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Two domains of the smoothelin-like 1 protein bind apo- and calcium–calmodulin independently

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ABSTRACT

The smoothelin-like 1 protein (SMTNL1) is a modulator of smooth and skeletal muscle contractility and can bind to calmodulin and tropomyosin. Calmodulin is the major calcium sensor of eukaryotic cells and it can cycle between calcium-free (apo-CaM) and calcium-bound (Ca-CaM) forms. Bioinformatic screening of the SMTNL1 sequence predicted a second CaM-binding region (CBD1) that is located N-terminal to the previously defined apo-CaM-binding site (CBD2). Pull-down assays, surface plasmon resonance, isothermal calorimetry and NMR techniques were used to determine that CBD1 associated preferentially to Ca-CaM while CBD2 bound preferentially to apo-CaM. Mutation of hydrophobic residues abolished Ca-CaM-binding to CBD1 while acidic residues in CBD2 were necessary for apo-CaM-binding to CBD2. The dissociation constant (K_d) for Ca-CaM-binding to a CBD1 peptide was $26 * 10^{-6}$M while the value for binding to a longer protein construct was $0.5 * 10^{-6}$ M. The binding of SMTNL1 to both apo-CaM and Ca-CaM suggests that endogenous CaM is continuously associated with SMTNL1 to allow for quick response to changes in intracellular calcium levels. We also found that the intrinsically disordered N-terminus of SMTNL1 can reduce binding to apo-CaM and increase binding to Ca-CaM. This finding suggests that an additional CaM-binding region may exist and/or that intramolecular interactions between the N-terminus and the folded C-terminus reduce apo-CaM-binding to CBD2. Intriguingly, CBD1 is located close to the SMTNL1 phosphorylation site and tropomyosin-binding region. We discuss the possibility that all three signals are integrated at the region surrounding CBD1.

1. Introduction

The ubiquitous calcium regulatory protein calmodulin (CaM) is the primary cellular signal transducer that provides highly specific and localized responses to changes in the calcium concentration (reviewed in [1–3]). CaM has a dumbbell structure composed of two calcium-binding domains (N- and C-lobes) which are connected by a flexible linker region. Each lobe comprises two EF-hands that bind to calcium. Calcium binding leads to major conformational rearrangements in each lobe and the formation of large hydrophobic patches that accept bulky hydrophobic residues of target proteins [4]. In the most common protein–protein complex structures, calcium-saturated CaM (Ca-CaM) is wrapped around the CaM-binding domain (CBD) of a target protein. The association of CaM with a target protein enables selective alterations in enzymatic activities, intracellular localization and/or other biological events. Ca-CaM most often binds to a classical CBD that consists of a basic, amphipathic ~20 residue α-helix with large hydrophobic amino acids [4,5].

The regulation of cellular events is most commonly associated with Ca-CaM; however, it is also recognized that calcium-free CaM (apo-CaM) has important signaling properties by associating with distinct apo-CaM-binding regions of target proteins. The usual apo-CaM-binding domain possesses an IQ-motif sequence that is named for the conserved IQ-residues that initiate the "IQXXXRGXXXR" consensus sequence (X, any amino acid) [6,7]. Various three-dimensional structures of CaM-target complexes highlight the enormous versatility of complex formation that is facilitated by CaM [2]. CBDs can be located close to phosphorylation sites (and/or other post-translational modification sites), protein–protein interaction domains or auto-inhibitory domains,

Abbreviations: aa, amino acids; bp, base pair; C-terminal, carboxy-terminal; CaM, calmodulin; Ca-CaM, calcium-saturated calmodulin; apo-CaM, calcium-free calmodulin; CBB, coomassie brilliant blue; CBD, aM-binding domain; CH, calponin homology; ΔCH, deletion of the calponin homology domain; CSP, chemical shift perturbation; Δ4K, deletion of KTKKK; GST, glutathione-*S*-transferase; HSQC, heteronuclear single quantum correlation; ITC, isothermal titration calorimetry; kDa, kilodalton; K_d, equilibration dissociation constant; MARCKS, myristoylated alanine-rich protein kinase C substrate; MLCK, myosin light chain kinase; N-terminal, amino-terminal; NaCl, sodium chloride; [NaCl], sodium chloride concentration; pxIDR, proximal intrinsic-disordered region; SMTNL1, smoothelin-like 1 protein; SPR, surface plasmon resonance; TMB, tropomyosin-binding; VGCC, voltage-gated calcium channel; WT, full-length wild-type SMTNL1

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which offers an additional explanation for how CaM-binding can contribute to the regulation of the cellular activity of a diverse array of targets.

The smoothelin-like 1 (SMTNL1) protein, originally termed calponin homology-associated with smooth muscle (CHASM), was discovered as a protein phosphorylated during calcium desensitization in the smooth muscle [8,9,15]. Several studies have implicated SMTNL1 in the modulation of vascular smooth muscle contractile activity as well as cardiovascular and skeletal adaptation to exercise, development and pregnancy [16–20]. A global SMTNL1 knock-out mouse has allowed the study of its physiological role [16]. The first investigations focused on the cardiovascular system and showed an exercise-adapted phenotype [16]. Newer data suggest that SMTNL1 may provide adaptive regulation of blood vessel contractile capacity through transcriptional/translational effects on protein abundance [18,19]. We have previously reported that SMTNL1 possesses an IQ-motif located within the terminal α-helix of its CH domain [12]. Calponin homology (CH)-domains are protein–protein interaction modules that are involved in cytoskeletal dynamics and signal transduction (for review, see [10,11]). The CH-domain of SMTNL1 is integral to several functional properties of the protein, including its association with apo-CaM [12]. The CH-domain together with a portion of the intrinsically disordered N-terminal region forms a tropomyosin-binding domain that was necessary for association with contractile filaments [13,14]. Furthermore, the SMTNL1 IQ-motif enabled the interaction with apo-CaM but not Ca-CaM. Interestingly, the interaction with the SMTNL1 IQ-motif was mediated by the two EF-hands in the C-lobe of apo-CaM rather than the flexible linker region. Many of the CaM side chains that also serve as calcium-binding ligands formed electrostatic contacts with SMTNL1. Thus we rationalized that binding of calcium to the EF-hand disrupts the interactions with the IQ-motif by requiring the same binding region.

In the current study in silico analysis of the SMTNL1 sequence revealed a potential additional CBD that was expected to associate with Ca-CaM. This CBD is located upstream of the CH-domain and within the tropomyosin-binding region near the S301 phosphorylation site. In this work, we verified the binding activity of the newly identified CBD within SMTNL1. Using pull-down studies, isothermal calorimetry, surface plasmon resonance and NMR spectroscopy we demonstrate that SMTNL1 possesses two CBDs, a novel site that specific for Ca-CaM and the previously described IQ-motif that is specific for apo-CaM. The vast majority of proteins regulated by CaM bind either to its calcium-free or -saturated form, creating a calcium-dependent regulatory switch. Intriguingly, SMTNL1 has the ability to associate with both apo-CaM and Ca-CaM, suggesting a novel regulatory mechanism.

2 . Material and methods

2.1. Materials

Mammalian CaM was expressed and purified from *Escherichia coli* strain BL21 (DE3) as described previously [21]. The CBD1 peptide (aa 307–329, Ac-RGPRAQNRKAIMDKFGGAASGPT-NH_2) was synthesized by the University of Calgary Peptide Synthesis Facility (Calgary, AB), confirmed by mass spectrometry and shown to be >95% pure by analytical HPLC. PreScission protease, glutathione-Sepharose 4B, CNBr-activated CH Sepharose and pGEX-6P1 were purchased from GE Healthcare (Piscataway, NJ). All other chemicals were purchased from VWR Scientific (Edmonton, AB) or Sigma Chemical Company (St. Louis, MO).

2.2. Expression and purification of recombinant SMTNL1 proteins

Various SMTNL1 mutants derived from mouse *Smtnl1* (GenBank ID: EDL27304.1) were utilized (see Fig. 1C for an overview): Full-length SMTNL1 (WT, base pairs (bp) 1–1377/amino acids (aa) 1–459) CH-domain (CH, lacking the intrinsically disordered domain, bp 1038–1380/aa 346–459), tropomyosin-binding fragment (TMB lacks the initial 194 amino acids, bp 583–1380/aa 195–459), deletion of CH-domain (ΔCH, bp 1–1038, aa 1–346) and proximal intrinsically disordered region (pxIDR, lacking the CH-domain, aa 583–1038/aa 195–346; termed ΔTBD/ΔCT in [14]) were described previously [9,13,14]. Several novel constructs, including CBD1/2 (bp 879–1377/aa 293–459) that contains both CBD1 and CBD2 but lacks the intrinsically disordered N-terminus, were newly generated using standard PCR techniques. The Δ4K truncations that lack the terminal five amino acids of CBD2 (KTKKK) were generated using primers with a premature stop-codon. Various single and double amino acid point mutants of WT-SMTNL1, TMBΔ4K and CH were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All SMTNL1 variants were cloned into the pGEX-6P1 vector (GE Healthcare) to generate N-terminal glutathione-S-transferase (GST)-fusion proteins. Proteins were expressed in *E. coli* strain BL21 (DE3) and isolated using glutathione-Sepharose following the manufacturer's protocol. In most cases, the N-terminal GST was removed by cleavage with PreScission protease followed by MonoQ or glutathione-Sepharose chromatography. The purified SMTNL1 proteins all contain the cloning artifact "GPLGS" at the N-terminus. Purified proteins were concentrated with an Amicon centrifugal filter (Millipore, Billerica, MA) and the buffer exchanged if necessary for the ensuing experiments. Proteins were not aggregated as judged from native gel-electrophoresis and dynamic light scattering (Supplementary Fig. 1).

2.3. Binding of CaM to immobilized GST-SMTNL1

Pull-down experiments with various GST-SMTNL1 proteins were performed to demonstrate CaM-binding. GST-SMTNL1 proteins or GST alone (200 μg) was immobilized on glutathione-Sepharose (20 μL) and then incubated for 2 h with purified CaM (100 μg) in buffer either containing 2 mM EDTA or 5 mM $CaCl_2$ plus 25 mM Tris, pH 7.2 and 50 mM NaCl. After extensive washing, bound CaM was eluted with SDS-PAGE loading buffer, subjected to SDS-PAGE and visualized by western blotting with anti-CaM antibody (05–173, Millipore). Equal loadings of SMTNL1 proteins onto GST-Sepharose were verified by Coomassie brilliant blue (CBB) staining of SDS-PAGE gels run in parallel.

2.4. Binding of SMTNL1 mutants to CaM-Sepharose

We completed pull-down experiments with CaM-Sepharose which was prepared by covalently coupling CaM to CNBr-activated CH Sepharose (GE Healthcare) following the manufacturer's recommendations. Blank-Sepharose was generated as a negative control by immobilizing with Tris. Purified SMTNL1 proteins (GST-tag removed) were incubated with CaM-Sepharose or blank-Sepharose in 20 mM HEPES, pH 7.0, in the presence of 5 mM $CaCl_2$ or 2 mM EDTA. After overnight incubation at 4 °C, the Sepharose was washed extensively. One-third of the resin was eluted with SDS-PAGE loading buffer and the remainder extensively washed in the same buffer plus 50 mM NaCl. Next, one-half of the resin was eluted as before and the remainder washed with the same buffer plus 150 mM NaCl and eluted. Equal volumes of all elutions were separated on SDS-PAGE, and SMTNL1 proteins were visualized by CBB staining. The gels were imaged with an LAS4000 luminescent image analyzer (GE Healthcare) and bands containing proteins quantified using Image Quant TL (GE Healthcare). To avoid inaccuracies introduced by variations in CBB staining, each band intensity was normalized to the sum of all detected protein. Statistical significance was calculated for binding to CaM-Sepharose, blank-Sepharose, presence or absence of calcium, as well as the binding dependency on [NaCl]. Binding to CaM-Sepharose was considered significant if it was different from blank-Sepharose values at the same [NaCl] and calcium level. GraphPad-PRISM was used for all statistical calculations, using two-way ANOVA with Bonferroni post hoc test, $p < 0.05$, $n \geq 4$, unless otherwise noted.

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