Kv Channel S1-S2 Linker Working as a Binding Site of Human β-Defensin 2 for Channel Activation Modulation*

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Background: The functional role of the Kv channel S1-S2 linker remains unclear.

Results: The S1-S2 linker-hBD2 interaction modifies Kv1.3 channel activation through electrostatic repulsion between positively charged hBD2 and the channel S4 segment.

Conclusion: The Kv1.3 channel S1-S2 linker is a novel peptide-binding site.

Significance: These findings could define the function of S1-S2 linkers in Kv channel gating modification among different Kv channels.

Among the three extracellular domains of the tetrameric voltage-gated K⁺ (Kv) channels consisting of six membrane-spanning helical segments named S1–S6, the functional role of the S1-S2 linker still remains unclear because of the lack of a peptide ligand. In this study, the Kv1.3 channel S1-S2 linker was reported as a novel receptor site for human β-defensin 2 (hBD2). hBD2 shifts the conductance-voltage relationship curve of the human Kv1.3 channel in a positive direction by nearly 10.5 mV and increases the activation time constant for the channel. Unlike classical gating modifiers of toxin peptides from animal venoms, which generally bind to the Kv channel S3-S4 linker, hBD2 only targets residues in both the N and C termini of the S1-S2 linker to influence channel gating and inhibit channel currents. The increment and decrement of the basic residue number in a positively charged S4 sensor of Kv1.3 channel yields conductance-voltage relationship curves in the positive direction by −31.2 mV and 2–4 mV, which suggests that positively charged hBD2 is anchored in the channel S1-S2 linker and is modulating channel activation through electrostatic repulsion with an adjacent S4 helix. Together, these findings reveal a novel peptide ligand that binds with the Kv channel S1-S2 linker to modulate channel activation. These findings also highlight the functional importance of the Kv channel S1-S2 linker in ligand recognition and modification of channel activation.

Voltage-gated K⁺ (Kv)4 channels are molecular sensors of membrane potential (1) and play critical roles in cellular signal-

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3 The abbreviations used are: Kv, voltage-gated K⁺; hBD2, human β-defensin 2; Co-IP, co-immunoprecipitation.
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FIGURE 1. Kv1.3 channel activation modified by hBD2. A and B, voltage-dependent inhibition of Kv1.3 channel currents by hBD2. 69.8 ± 2.4% and 40.9 ± 0.8% of Kv1.3 channel currents were inhibited by 10 pM hBD2 at −30 and 0 mV, respectively. The voltage protocol is placed at the top. C, activation G-V curves of the Kv1.3 channels modified by hBD2. Peak current amplitude during each activation step was used to generate G-V curves. Activation G-V curves of the Kv1.3 channels were plotted with the Boltzmann equation. The V_{1/2} values were −32.75 ± 1.01 and −22.27 ± 0.60 mV in the absence and presence of 10 pM hBD2, respectively. D, activation time constants of Kv1.3 channels are plotted as a function of voltages before (black) and after (red) applying 10 pM hBD2.

QuikChange Lightning multi site-directed mutagenesis kit (Stratagene) based on a wild type Kv1.2 plasmid. All plasmids were verified with DNA sequencing before protein expression.

Cell Cultures—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO2 incubator at a temperature of 37 °C. The cells were precleared under rotation for 4 h at 4 °C. Subsequently, precleared protein was obtained by Transmembrane protein extraction kit according to the manufacturer’s instructions (Merck). Precleared protein was obtained by incubating the cell supernatants with a 40-μl protein G-Sepharose bead slurry (GE Healthcare) for 1 h at 4 °C to reduce nonspecific binding of proteins to the Sepharose beads. After centrifugation, adequate soluble hBD2 was added to the supernatant and incubated under rotation for 4 h at a temperature of 4 °C. Subsequently, 50 μl of the protein G-Sepharose beads and 5 μl of an anti-FLAG
antibody (Sigma) were added to the samples, and rotation was continued overnight. After the overnight incubation, the beads were washed three times with ice-cold modified radioimmune precipitation assay buffer, followed by resuspension of the Sepharose beads in 2x SDS sample buffer. The samples were separated by SDS-PAGE and then transferred onto Immobilon-P membranes (Millipore) for Western blot analysis.

Results

**hBD2 Regulates Kv1.3 Channel Activation Kinetics**—hBD2 recruits T cells and influences the cytokine secretion of T cells through interactions with different receptors (7, 11, 12). Our recent report showed that hBD2 inhibits Kv1.3 channel currents by interacting with the channel extracellular pore region (7). Surprisingly, the Kv1.3 currents were more significantly inhibited by hBD2 at −30 mV than at 0 mV (Fig. 1, A and B), which suggests that in addition to serving as a channel pore blocker, hBD2 could also regulate Kv1.3 activation kinetics. In contrast to the voltage-independent inhibition of Kv1.3 channel currents by channel pore-blocking toxins (3, 8, 13), voltage-dependent inhibition is a hallmark of gating-modifier toxins for Kv channels, which suggests that hBD2 has other effects on Kv1.3 in addition to being a channel pore blocker. Consistent with a channel gating modifier, hBD2 shifted the midpoint of activation ($V_{50}$) of the Kv1.3 activation G-V...
curves toward the positive direction by nearly 10.5 mV (Fig. 1C). Furthermore, hBD2 significantly increased the activation time constant (τ) of the Kv1.3 channel at different voltages (Fig. 1D). These results suggested that hBD2 modifies human Kv1.3 channel activation rather than blocking the channel pore.

**The Kv1.3 Channel S1-S2 Linker Is the Binding Site of hBD2**—Next, we explored the structural requirements for hBD2 regulation of the Kv1.3 channel activation. Based on the insensitivity of the human Kv1.2 channel to hBD2 (7), we constructed chimeric channels using the human Kv1.2 and Kv1.3 channels (Fig. 2A). Because the differences in the amino acid sequences are mainly located in three extracellular domains (the S1-S2 linker, the S3-S4 linker, and the S5-S6 linker) and hBD2 is likely to interact with amino acid residues on the extracellular regions, we made chimeric channels by swapping these three regions between the human Kv1.2 and Kv1.3 channels (Fig. 2A).
Consistent with our previous findings that the Kv1.3 pore region is critical to hBD2 inhibition (7), the S5-S6 linker chimeric in which the S5-S6 linker of Kv1.2 channel was replaced with the equivalent region from the Kv1.3 channel exhibited marked inhibition by 10 nM hBD2 (Fig. 2B). However, the potency of this channel (IC$_{50}$ = 8.8 ± 3.9 nM) was ~400-fold less than the potency of the wild type Kv1.3 channel (Fig. 2, E and F), which suggests that other extracellular domains of the Kv1.3 channel might also be required for hBD2 inhibition of Kv1.3.

Although the S3-S4 linker is a well known binding interface for animal gating-modifying toxins (5, 14, 15), replacement of the S3-S4 linker of Kv1.2 channel with an equivalent region from the Kv1.3 channel resulted in a chimeric channel that lacked sensitivity to inhibition by hBD2 (9.2 ± 1.9% at 1000 nM) (Fig. 2C), which suggests that the S3-S4 linker was not essential to hBD2 inhibition of the Kv1.3 channel.

Interestingly, the S1-S2 linker chimeric channel, produced by substituting the S1-S2 linker in the Kv1.2 channel with the corresponding Kv1.3 channel domain, exhibited potent inhibition by 1 nM hBD2 (Fig. 2D). The potency of hBD2 inhibition (IC$_{50}$ = 0.58 ± 0.11 nM) was ~26-fold less potent than inhibition of the wild type Kv1.3 channel and ~15-fold more potent than the S5-S6 linker chimeric channel (Fig. 2, E and F). Taken together, these results suggest that the Kv1.3 channel S1-S2 linker is a novel interaction site for hBD2. To further confirm the interaction between hBD2 and the S1-S2 linker of the Kv1.3 channel, a co-immunoprecipitation (Co-IP) assay was performed. We immobilized different FLAG-tagged channels on protein G-Sepharose beads and detected whether the channels could retain hBD2 through Western blotting. Our results showed that the Kv1.3 channel could retain hBD2 and Kv1.2 channels retained much less hBD2 than the Kv1.3 channels, which was consistent with our previous studies (7). When the Kv1.3 channel S1-S2 linker was transferred to the Kv1.2 channel, the binding affinity of hBD2 and the S1-S2 linker channel chimera was higher than the binding affinity for the Kv1.2 channel (Fig. 2G). This Co-IP assay further confirmed the interaction between hBD2 and the S1-S2 linker of the Kv1.3 channel.

**Table 1**

| Channel     | −hBD2 | +hBD2 | ΔV$_{50}$ |
|-------------|-------|-------|-----------|
| WT          | −32.75 ± 1.01 | −22.27 ± 0.60 | 10.48     |
| E206A       | −34.96 ± 0.83 | −27.47 ± 0.79 | 7.49      |
| F207A       | −32.60 ± 0.67 | −28.17 ± 0.55 | 4.43      |
| R208A       | −36.91 ± 0.80 | −28.99 ± 0.92 | 7.92      |
| D209A       | −34.83 ± 0.88 | −31.74 ± 1.30 | 3.09      |
| E210A       | −34.21 ± 1.02 | −32.20 ± 0.99 | 2.01      |
| K211A       | −32.86 ± 0.64 | −30.65 ± 0.67 | 2.21      |
| D212A       | −37.09 ± 0.89 | −25.65 ± 0.70 | 11.44     |
| Y213A       | −34.62 ± 0.98 | −32.06 ± 0.82 | 2.56      |
| S218A       | −37.99 ± 0.66 | −29.76 ± 0.95 | 8.23      |
| Q219A       | −35.00 ± 0.95 | −28.59 ± 0.73 | 6.71      |
| D220A       | −33.91 ± 0.74 | −27.55 ± 0.79 | 6.36      |
| S221A       | −32.49 ± 0.98 | −24.28 ± 0.66 | 8.21      |
| E223A       | −32.16 ± 1.12 | −26.22 ± 1.04 | 5.94      |
| S230A       | −33.76 ± 0.86 | −25.77 ± 0.68 | 7.99      |
| S232A       | −37.92 ± 0.51 | −33.49 ± 0.43 | 4.43      |
| R233A       | −34.92 ± 0.81 | −22.23 ± 0.47 | 12.69     |
| G235A       | −35.68 ± 0.46 | −33.19 ± 1.22 | 2.49      |
| S237A       | −34.22 ± 0.83 | −24.06 ± 1.19 | 10.16     |
| S238A       | −33.07 ± 0.13 | −30.17 ± 0.73 | 2.90      |
| F239A       | −34.19 ± 0.77 | −36.64 ± 0.68 | 2.54      |
| D240A       | −36.93 ± 1.05 | −26.29 ± 1.05 | 10.64     |
| D241A       | −34.93 ± 0.76 | −32.63 ± 1.11 | 2.30      |
| P242A       | −30.98 ± 0.72 | −30.06 ± 0.70 | 0.92      |

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**Table 1**

**Determination of the interaction sites in the Kv1.3 channel S1-S2 linker mediating hBD2 interaction**

**Figure 2**

- **Panel A** shows the shift in the activation curves of the wild type Kv1.3 (WT) and Kv1.2 (C) channels (Fig. 2F). The Kv1.3 channel (WT) exhibited potent inhibition by 10 nM hBD2 (Fig. 2G). This Co-IP assay further confirmed the interaction between hBD2 and the S1-S2 linker of the Kv1.3 channel.

**Kv1.3 Channel S1-S2 Linker-hBD2 Interaction Modifies Channel Activation**—Next, we asked whether the Kv1.3 channel S1-S2 linker-hBD2 interaction could affect channel activation. As shown in Fig. 3 (A and B), Kv1.2 channel activation could not be modified by hBD2; however, activation of the S1-S2 linker chimeric channel, which was produced by substituting the S1-S2 linker in the Kv1.2 channel with the equivalent region from the wild type Kv1.3 channel, was markedly influenced by hBD2, and the shift in the G-V curves, ΔV$_{50}$, was ~10.5 mV, which is similar to the shift of G-V curves from wild type Kv1.3 (Figs. 1C and 3E). Generally, the S3-S4 linker-gating modifier toxin interactions shift the Kv channel G-V curves (15–17). However, no significant shift of the G-V curves was observed for the S3-S4 linker chimeric channel in the presence of hBD2 (Figs. 3, C and E), which is likely due to a much weaker interaction between this chimeric channel and hBD2 (Fig. 2C). Additionally, the channel pore region-hBD2 interaction had less effect on the activation curve of the S5-S6 linker chimeras (Fig. 3, D and E). These data clearly support a unique role for the S1-S2 linker in the modification of Kv1.3 activation by hBD2.

**Functional Residues in the Channel S1-S2 Linker Influence hBD2 Binding**—We used alanine scanning mutagenesis to further identify the amino acid residues in the Kv1.3 S1-S2 linker that are required for the interaction between hBD2 and Kv1.3. Table 1 shows the V$_{50}$ values of channel activation for the wild type and 23 mutant Kv1.3 channels in which each individual amino acid was replaced with alanine in the absence and presence of hBD2. In comparison with the ΔV$_{50}$ value of 10.5 mV for wild type Kv1.3 modified by hBD2, the ΔV$_{50}$ values of the nine mutant channels were significantly decreased by ~70–91%, which suggests that residues, including Asp-209, Glu-210, Lys-211, Tyr-213, Gly-235, Ser-238, Phe-239, Asp-241, and Pro-242, played an essential role in the hBD2 interaction with the S1-S2 linker of Kv1.3. The ΔV$_{50}$ values of the Kv1.3-F207A and Kv1.3-S232A channels were ~4.4 mV, which suggests that Phe-207 and Ser-232 moderately affect hBD2 interaction with Kv1.3. In contrast, the other 12 residues, including Glu-206, Arg-208, Asp-212, Ser-218, Gln-219, Asp-220, Ser-221, Glu-223, Ser-230, Arg-233, Ser-237, and Ser-240, had less effect on hBD2 interaction with Kv1.3 because the ΔV$_{50}$ values of their respective mutant channels were much closer or similar to the ΔV$_{50}$ value of the wild type Kv1.3 (Table 1). To verify the differential effects of the S1-S2 linker residues on hBD2 binding, we performed Co-IP experiments with three representative mutants, D209A, F207A, and D220A, and found remarkable, moderate, and small reductions in ΔV$_{50}$ values, respectively. In line with their ΔV$_{50}$ values, the channel mutants D209A, F207A, and D220A retained more and more hBD2 (Fig. 4C). Together, these results indicated that the amino acid residues in the Kv1.3 channel S1-S2 linker acted differentially to interact with hBD2, which resulted in modification of the channel activation kinetics.

**Kv1.3 Channel Activation Modification by Electrostatic Repulsion between S4 and hBD2**—Our results showed that hBD2 can anchor in two terminals of the Kv1.3 channel S1-S2
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linker for channel activation modulation (Table 1 and Fig. 4, A and E). Similar to other classical Kv channels, Kv1.3 activation from resting state is accompanied by a voltage-dependent outward movement of the S4 segment, which is enriched with conserved basic amino acid residues such as arginine and lysine (Fig. 4A) (18). We hypothesize that hBD2 modification of Kv1.3 channel activation is caused by an electrostatic repulsion between S4 and basic hBD2 anchoring in the S1-S2 linker (Fig. 4B).
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4F). To test this hypothesis, we constructed three mutant Kv1.3 channels by altering the positive electric charges in the S4 segment. The Kv1.3-A309R channel has an additional arginine residue in the S4 N-terminal; the Kv1.3-R312H/R315H channel had two arginine residues adjacent to S4 N-terminal replaced by histidine residues that partially maintained the positive electric charges; and the Kv1.3-R312S/R315S channel had two arginine residues replaced by polar serine residues. When the electrostatic repulsion force between hBD2 and S4 was increased in the Kv1.3-A309R channel, the G-V curve was shifted to a much more depolarized voltage in the presence of hBD2 (Fig. 4B). The corresponding $\Delta V_{50}$ value was ~31.2 mV, which is much larger than the $\Delta V_{50}$ value of wild type Kv1.3 (Fig. 1). However, whereas the electrostatic repulsion force between hBD2 and S4 was decreased in the Kv1.3-R312H/R315H and Kv1.3-R312S/R315S channels, there was no obvious shift in the G-V curves (Fig. 4, B and F). The $\Delta V_{50}$ values were ~4.4 mV and 2.2 mV for the Kv1.3-R312H/R315H and Kv1.3-R312S/R315S channels, respectively. In comparison with a $\Delta V_{50}$ value of 10.5 mV for the wild type Kv1.3 channel modified by hBD2 (Fig. 1), these $\Delta V_{50}$ values of the Kv1.3 S4 segment mutants corresponded to changes in the positive electric charges in the S4 segment of the mutant channels. In agreement with the minor effect of the S3–S4 linker on hBD2 binding (Fig. 2, C and F), the Co-IP experiments showed that all three S4 segment mutants with either more and fewer positive electric charges did not obviously impair hBD2 binding (Fig. 4D). These results highlight a critical role of electrostatic repulsion between hBD2 and S4 in Kv1.3 activation (Fig. 4F).

Discussion

The Kv Channel S1–S2 Linker Is a Novel Binding Site for a Peptide Ligand—The extracellular domains of Kv channels are critical interaction sites for both endogenous and exogenous molecules. Among the three extracellular domains of Kv channels, the functions of the S3–S4 linker and the pore region have been well documented by animal toxin studies in past years (19) (Fig. 5, A and B). The channel S3–S4 linker was targeted by the spider toxin hanatoxin (5), and the channel pore region was bound by the scorpion toxin charybdotoxin (3, 20). However, whether the channel S1–S2 linker can be recognized by a peptide ligand remains unknown. Our present work showed that the channel S1–S2 linker is a novel binding site of the hBD2 ligand.

We demonstrated that the S1–S2 linker of the Kv1.3 channel was critically involved in hBD2 interaction and modification of the Kv1.3 activation. By constructing chimeric channels in addition to site-directed mutagenesis and Co-IP experiments, we showed that the S1–S2 linker was responsible not only for hBD2 binding (Fig. 2) but also for hBD2-elicited regulation of the Kv channel activation kinetics (Fig. 3). Furthermore, we identified 9 amino acid residues in the S1–S2 linker that are required for hBD2 modification of Kv1.3 activation (Table 1). Although both the S1–S2 linker and the S3–S4 linker are located...
in the vicinity of the Kv channel voltage sensor domain, the S1-S2 linker of the Kv1.3 channel is much longer than the S3-S4 linkers of the Kv2.1 or Kv4.3 channels that are targeted by gating-modifier toxins (Figs. 4F and 5B) (5, 16, 17, 21), which likely results into different structural features of the channel receptor sites. Unlike residues in the C-terminal half of the S3 segment of the Kv channel S3-S4 linker, which generally affect toxin binding (21–23) (Fig. 5B), we found that essential amino acid residues required for hBD2 interaction with Kv1.3-S1-S2 linker are located at both terminals (Table 1 and Figs. 4A and 5). In fact, many residues in the middle of the S1-S2 linker had a minor effect on hBD2 interaction with Kv1.3 (Table 1 and Fig. 4). This feature of the hBD2-Kv1.3 interaction interface suggests that the Kv1.3 channel S1-S2 linker likely forms an extended loop similar to the loop in Kv1.2, which was observed by normal-mode-based x-ray crystallographic refinement (24) (Fig. 4). Unlike the known roles of each Kv channel extracellular domain individually targeted by one type of animal toxin (Fig. 5, A and B), both the S1-S2 linker and the pore region of Kv1.3 channel are binding sites for hBD2 (Fig. 5B), and the corresponding features of the channel-interacting interfaces in hBD2 would be interesting to explore.

The Kv Channel S1-S2 Linker Plays a Role of a Bridge Pier in Channel Activation Modulation—So far, no evidence shows an interaction between the S1-S2 linker and the S3-S4 linker in Kv channels. The structural analysis would help to depict the functional role of channel S1-S2 in the modulation of channel activation. The crystal structure of the Kv1.2 channel indicates that transmembrane S1, S2, S3, and S4 helices look like two rows of bridge piers with unknown signal communication (25) (Fig. 4F). When hBD2, which is a classically positively charged peptide ligand, binds both terminals of the channel S1-S2 linker, it is anchored in the S1-S2 bridge pier and shortens the spatial distance from the positively charged S4, which is strongly supported by the modification of Kv1.3 activation with increasing and decreasing positive charges in the channel S4 helix (Fig. 4). Therefore, the channel S1-S2 linker likely plays the novel role of a bridge pier in the modulation of Kv1.3 channel activation through electrostatic repulsion forces between the anchored hBD2 and the channel S4. Additionally, the preference of S1-S2 linkers as bridge piers between the Kv1.2 and Kv1.3 channels is an aspect of Kv channels that requires further investigation in the future.

In conclusion, we found a novel functional role for the Kv1.3 channel S1-S2 linker in channel gating as revealed by interactions between the linker and an endogenous gating-modifier, hBD2. Our work not only reveals a potent S1-S2 linker-hBD2 interaction and demonstrates its effect on Kv channel activation but also elucidates the distinct “electrostatic repulsion mechanism” underlying the modification of Kv1.3 channel activation. These findings might be important for defining the function of S1-S2 linkers in the modification of Kv channel gating among different Kv channels in future studies.

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