NMR insight into myosin-binding subunit coiled-coil structure reveals binding interface with protein kinase G-1α leucine zipper in vascular function

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Nitrovasodilators relax vascular smooth-muscle cells in part by modulating the interaction of the C-terminal coiled-coil domain (CC) and/or the leucine zipper (LZ) domain of the myosin light-chain phosphatase component, myosin-binding subunit (MBS), with the N-terminal LZ domain of protein kinase G (PKG)-1α. Despite the importance of vasodilation in cardiovascular homeostasis and therapy, our structural understanding of the MBS CC interaction with LZ PKG-1α has remained limited. Here, we report the 3D NMR solution structure of homodimeric CC MBS in which amino acids 932–967 form a coiled-coil of two monomeric α-helices in parallel orientation. We found that the structure is stabilized by non-covalent interactions, with dominant contributions from hydrophobic residues at a and d heptad positions. Using NMR chemical-shift perturbation (CSP) analysis, we identified a subset of hydrophobic and charged residues of CC MBS (localized within and adjacent to the C-terminal region) contributing to the dimer-dimer interaction interface between homodimeric CC MBS and homodimeric LZ PKG-1α. 15N backbone relaxation NMR revealed the dynamic features of the CC MBS interface residues identified by NMR CSP. Paramagnetic relaxation enhancement- and CSP-NMR-guided HADDOCK modeling of the dimer-dimer interface of the heterotetrameric complex exhibits the involvement of non-covalent intermolecular interactions that are localized within and adjacent to the C-terminal regions of each homodimer. These results deepen our understanding of the binding restraints of this CC MBS/LZ PKG-1α low-affinity heterotetrameric complex and allow reevaluation of the role(s) of myosin light-chain phosphatase partner polypeptides in regulation of vascular smooth-muscle cell contractility.

Contraction and relaxation of vascular smooth-muscle cells (VSMC) are mechanistically coupled to localized changes in blood vessel stretch and luminal hydrostatic pressure (1–5). Perturbations of vasoconstriction or vasodilation leading to VSMC dysfunction can result in hypertension or hypotension (5, 6). VSMC contraction is promoted via myosin light chain (MLC) phosphorylation by MLC kinase, whereas VSMC relaxation is stimulated through MLC dephosphorylation by the competing activity of MLC phosphatase (MLCP) (1, 3, 7). The MLCP holoenzyme is a heterotrimeric complex of the ~38-kDa protein phosphatase catalytic subunit (PP1c8), the 110–130-kDa myosin-binding subunit (MBS or MYPT1, a phosphatase regulatory domain), and a little-studied 20-kDa subunit (1, 7–9).

Several lines of evidence have established that nitric oxide (NO) and other nitrovasodilators induce VSMC relaxation, in part by cGMP-dependent protein kinase G-1α (PKG-1α)-mediated activation of MLCP (1, 10, 11). Gaseous NO produced by endothelial NO synthase diffuses from the endothelium to adjacent VSMC to activate soluble guanylate cyclase and generate cGMP. cGMP activates PKG-1α, which then binds, phosphorylates, and activates MBS of MLCP to promote VSMC relaxation (11). The protein-protein interaction (PPI) between MBS and PKG-1α is regulated and/or modulated by the C-terminal 180-amino acid (aa) domain of MBS (MBSCT180) and by the N-terminal 59-aa leucine zipper (LZ) domain (LZ PKG-1α) of PKG-1α (1, 9, 12–14). MBSCT180 comprises three predicted subdomains: a non-coiled-coil (non-CC) domain of aa 851–930, a typical CC domain encompassing aa 931–980, and an LZ domain including aa 1007–1028 of MBS (9, 13, 14) (aa numbering per Ref. 15).

LZ PKG-1α has been reported to interact with individual or with multiple subdomains of MBSCT180. LZ PKG-1α was first
shown to interact with the non-CC region (aa 888–928) of MBS (12). Both MBS fragments containing and lacking the LZ domain were subsequently shown to bind LZ PKG-1α in avian smooth-muscle tissue lysates, suggesting that the MBS LZ domain is not required for MBS interaction with LZ PKG-1α (9, 13, 14). Still other studies suggested that LZ PKG-1α binds to LZ and/or CC domains of MBS in the context of additional upstream regions of MBS to form a heterotetramer (9, 14). CC MBS indeed formed a low-affinity heterotetrameric complex with LZ PKG-I, and a more extended MBS construct including both CC and LZ domains (CCLZ MBS) bound LZ PKG-1α with higher affinity (9, 14). An MBS domain lacking its CC failed to bind PKG-1α (9). These studies taken together suggest the importance of both CC and LZ domains of MBS in formation of the PKG-I-1α:CC MBS complex. However, these results provide no atomic scale insight into the binding mechanism, due to the lack of 3D structural information for the subdomains of MBS_180.

We previously reported the structural characterization of the LZ PKG-1α homodimer and identified critical residues of LZ PKG-1α mediating its binding with CC MBS (14). However, the absence of 3D structural information for CC MBS and lack of knowledge of the core residues involved in its binding to PKG-1α have limited our structural understanding of the CC MBS-LZ PKG-1α complex. We and others have demonstrated that CC MBS is a folded homodimer in solution (9, 14, 16) and exhibits a well-dispersed 2D 1H-15N HSQC spectrum (16). We also reported 1H, 13C, and 15N NMR assignments for CC MBS and identified critical amino acid residues involved in formation of the α-helical coiled-coil region of CC MBS (17).

Here we report a 3D solution NMR structure of the CC MBS of MLCP. Using NMR chemical-shift perturbation (CSP)- and paramagnetic relaxation-enhancement (PRE)-driven intermolecular distances, we identify the critical hydrophobic and electrostatic residues of CC MBS constituting the dimer-dimer interaction interface of the CC MBS-LZ PKG-1α complex. We apply 15N backbone relaxation NMR and molecular dynamics simulations to validate the CC MBS interface residues identified by NMR CSP and report a HADDOCK model of this heterotetrameric complex of two homodimers. The combined technical approaches deepen our understanding of the binding interaction between CC MBS and LZ PKG-1α and set the stage for reevaluation of the interaction’s role in VSMC contractility and its regulation.

Results

The interaction between MBS/MYPT1 and PKG-1α is currently modeled between the N-terminal LZ domain of PKG-1α and the C-terminal MBS_180 domain of MLCP (see schematic in supplemental Fig. S1). Although CC MBS binds LZ PKG-1α, the structural basis of this interaction is not well-understood. Our results presented below describe the solution NMR structure of CC MBS and define at the molecular level the individual amino acid residues and dimer-dimer interface involved in the CC MBS-LZ PKG-1α complex of VSMC.

Protein purification and NMR spectroscopy

Overexpressed CC MBS polypeptide (aa 931–980) was predominantly soluble and was purified from soluble fractions (Fig. 1A). Size-exclusion chromatography (SEC) shows that the purified CC MBS polypeptide adopts a dimeric conformation in solution (Fig. 1B; extrapolated dimer mass of 13,275 Da from the calibration curve agrees well with theoretical mass of 13,142.2 Da). 13C/15N- or 15N-labeled CC MBS polypeptides of >96% apparent purity migrated as a single homodimeric peak on SEC (Fig. 1B), consistent with previous results (16). Fig. 1C shows the well-dispersed 2D 1H-15N HSQC spectrum of 13C/15N labeled CC MBS, including the previously unassigned side-chain 1H-15N correlation cross-peaks for 2 Asn and 5 Gln residues. The excellent dispersion of observed cross-peaks within the 1H-chemical shift region 8.95–7.33 ppm confirmed the well-folded state of CC MBS polypeptide. Expression and purification of unlabeled and 15N-labeled LZ PKG-1α followed protocols described previously (14). CD and 1D 1H NMR spectroscopy confirmed intact protein folding of LZ PKG-1α (supplemental Fig. S2).

Previously reported NMR assignments of CC MBS (17) were confirmed in 3D 1H-15N NOESY-HSQC (supplemental Fig. S3, A and B) and in 3D 1H-13C NOESY-HSQC spectra. 831 intra-chain NOEs were easily assigned in these highly resolved NOESY spectra. Sequential and medium range NOEs reveal (Fig. 2A) that aa Phe932–Ala967 constitute an α-helical coiled-coil region in CC MBS. An independent investigation of 15N relaxation rate ratios (T_1/T_2) validates identification of the coiled-coil region (supplemental Fig. S4A). Heteronuclear NOE values (supplemental Fig. S4B) suggest that this coiled-coil α-helical region is rigid in solution, where-as the remaining C-terminal loop region adopts a flexible conformation.

Assignment of NOESY data revealed no inter-subunit NOEs for anti-parallel packing of two monomer units. High cross-peak symmetry prevented assignment of unambiguous NOEs for parallel packing. Isotope-filtered 3D and 2D NOESY experiments conducted with an isotopically mixed CC MBS sample allowed unambiguous assignment of 271 intersubunit NOEs, identifying a parallel packing mode of two monomer units in the CC MBS structure. (For this experiment, equimolar equivalents of 13C/15N-labeled and 12C/14N-labeled samples were denatured and then refolded, with confirmation of folding by CD spectroscopy (supplemental Fig. S3C; see also “Experimental procedures”).) Fig. 2B shows strip plots for select residues eliciting numerous intersubunit NOEs in the isotopo-filtered 3D NOESY experiments (see also supplemental Fig. S3D). These NOEs were easily verified in isotope-filtered 2D NOESY experiments. Fig. 2C summarizes intersubunit NOE contacts between heptad positions a and d’, a and g’, and d and e’ in the parallel homodimer of CC MBS.

Structural restraints and structure determination of CC MBS

The NMR-derived experimental restraints consisted of 1933 unique distance restraints deduced from NOEs, 132 H-bond restraints, and 70 (ϕ, ψ)-backbone dihedral angle restraints (Table 1). Of 271 interchain NOEs assigned, 220 involved hydrophobic residues. Aromatic residues Phe932 and Tyr936...
shared a total of 50 interchain NOEs. Predominant interchain contacts included almost equally distributed backbone-side chain and side chain-side chain interactions. No interchain NOEs were observed C-terminal to Gln969, reflecting the region’s flexibility.

Fig. 3A shows an ensemble of the 20 best 3D structural conformers of the CC MBS parallel homodimer. These structures were selected based on lowest target functions, absence of upper distance NOE violations of <0.5 Å, and absence of dihedral angle violations of >5°, indicating good correlation between NMR experimental data and calculated structures. The mean structure is composed of two well-defined \( \alpha \)-helices (Phe\(^932\)–Ala\(^967\)) packed in parallel. C-terminal aa residues Thr\(^968\)–Phe\(^974\) constitute a flexible loop region. The CC dimer interface of the native 3D fold is packed and stabilized primarily by hydrophobic interactions. This tight packing is also reflected in a low RMSD value of all individual structures from the mean structure.

The representative CC MBS ribbon structure shown in Fig. 3A highlights the hydrophobic and charged CC residue locations at heptad positions \( a \) and \( d \) and heptad positions \( e \) and \( g \), respectively. In addition to several hydrophobic residues, heptad positions \( a \) and \( d \) also register polar residues. The hydrophobic phenyl ring protons of Tyr\(^936\) at heptad position \( a \) make several \( a-d' \) and \( a-g' \) interchain contacts. Although the presence of polar residue Asn\(^943\) at heptad \( a \) could partially destabilize its local environment, the residue’s several \( a-d' \) interchain contacts probably foster parallel packing of monomers within the coiled-coil structure. Thr\(^953\) at heptad \( d \) also makes \( d-a' \) interchain contacts, alongside similar contacts elicited by the classical hydrophobic residues contributing to core packing.

The dimer interface near its N terminus is stabilized by several interchain contacts conferred by Phe\(^932\) at position \( d \) and by Tyr\(^936\) at position \( a \). The aromatic rings of Phe\(^932\) and...
Phe^932^ makes a \( \pi-\pi \) stacking interaction, whereas Tyr^936^ engages Phe^932^ and Leu^935^ at \( g \) and Ile^939^ at \( d \) through several interchain contacts. Additional hydrophobic interactions arising from residues located at \( a \) and \( d \) positions in heptad repeats 3–5 stabilize dimer interface packing in middle and C-terminal regions. Interestingly, no electrostatic interactions were observed despite the placement of two Lys residues at \( e \) and two Glu residues at \( g \) heptad positions.

The electrostatic potential surface of CC MBS (Fig. 3C) reveals a near-C-terminal region occupied by several negatively charged residues and a near-N-terminal region of positive charge (overall pI, 5.72; net surface charge, -1). The positive electrostatic surface potential of the nearby LZ PKG-1\( \alpha \) C terminus (overall pI, 8.91; net surface charge, +2) is probably important in its binding to CC MBS.

**MD simulations of structural conformations of CC MBS and of LZ PKG-1\( \alpha \)**

We applied molecular dynamics (MD) to examine the conformational stability of the homodimeric NMR structures of
 Structural basis of CC MBS-LZ PKG-ια interaction

Table 1

Structural statistics for ensemble of 20 refined structures of CC MBS

| Parameters                                | Values       |
|--------------------------------------------|--------------|
| NOE restraints                             | 1933         |
| Total                                       | 1933         |
| Intraresidue (| | ) = 0          | 678          |
| Sequential (| | | ) = 1        | 488          |
| Medium range (1 < | | | < 5)          | 496          |
| Long range (| | | | ) ≥ 5        | 0            |
| Intermonomer                                | 271          |
| Hydrogen bonds                              | 132          |
| Average distance restraints/residuea        | 24.0         |
| Dihedral angles (per monomer subunit)       |              |
| φ                                          | 35           |
| ω                                          | 35           |
| Restraint violation (Å)                     | 0            |
| Upper distance (>0.5)                       | 0            |
| Dihedral angle (>5°)                        | 0            |
| Average RMSD from mean coordinatesb (Å)     |              |
| Backbone atoms (N, Cα, C)                   | 0.385 ± 0.13 |
| All heavy atoms                             | 0.775 ± 0.19 |
| Ramachandran map statistics (%)b            |              |
| Most favored regions                        | 97.0         |
| Additionally allowed regions                | 2.1          |
| Generously allowed regions                  | 0.9          |
| Disallowed regions                          | 0.0          |
| Mean energies (kcal/mol)                   | –2676.25 ± 108.88 |
| Van der Waals                               | –617.31 ± 15.62 |
| Electronic                                  | –3567.07 ± 100.74 |
| RMSD from idealized geometry                |              |
| Bond (Å)                                   | 0.014 ± 0.0003 |
| Angle (degrees)                            | 1.05 ± 0.0271 |
| NOE (Å)                                    | 0.029 ± 0.0023 |

a Per monomer subunit.

b Values for ordered structures (aa 931–967).

CC MBS and LZ PKG-ια. Within the coiled-coil region, the narrow distributions of values of radius of gyration (Rg) for CC MBS (1.76 nm) and LZ PKG-ια (1.62 nm) suggest overall stability with persistence of conformational fold throughout the simulation period (Fig. 4, A and B). Fig. 4, C and D, show the trajectory time-dependent Ca–Ca distance between the two chains of each homodimer (d1–2). In CC MBS and LZ PKG-ια, the distributions of d1–2 values remain closer to their overall averages of 4.6 Å (Fig. 4C) and 5.7 Å (Fig. 4D), respectively, and remain stable throughout their trajectories. Fig. 4, E and F, show average RMSD evolution across the simulation trajectory for CC MBS (0.37 nm) and LZ PKG-ια (0.26 nm). The similar initial RMSD values exhibit minor fluctuations at later simulation times. The initial RMSD values for CC MBS oscillates within a narrow distribution, whereas the variation for LZ PKG-ια is slightly larger throughout the simulation. These results demonstrate that tertiary conformations of the coiled-coil regions of these binding partners remain stable in solution during the simulation.

CC MBS-LZ PKG-ια interaction as detected by heteronuclear NMR

We previously reported conformational perturbation of LZ PKG-ια residues Leu12, Leu36, Lys37, Leu40, Cys43, Glu44, His54, and Gly55 upon binding of CC MBS, based on 2D 1H–15N HSQC chemical-shift perturbation and cross-peak intensity decrease (supplemental Fig. S3A). These CSP in LZ PKG-ια reflected reversible binding of CC MBS with Kd of 178 μM, as determined by isothermal titration calorimetry (14). We have now applied CSP analysis from 2D 1H–15N HSQC titration experiments to identify those CC MBS residues significantly perturbed upon binding of LZ PKG-ια. Fig. 5A shows 2D 1H–15N HSQC overlays of CC MBS resonances in the absence and presence of LZ PKG-ια at increasing stoichiometric ratios. Significant amino acid perturbations within CC MBS were evident in the presence of LZ PKG-ια at a 1:1 molar ratio. Further increase of LZ PKG-ια concentration caused no further change in chemical shift, suggesting binding saturation. The residual perturbation in CC MBS was measured by examining chemical shift change as well as decrease in peak intensity.

Effective chemical shift changes upon the addition of LZ-PKG-ια at increasing concentrations (Fig. 5 and supplemental Fig. S6) were observed for 10 residues: Ala948, Leu950, His951, Thr953, Asn954, Met955, Thr958, Asp959, Leu962, and Glu965. The Glu956 cross-peak disappeared from the complex spectra at higher ligand concentrations (Fig. 5B). Residues Asp952, Met955, and Glu963 exhibited decreased peak intensity (Fig. 5C). Residues Asp952 and Met955 displayed decreases in both CSP and peak intensity, whereas other residues showed only CSP change. The decreased peak intensities probably reflect slowed rotational tumbling of the ~25-kDa CC MBS-LZ PKG-ια complex, due to the larger Stokes radius associated with the elongated CC structure (18, 19).

The appearance of new cross-peaks in CC MBS upon the addition of LZ-PKG-ια probably reflects altered conformational exchange in CSP residues or decreased flexibility of undetected loop residues Ala975–Leu980. The observed CC MBS interactions span the faster-intermediate exchange regime on the NMR time scale (Fig. 5A). These results together suggest that the above-noted residues are at or adjacent to the CC MBS-LZ PKG-ια interaction interface. These PKG-ια-interacting residues map to the mid- to C-terminal region of the homodimer backbone of CC MBS along its C2 axis of symmetry (Fig. 5D) and positions a–f within the CC heptad repeats (Fig. 5E).

Backbone dynamics of CC MBS in the absence and presence of LZ PKG-ια

The well-resolved spectra allowed unambiguous recording of T1 (42 residues), T2, and 1H-15N NOEs (41 residues). Fig. 6 shows the CC MBS relaxation parameters as a function of residue number in the absence and presence of LZ PKG-ια. In the absence of ligand, relaxation parameters of CC MBS coiled-coil aa 932–967 were tightly distributed, indicative of a well-structured region. Lower values of T1 and heteronuclear NOE and higher values of T2 for the downstream aa 968–974 indicated greater conformational flexibility of this loop region.

Decreased spectral resolution of CC MBS in the presence of saturating LZ PKG-ια limited unambiguous assignments of T1, T2, and 1H-15N NOEs to 39, 37, and 33 residues, respectively. The decreased spectral resolution probably reflects both slowed rotational tumbling of the complex and increased line broadening of select CC MBS residues.

Complex formation modestly decreased CC MBS average T1 values (in s) from 1203.08 to 1182.45 and increased T2 values (in...
s) from 35.13 to 43.88, whereas NOE values were unaltered (from 0.73 to 0.72). These changes in average relaxation parameters were not globally distributed but were conferred largely through residues at or adjacent to the interaction interface. As shown in Fig. 6, $T_1$ and NOE values decreased and $T_2$ values increased for most CSP residues in CC MBS upon complex formation with LZ PKG-1α, suggesting modest local plasticity due to increased faster-time scale internal motions (picoseconds to nanoseconds). The results probably reflect conformational cooperativity elicited by these CC MBS residues in accommodating formation of the intermolecular interface (see HADDOCK calculations below).
NMR relaxation parameters are sensitive to both molecular shape and mass. Because amide N–HN bond vectors usually orient along the long axis of the molecule, 15N relaxation rates of elongated coiled-coil structures can be unusually elevated (20, 21). The elongated nature of the CC MBS coiled-coil region thus renders the higher values of $T_1/T_2$ ratio and total rotational correlation time ($\tau_R$) of its residues unreliable in estimating order parameter ($S^2$) (supplemental Fig. S4C). Therefore, $S^2$ values of CC MBS residues were deduced from the MD simulation trajectory. The coiled-coil helical residues exhibited higher $S^2$ values (0.85–0.95). The lower $S^2$ values of the C-terminal residues reflect their flexible loop conformation. Most residues that do not undergo CSP exhibit below- or near-average relaxation parameters and experience a rigid environment before and after complex formation with LZ PKG-Iα. NMR relaxation studies of LZ PKG-Iα residues in the presence of CC MBS will provide additional insight into conformational cooperativity near the dimer-dimer interface.

**CC MBS-LZ PKG-Iα interaction as detected by PRE NMR**

LZ PKG-Iα residues constituting the PPI interface with CC MBS were also identified by PRE NMR, using six single-Cys substitution mutants of CC MBS (Lys933, Gln938, His951, Met955, Thr958, and Lys966) (Fig. 7). CD spectra and SEC confirmed that the recombinant CC MBS constructs were well-folded (supplemental Fig. S7). Individual unlabeled and spin-labeled mutant CC MBS polypeptides were titrated into a solution of 15N LZ PKG-Iα. Spin labeling was with 1-oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-methyl-methanethiosulfonate (MTSL).

PRE (cross-peak intensity) of 15N LZ PKG-Iα decreased in the presence of only those CC MBS mutants with spin label MTSL incorporated at Met955, Thr958, or Lys966 (Tables 2 and 3 and supplemental Table S1). Fig. 7 shows representative TROSY spectra of LZ PKG-Iα in the presence of CC MBS without or with incorporated spin label at M955C (Fig. 7B). Titration of CC MBS Cys-substituted at Thr958 and Lys966 also elicited MTSL-dependent PRE in 15N LZ-PKG-Iα, whereas CC MBS Cys-substituted at His951, Gln938, or Lys933 did not elicit spin label-dependent PRE. The data suggest that a region extending from the middle through the C-terminal portion of CC MBS constitutes the PPI interface with LZ PKG-Iα, a finding consistent with our NMR CSP data.
The LZ PKG-1α residues exhibiting PRE effects are located within the Leu30–Gly55 C-terminal region (supplemental Table S1). The data together indicate that the PPI between CC MBS and LZ PKG-1α involves their C-terminal aa regions. PRE-based distance restraints were generated (see “Experimental procedures”) and used for determination of protein-protein complex structures in HADDOCK.

**HADDOCK structure of the CC MBS-LZ PKG-1α complex**

NMR CSP values from the titration experiments and PRE-based distances were used as interaction restraints for building the model of the CC MBS-LZ PKG-1α complex using data-driven docking. From each CC MBS subunit’s 13 CSP residues within the CC MBS homodimer, 9 residues in subunit 1 and 11 residues in subunit 2 qualified as active residues (Table 2; see “Experimental procedures”). Similar criteria applied to the LZ PKG-1α homodimer’s 8 CSP residues in each monomer yielded 5 active residues in subunit 1 and 7 active residues in subunit 2 (Table 2 and supplemental Fig. S5, A and B) (14). 23 PRE restraints were applied for the complex of CC MBS-LZ PKG-1α (9 for subunits 1, and 14 for subunits 2). These residues were entered into the ambiguous interaction restraints (AIR) file for calculations, whereas residues with non-qualifying CSP were excluded from docking calculations.

HADDOCK Z-score and stereochemical quality analysis yielded a top cluster of 17 structures (Fig. 8A) from the ensemble of 70 best structures (Table 3). A lowest energy conformation was chosen as the representative model of the complex (Fig. 8B). The CC regions of these homodimeric binding partners are packed in a V-shape with principal axes oriented at an angle of 57.3°. The convergence and molecular packing are consistent for each of these best 17 conformers. The buried interaction surface area is 1399.9 ± 91.2 Å² for the ensemble of 17 structures and 1332.21 ± 63.5 Å² for the best structures, with minimal variance among the docked complex structures.

The interaction interface of the complex involves the near mid-region of CC MBS and a contiguous C-terminal region of each molecule. The dimer-dimer interface of the complex (Fig. 8C) involves the electronegative surface of CC MBS (Fig. 3C) and the electropositive surface of the LZ PKG-1α located near C-terminal regions (supplemental Fig. S5C). The dimer-dimer interface of the heterotetrameric complex is stabilized by extensive non-covalent intermolecular interactions between CC MBS and LZ PKG-1α involving (on average per heterotetramer) >20 salt bridges, >20 hydrophobic interactions, and >15 H-bond contacts. These interactions involve CC MBS residues that are conformationally perturbed and elicit lower T1 and higher T2 values in complex with LZ PKG-1α. A complete list of key residues mediating interaction between CC MBS and LZ PKG-1α is shown in (supplemental Table S2).

**Discussion**

MBS/MYPT1 is a regulatory targeting subunit of the heterooligomeric MLCP. Modulation of MBS/MLCP activity influences subunit isoform splicing, subunit protein phosphorylation at multiple sites, and binding of scaffolding proteins, all of which regulate VSMC contractility through control of the phosphorylation state of myosin light chain (22). One regulatory pathway of MLCP involves the interaction between MBS and cGMP-dependent PKG-1α. This interaction is mediated by MBSCT180 of MBS and the LZ domain of PKG-1α. We and others have reported that the CC and/or LZ domains of MBS within MBSCT180 can bind to and functionally influence LZ PKG-1α. However, in the absence of 3D structural information for the CC or LZ domains of MBS, the structural basis and mechanism of the MBS-LZ PKG-1α interaction have remained incompletely understood.

In this paper, we have reported our solution of the tertiary structure of the homodimeric parallel coiled-coil of MBS and have characterized its interaction with the homodimeric parallel coiled-coil of LZ PKG-1α. Using NMR titration and PRE-guided restraints, we have solved the structure of the heterotetrameric complex of CC MBS-LZ PKG-1α, demonstrating critical intermolecular features of the interaction interfaces between and among these binding partners.

The C-terminal residues (residues 932–967) of MLCP represent a canonical CC homodimer with five heptad repeats, as illustrated by a 2D helical wheel representation (Fig. 5E). This structure is formed and stabilized by the packing of non-covalent interactions, with dominant contributions from hydrophobic residues at the a and d positions of two CC MBS monomers that associate to form a parallel homodimer with an extended interface assuming a slight left-handed helical twist or supercoil (PDB entry 5HUZ). The hydrophobic network of interactions is arranged in canonical “knobs-into-holes” fashion. No salt-bridge interaction is evident in the CC MBS tertiary fold. In addition to hydrophobic residues, polar and charged residues are also involved in the formation of interchain interactions. These interchain restraints emerge primarily from the heptad residues located at a, d, e, and g interfaces.
Our NMR data register CC MBS such that three Leu residues reside in the \(a\) position (heptad units 3–5), whereas additional Leu/Ile and Phe residues align within the \(d\) position. Although most of the \(a\) and \(d\) residues are hydrophobic, the partially exposed Tyr936 in the \(a\) position is tolerated due to a hydrophobic contribution from its aromatic ring atoms with other hydrophobic side chains at the interface and to stabilization of the dimer fold through extensive hydrogen bonding between adjacent helical monomers (23). The bonding network of Asn943 at position \(a\) in the second heptad repeat could favor parallel packing and homodimerization of the CC MBS subunits (24).

The polar and hydrophobic residues flanking this predominantly hydrophobic face (\(e\) and \(g\) positions) provide additional van der Waals interactions contributing to CC MBS dimer stability.

NMR (21) and X-ray crystal structures of LZ PKG-I\(\alpha\) (25) have been reported. We find that the homodimer interface of CC MBS binding partner LZ PKG-I\(\alpha\) is stabilized by hydrophobic and electrostatic interactions. In addition to known hydrophobic and electrostatic interactions, the 2 Lys residues at position \(d\) form Lys15\(d\)/Glu16\(e\) and Lys28\(d\)/Glu29\(e\) interhelical ion pairs (25). The reported LZ-PKG-I\(\alpha\) crystal structure (PDB code 4R4L) is an asymmetric homotrimer in which a homodimeric component is associated with a more...
The physiological role of the MBSCT100 interaction with LZ PKG-1α has permitted our interrogation of their interactions at atomic-level resolution, revealing a heterotetrameric complex formation (Fig. 8), in good agreement with CSP data. The increased flexibility of chemically perturbed CC MBS residues 954, 955–958, and 960 suggests that their dynamic properties probably recruit neighboring residues near the dimer–dimer interface, including aa 856, 960, 961, and 963 of CC MBS and aa 35 and 41 of LZ PKG-1α. This CSP residue cooperativity probably accommodates juxtaposed residues of both partner proteins to increase packing density at the dimer–dimer interface by a mechanism similar to that previously reported by Harbury et al. (26). The non-CC and the LZ domains of MBS adopt loop conformations between pH 7 and 8 (9, 14, 16). LZ MBS adopts a helical conformation at pH 4.5 (16). These data support the classical CC of MBS as the major mediator of MBS dimerization (7, 13). Future structural investigation of larger CC MBS constructs will provide additional insight into possible dynamic contributions of the non-CC and/or LZ domains in binding to LZ PKG-1α.

The physiological role of the MBSCT100 interaction with LZ PKG-1α was evaluated in PKG-1α knock-in mice in which critical Leu/Ile residues within the PKG-1α heptad repeat were
mutated to Ala (5). Direct interaction between heptad-mutant PKG-1α and myosin phosphatase was disrupted, despite retention of the VSMC relaxation response to cGMP (5). Similar previously studied PKG-1α substitutions preserved LZ PKG-1α dimer formation in vitro, but not the interaction between PKG-1α and AL9 (MBSCT180) (7). Differential PKG-1α binding to MBS in VSMC from wild-type mice and PKG-1α LZ domain Leu-Ile-to-Ala knock-in mice was validated by GST-fusion protein pulldown. In support of the interaction interface proposed here, GST-MBS failed to pull down PKG-1α fusion protein from VSMC of LZ PKG-1α CC domain Leu-Ile-to-Ala knock-in mouse (5).

In conclusion, we have presented here the solution structure of the CC MBS parallel homodimer (aa 932–967) and probed its structural features using 15N relaxation NMR and MD stimulation. CSP, PRE, and 15N relaxation NMR experiments identified CC MBS residues that contribute to interaction with LZ PKG-1α. Integration of these results with previously reported biophysical and biochemical data has allowed detection of a heterotetrameric dimer-of-dimers complex between CC MBS and LZ PKG-1α and provided important atomistic details supporting a role for these coiled-coil domains in the MBS-PKG-1α interaction. Spatial or temporal impairment of this interaction may contribute to hypertension and other complications of atherosclerosis (27). Elucidation of additional structural details of this MLC contractile complex will increase our understanding of its roles in vascular biology and contributions to cardiovascular disease.

**Experimental procedures**

**Expression and purification of PKG-1α LZ domain (PKG-1α 1–59) and MBS LZ domains**

The 177-bp cDNA encoding the LZ domain (aa 1–59) of PKG-1α (LZ PKG-1α) was cloned into GST-fusion vector pGEX-2T (GE Healthcare), expressed and purified as described previously (14, 21). The 150-bp MLCP MBS CC was cloned into His6-fusion vector pET28-a (EMD Chemicals), transformed into Escherichia coli, grown at 37°C to an A600 of 0.6–0.8, and then induced with 0.25 mM isopropyl-β-D-galactopyranoside for 4 h (16). Uniformly 13C/15N-enriched or 15N-enriched CC MBS polypeptides were expressed by growth in M9 minimal medium supplemented with 1% Bioexpress cell growth medium (Cambridge Isotopes Laboratories) plus [13C]glucose (2 g/liter) as the sole carbon source and/or [15NH4Cl (1 g/liter) as the sole nitrogen source and then stored as cell pellets at −80°C until further use. Isotopically labeled and unlabeled polypeptides were purified from soluble fractions. Tag-free LZ PKG-1α and CC MBS were obtained by thrombin cleavage and subsequent purification by SEC (14, 16). Purity and homogeneity of unlabeled or 13C/15N- or 15N-labeled polypeptides were assessed by SDS-PAGE and by analytical mass spectrometry.

**Size-exclusion chromatography**

Protein samples of recombinant wild-type and mutant CC MBS and LZ PKG-1α were dialyzed in buffer A (25 mM potassium phosphate, 10 mM NaCl, pH 7.0) and then clarified by centrifugation, loaded onto a pre-equilibrated Superdex 75 SEC column, and eluted in buffer A at a flow rate of 1 ml/min.

**MALDI-TOF MS**

Polypeptide identity was confirmed by MALDI-TOF MS on an AB/MDS Sciex 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems) by determination of m/z ratio as described (16).

**Far-UV CD spectroscopy**

Polypeptide solutions of wild-type and mutant CC MBS and of LZ PKG-1α (25 μM) were clarified by centrifugation. Protein folding was assessed by far-UV (260–190 nm) CD at 25°C with Jasco-810 or Jasco-815 spectropolarimeters (Jasco, Easton, MD) purged with N2 gas in a 1-mm path-length quartz cuvette (Starna Cells Inc.) with four scans in continuous mode at 1-nm bandwidth, 2-s response time, and 20 nm/min scan speed.

**NMR sample preparation**

Size-exclusion chromatography fractions of CC MBS domain were concentrated with Amicon Ultra-15 spin filters. Nominally pure samples were prepared for NMR in 25 mM potassium phosphate, 10 mM NaCl, pH 7.0, in 92% H2O, 8%
D₂O. Samples also contained 1 mM DTT-d₄₀, 0.25 mM DSS as internal standard and 0.05% (w/v) NaN₃.

**NMR spectroscopy**

Experiments were performed at 303 K with a Bruker 800-MHz spectrometer equipped with a 5-mm TCI cryoprobe, a Bruker 700-MHz spectrometer equipped with 5-mm TCI cryoprobe, and a 600-MHz Bruker spectrometer equipped with a triple resonance PFG (z axis) probe. All NMR data were acquired in gradient-selected, sensitivity-enhanced mode. NMR data were processed by NMRPipe/NMRDraw (28) and analyzed by ANSIG (29) and CCPNMR (30). Protein backbone and side chain resonances of ¹H, ¹³C, and ¹⁵N were assigned with standard 2D, 3D, and triple resonance experiments (31–34). HBCBCDCE was used for correlations between ¹³C assignments, including those in aromatic moieties (34); ¹³C times of 120 and 150 ms; 3D NOESY (X double half-filter) experiments (36) with mixing and side chain resonances of ¹H, ¹³C, and ¹⁵N were assigned and analyzed by ANSIG (29) and CCPNMR (30). Protein backbone NMR data were processed by NMRPipe/NMRDraw (28) and acquired in gradient-selected, sensitivity-enhanced mode. Triple resonance PFG (z axis) probe, and a 600-MHz Bruker spectrometer equipped with a 5-mm TCI cryoprobe, a 700-MHz spectrometer equipped with 5-mm TCI cryoprobe, and a 800-MHz spectrometer equipped with a 5-mm TCI cryoprobe.

**MD simulations of structural conformations of CC MBS and of LZ PKG-ια**

Simulations were performed for the three best NMR structural models of CC MBS and of LZ PKG-ια using GROMACS version 5.0 (45) with a GROMOS96 54a7 force field (46) on a 12-processor Linux PC on Orchestra clusters (see **Supplemental Methods** for details). A 300 K reference temperature was set using a Berendtsen thermostat. Bond lengths were constrained by the LINCS algorithm (47). The system was equilibrated under position restraints with 250-ps NVT simulations followed by 250-ps NPT simulations in the presence of Parrinello-Rahman pressure coupling. Production runs were executed for 50 ns. Simulation trajectories were analyzed by GROMACS routine utility and in-house scripts.

**CC MBS-LZ PKG-ια interaction using heteronuclear NMR**

The CC MBS interaction with LZ PKG-ια was measured with a series of 2D ¹H-¹⁵N TROSY experiments. CC MBS was titrated with increasing concentrations of LZ PKG-ια until achieving a 1:1 volumetric stoichiometry. CSP data were determined using weighted-average chemical shifts (Δδ_weighted) for each amino acid residue (Δδ_weighted = ((Δ¹H)² + (Δ¹⁵N/5)²)½; see **Supplemental Methods** and by monitoring decreases in ¹H-¹⁵N correlation cross-peak intensity in 2D ¹H-¹⁵N TROSY spectra.

**CC MBS-LZ PKG-ια interaction analyzed by PRE NMR**

Six recombinant CC MBS polypeptides harvesting single Cys substitutions were incubated with 10 mM DTT for ~4 h at 4 °C to ensure complete reduction, after which DTT was removed by SEC. Polypeptides were then nitroxide-spin-labeled using MTSL (Toronto Research Chemicals). Fresh stock solutions of 150 mM MTSL were prepared by dissolving 10 mg in 250 µl of acetone. A reaction mixture comprising a 1:7 molar ratio of protein/MTSL (protein concentration of 250 µM) was gently agitated overnight at room temperature. Illustra NAP-5 columns (GE Healthcare) pre-equilibrated with buffer A were used to remove excess MTSL, as described elsewhere (48). Spin-label efficiency was determined by MALDI mass spectrometry. All six constructs of CC MBS were fully spin-labeled, and no traces of free MTSL were found. Samples for PRE NMR measurements included 0.3 mM ¹⁵N-labeled PKG-ια mixed with 0.3 mM MTSL-labeled CC MBS (paramagnetic or oxidized sample). Control samples (diamagnetic or reduced) included 0.3 mM ¹⁵N-labeled PKG-ια mixed with 0.3 mM unlabeled CC MBS. PRE was calculated for non-overlapping LZ PKG-ια residues by determining cross-peak intensity ratios (I_parallel/I_diagonal) in 2D ¹H-¹⁵N TROSY spectra.
acquired at 800 MHz for paramagnetic versus diamagnetic samples using identical solution conditions and acquisition parameters. Data collection time for each spectrum was 1 h 35 min. LZ PKG-Î± resonances eliciting significantly reduced ratios of $I_{para}/I_{dia}$ allowed PRE-based distance measurements using the methods described (48–51).

**Backbone dynamics of CC MBS**

$T_1$, $T_2$, and [1H]-15N heteronuclear NOE were measured for CC MBS in the absence and presence of saturating LZ PKG-Î± concentrations on a Bruker Avance 600-MHz spectrometer using pulse programs as described elsewhere (52, 53). Relaxation times $T_1$ and $T_2$ were determined by fitting peak heights of respective experimental data sets to a single exponential decay using the nonlinear least-squares routine in NMRPipe/ NMRDraw. [1H]-15N heteronuclear NOEs were determined as the ratio of spectral peak height recorded with 1H saturation (NOE) to that recorded without 1H saturation (NONOE). Errors in data fit were estimated from duplicate measurements (see supplemental material for details).

**CCMBS-LZ PKG-Î± complex structure using HADDOCK**

For HADDOCK calculation (54) of the structure of the CC MBS-LZ PKG-Î± complex, active residues constituting the CC MBS-LZ PKG-Î± interaction interface were selected based on significant CSP (change in cross-peak position and decrease in cross-peak intensity), PRE-driven distances, and monomer unit solvent accessibility (>35% as determined by Naccess (55), ASAView (56), and APBS (57)). These active residues were used to generate AIR for structure calculation. Passive residues were picked automatically by HADDOCK. The Guru interface of HADDOCK calculated a total of 200 structures using mostly default parameters. These CNS-driven, water-refined structures were analyzed and presented.

**Electrostatic potential surface**

Electrostatic potential surfaces for the structures of CC MBS, LZ PKG-Î±, and their complex were calculated using PDB2PQR (58) and APBS (57). Figs. 3C and 8C highlight regions of positive (blue) and negative charge (red).

**Author contributions**—A. K. S., S. L. A., and A. C. R. conceived and designed the research. A. K. S., G. B., and C. A. performed NMR experiments. A. K. S. conducted NMR data processing and simulations. A. K. S., G. B., and S. L. A. analyzed and interpreted results. A. K. S. drafted the manuscript. All authors revised and approved the manuscript.

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