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^1H , ^{13}C , and ^{15}N backbone resonance assignments of the 37 kDa voltage-gated Ca^{2+} channel $\beta 4$ subunit core SH3-GK domains

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Abstract

The β subunit of the voltage-gated Ca^{2+} channel (α_1 , $\alpha_2\delta$, and β subunits) is a member of the MAGUK family of proteins and plays an essential role in regulating Ca^{2+} channel trafficking and gating. It also serves as a central interaction partner for various Ca^{2+} channel regulatory proteins. We report here the nearly complete ^1H , ^{13}C , and ^{15}N backbone resonance assignments of the 37 kDa core SH3-GK domains of the $\beta 4$ subunit. This is the first report of solution assignments for β subunits, and as such will lay the foundation for future investigations of interaction site mapping, functional dynamics, and protein complex structure determination.

Keywords

NMR resonance assignment; Ca^{2+} channel β subunit; Membrane associated guanylate kinase (MAGUK); SH3-domain; Guanylate-kinase domain

Biological context

Voltage-gated Ca^{2+} channels (VGCC) are large protein complexes found in the plasma membranes of excitable cells. They play critical roles in many cellular events such as excitation-contraction coupling, neurotransmitter release, and Ca^{2+} -dependent gene transcription (Catterall 2011). The VGCC is composed of a pore-forming α_1 subunit and auxiliary $\alpha_2\delta$ and β subunits. The β subunit is entirely cytosolic and plays an essential role in regulating α_1 trafficking and gating properties. These functions are thought to occur mainly through a high-affinity interaction between the β subunit and the α_1 interaction domain (AID) of the intracellular loop between α_1 repeat motif I and II (Pragnell et al. 1994). The β subunits are encoded by four distinct genes ($\beta 1$ – $\beta 4$), each with different splicing variants. An initial modeling study revealed that β subunits belong to the MAGUK family of proteins, containing conserved Src homology (SH3) and guanylate kinase (GK)-like domains connected by a variable hook sequence and flanked by variable sequence on both N and C termini (Hanlon et al. 1999). X-ray crystallography studies confirmed the conserved SH3-GK core domains in different β subunits and also revealed that the α_1 AID

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binds to a hydrophobic groove in the β subunit GK domain (Chen et al. 2004; Opatowsky et al. 2004; Van Petegem et al. 2004).

Apart from the specific interaction between $\alpha 1$ and β subunits, a large β subunit-centered protein interaction network has recently been identified (reviewed in Buraei and Yang 2010). This suggests that a variety of signaling pathways can influence Ca^{2+} channel regulation via direct interaction with β subunits. For example, RGK (Rem, Gem/Kir) proteins, a group of small GTPases, interact with β subunits to induce marked inhibitory effects on Ca^{2+} channel currents (Beguín et al. 2001), and the presynaptic active zone protein Rab interacting molecule 1 (RIM1) interacts with the β subunit to generate a slowed inactivation of $\alpha 1$ and enhanced neurotransmitter release (Kiyonaka et al. 2007). However, due to lack of atomic-level structural studies, the underlying molecular mechanisms of these interactions remain unclear.

We aim to use solution NMR to study the interactions between the VGCC β subunit and its various interaction partners. As a start, we report here a nearly complete ^1H , ^{13}C and ^{15}N backbone resonance assignment of the 37 KDa core SH3-GK domains of the VGCC $\beta 4$ subunit.

Materials and methods

A DNA construct encoding human VGCC $\beta 4$ SH3 (16–135) and GK (166–374) domains separated by two serine residues was synthesized using overlap extension PCR and subcloned into the pET15b expression vector (Novagen). The two serine residues in addition to the intrinsically disordered region 166–174 (AKQKQKVTE) (Van Petegem et al. 2004; Xu et al. 2011) serve as a flexible linker for connecting SH3 and GK domains. The construct was expressed in *E. coli* BL21 Rosetta (DE3) pLysS (Novagen) as an N-terminal His₆-tag fusion protein. Two additional Cys to Ser mutations ($\beta 4$ C70S and $\beta 4$ C112S) were performed to improve protein stability. Freshly transformed cells were first grown overnight in 10 mL LB media at 37 °C before being transferred into 50 mL M9 media supplemented with $^{15}\text{NH}_4\text{Cl}$, $\text{U-}^2\text{H-}^{13}\text{C}$ -glucose (Cambridge Isotope Laboratories, Andover MA) dissolved in 80 % D_2O . The cells were then grown for 12 h and centrifuged at 5,000 RPM. The pellet was resuspended into 1 L M9 with $^{15}\text{NH}_4\text{Cl}$, $\text{U-}^2\text{H-}^{13}\text{C}$ -glucose in 99.8 % D_2O and grown at 37 °C with vigorous shaking until OD₆₀₀ 0.5 was reached. One gram of ^2H , ^{13}C , and ^{15}N labeled Celtone amino acid mixture (Cambridge Isotope Laboratories) was dissolved in 5 mL D_2O , filtered, and added to the media. Protein expression was induced by adding IPTG to a final concentration 0.5 mM. After 16 h induction at 30 °C, cells were harvested by centrifugation at 5,000 RPM, resuspended in a binding buffer containing 50 mM Tris-HCl (pH 8.0) and 0.5 M NaCl, and lysed by sonication. The lysate was incubated with 50 units of DNase I (Bio-Rad) for 30 min and centrifuged at 17,000 RPM for 30 min. The supernatant was filtered and then loaded onto a 15-ml Ni^{2+} resin column (Bio-Rad) pre-equilibrated with 100 ml of binding buffer. After washing with eight column volumes of binding buffer, the protein was eluted with increasing concentrations of imidazole (up to 300 mM). Guanidine hydrochloride was added to the concentrated protein fractions to allow fast amide deuterium-proton exchange in the unfolded state. The protein was refolded by rapid dilution into buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 5 mM DTT.

Guanidine hydrochloride was removed by dialysis against the same buffer at 4 °C. The final protein purification was achieved by FPLC gel filtration using a Sephacryl S-200 HR column (AKTA, GE Healthcare) in 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl.

The ^2H , ^{15}N and ^{13}C labeled $\beta 4$ core domain sample was concentrated to 0.5 mM and exchanged into 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl, 2 mM DTT, 0.02 % NaN_3 , 50 mM Glu/Arg, and 10 % D_2O by repeated centrifugation using an Amicon Ultra-15 30 K filter unit (EMD Millipore) at 3,000 RPM. All NMR experiments were performed at 298 K on Varian Inova 500 MHz, 600 MHz, and 900 MHz spectrometers equipped with triple resonance cryogenic probes. Backbone assignments were made from TROSY type ^1H - ^{15}N HSQC, HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HNCO, and HN(CA)CO. A TROSY- ^1H - ^{15}N HSQC_NOESY was also recorded to verify the assignments by checking the sequential or inter-strand NH-NH NOEs. All spectra were processed with NMRPipe (Delaglio et al. 1995) and analyzed using CCPNMR (Vranken et al. 2005).

Resonances assignment and data deposition

Ninety-two percent of expected backbone amide pairs (286 out of 312 non-proline residues) have been assigned, excluding the 21 residues of the N-terminal His₆-tag and the two serines used to bridge SH3 and GK (Fig. 1). Additionally, 92 % of C_α (305 out of 329), 91 % of C' (300 out of 329) and 89 % of C_β (282 out of 318) resonances have been assigned. Residues without assignments include N216-S219, S227-N228, K295-S298, D309-L311 and the C-terminal tail, T349-S351. The missing assignments could be due to either chemical exchange or significant resonance overlapping.

Consensus chemical shift index analysis (Wishart and Sykes 1994) based on assigned C_α , C_β and C' chemical shifts was used to predict the secondary structure shown in Fig. 2. The results are in close agreement with $\beta 4$ core domain secondary structural elements observed in a crystallography study (Chen et al. 2004). Interestingly, there is a notable difference between $\beta 3$ and $\beta 4$ in the loop region formed by K220-R227 that may be due to a single amino acid exchange. Chemical shift index, NH-NH NOE analysis, and X-ray crystallography indicate that there is no regular secondary structure for this region in $\beta 4$. By contrast, the highly homologous $\beta 3$ core crystal structure shows an α -helix in this region (Chen et al. 2004; Opatowsky et al. 2004). The backbone ^{15}N , ^1H , $^{13}\text{C}'$, $^{13}\text{C}_\alpha$ and side chain $^{13}\text{C}_\beta$ chemical shifts have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 19501.

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Fig. 1. ^1H - ^{15}N TROSY-HSQC spectrum of 0.5 mM $\beta 4$ SH3-GK core in 50 mM phosphate buffer (pH 7.0) and 100 mM NaCl, recorded at 298 K with a 900 MHz spectrometer. Cross-peaks are marked with residue type and sequence numbers. The crowded central region is enlarged and shown in the *inset*

Fig. 2. Secondary structure prediction from Consensus Chemical shift index analysis based on C_{α} , C_{β} and C' chemical shifts. Bars are used to represent the C chemical shift differences relative to their random coil values