

# Molecular basis for differential modulation of BK channel voltage-dependent gating by auxiliary $\gamma$ subunits

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Large conductance  $\text{Ca}^{2+}$ - and voltage-activated potassium (BK) channels are comprised of pore-forming  $\alpha$  subunits and various regulatory auxiliary subunits. The BK channel auxiliary  $\gamma$  (BK $\gamma$ ) subunits are a newly identified class of proteins containing an extracellular leucine-rich repeat domain (LRRD), a single transmembrane (TM) segment, and a short cytoplasmic C-terminal tail (C-tail). Although each of the four BK $\gamma$  proteins shifts the voltage dependence of BK channel activation in a hyperpolarizing direction, they show markedly different efficacies, mediating shifts over a range of 15–145 mV. Analyses of chimeric BK $\gamma$  subunits created by swapping individual structural elements, and of BK $\gamma$  deletion and substitution mutants, revealed that differential modulation of BK gating by the four BK $\gamma$  subunits depends on a small region consisting of the TM segment and the adjacent intracellular cluster of positively charged amino acids. The  $\gamma 1$  and  $\gamma 2$  TM segments contributed approximately  $-100$  mV, and the  $\gamma 1$  and  $\gamma 3$  C-tails contributed approximately  $-40$  mV, to shifting the voltage dependence of BK channel activation, whereas the  $\gamma 3$  and  $\gamma 4$  TM segments and the  $\gamma 2$  and  $\gamma 4$  C-tails contributed much less. The large extracellular LRRDs were mainly functionally interchangeable, although the  $\gamma 1$  LRRD was slightly less effective at enhancing (or slightly more effective at attenuating) the shift in BK channel voltage-dependent gating toward hyperpolarizing potentials than those of the other BK $\gamma$  subunits. Analysis of mutated BK $\gamma$  subunits revealed that juxta-membrane clusters of positively charged amino acids determine the functions of the  $\gamma 1$  and  $\gamma 3$  C-tails. Therefore, the modulatory functions of BK $\gamma$  subunits are coarse- and fine-tuned, respectively, through variations in their TM segments and in the adjacent intracellular positively charged regions. Our results suggest that BK channel modulation by auxiliary  $\gamma$  subunits depends on intra- and/or juxta-membrane mechanisms.

## INTRODUCTION

The large conductance  $\text{Ca}^{2+}$ - and voltage-activated potassium (BK) channel is widely expressed and plays important roles in many physiological processes, including neuronal firing and neurotransmitter release (Gribkoff et al., 2001), and frequency tuning of auditory hair cells (Ramanathan et al., 1999). The BK channel features exceptionally large single-channel conductance and dual activation by membrane depolarization and elevation in intracellular free calcium ( $[\text{Ca}^{2+}]_i$ ). Auxiliary membrane proteins are potent and critical regulators of ion channels (Gurnett and Campbell, 1996; Sun et al., 2012; Yan and Tomita, 2012). BK channels consist of the homotetrameric pore-forming voltage- and calcium-sensing  $\alpha$  subunits (BK $\alpha$ ) alone or in association with regulatory tissue-specific auxiliary  $\beta$  or  $\gamma$  subunits. The newly identified BK channel auxiliary  $\gamma$  (BK $\gamma$ ) subunits are a group of leucine-rich repeat (LRR)-containing membrane proteins, called  $\gamma 1$  (LRRC26),  $\gamma 2$  (LRRC52),  $\gamma 3$  (LRRC55),

and  $\gamma 4$  (LRRC38) (Yan and Aldrich, 2010, 2012). The four BK $\gamma$  subunits have different tissue-specific mRNA expression and may broadly modulate BK channels in different tissues, including brain, which expresses the  $\gamma 1$  and  $\gamma 3$  mRNAs (Yan and Aldrich, 2012). The as yet limited studies on the mechanisms and physiological functions of BK $\gamma$  subunits have mainly focused on  $\gamma 1$ , which has been reported to regulate BK channels in prostate cancer (Gessner et al., 2006; Yan and Aldrich, 2010), salivary gland cells (Almassy and Begenisich, 2012), and also likely in airway epithelial cells (Manzanares et al., 2014) and arterial smooth muscle cells (Evanson et al., 2014). The mouse  $\gamma 2$  subunit was found to function as an accessory subunit of the sperm-specific mouse Slo3 channels (Yang et al., 2011).

The BK $\gamma$  subunits are structurally unrelated to the double-membrane-spanning BK channel  $\beta$  subunits. The four BK $\gamma$  subunits are similar to each other in terms of their overall protein sequences (Fig. 1), and they all contain an N-terminal signal peptide, a relatively large

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Abbreviations used in this paper: BK, large conductance  $\text{Ca}^{2+}$ - and voltage-activated potassium;  $[\text{Ca}^{2+}]_i$ , intracellular free calcium; C-tail, C-terminal tail; HA, Horrigan and Aldrich; HEK, human embryonic kidney; LRR, leucine-rich repeat; LRRD, LRR domain; TM, transmembrane.

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extracellular LRR domain (LRRD), a single transmembrane (TM) segment, and a short intracellular C-terminal tail (C-tail) (Figs. 1 and 2). Unlike the complex effects and mechanisms of different BK channel  $\beta$  (BK $\beta$ ) subunits on many aspects of BK channel gating (Wallner et al., 1999; Brenner et al., 2000; Meera et al., 2000; Xia et al., 2000; Zeng et al., 2003; Savalli et al., 2007; Contreras et al., 2012; Sun et al., 2012), the action of the  $\gamma$ 1 subunit is remarkable in its mechanistic simplicity and modulatory magnitude (Yan and Aldrich, 2010; Zhang and Yan, 2014). An analysis of the  $\gamma$ 1 subunit's effects on different BK channel gating parameters within the framework of an allosteric Horrigan and Aldrich (HA) model (Horrigan and Aldrich, 2002) suggested that this subunit modulates the BK channel mainly by an  $\sim$ 20-fold enhancement in the allosteric coupling factor between voltage sensors and the channel pore (Yan and Aldrich, 2010). The  $\gamma$ 1 subunit also exhibited an "all-or-none" regulatory effect on BK channels independent of the molar ratio of injected BK $\alpha$ / $\gamma$ 1 RNA in *Xenopus laevis* oocytes (Gonzalez-Perez et al., 2014). This action is fundamentally different from that of BK $\beta$  subunits, which regulate the voltage dependence of BK channel activation in a titration-dependent mode (Wang et al., 2002).

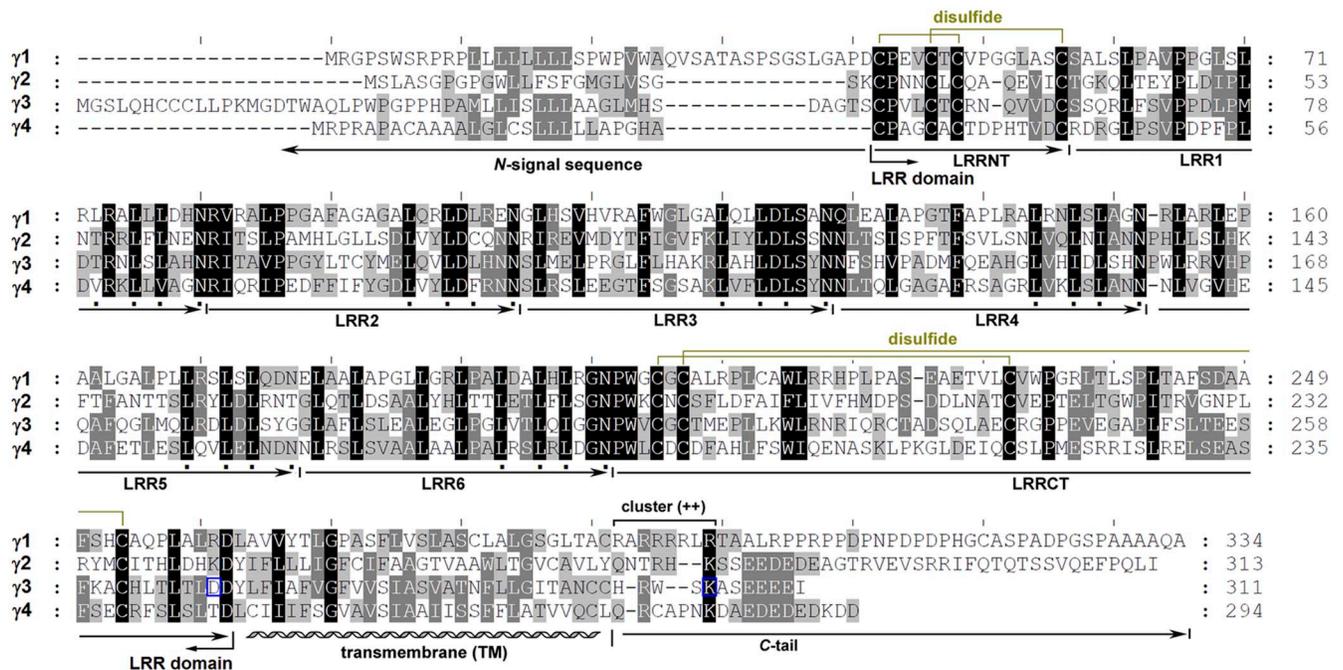
In spite of the above interesting structural and functional features of the BK $\gamma$  subunits, the molecular basis and mechanisms underlying BK channel regulation by BK $\gamma$  subunits are largely unknown. The four BK $\gamma$  subunits have distinct capabilities in shifting the voltage

dependence of BK channel activation toward hyperpolarizing voltages by  $\sim$ 140 mV ( $\gamma$ 1), 100 mV ( $\gamma$ 2), 50 mV ( $\gamma$ 3), and 20 mV ( $\gamma$ 4) in the absence of calcium (Yan and Aldrich, 2010, 2012). Identification of key structural elements underlying BK $\gamma$  subunits' different modulatory effects on BK channels could provide important insight into the molecular basis and mechanisms underlying BK channel regulation by BK $\gamma$  subunits. In this study, we generated chimeric BK $\gamma$  proteins by swapping individual structural elements among the four BK $\gamma$  subunits. Together with deletion and point mutations, we systematically analyzed the functional contributions of different structural elements to the BK $\gamma$  subunits' modulatory effects on BK channel voltage-dependent gating. We found that the differential modulatory functions of BK $\gamma$  subunits are mainly determined by a small region consisting of the TM segment and its adjacent intracellular cluster of positively charged residues. Our results suggest involvement of major intra- and/or juxta-membrane mechanisms in the auxiliary  $\gamma$  subunits' activating effects on BK channels.

## MATERIALS AND METHODS

### Expression of BK $\alpha$ and BK $\gamma$ proteins in human embryonic kidney (HEK)-293 cells

Recombinant cDNA constructs of human BK $\alpha$  (hSlo),  $\gamma$ 1 (LRRC26),  $\gamma$ 2 (LRRC52),  $\gamma$ 3 (LRRC55), and  $\gamma$ 4 (LRRC38) subunits were used for heterologous expression studies in HEK-293 cells. To divide

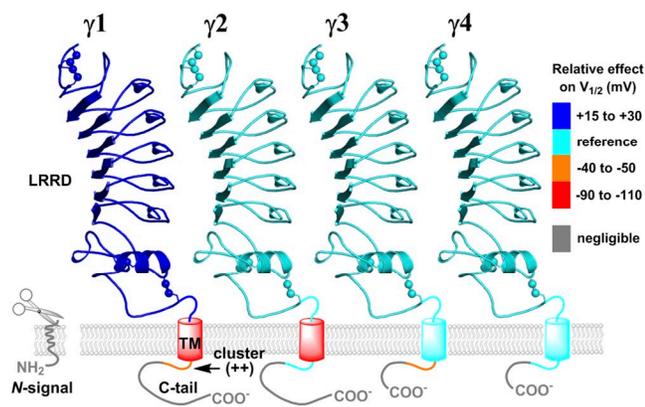


**Figure 1.** Protein sequence alignment of BK $\gamma$  subunits in humans. The corresponding amino acid sequences for different domains or segments are assigned and used for the generation of chimeric proteins. Cluster (++) refers to the cluster of positively charged amino acids. The key residues of the consensus sequence (LxxLxLxxN) in each LRR unit are marked by black squares below. Four cysteine pairs for potential disulfide formation are indicated. The two residues in  $\gamma$ 3 used for point mutation analyses, D269 and K304, are outlined in blue.

each BK $\gamma$  subunit into four structural elements for generation of chimeric BK $\gamma$  proteins, we defined the N-signal peptide sequence as the N-terminal sequence before the first conserved disulfide-forming Cys residue of the LRRNT unit, the TM segment as a relatively hydrophobic region of 29-amino acid residues flanked by charged amino acid residues, LRRD as the sequence located between N-signal peptide and the TM segment, and C-tail as the sequence on the C-terminal side of the TM segment (Fig. 1). Synthetic cDNA sequences of chimeric BK $\gamma$  subunits were subcloned into the mammalian expression vector of pCDNA6, with V5 tags attached at their C termini. As described previously (Yan and Aldrich, 2010, 2012), fusion cDNA constructs, which encode precursor fusion proteins of human BK $\alpha$  and C-terminal-tagged BK $\gamma$  proteins, were generated with the pCDNA6 vector and used to facilitate the co-translational assembly of BK $\alpha$ -BK $\gamma$  protein complexes after endogenous cleavage by peptidases at the linker (signal peptide) region in the mature proteins. This previously established co-translational assembly strategy produced comparably reproducible results as the other strategies that generated over- or super-expression of BK $\gamma$  relative to BK $\alpha$  in a single HEK-293 cell using either an IRES (internal ribosome entry site)-based single bicistronic expression method or BK $\alpha$ -stable cell line method (Yan and Aldrich, 2010, 2012). HEK-293 cells were obtained from ATCC, transfected with plasmids using Lipofectamine 2000 (Invitrogen), and subjected to electrophysiological assays 16–72 h after transfection.

### Electrophysiology

To record the BK channel currents, we used patch-clamp recording in excised inside-out patches of HEK-293 cells with symmetric internal and external solutions of 136 mM KMeSO<sub>3</sub>, 4 mM KCl, and 20 mM HEPES, pH 7.20. The external solution was supplemented with 2 mM MgCl<sub>2</sub>, and the internal solution was supplemented with 5 mM HEDTA without Ca<sup>2+</sup> to create a virtually Ca<sup>2+</sup>-free solution. Steady-state activation was expressed as the normalized conductance (G/G<sub>max</sub>) calculated from the relative amplitude of the tail currents (deactivation at -120 mV). The voltage of half-maximal activation ( $V_{1/2}$ ) and the equivalent gating charge ( $z$ ) were obtained by fitting the relations of G/G<sub>max</sub> versus voltage with the single-Boltzmann function  $G/G_{max} = 1/(1 + e^{-z(V-V_{1/2})/RT})$ .



**Figure 2.** Schematic of the structure, membrane topology, and generalized relative functional contributions of individual structural elements in BK $\gamma$  subunits. The structural models of the LRRDs were built as described previously (Yan and Aldrich, 2012). The different colors indicate the structural elements' relative contributions (in millivolts) to the BK $\gamma$  subunit-induced shifts of voltage dependence of BK channel activation. "Reference" means "being used as a reference for comparison," and "negligible" means "can be deleted without influence on modulatory function."

TABLE 1

*Boltzmann-fit parameters of the voltage-dependent BK channel activation in the presence of auxiliary BK $\gamma$  wild types, chimeras, and mutants*

Expression	Boltzmann fit parameters		
	$mV$	$z$	$n$
BK $\alpha$ alone	167 $\pm$ 2	1.26 $\pm$ 0.07	8
+ $\gamma$ 1	22 $\pm$ 4	1.75 $\pm$ 0.09	7
+ $\gamma$ 2	61 $\pm$ 3	1.17 $\pm$ 0.07	8
+ $\gamma$ 3	115 $\pm$ 2	1.36 $\pm$ 0.05	6
+ $\gamma$ 4	154 $\pm$ 3	1.27 $\pm$ 0.07	7
+ $\gamma$ 1/ $\gamma$ 4-signal	13 $\pm$ 3	1.49 $\pm$ 0.06	4
+ $\gamma$ 3/ $\gamma$ 1-signal	121 $\pm$ 5	1.37 $\pm$ 0.06	3
+ $\gamma$ 4/ $\gamma$ 1-signal	155 $\pm$ 1	1.32 $\pm$ 0.03	4
+ $\gamma$ 1/ $\gamma$ 2-LRRD	-3 $\pm$ 3	1.38 $\pm$ 0.04	6
+ $\gamma$ 1/ $\gamma$ 3-LRRD	6 $\pm$ 3	1.52 $\pm$ 0.09	8
+ $\gamma$ 1/ $\gamma$ 4-LRRD	-12 $\pm$ 3	1.66 $\pm$ 0.05	11
+ $\gamma$ 1/ $\gamma$ 2-TM&tail	90 $\pm$ 2	1.28 $\pm$ 0.15	4
+ $\gamma$ 1/ $\gamma$ 3-TM&tail	151 $\pm$ 3	1.48 $\pm$ 0.07	6
+ $\gamma$ 1/ $\gamma$ 2-tail	143 $\pm$ 3	1.26 $\pm$ 0.05	4
	25 (75%) <sup>a</sup>	1.71	3
+ $\gamma$ 1/ $\gamma$ 3-tail	126 (25%)	1.06	
	28 (16%)	1.33	8
	150 (84%)	0.95	
+ $\gamma$ 1/ $\gamma$ 4-tail	147 $\pm$ 3	1.23 $\pm$ 0.08	5
+ $\gamma$ 2/ $\gamma$ 1-tail	17 $\pm$ 2	1.86 $\pm$ 0.10	3
+ $\gamma$ 2/ $\gamma$ 3-tail	21 $\pm$ 2	1.67 $\pm$ 0.12	4
+ $\gamma$ 2/ $\gamma$ 4-tail	68 $\pm$ 2	1.12 $\pm$ 0.08	5
+ $\gamma$ 3/ $\gamma$ 1-tail	120 $\pm$ 2	1.81 $\pm$ 0.20	3
+ $\gamma$ 3/ $\gamma$ 2-tail	141 $\pm$ 5	1.66 $\pm$ 0.20	3
+ $\gamma$ 3/ $\gamma$ 4-tail	156 $\pm$ 4	1.40 $\pm$ 0.07	3
+ $\gamma$ 1/ $\gamma$ 2-TM	32 $\pm$ 3	1.40 $\pm$ 0.07	5
+ $\gamma$ 1/ $\gamma$ 3-TM	135 $\pm$ 2	1.24 $\pm$ 0.02	3
+ $\gamma$ 1/ $\gamma$ 4-TM	155 $\pm$ 4	1.30 $\pm$ 0.03	5
+ $\gamma$ 2/ $\gamma$ 1-TM	155 $\pm$ 4	1.33 $\pm$ 0.09	6
+ $\gamma$ 2/ $\gamma$ 3-TM	155 $\pm$ 5	1.54 $\pm$ 0.07	4
+ $\gamma$ 2/ $\gamma$ 4-TM	158 $\pm$ 7	1.46 $\pm$ 0.10	4
+ $\gamma$ 3/ $\gamma$ 1-TM	165 $\pm$ 4	1.14 $\pm$ 0.05	5
	119	1.11	1
+ $\gamma$ 3 <sup>D269R</sup> / $\gamma$ 1-TM	138 $\pm$ 6 (36%)	1.12 $\pm$ 0.10	7
	62 $\pm$ 3 (64%)	1.11 $\pm$ 0.05	
+ $\gamma$ 3 <sup>D269R</sup>	117 $\pm$ 3	1.36 $\pm$ 0.11	5
+ $\gamma$ 3/ $\gamma$ 2-TM	26 $\pm$ 2	1.44 $\pm$ 0.06	5
+ $\gamma$ 3/ $\gamma$ 4-TM	160 $\pm$ 4	1.38 $\pm$ 0.06	4
+ $\gamma$ 4/ $\gamma$ 2-TM	113 $\pm$ 4	1.15 $\pm$ 0.11	4
+ $\gamma$ 4/ $\gamma$ 2-TM	72 $\pm$ 3	1.27 $\pm$ 0.06	5
+ $\gamma$ 1- $\Delta$ tailC <sup>302-334</sup>	29 $\pm$ 4	1.40 $\pm$ 0.09	4
+ $\gamma$ 1- $\Delta$ tailN <sup>291-298</sup>	168 $\pm$ 3	1.22 $\pm$ 0.11	5
+ $\gamma$ 2- $\Delta$ tailN <sup>277-279</sup>	73 $\pm$ 3	1.12 $\pm$ 0.04	7
+ $\gamma$ 2- $\Delta$ tailC <sup>280-313</sup>	63 $\pm$ 2	1.28 $\pm$ 0.09	4
+ $\gamma$ 2- $\Delta$ tail <sup>277-313</sup>	166 $\pm$ 6	1.16 $\pm$ 0.01	3
+ $\gamma$ 3- $\Delta$ tailC <sup>305-311</sup>	109 $\pm$ 3	1.56 $\pm$ 0.12	4
+ $\gamma$ 3- $\Delta$ tailN <sup>298-304</sup>	180 $\pm$ 3	1.26 $\pm$ 0.12	5
+ $\gamma$ 3 <sup>K304N</sup>	144 $\pm$ 2	1.34 $\pm$ 0.12	4

$n$  values are the number of recorded excised inside-out patches from different HEK-293 cells.

<sup>a</sup>The indicated percentage in parentheses refers to the portion of the channels' subpopulation that was obtained from a double-Boltzmann function fit.

or with the double-Boltzmann function  $G/G_{\max} = a/(1 + e^{-z\Delta F(V-V_{a1/2})/RT}) + (1 - a)/(1 + e^{-z\Delta F(V-V_{b1/2})/RT})$ . Experimental values are reported as the mean  $\pm$  the SEM.

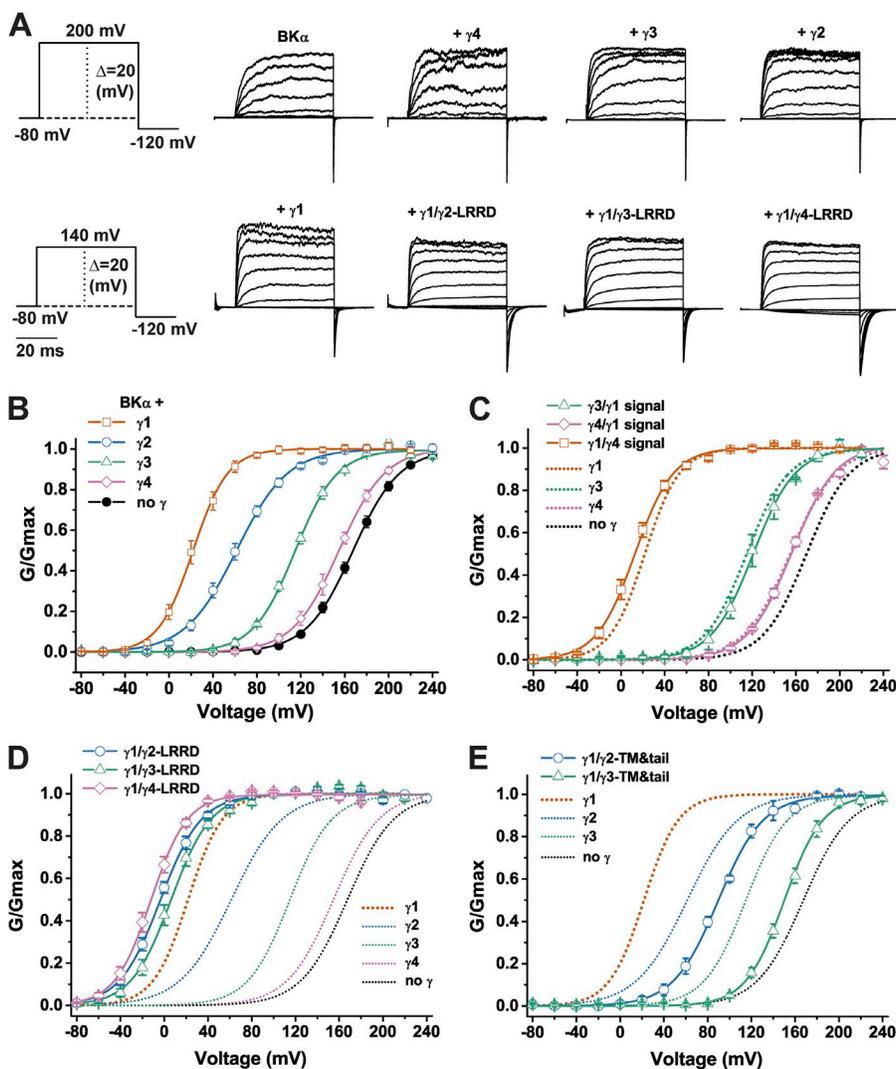
## RESULTS

### Differential modulation of BK channel voltage-dependent gating by the BK $\gamma$ subunits

We observed that the four BK $\gamma$  subunits, when heterologously expressed in HEK-293 cells, produced distinct shifts toward hyperpolarizing potentials in the voltage dependence of BK channel activation (Fig. 3, A and B), which is similar to the findings of a previous report (Yan and Aldrich, 2012). The half-activation voltages ( $V_{1/2}$ ) of BK channels were shifted by 145  $\pm$  5 mV by  $\gamma 1$  ( $V_{1/2} = 22 \pm 4$ ), 107  $\pm$  4 mV by  $\gamma 2$  ( $V_{1/2} = 61 \pm 3$  mV), 52  $\pm$  3 mV by  $\gamma 3$  ( $V_{1/2} = 115 \pm 2$  mV), and 13  $\pm$  4 mV by  $\gamma 4$  ( $V_{1/2} = 154 \pm 3$  mV), compared with the BK $\alpha$  channel alone ( $V_{1/2} = 167 \pm 2$  mV) in the virtual absence of  $[Ca^{2+}]_i$ . The equivalent gating charge ( $z$ ) of BK channel voltage gating

was largely unaffected by the  $\gamma 2$  ( $z = 1.17 \pm 0.07 e$ ),  $\gamma 3$  ( $z = 1.36 \pm 0.05 e$ ) and  $\gamma 4$  ( $z = 1.27 \pm 0.07 e$ ) subunits but markedly increased by the  $\gamma 1$  subunit ( $z = 1.75 \pm 0.09e$ ), compared with the BK $\alpha$  channel alone ( $z = 1.26 \pm 0.07 e$ ) in the virtual absence of  $[Ca^{2+}]_i$ . For comparison, the Boltzmann fit parameters of the voltage-dependent BK channel activation in the presence of auxiliary BK $\gamma$  wild types, as well as all chimeras and mutants discussed below, are given in Table 1.

We first determined whether the BK $\gamma$  subunits' distinct capabilities in modulating BK channel voltage-dependent gating are intrinsic properties of their mature proteins or are caused by differences in their signal peptide functions. We generated chimeric BK $\gamma$  proteins of the  $\gamma 3/\gamma 1$ -signal and  $\gamma 4/\gamma 1$ -signal by replacing the N-terminal signal peptide sequences in  $\gamma 3$  and  $\gamma 4$  with that of  $\gamma 1$ . Similarly, we generated chimeric BK $\gamma$  proteins of the  $\gamma 1/\gamma 4$ -signal by using the  $\gamma 4$  N-terminal signal peptide sequence to guide the expression of  $\gamma 1$  protein. We observed that swapping the N-terminal signal peptide regions, which elicited changes of <10 mV in the BK channel



**Figure 3.** Effects of swapping N-signal peptides and LRRDs on the modulatory functions of BK $\gamma$  subunits. (A) Representative traces of recorded BK channel currents in response to the depolarization of the membrane potential from  $-80$  mV in 20-mV steps for selected expression constructs. (B–E) Voltage dependence of BK channel activation (plotted from tail currents at  $-120$  mV) in the absence and presence of wild-type BK $\gamma$  subunits (B); in the presence of  $\gamma 1$  N-signal peptide-swapping chimera (C); in the presence of LRRD-swapping chimera (D); and in the presence of TM and C-tail region-swapping chimera, whose main bodies were from  $\gamma 1$  and whose TM and C-tail regions together were from other BK $\gamma$  subunits (E). For all indicated given names of chimeric BK $\gamma$  subunits, the acceptor subunit used to generate the main protein body appears before the slash, and the donor subunit used for a swapped domain or region together with the name of swapped domain or region appears after the slash. All BK channel currents were recorded in the virtual absence of  $[Ca^{2+}]_i$  and plotted as the normalized conductance ( $G/G_{\max}$ ) against different membrane voltages. Thick dot lines are used for the major wild-type BK $\gamma$  protein(s) intended for comparison. Error bars represent  $\pm$  SEM.

$V_{1/2}$  values, had no significant effect on the BK $\gamma$  subunits' modulatory functions (Fig. 3 C). The BK channel  $V_{1/2}$  values in the virtual absence of  $[Ca^{2+}]_i$  were  $121 \pm 5$  mV with the  $\gamma 3/\gamma 1$ -signal,  $V_{1/2} = 155 \pm 1$  mV with the  $\gamma 4/\gamma 1$ -signal, and  $V_{1/2} = 13 \pm 3$  mV with the  $\gamma 1/\gamma 4$ -signal (Table 1). These results indicate that the much weaker modulatory functions of the  $\gamma 3$  and  $\gamma 4$  subunits, as compared with that of the  $\gamma 1$  subunit, are intrinsic properties of the subunits' mature proteins and independent of their N-terminal signal peptide sequences.

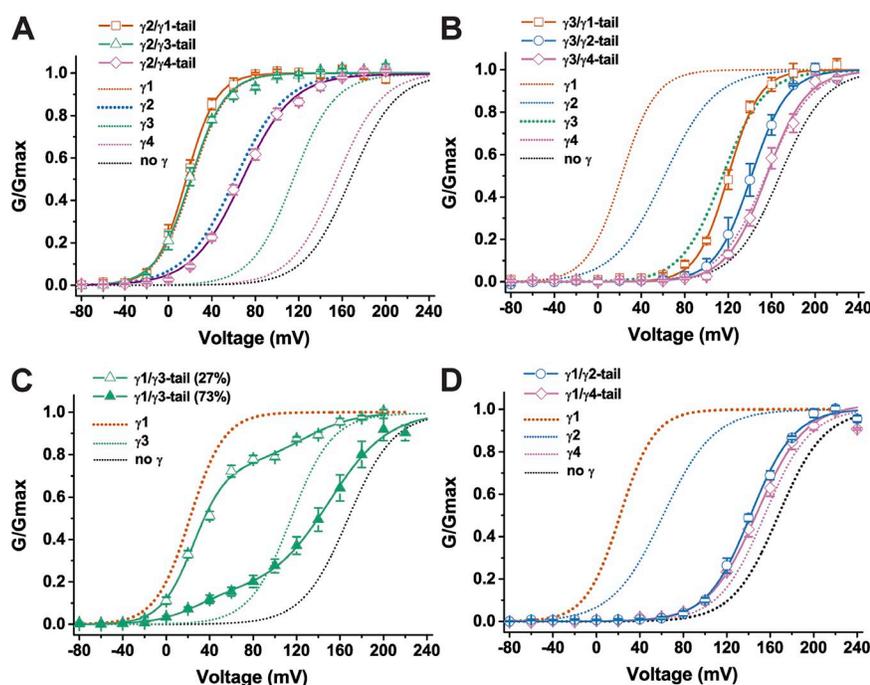
#### LRRD does not determine the modulatory functions of different BK $\gamma$ subunits

The relatively large LRRD comprises six tandem LRR structural units (LRR1–6) and two cysteine-rich modules, LRRNT and LRRCT, which are capped on the N- and C-terminal sides, respectively (Fig. 1). We found that substituting the LRRD of  $\gamma 1$  with those of the other three BK $\gamma$  subunits resulted in no reduction but instead slight to moderate gain-of-function in the  $\gamma 1$  subunit's modulatory effect on BK channel voltage-dependent gating (Fig. 3, A and D). Compared with the unaltered  $\gamma 1$  protein ( $V_{1/2} = 22 \pm 4$  mV), the resultant LRRD-swapping chimeras of  $\gamma 1/\gamma 2$ -LRRD ( $V_{1/2} = -3 \pm 3$  mV),  $\gamma 1/\gamma 3$ -LRRD ( $V_{1/2} = 6 \pm 3$  mV), and  $\gamma 1/\gamma 4$ -LRRD ( $V_{1/2} = -12 \pm 3$  mV) caused further shifts of 25, 16, and 34 mV, respectively, in the voltage dependence of BK channel activation toward hyperpolarizing potentials (Fig. 3 D). Because the other BK $\gamma$  subunits are weaker modulators than  $\gamma 1$  in terms of shifting the voltage dependence of BK channel activation, this result contradicts what one would expect if the LRRD were a determinant of the subunits' different modulatory functions. In contrast, replacing the TM and

C-tail regions of  $\gamma 1$  with those of  $\gamma 2$  or  $\gamma 3$  caused a 47% reduction ( $V_{1/2} = 90 \pm 2$  mV with  $\gamma 1/\gamma 2$ -TM&tail) or a 89% loss ( $V_{1/2} = 151 \pm 3$  mV with  $\gamma 1/\gamma 3$ -TM&tail) of the  $\gamma 1$  subunit's modulatory function (Fig. 3 E), which suggests that the TM and C-tail regions determine the BK $\gamma$  subunits' modulatory functions. The modulatory effects of the  $\gamma 1/\gamma 2$ -TM&tail and  $\gamma 1/\gamma 3$ -TM&tail chimeras were even smaller than those of the unaltered  $\gamma 2$  and  $\gamma 3$  subunits, respectively. These results suggest that compared with the LRRDs of other BK $\gamma$  subunits, the  $\gamma 1$  LRRD plays a slightly larger inhibitory or less stimulatory role in shifting the BK channel voltage-dependent gating toward hyperpolarizing potentials (Fig. 2).

#### C-tails partially contribute to the difference in modulatory functions among BK $\gamma$ subunits

The short C-tails of the four BK $\gamma$  subunits have different lengths and very low sequence similarities. According to our assignments of the C-terminal borders of the neighboring TM segments, the  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ , and  $\gamma 4$  C-tails have 44, 40, 12, and 18 amino acid residues, respectively (Fig. 1). Substituting the C-tail of  $\gamma 2$  with those of the other three BK $\gamma$  subunits produced fully functional chimeric proteins (Fig. 4 A). The modulatory effect of the  $\gamma 2/\gamma 4$ -tail chimera ( $V_{1/2} = 68 \pm 2$  mV) was nearly the same as that of unaltered  $\gamma 2$ , which suggests that the  $\gamma 4$  C-tail functions similarly as the  $\gamma 2$  C-tail. In contrast, both the  $\gamma 1$  and  $\gamma 3$  C-tails potentiated the  $\gamma 2$  acceptor subunit's modulatory function by approximately  $-40$ -mV shifts in the BK channel  $V_{1/2}$  ( $17 \pm 2$  mV with  $\gamma 2/\gamma 1$ -tail and  $21 \pm 2$  mV with  $\gamma 2/\gamma 3$ -tail), which became more like that produced by  $\gamma 1$ . These modifications in  $V_{1/2}$  were accompanied by an increase in the steepness of the G-V



**Figure 4.** Effects of swapping C-tail regions on the modulatory functions of BK $\gamma$  subunits. Voltage dependence of BK channel activation in the presence of BK $\gamma$  subunit chimeras, whose C-tail regions were from different BK $\gamma$  subunits and whose main bodies were from  $\gamma 2$  (A),  $\gamma 3$  (B), and  $\gamma 1$  (C and D). Thick dot lines are used for the major wild-type BK $\gamma$  protein(s) intended for comparison. Error bars represent  $\pm$  SEM.

relationship curve ( $z = 1.86 \pm 0.1$  e with  $\gamma 2/\gamma 1$ -tail and  $z = 1.67 \pm 0.12$  e with  $\gamma 2/\gamma 3$ -tail, as compared with  $z = 1.17 \pm 0.07$  e with  $\gamma 2$ ), which approached that of  $\gamma 1$  ( $z = 1.75 \pm 0.09$  e). These results suggest that the  $\gamma 1$  and  $\gamma 3$  C-tails are similarly effective, whereas the  $\gamma 2$  and  $\gamma 4$  C-tails are similarly less effective or ineffective, in their contributions to the BK $\gamma$  subunits' modulatory functions.

Consistent with the above inference, the  $\gamma 1$  C-tail did not cause a significant (i.e.,  $\geq 15$  mV in  $V_{1/2}$ ) change in the  $\gamma 3$  acceptor subunit's function ( $V_{1/2} = 120 \pm 2$  mV with  $\gamma 3/\gamma 1$ -tail). In contrast, the  $\gamma 2$  and  $\gamma 4$  C-tails both markedly reduced the  $\gamma 3$  acceptor subunit's modulatory function ( $V_{1/2} = 141 \pm 5$  mV with  $\gamma 3/\gamma 2$ -tail and  $V_{1/2} = 156 \pm 4$  mV with  $\gamma 3/\gamma 4$ -tail) to levels similar to that of the weaker  $\gamma 4$  ( $V_{1/2} = 154 \pm 3$  mV) (Fig. 4 B). The  $\sim 40$ -mV difference between the pair of the  $\gamma 1$  and  $\gamma 3$  C-tails and the pair of the  $\gamma 2$  and  $\gamma 4$  C-tails in their influences on the  $\gamma 2$  or  $\gamma 3$  acceptor's modulatory functions agrees well with the differences between  $\gamma 1$  and  $\gamma 2$  and between  $\gamma 3$  and  $\gamma 4$  in their abilities to shift the voltage dependence of BK channel activation. These results indicate that although the C-tail region is not a major determinant of the overall large difference in modulatory functions across the four BK $\gamma$  subunits, it is very likely a key determinant of the functional differences between  $\gamma 1$  and  $\gamma 2$ , and between  $\gamma 3$  and  $\gamma 4$ .

#### $\gamma 1$ TM segment and other BK $\gamma$ subunits' C-tails are incompatible

The above results with C-tail-swapped chimeric BK $\gamma$  proteins showed that the C-tails of  $\gamma 1$  and  $\gamma 3$  function similarly in the presence of the main bodies (i.e., the LRRDs and TM segments) of  $\gamma 2$  and  $\gamma 3$ . However, upon replacement of the  $\gamma 1$  C-tail with the  $\gamma 3$  C-tail, the resultant  $\gamma 1/\gamma 3$ -tail chimera caused the G-V relationships of BK channels to be fitted to at least two populations of BK channels in all recorded 11 excised patches (Fig. 4 C). In three patches, the G-V relationships were best fitted to a major population ( $\sim 75\%$ ) of low voltage-activated channels ( $V_{1/2}$  of  $\approx 25$  mV), similar to those associated with the unaltered  $\gamma 1$  subunit (BK $\alpha/\gamma 1$ ) and to a minor population ( $\sim 25\%$ ) of high voltage-activated channels more similar to those of BK $\alpha$  alone. In the other eight patches, the G-V relationships were best fitted to a minor population (16%) of low voltage-activated BK $\alpha/\gamma 1$ -type channels and to a major population (84%) of high voltage-activated BK $\alpha$ -type channels. These two distinct G-V relationships for different membrane patches have been observed in the same batch of transfected cells. These results suggest that the  $\gamma 3$  C-tail did function as the  $\gamma 1$  C-tail in the  $\gamma 1/\gamma 3$ -tail chimera in about half of the recorded BK channels. However, in the other half of the recorded BK channels, the  $\gamma 3$  C-tail caused a drastic reduction (72–88%) in the  $\gamma 1$ 's modulatory function. Moreover, both the  $\gamma 2$  and  $\gamma 4$  C-tails caused an  $\sim 85\%$  loss of the  $\gamma 1$  acceptor subunit's modulatory function

( $V_{1/2} = 143 \pm 3$  mV with  $\gamma 1/\gamma 2$ -tail and  $V_{1/2} = 147 \pm 3$  mV with  $\gamma 1/\gamma 4$ -tail) (Fig. 4 D). Given that the  $\gamma 1$  C-tail in the presence of  $\gamma 2$  and  $\gamma 3$  TM segments produced only a limited contribution (i.e., approximately  $-40$  mV) to the shift of the BK channel  $V_{1/2}$ , the  $\gamma 1$  C-tail is unlikely a sole determinant of  $\gamma 1$ 's modulatory function. The drastic reduction in the  $\gamma 1$  subunit's modulatory function upon replacement of its C-tail with those of the other  $\gamma$  subunits was likely caused by some structural or functional incompatibility (i.e., antagonistic effect) between the other  $\gamma$  subunits' C-tails and other regions of the  $\gamma 1$  subunit.

Although both  $\gamma 1$  and  $\gamma 2$  modulate the BK channel very effectively, the  $\gamma 2/\gamma 1$ -TM chimera, which resulted from  $\gamma 1$ 's TM segment being in the presence of  $\gamma 2$ 's LRRD and C-tail, was ineffective (i.e., the resultant  $V_{1/2}$  is within 15 mV of that produced by BK $\alpha$  alone) in modulating BK channel voltage gating ( $V_{1/2} = 155 \pm 4$  mV) (Fig. 5 A), suggesting that incompatibility exists between the  $\gamma 1$  TM segment and either the  $\gamma 2$  LRRD or the  $\gamma 2$  C-tail. Given that the  $\gamma 2$  LRRD was fully compatible (i.e., no obvious antagonistic effect) with the rest of  $\gamma 1$  in the  $\gamma 1/\gamma 2$ -LRRD chimera (Fig. 3 D) and that the  $\gamma 1/\gamma 2$ -tail chimera was only slightly effective (i.e., the resultant  $V_{1/2}$  is within 15–30 mV of that produced by BK $\alpha$  alone) in modulating the BK channel (Fig. 4 D), this incompatibility must exist between the  $\gamma 1$  TM segment and the  $\gamma 2$  C-tail.

Unexpectedly, replacing the TM segment of  $\gamma 3$  with that of  $\gamma 1$  created a  $\gamma 3/\gamma 1$ -TM chimera that completely failed to potentiate  $\gamma 3$ 's modulatory function and instead caused a loss of  $\gamma 3$ 's modulatory function in most recorded excised patches ( $V_{1/2} = 165 \pm 5$  mV,  $n = 5$ ;  $V_{1/2} = 119$  mV,  $n = 1$ ) (Fig. 5 A and Table 1). This outcome suggests that in addition to the partial incompatibility that exists between the  $\gamma 1$  TM segment and the  $\gamma 3$  C-tail, functional incompatibility exists between the  $\gamma 1$  TM segment and the  $\gamma 3$  LRRD.  $\gamma 3$  has a negatively charged residue, D269, which in  $\gamma 1$  is a positively charged residue, R260, located immediately on the N-terminal side of the TM segment (Fig. 1). We arbitrarily included these residues' positions in the LRRDs to construct chimeric BK $\gamma$  proteins (Fig. 1). The D269R mutation itself had no effect on  $\gamma 3$ 's modulatory function (Fig. 5 A), but the  $\gamma 1$  TM segment became partially effective in potentiating the  $\gamma 3^{\text{D269R}}$  mutant's modulatory function. The resultant G-V relationship of the BK channels in the presence of the  $\gamma 3^{\text{D269R}}/\gamma 1$ -TM chimera could be fitted with a double-Boltzmann function to a major population (64%) of low voltage-activated channels ( $V_{1/2} = 62$  mV,  $z = 1.1$  e) and to a minor population (36%) of high voltage-activated channels ( $V_{1/2} = 138$  mV,  $z = 1.1$  e) (Fig. 5 A and Table 1). Therefore, in the presence of the  $\gamma 3$  C-tail, the  $\gamma 1$  TM segment is also incompatible with the extracellular  $\gamma 3$  juxta-membrane charged residue D269. Overall, the  $\gamma 1$  subunit has a special TM segment that is

fully compatible with the  $\gamma 1$  C-tail, partially compatible with the  $\gamma 3$  C-tail, and fully incompatible with the  $\gamma 2$  and  $\gamma 4$  C-tails.

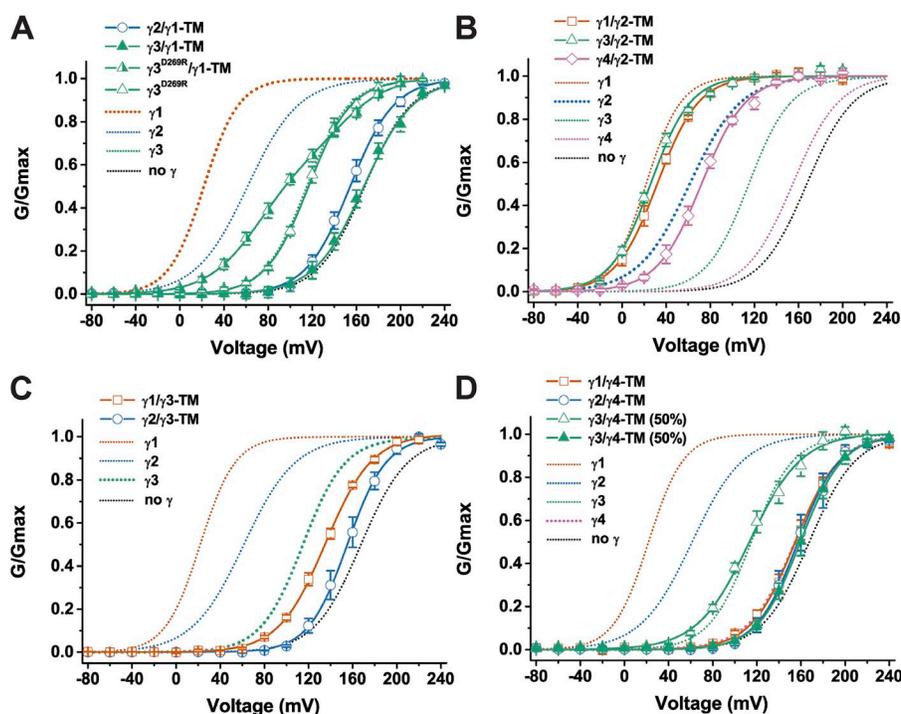
#### The TM segment is a key determinant of BK $\gamma$ subunits' different modulatory functions

The TM segment of  $\gamma 2$ , unlike that of  $\gamma 1$ , was fully compatible with other BK $\gamma$  subunits' C-tails. Upon being swapped with other BK $\gamma$  subunits, the  $\gamma 2$  TM segment fully potentiated the  $\gamma 3$  and  $\gamma 4$  subunits' modulatory functions ( $V_{1/2} = 26 \pm 2$  mV with  $\gamma 3/\gamma 2$ -TM and  $V_{1/2} = 72 \pm 3$  mV with  $\gamma 4/\gamma 2$ -TM) to levels nearly as high as those of  $\gamma 1$  and  $\gamma 2$ , respectively, but caused little change in  $\gamma 1$ 's function ( $V_{1/2} = 32 \pm 3$  mV with  $\gamma 1/\gamma 2$ -TM) (Fig. 5 B). This observation indicates that in the presence of the  $\gamma 1$  or  $\gamma 3$  C-tail, the  $\gamma 2$  TM segment is as effective as the  $\gamma 1$  TM segment in modulating BK channel gating. However, replacing the TM segments of  $\gamma 1$  and  $\gamma 2$  with those of  $\gamma 3$  and  $\gamma 4$  resulted in a 78–92% reduction of  $\gamma 1$  and  $\gamma 2$ 's modulatory functions, which became more similar to those of  $\gamma 3$  and  $\gamma 4$  (Fig. 5, C and D). The resultant BK channel  $V_{1/2}$  values were  $135 \pm 2$  mV with  $\gamma 1/\gamma 3$ -TM,  $155 \pm 4$  mV with  $\gamma 1/\gamma 4$ -TM,  $155 \pm 5$  mV with  $\gamma 2/\gamma 3$ -TM, and  $158 \pm 7$  mV with  $\gamma 2/\gamma 4$ -TM (Table 1). The smaller modulatory effect of the  $\gamma 1/\gamma 3$ -TM chimera compared with that of  $\gamma 3$  was likely caused by the functional difference between the  $\gamma 1$  and  $\gamma 3$  LRRDs. Therefore, the  $\gamma 3$  and  $\gamma 4$  TM segments are similarly ineffective in modifying BK channel voltage gating in the presence of the  $\gamma 2$  or  $\gamma 4$  C-tails, as indicated by similarly high  $V_{1/2}$  values with  $\gamma 4$  and chimeras of  $\gamma 2/\gamma 3$ -TM,  $\gamma 2/\gamma 4$ -TM, and  $\gamma 3/\gamma 4$ -tail.

Nevertheless, in the presence of the  $\gamma 1$  or  $\gamma 3$  C-tail, the  $\gamma 3$ -TM segment appeared to be slightly more effective than the  $\gamma 4$ -TM segment. There was approximately  $-20$ -mV difference in the resultant BK channel  $V_{1/2}$  between  $\gamma 1/\gamma 3$ -TM and  $\gamma 1/\gamma 4$ -TM. Likewise, replacing the TM segment of  $\gamma 3$  with that of  $\gamma 4$  caused the functional conversion of  $\gamma 3$  to be more like that of  $\gamma 4$  ( $160 \pm 4$  mV with  $\gamma 3/\gamma 4$ -TM) in four of the eight examined excised patches (Fig. 5 D). Collectively, the above results suggest that the TM segment is a key determinant of the difference in the modulatory functions between the potent modulators  $\gamma 1$  and  $\gamma 2$  and the much weaker modulators  $\gamma 3$  and  $\gamma 4$ .

#### Effects of the TM segment, C-tail, and LRRD on BK channel voltage-dependent gating

To thoroughly compare the contributions of the LRRD, C-tail, and TM segment to the BK $\gamma$  proteins' modulatory functions on BK channels, we grouped and plotted the shifts in  $V_{1/2}$  ( $\Delta V_{1/2}$ ) of the BK channels caused by all wild-type and chimeric BK $\gamma$  proteins, except those containing the incompatible  $\gamma 1$ -TM segment and other BK $\gamma$  subunits' C-tails (Fig. 6). The BK channel  $V_{1/2}$  values are first determined by the TM segment. The differences between the  $\gamma 1$ - and  $\gamma 2$ -TM segments in their effects on BK channel  $V_{1/2}$  were indistinguishable (i.e.,  $<10$  mV) or small (i.e.,  $10$ – $20$  mV), i.e.,  $-9$  mV between  $\gamma 1$  and  $\gamma 1/\gamma 2$ -TM and  $-20$  mV between  $\gamma 1/\gamma 2$ -LRRD and  $\gamma 2/\gamma 1$ -tail (Fig. 6). As noted above, the differences between the  $\gamma 3$ - and  $\gamma 4$ -TM segments in their effects on the BK channel  $V_{1/2}$  in the presence of the  $\gamma 1$  or  $\gamma 3$  C-tail were limited.



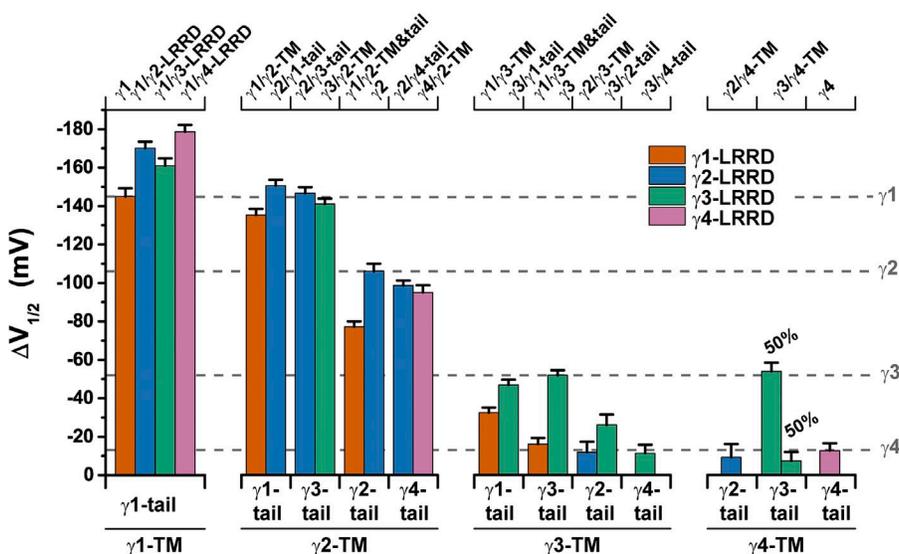
**Figure 5.** Effects of swapping TM segments on the modulatory functions of BK $\gamma$  subunits. Voltage dependence of BK channel activation in the presence of BK $\gamma$  subunit chimeras, whose main bodies were from different  $\gamma$  subunits and whose TM segments were from  $\gamma 1$  (A),  $\gamma 2$  (B),  $\gamma 3$  (C), and  $\gamma 4$  (D). Thick dot lines are used for the major wild-type BK $\gamma$  protein(s) intended for comparison. Error bars represent  $\pm$  SEM.

However, the difference between these two pairs of TM segments ( $\gamma 1$  and  $\gamma 2$  vs.  $\gamma 3$  and  $\gamma 4$ ) in their effects on the BK channel  $V_{1/2}$  was very large ( $\sim 100$  mV). The differences between the  $\gamma 1$ - and  $\gamma 3$ -TM segments in their effects on the BK channel  $V_{1/2}$  were more than  $-110$  mV, for example, a  $-112$ -mV difference between  $\gamma 1$  and  $\gamma 1/\gamma 3$ -TM and a  $-114$ -mV difference between  $\gamma 1/\gamma 3$ -LRRD and  $\gamma 3/\gamma 1$ -tail. Similarly, the average difference between the  $\gamma 2$ - and  $\gamma 3$ -TM segments in their effects on BK channel  $V_{1/2}$  was about  $-95$  mV, with differences of  $-103$  mV between  $\gamma 1/\gamma 2$ -TM and  $\gamma 1/\gamma 3$ -TM,  $-89$  mV between  $\gamma 3/\gamma 2$ -TM and  $\gamma 3$ , and  $-94$  mV between  $\gamma 2$  and  $\gamma 2/\gamma 3$  TM. The differences between the  $\gamma 2$ - and  $\gamma 4$ -TM segments in their effects on the BK channel  $V_{1/2}$  were  $-97$  mV between  $\gamma 2$  and  $\gamma 2/\gamma 4$ -TM and  $-82$  mV between  $\gamma 4/\gamma 2$ -TM and  $\gamma 4$ .

The shifts in BK channel  $V_{1/2}$  values were second determined by the C-tail regions (Fig. 6). The differences between the  $\gamma 1$  and  $\gamma 3$  C-tails and between the  $\gamma 2$  and  $\gamma 4$  C-tails in their effects on the BK channel  $V_{1/2}$  were nearly indistinguishable (i.e.,  $<10$  mV) in the presence of the  $\gamma 2$ -TM segment; for example, the differences between  $\gamma 2/\gamma 1$ -tail and  $\gamma 2/\gamma 3$ -tail and between  $\gamma 2$  and  $\gamma 2/\gamma 4$ -tail were  $-4$  and  $-7$  mV, respectively. Nevertheless, the differences between these two pairs of C-tails in their effects on BK channel  $V_{1/2}$  were at least as high as  $45$  mV, including differences of  $-58$  mV between  $\gamma 1/\gamma 2$ -TM and  $\gamma 1/\gamma 2$ -TM and C-tail ( $\gamma 1$  C-tail vs.  $\gamma 2$  C-tail),  $-45$  mV between  $\gamma 2/\gamma 1$  C-tail and  $\gamma 2$  ( $\gamma 1$  C-tail vs.  $\gamma 2$  C-tail), and  $-48$  mV between  $\gamma 2/\gamma 3$ -tail and  $\gamma 2/\gamma 4$ -tail ( $\gamma 3$  C-tail vs.  $\gamma 4$  C-tail). In the presence of the  $\gamma 3$  TM segment, the differences between the C-tails of individual subunits within each subunit pair (i.e.,  $\gamma 1$  and  $\gamma 3$ ;  $\gamma 2$  and  $\gamma 4$ ) in their effects on the BK channel  $V_{1/2}$  were either small or indistinguishable (e.g.,  $5$  mV between  $\gamma 3/\gamma 1$ -tail and  $\gamma 3$  and  $-16$  mV between  $\gamma 1/\gamma 3$ -TM and  $\gamma 1/\gamma 3$ -TM and C-tail for the difference between the

$\gamma 1$  and  $\gamma 3$  C-tails, and  $-15$  mV between  $\gamma 3/\gamma 2$ -tail and  $\gamma 3/\gamma 4$ -tail for the difference between the  $\gamma 2$  and  $\gamma 4$  C-tails). The differences between these two pairs of C-tails in their effects on the BK channel  $V_{1/2}$  were still significant ( $\sim 20$ – $40$  mV) in the presence of the  $\gamma 3$ - or  $\gamma 4$ -TM segments (e.g.,  $-41$  mV between  $\gamma 3$  and  $\gamma 3/\gamma 4$ -tail for the difference between  $\gamma 3$  and  $\gamma 4$  C-tails,  $-21$  mV between  $\gamma 3/\gamma 1$ -tail and  $\gamma 3/\gamma 2$ -tail for the difference between the  $\gamma 1$  and  $\gamma 2$  C-tails, and  $-26$  mV between  $\gamma 3$  and  $\gamma 3/\gamma 2$  C-tail for the difference between the  $\gamma 3$  and  $\gamma 2$  C-tails), although overall they were smaller than those in the presence of the  $\gamma 2$ -TM segment. Therefore, in the presence of different BK $\gamma$  (i.e.,  $\gamma 2$ ,  $\gamma 3$ , and  $\gamma 4$ ) TM segments, we observed a similar trend: the C-tails of individual subunits within each subunit pair (i.e.,  $\gamma 1$  and  $\gamma 3$ ;  $\gamma 2$  and  $\gamma 4$ ) had more similar functionality, whereas the two pairs themselves differed significantly in functionality.

Collectively, the shifts in BK channel  $V_{1/2}$  values were least affected by differences in LRRDs (Fig. 6). An analysis of six pairs of comparable chimeras formed with a TM segment and C-tail from the same BK $\gamma$  subunit indicated that the LRRDs of  $\gamma 2$ ,  $\gamma 3$ , and  $\gamma 4$  had little difference in their influences on the BK channel  $V_{1/2}$  values, eliciting an average change of only  $10$  mV. A slightly larger difference of  $18$  mV was observed only between the  $\gamma 3$  and  $\gamma 4$  LRRDs in the presence of the  $\gamma 1$ -TM segment and C-tail (i.e.,  $\gamma 1/\gamma 3$ -LRRD vs.  $\gamma 1/\gamma 4$ -LRRD). However, compared with the LRRDs of the other three BK $\gamma$  subunits, the  $\gamma 1$  LRRD caused a larger shift in the BK channel  $V_{1/2}$  of  $14$ – $36$  mV (mean,  $23$  mV) toward depolarizing voltages among seven pairs of comparable chimeras. Therefore, LRRDs are not major determinants of the BK $\gamma$  subunits' different modulatory functions, and they instead may slightly alleviate the functional difference between  $\gamma 1$  and other BK $\gamma$  subunits.



**Figure 6.** Shifts in BK channel  $V_{1/2}$  ( $\Delta V_{1/2}$ ) values caused by different wild-type, chimeric, or mutant BK $\gamma$  proteins. The names of the BK $\gamma$  proteins appear above the graph, and the corresponding compositions of the TM and C-tail regions from different BK $\gamma$  subunits appear below the graph. Each LRRD is indicated by a different color. The  $\Delta V_{1/2}$  values with BK $\gamma$  wild types are indicated by dotted gray lines. Error bars represent  $\pm$  SEM.

Juxta-membrane positively charged residues determine the C-tail functions of  $\gamma 1$  and  $\gamma 3$

Each of the four BK $\gamma$  subunits' C-tails contains a cluster of positively charged residues that is adjacent to the C terminus of the TM segment (Fig. 1). This residue cluster might be involved in the proper insertion and orientation of the TM segment, which follows the general "positive-inside rule" (White and von Heijne, 2004). The juxta-membrane cluster of positively charged amino acids in  $\gamma 1$  is heavily charged with six arginine residues within a peptide sequence of eight residues ( $^{291}\text{RARRRRLR}^{298}$ ), whereas the clusters in  $\gamma 2$  and  $\gamma 3$  contain only one arginine, one lysine, and one histidine in short sequences of  $^{277}\text{RHK}^{279}$  ( $\gamma 2$ ) and  $^{298}\text{HRWSK}^{304}$  ( $\gamma 3$ ). To determine whether these clusters are major contributors to the modulatory functions of C-tails, we performed deletion and substitution mutations in the C-tail regions and assessed the mutated C-tails' effects on BK channel voltage-dependent gating. Deleting most or all residues on the C-terminal side of the clusters in  $\gamma 1$  and  $\gamma 3$  did not significantly change the subunits' modulatory functions ( $V_{1/2} = 29 \pm 4$  mV with  $\gamma 1\text{-}\Delta\text{tailC}^{302-334}$  and  $V_{1/2} = 109 \pm 3$  mV with  $\gamma 3\text{-}\Delta\text{tailC}^{305-311}$ ) (Fig. 7 A). However, similar to a previous report (Yan and Aldrich, 2012), deletion of the polyarginine region in  $\gamma 1$  caused a 100% loss of  $\gamma 1$ 's modulatory function ( $V_{1/2} = 168 \pm 3$  mV with  $\gamma 1\text{-}\Delta\text{tailN}^{291-298}$ ). These results suggest that the juxta-membrane positively charged region is more than an important contributor to the  $\gamma 1$ -induced shift in BK channel voltage-dependent gating; rather, the region is essential for the whole  $\gamma 1$  protein function, presumably by determining the protein's proper folding, surface expression, or assembly with BK $\alpha$ . Similarly, deletion of the positively charged region  $^{298}\text{HRWSK}^{304}$  in  $\gamma 3$  resulted in a loss of  $\gamma 3$ 's modulatory function, yielding a  $V_{1/2}$  ( $180 \pm 3$  mV with  $\gamma 3\text{-}\Delta\text{tailN}^{298-304}$ ) close to that of BK $\alpha$  alone (Fig. 7 A). Neutralization of the last lysine residue (K304) in this cluster also reduced  $\gamma 3$ 's modulatory effect on the BK channel  $V_{1/2}$  by nearly 30 mV ( $V_{1/2} = 144 \pm 2$  mV with  $\gamma 3^{\text{K304N}}$  mutant vs.  $115 \pm 2$  mV with wild type) (Fig. 7 A).

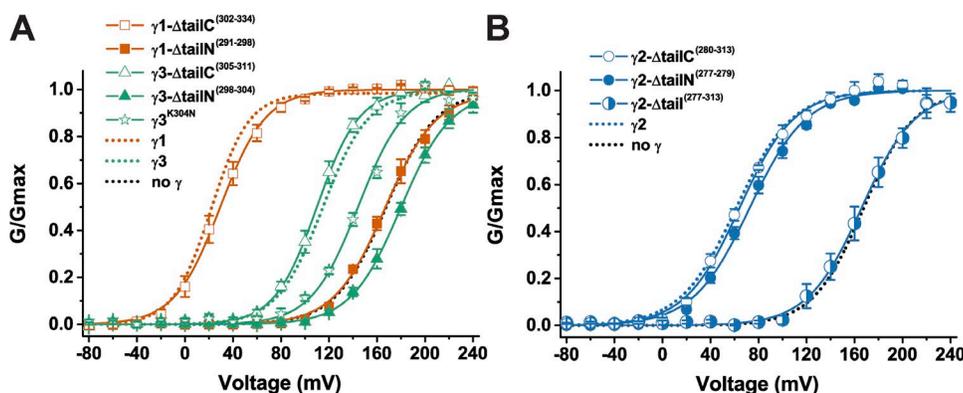
For the  $\gamma 2$  C-tail, the deletion of either the three-residue cluster  $^{277}\text{RHK}^{279}$  or the rest of the C-tail region to

the C-terminal end alone did not significantly alter  $\gamma 2$ 's modulatory function ( $V_{1/2} = 73 \pm 3$  mV with  $\gamma 2\text{-}\Delta\text{tailN}^{277-279}$  and  $V_{1/2} = 63 \pm 2$  mV with  $\gamma 2\text{-}\Delta\text{tailC}^{280-313}$ ). However, the deletion of both regions resulted in a 100% loss of  $\gamma 2$ 's modulatory function ( $V_{1/2} = 166 \pm 6$  mV with  $\gamma 2\text{-}\Delta\text{tail}^{277-313}$ ) (Fig. 7 B). These results suggest that some amino acid residues on the C-tail—not necessarily the positively charged residues immediately following the TM segment—are still needed for  $\gamma 2$  to be functional. There are multiple positively charged and negatively charged residues on the C-terminal side (residues 280–313) of the three-residue cluster  $^{277}\text{RHK}^{279}$  in  $\gamma 2$ 's C-tail region.

## DISCUSSION

In this study, by swapping structural elements among BK $\gamma$  subunits and by performing deletion and substitution mutations, we were able to systematically analyze the functional contributions of the BK $\gamma$  subunits' individual structural elements to the subunits' different modulatory functions. We identified the TM segment and the adjacent intracellular clusters of positively charged amino acids as key determinants of the distinct effects that different BK $\gamma$  subunits have on BK channel voltage gating. Our results suggest that BK channel modulation by BK $\gamma$  subunits involves major intra- and/or juxta-membrane mechanisms. Our findings also reveal a strategy in which the modulatory functions of regulatory membrane proteins of voltage-gated ion channels can be coarse- and fine-tuned, respectively, through variations in their TM segments and adjacent intracellular positively charged region.

A previous study showed that deletion of a signal peptide, TM segment, or C-tail, or even a single LRR unit of the LRRD, resulted in a nearly 100% loss of the  $\gamma 1$ 's modulatory function (Yan and Aldrich, 2010), which could be complicated by disruptions in the subunit's 3-D structure. To maintain structural integrity, in this study, we systematically swapped individual structural elements among the four BK $\gamma$  subunits to identify the molecular determinants of their different modulatory



**Figure 7.** Effects of deletion and substitution mutations within the C-tail regions on the modulatory functions of BK $\gamma$  subunits. The voltage dependence of BK channel activation in the presence of  $\gamma 1$  and  $\gamma 3$  (A) or in the presence of  $\gamma 2$  (B) mutants is shown. Error bars represent  $\pm$  SEM.

functions. Of the 37 BK $\gamma$  chimera or mutants we examined, 20 effectively modulated BK channels and produced G-V curves that could be fitted by a single Boltzmann function with a normal slope ( $z > 1$ ) and more than 30-mV shifts in  $V_{1/2}$  compared with that formed by BK $\alpha$  alone. According to the previously described “all-or-none” gating shift for the  $\gamma 1$  subunit (Gonzalez-Perez et al., 2014), the property of effective shift in  $V_{1/2}$  and the resultant normal G-V slope is a good indication that these 20 BK $\gamma$  chimera or mutants formed functional complexes with BK $\alpha$  that reflect full stoichiometric assembly. Indeed, four of the other chimera and mutants resulted in two populations of channels, one with a significant shift in voltage-dependent gating and the other with a voltage dependence more similar to the channel formed by BK $\alpha$  alone. Similar variable ratio of two populations of BK channels, whose G-V curves were either shifted to a full extent or unchanged (“all-or-none”), was reported when the molar ratio of the injected BK $\alpha$ / $\gamma 1$  RNA to *Xenopus* oocytes was varied (Gonzalez-Perez et al., 2014). Therefore, these proteins were likely partially defective in folding, surface expression, or proper assembly with BK $\alpha$ . For three of these four chimera and mutants, we unexpectedly observed some patch-to-patch variation that resulted in two distinct G-V relationships; e.g., the recorded BK channels were dominated by a low  $V_{1/2}$  population in three excised membrane patches but by a high  $V_{1/2}$  population in another eight patches in the presence of  $\gamma 1$ / $\gamma 3$ -tail (Table 1). This variation might arise from patch-to-patch variation caused by differences in cell membrane composition (such as the presence or absence of lipid rafts) and/or cell-to-cell variation in protein trafficking machinery. The remaining 13 BK $\gamma$  chimera or mutants resulted in  $V_{1/2}$  values within 30 mV of that produced by the BK $\alpha$  alone; these proteins may be only slightly effective in modifying the BK channel voltage gating or may have failed to fold, express on the cell surface, or assemble with BK $\alpha$  properly. Because we designed the present study to identify key determinants of the apparent differential modulatory effects of the four BK $\gamma$  subunits on BK channels, we consider these non- or less-modulatory BK $\gamma$  chimera or mutants, regardless of the causes, still useful when comparing with other chimeras or mutants for the apparent differential modulatory effects.

The BK $\gamma$  subunits are type I single-pass TM proteins. Similar to the reported essential role of the N-terminal signal peptide in  $\gamma 1$  function (Yan and Aldrich, 2012), we expect that those of other BK $\gamma$  subunits also play a key role in the subunits’ maturation and proper surface expression. An ineffective signal peptide can cause a defect in cellular localization or protein folding, leading to a dysfunctional protein. The sequences of the four BK $\gamma$  subunits’ N-terminal signal peptide regions have very little similarity (Fig. 1). By swapping distinct signal peptide regions among BK $\gamma$  proteins, we first demonstrated

that the BK $\gamma$  proteins’ different modulatory functions are intrinsic properties of the proteins and are independent of the proteins’ signal peptide sequences. The four BK $\gamma$  subunits are closely related to each other mainly because of their LRRDs, which share a considerable level of sequence identity (31–37%) or similarity (43–48%) and have been predicted to have overall similar 3-D structures (Yan and Aldrich, 2012) (Figs. 1 and 2). We initially expected all four BK $\gamma$  subunits’ LRRDs to play a critical role in determining their subunits’ differential modulatory functions, but to our surprise, only the  $\gamma 1$  LRRD was slightly different from the other LRRDs in function in BK $\gamma$  chimeric proteins. The  $\gamma 1$  LRRD may play a slightly inhibitory role, or the other BK $\gamma$  subunits’ LRRDs may play a slightly stimulatory role, in BK channel voltage-gated activation.

On the basis of the results of our functional analyses of the LRRDs, TM segments, and C-tails in the BK $\gamma$  subunit chimeras, we concluded that the TM and the C-tail regions are the main determinants of the four BK $\gamma$  subunits’ differential modulatory functions. As seen in Fig. 6, the differences in the different  $\gamma$  subunit-induced shifts of the BK channel  $V_{1/2}$  are first determined by the TM segments, in which the  $\gamma 1$  and  $\gamma 2$  TMs produced low  $V_{1/2}$  BK channels, whereas the  $\gamma 3$  and  $\gamma 4$  TM segments all resulted in high  $V_{1/2}$  channels. The differences between the TM segments of  $\gamma 1$  and  $\gamma 2$  and between those of  $\gamma 3$  and  $\gamma 4$  in their effects on BK channel  $V_{1/2}$  were small, but the difference between the TM segments of the two BK $\gamma$  subunit pairs themselves was large ( $\sim 100$  mV). The C-tails further adjust the modulatory functions of the four BK $\gamma$  subunits. Although the differences between the C-tails of  $\gamma 1$  and  $\gamma 3$  and between those of  $\gamma 2$  and  $\gamma 4$  in their effects on BK $\gamma$  modulatory functions were small, the difference between the two pairs’ C-tails in their effects on BK channel  $V_{1/2}$  could be big ( $\sim 40$ – $50$  mV), which could be largely accounted for by the different modulatory functions between  $\gamma 1$  and  $\gamma 2$  and between  $\gamma 3$  and  $\gamma 4$ . We also found that the functions of the  $\gamma 1$  and  $\gamma 3$  C-tails are determined by the juxta-membrane clusters of positively charged amino acids. On the basis of these findings, we generalized the relative contributions of the four BK $\gamma$  subunits’ individual structural elements to the subunits’ modulatory functions in BK channel voltage-dependent gating (Fig. 2).

Our findings demonstrate that the TM segments of  $\gamma 1$  and  $\gamma 2$  play a major role in setting these subunits’ high modulatory efficacies, and that the  $\gamma 1$  and  $\gamma 3$  subunits’ juxta-membrane clusters of positively charged amino acids also significantly contribute to these subunits’ modulatory functions. It was reported previously that only the TM segment is critical for the physical association of  $\gamma 1$  with BK $\alpha$  (Yan and Aldrich, 2010). Therefore, the physical association or interaction between the BK $\gamma$  TM segments and BK $\alpha$  may be a main mechanism by which the  $\gamma 1$  and  $\gamma 2$  subunits modulate BK channel

voltage-dependent gating. In principle, this possibility is consistent with the previous notion that  $\gamma 1$  mainly affects the allosteric coupling (D-factor) between the voltage-sensor domain and the pore (Yan and Aldrich, 2010), which likely occurs in the membrane. The other BK $\gamma$  subunits, particularly  $\gamma 2$ , whose TM segment is similar to that of  $\gamma 1$  in function, may have a similar mechanism underlying their BK channel modulation. According to the HA allosteric model (Horrigan and Aldrich, 2002), an increase in D-factor may slightly steepen the slope of the G-V relationship curve, as reflected by an increased  $z$  value obtained from a Boltzmann fit. We found that, compared with other BK $\gamma$  subunits,  $\gamma 1$  increased the  $z$  value of the BK channels to a greater degree. When the C-tail of  $\gamma 2$  was replaced with that of  $\gamma 1$  or  $\gamma 3$ ,  $\gamma 2$  elicited  $V_{1/2}$  and  $z$  values approaching those of  $\gamma 1$ , suggesting that the C-tail has a large impact on the slope of the G-V curve. However, because a slight modification in the voltage-sensing parameters  $Z_j$  or  $J_0$  can have a marked effect on the slope of the G-V curve, de-convolving the G-V curve to identify contributions from different HA model gating parameters to its slope is difficult.

The way in which the BK $\gamma$  subunits' C-tails contribute to the subunits' modulatory functions remains unclear. The C-tails may contribute to the modulatory functions of BK $\gamma$  subunits through two distinct mechanisms. First, the C-tail or its juxta-membrane cluster of positively charged amino acids may be required for BK $\gamma$  to be functional, presumably by ensuring the proper insertion or orientation of the TM segment in accordance with the positive-inside rule (White and von Heijne, 2004). This possibility would explain why the deletion of C-tails or even the juxta-membrane clusters of positively charged amino acids led to a nearly 100% loss of the modulatory function of  $\gamma 1$  and  $\gamma 3$  in the present study. Second, through one or more unknown mechanisms, the juxta-membrane positively charged residues may directly affect BK channel gating independently of their influence on the TM segments. This would account for the additional shift in the BK channel  $V_{1/2}$  (approximately -40 mV) and the increase in the steepness of the G-V curve caused by the  $\gamma 1$  and  $\gamma 3$  C-tails as compared with the  $\gamma 2$  and  $\gamma 4$  C-tails in the presence of  $\gamma 2$ 's LRRD and TM segments. Alternatively, the juxta-membrane clusters of positively charged amino acids in  $\gamma 1$  and  $\gamma 3$  may indirectly affect BK channel gating by interacting with the TM segments. This indirect mechanism might be similar to the positive-inside rule mechanism in that it alters the structure of the TM segment to enhance the function of the BK $\gamma$  subunit, which would be consistent with our observation that the effects of individual C-tails on BK channel voltage gating somehow varied in a TM segment-dependent manner. The functional incompatibility of  $\gamma 1$ 's TM segment with other BK $\gamma$  subunits' C-tails suggests their strong interactions. However, a precise delineation of the juxta-membrane border between

the TM segment and intracellular C-tail will be needed in future studies to clearly define their separate modulatory functions and determine their interactions.

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