

A short motif in the C-terminus of mouse bestrophin 4 inhibits its activation as a Cl channel

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Abstract Bestrophins are a new family of anion channels. Here, we examined the Cl channel activity of mBest4. Surprisingly, wild type mouse bestrophin-4 (mBest4) did not induce functional Cl channels when over-expressed in HEK293 cells. However, deletion of part of the C-terminus (residues 353–669) produced large Cl currents, suggesting the presence of a C-terminal motif that inhibited Cl channel function. Deletion of a short motif (356–364) or substitution of certain residues in this motif with alanines also resulted in expression of robust Cl currents. The channel activity of the mBest4 protein lacking the C-terminus (residues 353–669) was specifically inhibited by co-expression of C-terminal fragments of mBest4 having the inhibitory motif, suggesting that the C-terminal motif blocked mBest4 channel activity probably by interacting with the channel pore.
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1. Introduction

Bestrophins are a new family of Cl channels [1,8,11,13] with a different structure than other well-studied Cl channels such as cystic fibrosis transmembrane conductance regulator, voltage-gated Cl channel family channels, and GABA receptors [2]. In humans, there are four bestrophin homologues (hBest1–4) [12,14]. hBest1 was positionally cloned from families with Best vitelliform macular dystrophy, an early-onset form of macular degeneration [5,7]. All four human bestrophins induce Cl currents when over-expressed [13].

Four bestrophin homologues also exist in mice [3]. However, only mBest2 has been reported to induce Cl currents in heterologous expression [8,9]. Wild type mouse bestrophin 4 (mBest4)¹ was first cloned from mouse heart [3]. Its first 363 amino acids are highly homologous to other bestrophins, but it has a unique C-terminus. Here, we identified a short motif

located in the mBest4 C-terminus, which blocked mBest4 Cl channel activity.

2. Materials and methods

2.1. Production of mouse bestrophin 4 mutations and heterologous expression

mBest4 (Vmd213) cDNA was cloned from mouse heart mRNA by RT-PCR. The sequence agreed with Genbank accession AY450426 and Swissprot accession Q6H1V1. The cDNA was subcloned into pcDNA3.1 (Invitrogen). Site-specific mutations and deletions were made using a PCR-based mutagenesis kit (Quickchange; Stratagene) as described previously [8]. The human ortholog of mBest4 (called hBest3 [14], Genbank accession AAR99656) was provided by Dr. Jeremy Nathans (Johns Hopkins University). cDNAs were cotransfected into HEK293 cells (ATCC) using FuGene-6 transfection reagent (Roche) and pEGFP (Invitrogen) to identify transfected cells. 0.1–0.3 µg cDNA was used to transfect one 35-mm culture dish.

2.2. Electrophysiology

Recordings were performed at 22–24 °C using the whole-cell configuration of the patch clamp [6]. Voltage clamp steps or ramps were used as indicated in the figures with a holding potential of 0 mV. The standard pipette solution (~4.5 µM free Ca) contained (in mM) 146 CsCl, 2 MgCl₂, 5 (Ca²⁺)-EGTA, 8 HEPES, 10 sucrose, pH 7.3, adjusted with NMDG. Low intracellular Ca solution (<20 nM free Ca) contained (in mM) 146 CsCl, 2 MgCl₂, 5 EGTA, 8 HEPES, 10 sucrose, pH 7.3. The standard extracellular solution contained (in mM) 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH 7.3 with NaOH. This combination of solutions set E_{rev} for Cl⁻ currents to zero, while cation currents carried by Na⁺ or Cs⁺ have very positive or negative E_{rev} , respectively. Anion permeability relative to Cl was determined by measuring the shift in E_{rev} upon changing the bath solution from one containing 150 mM Cl⁻ to another with 140 mM X⁻ and 10 mM Cl⁻, where X is the substitute anion [10]. The permeability ratio was estimated using the Goldman–Hodgkin–Katz equation:

$$P_x/P_{Cl} = [Cl^-]_i / ([X^-]_o \exp(\Delta E_{rev} F/RT)) - [Cl^-]_o / [X^-]_o,$$

where ΔE_{rev} is the difference between the reversal potential with the test anion X⁻ and that observed with symmetrical Cl⁻, and F, R, and T have their normal thermodynamic meanings.

3. Results and discussion

3.1. C-terminal deletions allow mBest4 to express functional Cl currents

Because the level of mBest4 transcript was reported to be high in mouse heart [3], we cloned mBest4 cDNA from this tissue to study its physiological function. However, it did not induce significant Cl currents when transiently transfected into HEK293 cells (Fig. 1A). This was surprising because the human

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¹ mBest4 constructs with truncated C-termini were named Δ(residue range), where “residue range” are the positions of the deleted amino acids. C-terminal fragments were named as mBest4(residue range) where “residue range” refers to the amino acid positions that were present in the construct.

Abbreviations: mBest4, mouse bestrophin 4; SCN, thiocyanate; HEK, human embryonic kidney; TMD, transmembrane domain

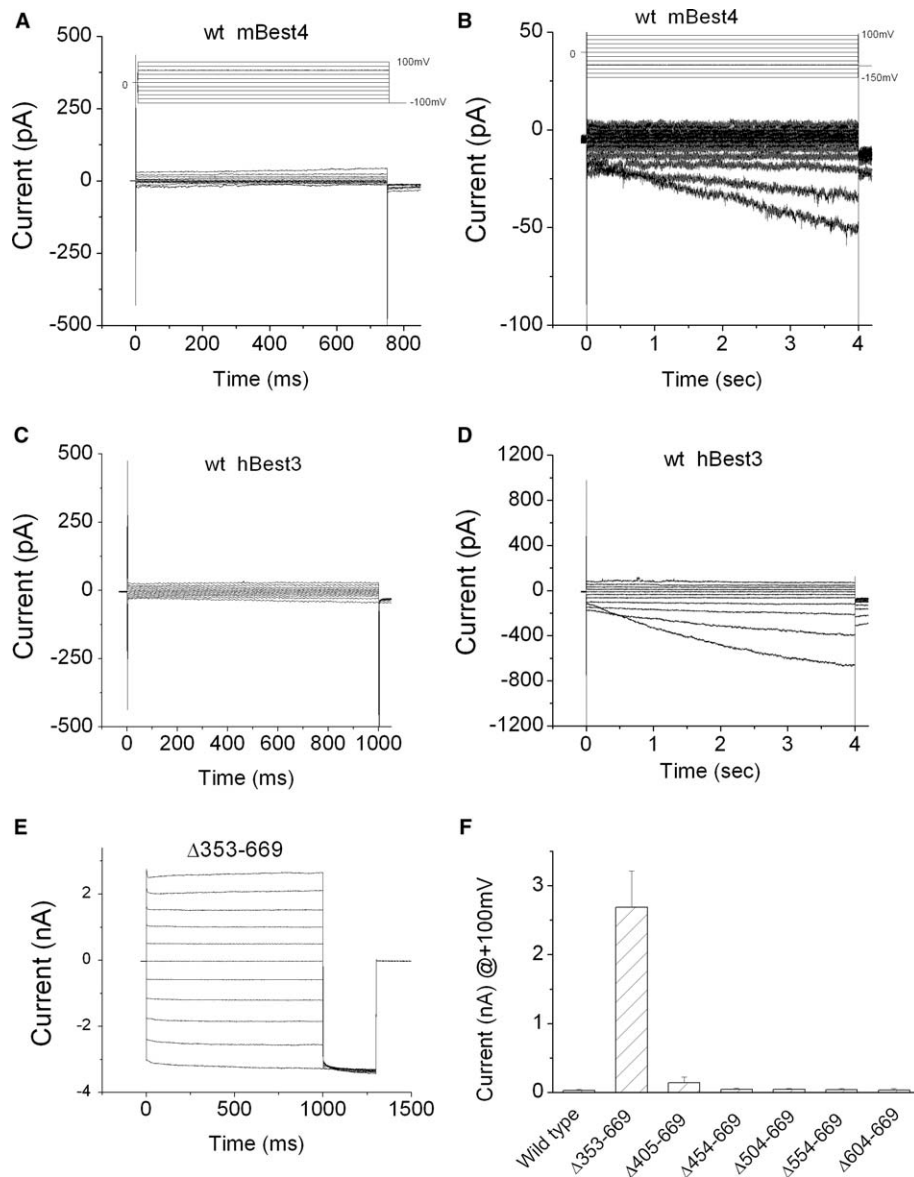


Fig. 1. Activation of mBest4 as a Cl channel in HEK293 cells by truncation of C-terminus. Whole cell currents in response to voltage steps (750 ms steps between -100 and 100 mV) in HEK293 cells transiently transfected with wild type mBest4 (A), wild type hBest3 (C) and mBest4- $\Delta 353-669$ (E); Four seconds steps between -150 and 100 mV with wild type mBest4 (B) or hBest3 (D). F Average Cl currents at the end of the $+100$ mV pulse for wild type mBest4 and six C-terminal truncation mutants. $0.3 \mu\text{g}$ cDNA was used for transfection for A, B, E and F, but $0.6 \mu\text{g}$ cDNA was used for C and D (n equals 3–7).

co-expressed mBest4(292–669) and mBest4(353–669) both of which contain the inhibitory motif (Fig. 3B). However, mBest4(292–669) with residues 353–404 deleted did not block the $\Delta 353-669$ -induced Cl currents (Fig. 3). This result supports our hypothesis that the motif in C-terminus inhibits mBest4 activation through its direct action on the channel pore.

3.4. mBest4-FLG/AAA mutant has a large conductance to SCN

To characterize the channel properties of mBest4, we examined the relative anion permeability and conductance of the mBest4-FLG/AAA mutant. Unlike hBest1 and mBest2 [8,11,13], mBest4-FLG/AAA did not require intracellular Ca to be activated (Fig. 4A). Although this experiment suggests that wild type mBest4 is Ca-independent, the possibility exists

that the FLG/AAA mutation alters the Ca-dependence of mBest4.

mBest4-FLG/AAA-induced whole-cell currents were largely voltage-independent but had a very small inactivating component at positive potentials and a small deactivating tail current upon hyperpolarization (Fig. 4B). The steady-state current–voltage ($I-V$) relationship was linear (Fig. 4C). With symmetrical [Cl] on both sides of the membrane, the current reversed close to zero as expected for Cl currents. The mBest4-FLG/AAA mutant showed a lyotropic order of anion permeability: $\text{SCN} > \text{Br} > \text{I} > \text{Cl}$ (Fig. 4D and E) like many other Cl channels including mBest2 [2]. The thiocyanate (SCN) conductance of the mBest4-FLG/AAA channel was greater than Cl conductance ($G_{\text{SCN}}/G_{\text{Cl}} = 2.0 \pm 0.05$) (Fig. 4D and F). This characteristic distinguishes mBest4 from mBest2 Cl currents which are

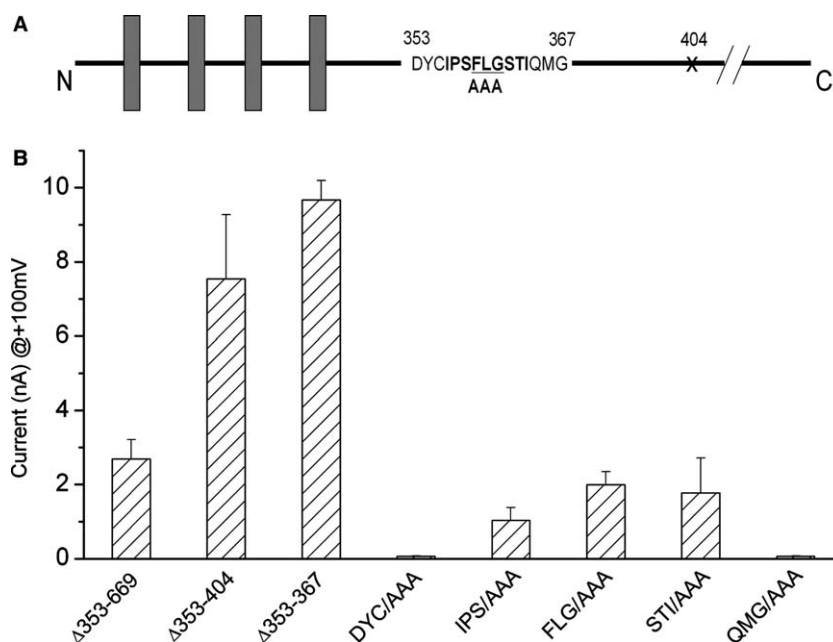


Fig. 2. Detection of an inhibitory motif in mBest4 C-terminus. (A) A schematic model for mBest4. Vertical rectangles represent TMDs. The amino acid sequence of the inhibitory motif is shown in single letter code. (B) Average Cl currents recorded at +100 mV from HEK293 transfected with mBest4 mutants (n equals 3–8). 0.3 μ g DNA was used for transfection of Δ 353–669 but 0.1 μ g was used for other mutants. Currents induced by all mutants were time and voltage-independent and had E_{rev} very close to E_{Cl} .

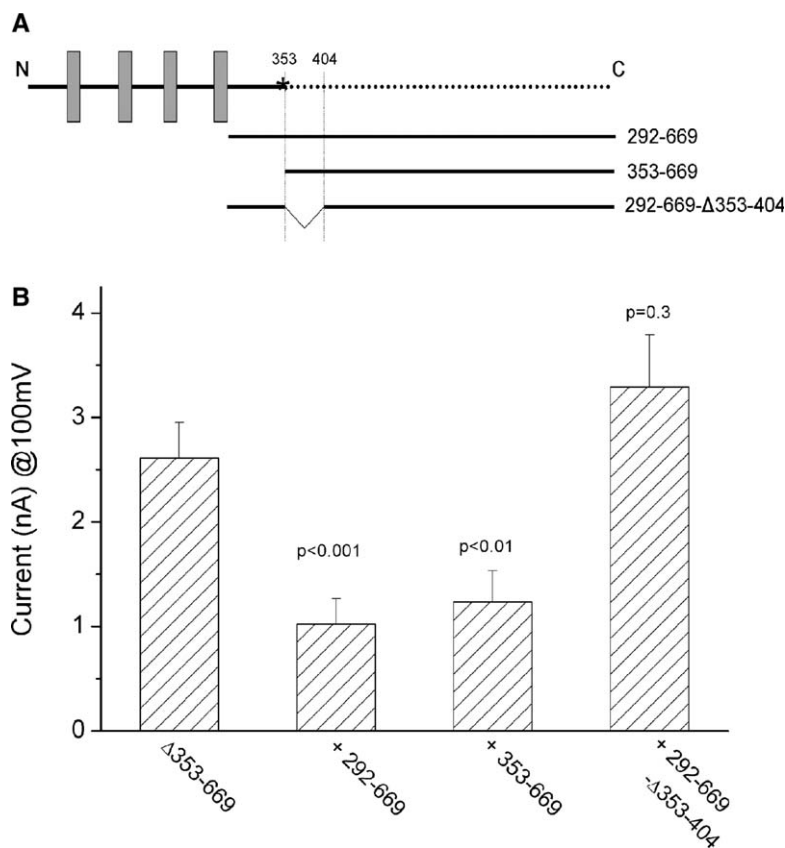


Fig. 3. Block of Δ 353–669-induced Cl currents by co-expression of mBest4 C-termini. (A) Design for mBest4 C-terminal domains co-transfected with Δ 353–669. (B) Average Cl currents from HEK293 cells transfected with Δ 353–669 alone or Δ 353–669 plus mBest4(292–669), mBest4(353–669) or mBest4(353–669 to Δ 353–404). Δ 353–669 mutant and mBest4 C-terminal domains were co-transfected to HEK293 cells with equal amounts of DNA (0.3 μ g) ($n = 9$ –17).

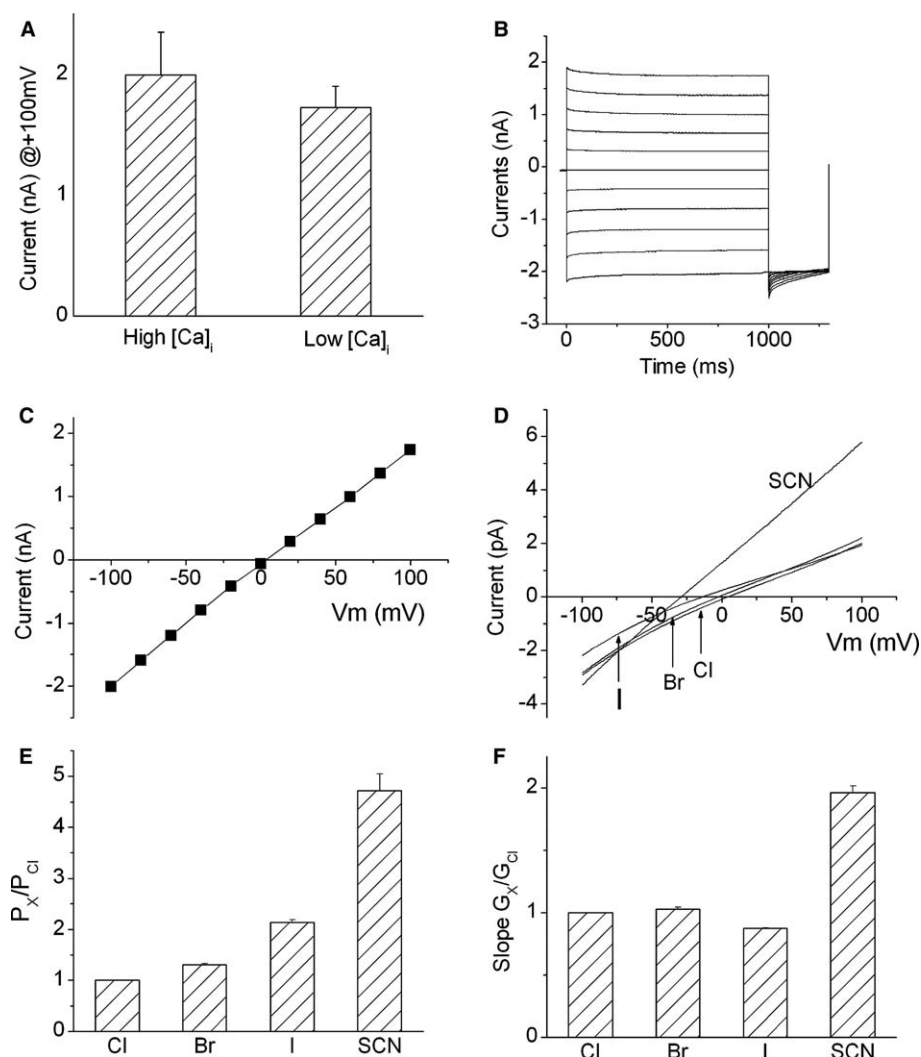


Fig. 4. *Ca*-independence and anion permeability and conductance of activated mBest4-FLG/AAA mutant. (A) mBest4-FLG/AAA-expressed Cl currents are not *Ca*-dependent. Whole-cell recordings were performed with a step voltage protocol described in 2. ($n = 6$ and 3, $P > 0.5$). (B) Steady-state currents induced by FLG/AAA mutant. (C) *I*-*V* curves of currents (B) plotted at the end of each trace. (D) Effect of external anions on the *I*-*V* relationships in FLG/AAA mutant. FLG/AAA-expressed Cl channels from a representative cell show a lyotropic sequence of anion permeability (SCN > I > Br > Cl), indicated by the shift of reversal potentials after shifting to different anions in the bath. (E) Average relative anion permeability ratios (P_{SCN}/P_{Cl}) in FLG/AAA mutant. The difference in reversal potentials between Cl and other anions in panel D was used for calculation of relative permeability ratios with the Goldman-Hodgkin-Katz equation (see 2). (F) Average slope relative conductance ratios (G_{SCN}/G_{Cl}) in FLG/AAA mutant. The G_{SCN}/G_{Cl} ratios were obtained from the measurement of the slope of the current-voltage relationship between -25 and $+25$ mV from reversal potentials in panel D ($n = 4-6$ for B-F).

blocked by SCN ($G_{SCN}/G_{Cl} = 0.14$) [8]. The FLG/AAA mutant had similar conductance to Cl, Br and I anions (Fig. 4D and F).

The difference in relative conductance for SCN and Cl between mBest2 and mBest4 might be explained by differences in amino acids in transmembrane domain 2 which we have shown plays a role in anion permeation in mBest2 [8,9]. For instance, S₇₄, L₇₅, S₇₉, and H₉₁ residues in mBest2 TMD2 are E₇₄, Q₇₅, T₇₉, and N₉₁ in mBest4 TMD2. The study implies that mBest2 and mBest4 may have distinctive channel pore structures and play different physiological roles.

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