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BRIEF COMMUNICATION

C-Terminal Membrane Association of Bestrophin 3 and Its Activation as a Chloride Channel

Xiaohua Han · Zhiqiang Qu · Junxia Xie · Hong Jiang

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Abstract Bestrophin 3 (Best3), a member of the bestrophin Cl^- channel family, is a candidate of cGMP-sensitive, Ca^{2+}-activated Cl^- channel in vascular smooth muscle cells. The Best3 channel was recently found to play an important role in vasomotion. However, the mechanism for its activation has not been clarified. In previous studies, we found that a Best3 C-terminal sequence (amino acids 353–404) was associated with the cellular membrane. The sequence includes an autoinhibitory domain (^{356}IPSFLGS^{362}) and a downstream basic residue domain (amino acids 384–397). In this study, we found that the sequence (368–383) between the two domains is actually a determinant for Best3 C-terminal membrane associability. Deletion of the sequence almost abolished the membrane association but did not activate the Best3 channel. Treatment of Best3-expressing HEK293 cells with the PI3Kα inhibitor IV (a Best3 activator) could not abolish but weakened the Best3 membrane association. The result supports the assumption that the positively charged basic residues in the Best3 C terminus are likely associated with the membranous negatively charged phospholipids, which plays a role in the regulation of Best3 activation. But the relationship between membrane associability and Best3 activation seems more complicated than expected.

X. Han (✉) · Z. Qu (✉) · J. Xie · H. Jiang
Department of Physiology, Qingdao University Medical College, Qingdao 266071, People's Republic of China
e-mail: xiaohua.han@163.com

Z. Qu
e-mail: zqu30033@yahoo.com

Z. Qu
Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA

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Introduction

The Ca^{2+}-activated Cl^- channels (CaCCs) play important roles in regulating the tone of vascular smooth muscle cells (VSMCs) (Large and Wang 1996). As a specific kind of CaCC in the vascular wall, the cGMP-sensitive, Ca^{2+}-activated Cl^- channel ($CaCC_{cGMP}$) has attracted broad attention recently. The molecular identity of the $CaCC_{cGMP}$ channel has been suggested to be the product of the bestrophin 3 (Best3) gene in VSMCs (Matchkov et al. 2008); another study has confirmed that Best3, which functions as an anion channel, was directly involved in vasomotion (Broegger et al. 2011). By transfecting rat mesenteric small arteries in vivo with Best3-specific siRNA, this study demonstrates that Best3 is important for synchronization of VSMCs, helping to generate vasomotion. Best3, however, is not important for tonic contraction in the arteries.

Because of the physiological importance of Best3 in vascular function, it is worthwhile to know how the channel is activated. Nonetheless, Best3 expressed in heterologous cells induced few Cl^- currents with a physiological range of stimulations (Tsunenari et al. 2003; Qu et al. 2006), and the mechanism for its activation remains elusive. In previous studies, we found a membrane-associated sequence (amino acids 353–404) which was important for Best3 channel activation (Qu et al. 2006, 2007). The low activity of the channel in heterologous cells was thought to be due to an autoinhibitory (AI) domain composed of seven amino acids (^{356}ISPFLGS^{362}). However, mutation or deletion of the AI domain from the Best3 C terminus did not abolish the membrane association

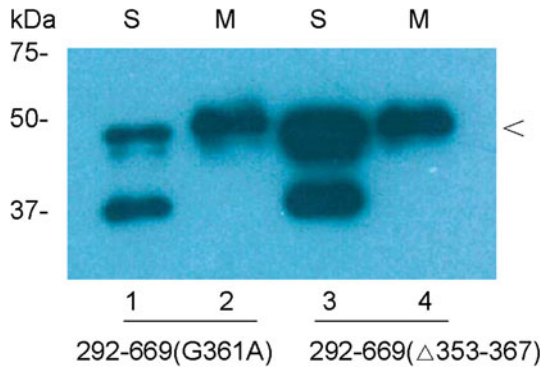

Fig. 1 Mutation or deletion of AI domain did not abolish mBest3 C-terminal membrane associability. The G361A mutation was introduced into the AI domain (^{356}IPSFLGS^{362}); the AI domain (Δ353–367) was deleted from the mBest3 C terminus (292–669) cDNA in pcDNA3.1 vector. Mutants were respectively transfected into HEK293 cells for 2 days. The cellular proteins in soluble (S) and membrane (M) fractions were extracted and subjected to Western blotting. The *arrowhead* indicates the expected protein bands (~45 kDa). Bands with smaller molecular masses (~37 kDa) in *lanes 1* and *3* were probably the protease-degraded proteins. One representative of three similar results is presented

(Fig. 1). This result led to the assumption that downstream of the AI domain some other amino acids may be involved in membrane associability and Best3 activation. As expected, we confirmed eight positively charged basic amino acids, from 384 to 397, which also functioned as regulators of Best3 activity (Qu et al. 2010). Mutations of these basic amino acids significantly activated Best3 as a Cl^- channel. Based on the assumption that the basic amino acids may directly associate with negatively charged membranous phospholipids, we discovered that the phosphatidyl inositol 3-kinase α (PI3Kα) inhibitor IV, which theoretically reduces the amount of the membranous phospholipid, could strongly activate Best3 (Qu et al. 2010).

In this study, we further observed the C-terminal membrane associability of mouse Best3 (mBest3) to explore the relationship between membrane associability and channel activation. We found that the intermediate part (368–383) between the AI and the basic residue domains was a determinant of mBest3 membrane associability. Deletion of the sequence almost abolished the membrane association but failed to activate the mBest3 channel. The result does not support our previous hypothesis that Best3 is activated when its C terminus is disassociated from the cellular membrane. However, when Best3-expressing HEK293 cells were treated with the PI3Kα inhibitor IV (a Best3 activator), the Best3 membrane association was not abolished but was weakened, supporting the assumption that the positively charged basic residues in the Best3 C terminus are likely associated with the membranous negatively charged phospholipids, which probably plays a role in the regulation of Best3 activation. Based on the above results, we believe that the relationship between the C-terminal membrane associability and Best3 channel activation is not straightforwardly correlated.

Materials and Methods

Materials

The PI3Kα inhibitor IV was purchased from Calbiochem (San Diego, CA), dissolved in DMSO as a stock solution (10 mM) and kept at −20 °C before use.

Generation of mBest3 Mutations and Expression in HEK293 Cells

mBest3 in pcDNA3.1 vector was mutated or deleted site-specifically using a PCR-based mutagenesis kit (Quick-change; Stratagene, La Jolla, CA) (Qu et al. 2006). cDNA expression vectors were cotransfected with enhanced green fluorescent protein (pEGFP) (Invitrogen, Carlsbad, CA) into HEK293 cells (ATCC, Rockville, MD) using FuGene-6 transfection reagent (Roche, Indianapolis, IN). About 0.3 μg of plasmid DNA was used to transfect HEK293 cells on one 35-mm culture dish. Transfected cells were identified by the pEGFP-expressed green fluorescence. Transfection efficiency was 20–30 %. The next day, trypsinized cells were placed on glass coverslips for whole-cell patch-clamp recordings.

Electrophysiology

Current recordings were performed using whole-cell voltage patch-clamp configuration at 22–24 °C, as described before (Qu et al. 2010). Briefly, the voltage-step protocol (from –100 to 100 mV, with 20-mV increment) was used with a holding potential of 0 mV. Standard pipette solution (~4.5 μM free Ca^{2+}) contained (in mM) 146 CsCl, 2 $MgCl_2$, 5 (Ca^{2+})-EGTA, 10 sucrose and 8 HEPES (pH 7.3). Standard extracellular solution contained (in mM) 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.3). This combination of solutions set E_{rev} for Cl^- currents to zero, while cation currents carried by Na^+ or Cs^+ had very positive or negative E_{rev}, respectively. The amplitude of whole-cell currents at the end of a −100-mV pulse was measured. Student's *t* test was used for statistical analysis.

Solubility of mBest3 C Termini

mBest3 C-terminal cDNA with or without point or deletion mutations subcloned into pcDNA3.1 vector (Qu et al. 2006) was cotransfected with pEGFP into HEK239 cells

using Lipofectamine 2000 (Invitrogen). About 3.0 μg DNA was used to transfect HEK293 cells on one 10-cm dish. Transfection efficiency was about 60 %. Two days later, transfected cells were harvested and sonicated in phosphate-buffered saline (PBS) solution with 1 mM DTT, 1 mM PMSF and 1/200 (volume) protease inhibitor set III (Calbiochem) and ultracentrifuged at 42,500×*g* (Beckman Optima TLX ultracentrifuge, TLS 55 rotor; Beckman Coulter, Fullerton, CA). The supernatant was collected as a soluble fraction (S). The pellet was washed with PBS, dissolved in a nonionic detergent lysis buffer (in mM: 1 % Triton X-100, 150 NaCl, 0.5 EGTA, 10 % glycerol, 1 DTT, 10 HEPES, pH 7.3, 1 PMSF, 1/200 protease inhibitor cocktail III) and centrifuged at 16,000×*g*. The resulting supernatant contained Triton-soluble membrane proteins (M). Protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, IL). An equivalent amount (10 μg/lane) of protein was used for SDS-PAGE and Western blotting, using an anti-mBest3 C-terminal antibody (05619) (Qu et al. 2007). Each experiment was repeated three times, and a representative result was presented.

Results and Discussion

The AI Domain is Not a Determinant of mBest3 C-Terminal Membrane Associability

Previously, we found that wild-type mBest3 C terminus (residues 292–669) could strongly associate with cellular membranes, but after deletion of amino acids 353–404 from the mBest3 C terminus, membrane associability was abolished and the mBest3 channel was activated robustly (Qu et al. 2006). The AI domain (^{356}ISPFLGS^{362}) was found to reside in the 353–404 sequence. Mutation of any amino acids in the domain activated mBest3 (Qu et al. 2006). Therefore, we hypothesized that the AI domain was related to the C-terminal cellular membrane association. To test the hypothesis, we took advantage of two C-terminal mutants, G361A and Δ353–367, which either mutated or deleted the AI domain. The two mutants were previously demonstrated to express robust Cl^- currents (Qu et al. 2006, 2007). To examine whether the activation of Best3 by the two AI domain mutations was due to interruption of the C-terminal membrane association, we expressed mBest3 C termini carrying either the G361A mutation or the Δ353–367 deletion in HEK293 cells. The soluble (S) and membrane (M) fractions of HEK293 cellular proteins were extracted and subjected to SDS-PAGE and Western blotting. Immunoblots were probed with anti-mBest3 antibody as described before (Qu et al. 2007). In Fig. 1, clearly proteins of the two C-terminal mutants remained in the membrane fractions (lanes 2 and 4), indicating that mutation or deletion of the AI domain could not abolish the membrane associability of the mBest3 C terminus. The result suggests that the AI domain may not be a determinant of C-terminal membrane association and that other residues within the 353–404 sequence may contribute to the membrane associability.

The 368–383 Sequence Determines Best3 C-Terminal Membrane Associability

In our previous study, we found that Best3 activation may be controlled by both the AI domain (^{356}ISPFLGS^{362}) and the basic residue domain within the 384–397 sequence. Since disruption of the AI domain did not abolish the membrane associability, we assumed that the basic residue domain may be involved in the C-terminal membrane association. The 353–404 sequence was divided into three parts: 353–367 (including the AI domain), 368–383 and 384–404 (including the basic residue domain). After mutation or deletion of sequences from the mBest3 C terminus, we performed cellular transfection, purification of C-terminal proteins in S and M fractions and Western blotting. In Fig. 2a, deletion of the 353–404 sequence (Δ353–404) made the membrane associability almost disappear (lanes 1 and 2) as expected, while deletion of the 384–404 sequence (Δ384–404) did not remove the membrane associability (lanes 3 and 4), indicating that the

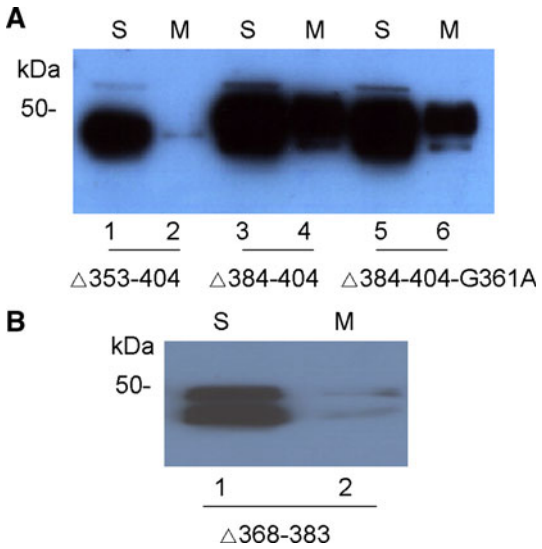

Fig. 2 Contribution of 368–383 and 384–404 domains to C-terminal membrane associability. **a** Amino acid 353–404 deletion (Δ353–404) from mBest3 C terminus (292–669) cDNA almost abolished membrane associability (*lane 2*), while the Δ384–404 and Δ384–404-G361A mutants did not (*lanes 4* and *6*). **b** The Δ368–383 deletion mutant almost abolished the membrane associability of mBest3 C terminus (*lane 2*). The figure is one representative of three experiments

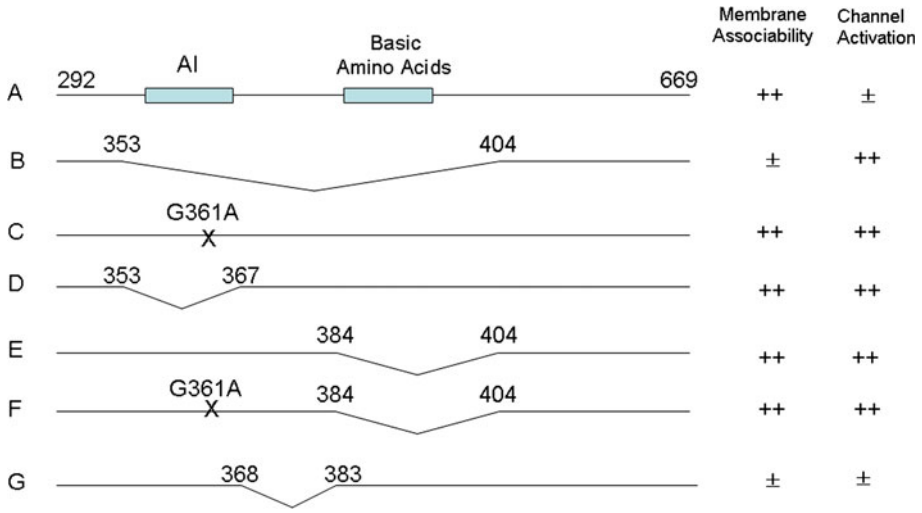

Fig. 3 Schematic map to describe the relation of the mBest3 C-terminal point or deletion mutations with changes in membrane associability and channel activation. *AI* an autoinhibitory domain (^{356}IPSFLGS^{362}), *Basic amino acids* eight positively charged residues within the 384–397 region; for membrane associability ++ cellular membrane associability, ± no associability; for channel activation ++ >−1,000 pA at −100 mV in whole-cell patch-clamp configuration, ± <−100 pA at −100 mV; × point mutations, V deletions of different lengths of sequence. Original recordings in **b–f** have been described previously (O'Driscoll et al. 2008; Qu et al. 2006, 2007)

384–404 domain was not a determinant of C-terminal membrane association either. Simultaneous destruction of the AI domain (G361A mutation) and the basic residue domain (Δ384–404) in the mBest3 C terminus also failed to remove the membrane association (lanes 5 and 6). The results suggest that the intermediate domain (residues 368–383) between the AI domain and the basic residue domain may be involved in membrane association. To test this suggestion, the 368–383 sequence was deleted from the C terminus. Figure 2b shows that the Δ368–383 mutant lost almost all membrane associability of the C terminus. However, deletion of the 368–383 sequence from mBest3 did not induce significant Cl^- currents at −100 mV (−70 ± 12 pA, $n = 3$, compared to wild-type: −76 ± 14 pA, $n = 3$, $P > 0.1$). This result suggests that the 368–383 sequence is a determinant of C-terminal membrane association but its association with the membrane is not directly correlated with channel activation (also see Fig. 3g). How the 368–383 sequence plays a role in the activation of Best3 remains to be revealed.

In summary (Fig. 3), the entire mBest3 C terminus is associated with the cellular membrane (++) (Fig. 3a). The 353–404 deletion (Δ353–404) almost abolished the membrane associability (±) (Fig. 3b). Within the 353–404 sequence, there are two important structures: the AI domain (356–362) and the basic residue domain (384–397). Two mutations in the AI domain (G361A and Δ353–367) could not remove the membrane associability (++) (Fig. 3c, d). The results suggest that the AI domain is not a determinant of C-terminal membrane associability. Removal of the basic residue domain (Δ384–404) or its combination with AI domain mutation (Δ384–404-G361A) could not remove the membrane associability (++) either (Fig. 3e, f). These results imply that the intermediate domain flanked by the AI domain and the basic residue domain may contribute to the membrane associability. Indeed, deletion of the intermediate domain (368–383) almost completely destroyed the membrane associability (±) (Fig. 3g). Therefore, among the three parts, the intermediate part (368–383) seems to be a determinant of membrane associability. However, deletion of the 368–383 intermediate domain had no effect on Best3 activation (as indicated in Fig. 3g), while either AI or basic residue destruction caused Best3 activation, recorded via patch clamp (Fig. 3b–f). These recordings have been published previously, and for original recordings in Fig. 3b–f, see previous publications (Qu et al. 2006, 2007, 2010). Combining the results from membrane associability and Best3 activation, we conclude that Best3 activation does not have to depend on C-terminal dissociation from the membrane. We hypothesize that the 368–383 domain controls the Best3 C-terminal membrane associability and may have functions other than channel activation. How the AI domain controls activation of Best3 requires further investigation.

PI3Kα Inhibitor IV Weakens C-Terminal Membrane Associability

According to previous results (Qu et al. 2006, 2007), we hypothesized that the basic amino acids may be involved in Best3 C-terminal membrane association via binding to

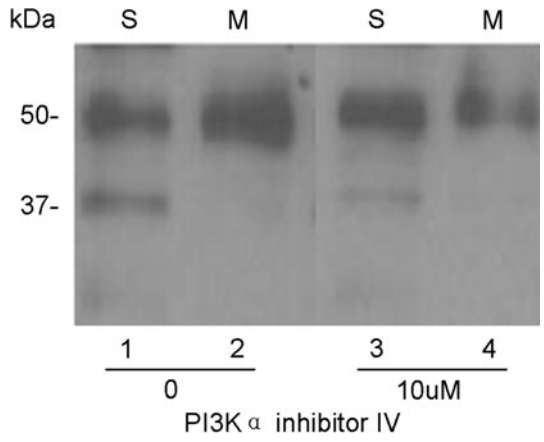

Fig. 4 PI3Kα inhibitor IV weakened Best3 C-terminal membrane associability. The Best3 C terminus in the pcDNA3.1 vector was transfected into HEK293 cells for 2 days. Before harvest, cells were treated with PI3Kα inhibitor IV (10 μM) for 15 min. Extraction of soluble and membrane fractions of cellular proteins and Western blotting was as described in Fig. 1. *Lane 4* shows that PI3Kα inhibitor IV treatment weakened membrane associability. The figure is one representative of three similar results

membranous phospholipids. Led by the hypothesis, we discovered that the PI3Kα inhibitor IV could strongly activate Best3 (Qu et al. 2010). Although the above results indicate that Best3 activation may not depend on C-terminal membrane association, we think that it is necessary to further test the hypothesis by way of immunobiochemistry. We treated Best3 C terminus–transfected cells with PI3Kα inhibitor IV (10 μM) for 15 min before harvesting and performed immunoblotting as described above. The results showed that the treatment moderately weakened the membrane associability (Fig. 4, lane 4).

The experiments provided evidence that Best3 C-terminal membrane association is mediated at least partially by membranous phospholipid metabolism. The evidence explains why the PI3Kα inhibitor is capable of activating Best3. It is well known that the cellular membrane contains a large amount of phospholipids whose phosphate bases make the cytosolic surface of the membrane negatively charged. The negative charges tend to recruit any residues charged positively. Therefore, the positively charged basic residues in the Best3 C terminus and membranous phospholipids attract mutually, leading to binding of the C terminus with the membrane. A PI3K inhibitor, which inhibits membranous phospholipid synthesis (e.g., PIP_3 and PIP_2) (Kong and Yamori 2009) and then theoretically reduces the amount of negative charge in the membrane, may interrupt the interaction between the basic residues and the membrane. The result in Fig. 4 supports the explanation: treatment with the PI3K inhibitor reduced the membrane-associated protein (lane 4). We assumed that dissociation of the 384–397 sequence from the cellular membrane eventually leads to activation of Best3 somehow. In Fig. 2b (lane 2) traces of proteins remained in the membranous phase after the 368–383 sequence was deleted. We assumed that the basic residue sequence contributed to the remaining membrane association. Therefore, the activating effect of PI3Kα inhibitor IV may be exerted through dissociation of the basic residue domain from the cellular membrane.

The way that the intermediate 368–383 sequence interacts with the cellular membrane may be different. Within the 368–383 sequence, basic residues do not exist. Therefore, PI3Kα inhibitor IV may not affect their membrane associability. Previously, we have demonstrated that the Best3 C-terminal sequence was not membrane integral since 0.1 M Na_2CO_3 (pH 11.5) solution made the C terminus totally dissociate from the membrane (Qu et al. 2010). However, a highly hydrophobic sequence does exist within the 368–383 sequence, which may form a hydrophobic bond with the membrane (Qu et al. 2010). This explains why a solution of high ionic strength (2 M NaCl) cannot dissociate the membrane from the Best3 C terminus.

The membrane-associating characteristics of Best3 and the role of the PI3Kα inhibitor IV have helped us to understand the mechanism of Best3 activation. A potential function of Best3 in the cardiovascular system has been suggested (Matchkov et al. 2008; O'Driscoll et al. 2008; Broegger et al. 2011). We believe that the newly found activator of the Best3 channel, PI3Kα inhibitor IV, will be a useful tool for the study of the rhythm of vascular contraction and relaxation.

In summary, the C-terminal membrane associability of mBest3 is determined by the 368–383 sequence flanked by the AI and basic residue domains. However, the association of the sequence with the cellular membrane is not a determinant of mBest3 activation. The basic residue domain regulates mBest3 channel activity probably through its interaction with the cellular membrane.

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