S.D., with $n = 7$.

**DISCUSSION**

The aim of this study was to gain further insights on the molecular determinant involved in coupling a voltage sensor and a pore domain. Our rationale considered the use of a gain-of-function approach aimed at endowing the voltage-insensitive prokaryotic KcsA channel with voltage dependence, by linking it to the proposed voltage sensor module of the eukaryotic Shaker channel. For this purpose, KcsA was in-
inserted between the junction of exons 8 and 9 at the N terminus of Shaker S5, and the conserved PVP motif at S6 of the eukaryotic channel (Fig. 1). Thus, the Shaker-KcsA chimera conserved the Shaker residues at both the N-end of S5 and the C-end of S6. Intriguingly, homomeric chimeric channels were not gated by voltage. The lack of channel activity was not because of a defect in protein biogenesis and trafficking, because heteromeric assemblies of Shaker wild type and chimera subunits give rise to voltage-gated channels. Thus, the absence of channel activity of the chimera was because of uncoupling of the voltage sensor and the activation gate. Hence, to determine which were the molecular determinants in both the C-end of S6 and N terminus of S5 that are critical for precise coupling of both the protein modules, we sequentially replaced the Shaker amino acids at both regions by the corresponding KcsA. This strategy gave rise to several findings: (i) the PVP domain present in Shaker S6 does not contribute to voltage sensing, consistent with other reports (37–39); (ii) mutation of the channel gate (Val478) gives rise to voltage-independent, [K+]o-dependent channel activity; (iii) the C-end region of S6 modulates the stability and energetics of the channel gate but does not participate in voltage sensing; and (iv) the N terminus domain of S5 is critical for endowing strong voltage sensitivity to the chimeras. Noteworthy, sequential substitution of Shaker residues at the N-end of S5 by those of KcsA created channels with voltage-dependent gating properties that progressively approached those characteristic of the eukaryotic channel. Furthermore, the most salient contribution of our study is the identification of region Phe401–Phe404 in the N terminus of the S5 segment as a critical structural determinant for providing competent voltage-gated channel activity to the chimeras. Indeed, replacement of Phe401 by Val notably hyperpolarized the \( V_{0.5} \) and reduced the \( \Delta G_{act} \) for activation, implying that the amino acid at this position is involved in protein-protein interactions with residues of the S4 segment. In addition, analysis of voltage-activated currents at high [K+]o, revealed that Phe402 and Phe404 are involved in the fine tuning of voltage gating, as demonstrated by the [K+]o-dependent hyperpolarization of
with the strong impact on voltage sensitivity of mutating these amino acids, and with the assigned role of S5 in regulating the final steps in activation that leads to channel opening (16, 48).

Thus, our functional findings on a hybrid Kv/KcsA channel also question the structural arrangement that emerged from the KvAP crystals, and favor a more conventional type of conformation for Kv channels. Although our structural model may account for most of the functional findings in the eukaryotic Kv channel, it does not favor a particular mode of voltage gating, being consistent with both upward translation of S4 or its tilting within the membrane. Our proposed model is also compatible with a recently proposed mode of voltage gating where the S3b-S4 voltage sensor paddle interacts with the C-end S5 segment of the adjacent subunit in the resting position, and flips across the intersubunit interface to interact with the S4 of its own subunit (31). Accordingly, coupling of the voltage sensor movement to the channel gate depends primarily on the flexibility of the S4-S5 linker and the interactions of the S5 N terminus, consistent with our results. Taken together all these findings suggest the notion that sensitive coupling of the voltage sensor to the pore domain requires a molecular compatibility between the N- and C-terminal regions of the S5 segment. A mismatch in the sequence results in a decrease in channel voltage sensitivity. Future studies will unravel the precise mechanism of how the electrical movement of the paddle induces the mechanical opening of the channel gate.

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Marco Caprini, Marianna Fava, Pierluigi Valente, Gregorio Fernandez-Ballester, Carmela Rapisarda, Stefano Ferroni and Antonio Ferrer-Montiel

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