Solution Structure Of Calmodulin Bound To The Binding Domain Of The HIV-1 Matrix Protein

Jiri Vlach,‡ Alexandra B. Samal‡ and Jamil S. Saad*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

Running Title: Structure of CaM bound to HIV-1 matrix peptide

Keywords: Calmodulin, HIV-1, Gag, Matrix, NMR, Peptides, viral replication

Capsule

Background: Calmodulin (CaM) binds to the matrix (MA) domain of HIV-1 Gag.

Results: We present the NMR structure of CaM bound to the minimal binding domain of MA.

Conclusion: Data offer a structural basis for Gag–CaM interactions during HIV-1 replication.

Significance: Characterization of MA–CaM interaction may help in understanding the functional role of CaM in HIV-1 replication.

SUMMARY

Subcellular distribution of calmodulin (CaM) in human immunodeficiency virus type-1 (HIV-1) infected cells is distinct from that observed in uninfected cells. CaM co-localizes and interacts with the HIV-1 Gag protein in the cytosol of infected cells. Although it has been shown that binding of Gag to CaM is mediated by the matrix (MA) domain, the structural details of this interaction are not known. We have recently shown that binding of CaM to MA induces a conformational change that triggers myristate exposure, and that the CaM-binding domain of MA is confined to a region spanning residues 8–43 (MA(8–43)). Here, we present the NMR structure of CaM bound to MA(8–43). Our data revealed that MA(8–43), which contains a novel CaM-binding motif, binds to CaM in an antiparallel mode with the N-terminal helix (α1) anchored to the CaM C-terminal lobe, and the C-terminal helix (α2) of MA(8–43) bound to the N-terminal lobe of CaM. The CaM protein preserves a semi-extended conformation. Binding of MA(8–43) to CaM is mediated by numerous hydrophobic interactions and stabilized by favorable electrostatic contacts. Our structural data are consistent with the findings that CaM induces unfolding of the MA protein to have access to helices α1 and α2. It is noteworthy that several of MA residues involved in CaM binding have been previously implicated in membrane binding, envelope incorporation and particle production. The present findings may ultimately help in identification of the functional role of CaM in HIV-1 replication.

During the late phase of human immunodeficiency virus type-1 (HIV-1) infection, the newly synthesized Gag polypeptides are targeted to the plasma membrane (PM) for assembly and formation of immature particles (1-4). During virus maturation, the Gag polypeptide is cleaved into several proteins including myristoylated matrix (MA), capsid (CA), nucleocapsid (NC), and short peptides (SP1, SP2, and P6) (1). One of the major roles of the MA domain of Gag during virus replication is targeting the Gag protein to specific sites on the PM for assembly. Gag association with the PM is critically dependent on the myristoyl group (myr) and a basic patch localized in the N-terminus of MA (1,5,6). Proper targeting and localization of HIV-1 Gag on the PM is dependent on phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P2) (7-9). Efficient binding of HIV-1 Gag and MA to membranes is sensitive to lipid composition and the hydrophobic environment of the bilayer (acyl chains and cholesterol)(10) Structural studies have shown that HIV-1 MA binds directly to PI(4,5)P2 and other PM lipids such as phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (11,12). Gag binding to the PM appears to be complex and is likely...
mediated by a network of interactions between the MA domain and various membrane lipids and/or contents.

Despite the significant progress made in elucidating the molecular determinants of HIV-1 Gag assembly and virus release, the pathway(s) by which Gag is trafficked to the assembly sites in the infected cell and its intracellular interactions are poorly understood. Several cellular proteins have been implicated in Gag intracellular trafficking, virus assembly, and Gag-envelope co-localization. These include the human adaptor protein complexes 1, 2 and 3 (13-15), tail interacting protein (16), Golgi-localized gamma-ear containing Arf-binding protein (17,18), ADP ribosylation factor (17), the suppressor of cytokine signaling 1 (19,20), Lck (a lymphoid specific Src kinase) (21), N-ethylmaleimide-sensitive factor receptor (22), Filamin A (23), vacuolar protein sorting-associated protein 18 (24), Mon2 (24), and Lyric (25). The majority of these proteins have been shown to play roles in Gag intracellular trafficking and/or assembly.

We have been particularly interested in understanding the functional role of calmodulin (CaM) in HIV-1 replication. It has been previously shown that the subcellular concentration of CaM in HIV-infected cells is distinct from that in uninfected cells (26). HIV-1 Gag was found to co-localize with CaM in a diffuse pattern in the cytoplasm (27). However, the exact role of CaM in virus replication has yet to be established. CaM is a highly conserved calcium-binding protein expressed in all eukaryotic cells and is implicated in numerous cellular functions (28-33). It can be localized in various subcellular locations, including the cytoplasm, within organelles, or associated with the PM (28-33). CaM is also known to interact with many myristoylated and phosphoinositide-binding proteins to regulate their membrane localization (34-47).

The N- and C-terminal lobes of CaM each possess two helix-loop-helix motifs called “EF-hands” (48-50). Binding of calcium to EF-hands induces major structural rearrangements in the N- and C-terminal lobes resulting in the opening of large binding pockets on the surface of each domain consisting of hydrophobic residues that are essentially buried in the apoprotein (29,32,33). CaM has a “dumbbell-like” architecture with the N- and C-terminal lobes connected by a flexible central linker, thus adopting a semi-extended conformation. The central linker can adopt many different conformations allowing the N- and C-terminal lobes to wrap around target sequences in many different orientations. CaM-binding motifs typically consist of a region of ~20 residues forming basic, often amphipathic helix. In many classical CaM-binding targets, hydrophobic residues involved in binding to CaM hydrophobic pockets, usually occupy conserved positions at 1-5-10 or 1-8-14 (30). Additional basic residues are responsible for stabilization of the complex via electrostatic interactions with CaM acidic residues. Although these patterns are found in many CaM-binding proteins, unclassified motifs have also been identified (30).

Attempts to identify specific roles of CaM in viral replication and infectivity are not limited to HIV-1. CaM possesses a functional role in budding of Ebola virus-like particles (VLPs) by interacting with the viral matrix protein VP40 (51). CaM also appears to play a role in simian immunodeficiency virus replication by interacting directly with the MA domain of Gag (27,34,37,52-55). In vivo and in vitro studies revealed that CaM interacts with additional HIV-1 proteins like Nef, Tat and gp160 (27,34,37,52,53,56). Small-angle x-ray scattering (SAXS) studies have provided a global picture of CaM bound to full-length MA (57) or to short peptides derived from the MA protein (58). Our lab (59) and others (57,60) have shown that CaM binds directly to the MA protein in a calcium-dependent manner. We have shown that binding of CaM induces a conformational change in MA, triggering myr exposure (59). By employing NMR, biophysical, biochemical and mass spectrometry methods we have identified the CaM-binding region to be a region spanning residues 8–43 (MA(8–43)) (61). Within MA, residues 11–19 form an α-helix (α1), residues 20–30 form a β-hairpin, and residues 31–45 form a second α-helix (α2) (11,62-64). Here, we present the NMR structure
of CaM in complex with MA(8–43). Our data reveal that MA(8–43) binds to CaM in an antiparallel mode with the N-terminal helix (α1) anchored to the C-terminal lobe of CaM while the C-terminal helix (α2) of MA(8–43) is docked on the N-terminal lobe. The interaction is mediated by numerous hydrophobic and electrostatic contacts. These findings may help in identification of the precise functional role of CaM in HIV-1 replication.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. CaM and MA(8–43) samples have been prepared as described (61). The CaM-MA(8–43) complex was prepared by mixing equimolar amounts of CaM and MA(8–43), which was then passed through a gel filtration column (Superdex 75, GE Healthcare). Fractions of the complex were pooled and concentrated as desired. All samples were stored in a buffer containing 50 mM Tris-d11 (pH 7), 100 mM NaCl and 5 mM CaCl2.

NMR Spectroscopy. Isotopically unlabeled and uniformly 13C-, 15N-, or 13C-/15N-labeled protein samples were prepared at ~400–500 μM concentrations. NMR data were collected at 35 °C on a Bruker Avance II (700 MHz 1H) spectrometer equipped with a cryogenic triple-resonance probe, processed with NMRPipe (65) and analyzed with NMRVIEW (66) or CCPN Analysis (67). The backbone and side-chain atom resonances of CaM-MA(8–43) complex were assigned using HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, 15N-edited NOESY- and TOCSY-HSQC, hCCH-TOCSY, and HcCH-TOCSY experiments. Assignments of aromatic signals were confirmed by the (H)CB(CGCC-TOCSY)Hαr experiment (68). Intramolecular NOE contacts were obtained from 3D 15N-edited NOESY-HSQC, 4D 13C-/15N-edited HMQC-NOESY-HSQC and 4D 13C-/13C-edited HMQC-NOESY-HMQC (120ms mixing time). Intermolecular NOEs were detected by 3D 13C-edited/half-filtered NOESY experiments (120ms mixing time). Assignments of the majority of intermolecular NOEs were cross-validated in the 13C-edited/half-filtered NOESY spectra collected on 12C-CaM:13C-MA(8–43) and 13C-CaM:12C-MA(8–43) samples. Combined 1H-15N chemical shift differences (Δδ) were calculated as ΔδHN = ((ΔδH)2 + (ΔδN/5)2)0.5 and similarly for 1H-13C as ΔδHC = ((ΔδH)2 + (ΔδC)2)0.5, where ΔδX is a chemical shift change of nucleus X. 1H, 13C and 15N chemical shifts were referenced to DSS (69).

Structure Calculations. Structure of the CaM-MA(8–43) complex was calculated with CYANA starting from random initial angles. Distance restraints with upper distance limits of 2.7, 3.3 and 5.0 Å were determined based on the intensities of NOE cross-peaks. Standard pseudo atom corrections were applied to groups of degenerate hydrogen atoms during calculations. Phi and psi dihedral angle restraints were generated in TALOS+ (70) and used with an uncertainty of ±2 × standard deviation or ±20 deg, whichever was larger, only for residues with a “Good” score. For MA(8–43), hydrogen bond restraints with O–H and O–N distances of 1.7–2.0 and 2.0–3.0 Å, respectively, were used in α-helical regions (residues 15–19 and 31–39) identified based on the chemical shift index (71). For CaM, hydrogen bond restraints were defined for the β-sheets between residues 27, 63, and residues 100, 136, as well as for several N-capping residues identified from characteristic chemical shifts (72). Positions of calcium atoms were restricted within 2.4±0.1 Å from known Ca2+-binding carboxyl and carbonyl oxygens (73). Low-weight torsion angle restraints were used for a general treatment of side-chain rotamers during the initial stages of simulated annealing. A standard protocol for simulated annealing and molecular dynamics minimization in torsion angle space was applied. In the final calculation cycle, 100 random input structures were minimized and 20 structures with the best agreement with the final restraint set were selected. Analysis and visualization of structures were performed using PROCHECK-NMR (74), VMD (75), and Pymol (The PyMOL Molecular Graphics System, Version 1.5.0.2 Schrödinger, LLC.). Electrostatic potential maps were generated using PDB2PQR and APBS software (76,77).

RESULTS
Comparison of Binding of MA and MA(8–43) to CaM. We have previously shown that the majority of \(^1\)H–\(^{15}\)N resonances in the NMR spectrum of MA exhibit significant chemical shift changes upon binding to CaM (59). Extensive loss and/or broadening of the NMR signals precluded determination of the solution structure of the complex. In a subsequent study, we have identified the minimal CaM-binding domain of MA by utilizing a proteolytic digestion assay (61). Analysis of the digestion products by mass spectrometry revealed that the abundant MA species resistant to proteolysis is a peptide spanning residues 8–43 (61). The formation of the complex between MA(8–43) and CaM has led to substantial chemical shifts changes for the vast majority of \(^1\)H and \(^{15}\)N signals in the HSQC spectrum of CaM (Fig. 1). These changes are very similar to those observed in the HSQC spectrum obtained for CaM when bound to the full-length MA protein (Fig. 2A and B), indicating that MA(8–43) binds to CaM in a manner similar to that observed for the full-length MA protein (61).

**NMR Signal Assignments.** Signal assignments were obtained for the vast majority of atoms. No backbone amide signals were detected in the \(^1\)H–\(^{15}\)N HSQC spectra for Lys30–Lys32 of MA(8–43) and Lys75–Met76 of CaM due to signal overlaps or possibly due to a conformational and/or chemical exchange on an intermediate NMR scale. Similar broadening of signals for residues within the central linker of CaM has been observed and attributed to the flexible nature of this region (78). The most significant chemical shift changes of backbone amide and side-chain methyl signals of CaM have been observed for methionine residues 36, 51, 71, 72, 109, 124, 144, and 145, which are crucial for target binding (30,33,79,80), and for many other residues in the hydrophobic pockets. The largest effects are observed for signals corresponding to CaM residues Ile52–Val55 and Met71–Arg74 located in the N-terminus, and residues Met124 and Met144 in the C-terminus (Fig. 2C). Despite the observation of substantial \(^1\)H, \(^{13}\)C and \(^{15}\)N chemical shift changes in the NMR spectra of CaM, the intramolecular NOE patterns remain unchanged upon peptide binding, indicating that the structure of CaM is not greatly altered upon binding of MA(8–43).

Numerous intermolecular NOEs between CaM and MA(8–43) residues were detected in the \(^{13}\)C-edited/half-filtered NOESY spectra. As shown in Figure 3A, the \(^{13}\)C-edited/half-filtered NOESY spectrum obtained on \(^{13}\)C-CaM/\(^{12}\)C-MA(8–43) shows NOEs between Trp16 of MA(8–43) and methyl groups of several hydrophobic residues in the C-terminal lobe of CaM such as Ile100, Met124 and Met144. Unambiguous NOEs between hydrophobic residues in the C-terminal helix (\(\alpha_2\)) of MA(8–43) and hydrophobic residues in the N-terminal lobe of CaM were also detected in a spectrum obtained for the reciprocally labeled \(^{12}\)C-CaM/\(^{13}\)C-MA(8–43) sample (Fig. 3B). MA(8–43) residues Leu21–Tyr29 and CaM residues Met76–Asp80 lack medium-range intramolecular NOE contacts indicative of a regular secondary structure. Backbone amide signals of residues Gly24\(^ M \)–Gln28\(^ M \) and Thr79\(^ C \)–Asp80\(^ C \) (M and C indicate MA(8–43) and CaM, respectively) exhibit moderately strong NOEs with water resonance, suggesting increased solvent accessibility and lack of secondary structure, consistent with the dynamic nature of these regions. No NOE contacts have been observed between the N- and C-terminal helices of MA(8–43), indicating the absence of intramolecular contacts between the two domains. Furthermore, no NOEs have been observed between the N- and C-terminal domains of CaM.

**Structure of the CaM-MA(8–43) Complex.** A summary of the NOE distance restraints is shown in Table 1. A total of 187 intermolecular NOEs detected between MA(8–43) and CaM residues were used to calculate the structure. An ensemble of the lowest-energy 20 structures calculated for the complex shows a good convergence and correspondingly low positional RMSD values within the structured regions (Fig. 4 and Table 1). Previous NMR and circular dichroism data have shown that the free MA(8–43) peptide exists in a random coil conformation, but adopts an \(\alpha\)-helical conformation when bound to CaM (61). Consistent with these observations, the CaM-MA(8–43) structure shows that residues Asp14–
Arg20 and Leu31–Glu40 of MA(8–43) are α-helical, whereas residues Leu21–Gln28 connecting the two MA α-helices, residues Leu8–Leu13, and Leu41–Arg43 lack a regular secondary structure (Fig. 5A). The linker regions were not restrained during structure calculations and the two domains in the resulting structures adopt pseudo-random orientations with respect to each other, limited only by steric factors.

The CaM protein interacts with MA(8–43) in an anti-parallel mode (Fig. 5). Surface representation of the CaM-MA(8–43) complex shows a large interaction interface (Fig. 5B). The CaM protein preserves its semi-extended conformation when bound to MA(8–43). Furthermore, an overall electrostatic compatibility between highly positively charged MA(8–43) and negatively charged CaM is clearly observed (Fig. 5C). The hydrophobic faces of α1 and α2 helices of MA insert into the C- and N-terminal hydrophobic grooves of CaM, respectively (Fig. 6). The major stabilizing factor of the interaction between α1 of MA(8–43) and CaM is the deep insertion of Trp16 and Ile19 side chains into the C-terminal pocket of CaM (Fig. 6A). As indicated by strong NOE cross-peaks (Fig. 3A), the indole ring of Trp16 is in a close proximity to CaM residues Ile100, Met124, Ile125, Ala128, Val136 and Met144 (Fig. 6A). Additionally, the side chain of Ile19M interacts with CaM residues Phe92, Val105, Met109, Leu112, Met124, Phe141 and Met145. Weak intermolecular NOEs were also observed between Leu13M and Leu21M and hydrophobic CaM residues; Leu13M interacts with Met109C and Met124C, while Leu21M interacts with Ala88C and Met144C. Hydrophobic interactions between α2 of MA(8–43) and CaM N-terminus involve MA(8–43) residues Tyr29, Leu31, Ile34, Val35, Ala37 and Leu41, and CaM residues Leu18, Phe19, Val35, Leu39, Met36, Met51, Val55, Phe68, Met71 and Met72 (Figs. 3B and 6B). Surprisingly, the aromatic side chain of Trp36, positioned in the middle of MA helix α2, is mostly solvent-exposed and does not participate in the interactions (Fig. 6).

In addition to favorable hydrophobic interactions, the complex is likely stabilized by several salt bridges as indicated by strong NOEs between the side chains of Lys εH and Glu CyH groups. As indicated by the NOE cross-peaks, Lys15M is in close proximity to Glu120C, while Lys18M is close to Glu114C (Fig. 6). An analogous structural arrangement is observed at the beginning of MA(8–43) α2 helix with Lys30M side chain in close proximity to Glu47C, similar to the Lys15M/Glu120C pair (Fig. 6). Salt bridges can also form within the central part of the complex as Lys26 and Lys27 located in the linker between α1 and α2 of MA(8-43) are favorably close to the highly acidic region in the central linker of CaM domains (Asp78–Glu87). However, unambiguous assignments of NOEs between the respective side chains have been precluded due to signal overlaps. Taken together, there is a pronounced complementarity in terms of the sizes of MA(8–43) and CaM interacting domains. The smaller helix α1 of MA binds the relatively small C-terminal domain of CaM, while helix α2 of MA, which is about twice as long as α1, is fully engaged in binding the larger N-terminal CaM domain. Moreover, the length of the linker between the two MA helices is sufficient to allow full contact of the interaction interfaces while retaining a flexibility of CaM and MA central linker regions.

DISCUSSION

We have previously shown that CaM interacts directly with the MA protein, inducing a conformational change that triggers myr exposure (59). We have identified the minimal CaM-binding domain of MA to a region spanning residues 8–43 (MA(8–43)) (61). The 36-residue region of MA is the largest CaM-binding motif discovered to date since the CaM-binding domain of target proteins is typically ~15-20 amino acids long (30). The positioning of the hydrophobic residues involved in binding to CaM indicates that MA(8–43) contains a novel CaM-binding motif, which does not belong to any of the previously characterized motifs.

The presence of two highly adaptable hydrophobic surfaces on the N and C-terminal domains of CaM is a prominent feature that, together with the flexibility of the protein central region, allows it to bind to numerous targets (33). Even though the CaM-interacting proteins are characteristic by a high variability of amino
acid sequences within binding regions, several motifs have been identified based on the relative positions of anchoring hydrophobic residues. Accordingly, MA residues Trp16 and Ile19 constitute a 1–4 binding motif, whereas Leu31, Ile34 and Val35 make a 1–4–5 motif. To the best of our knowledge, such motifs have not been previously identified. A tryptophan residue is however found as the first anchoring residue in the majority of peptides binding to the C-terminal lobe of CaM.

Besides target protein sequences, CaM–protein complexes exhibit high variability also in terms of overall structures. CaM exclusively binds to α-helical motifs but several distinct binding modes have been identified differing in the involvement and relative positions of CaM binding lobes. Depending on the sequence and length of the target protein, CaM interacts with the protein via either one or two lobes simultaneously. When both CaM lobes are involved, CaM typically adopts a compact structure by wrapping around an α-helix of the target protein. The binding mode represented in the CaM-MA(8–43) complex, in which one molecule interacts with CaM via two well separated helical motifs, is distinct from the vast majority of reported structures. Thus, MA(8–43) represents a rare target sequence that is substantially different (in length and composition) from almost all known CaM-binding domains, which confirms the enormous versatility of CaM-target complex formation. Among the ~40 structures of CaM-protein/peptide complexes deposited in the protein data bank, only one structure possesses features highly similar to those observed in the CaM-MA(8–43) structure. Bipartite binding motif has been identified for CaM bound to Munc13-1, a regulator of synaptic vesicle priming (81). Munc13-1 binds the C-terminal lobe of CaM via its long N-terminal helix that is connected by a long flexible linker to a very short α-helix, which binds the N-terminal lobe of CaM (81). In another structure of CaM complex with a peptide derived from vacuolar calcium-ATPase BCA1 protein, two CaM-interacting α-helices in the BCA1 peptide are connected by a very short and rather rigid linker (82); the central part of CaM-BCA1 complex lacks the flexibility observed in the structures of CaM bound to Munc13-1 or MA(8–43). Taken together, our structural findings show a rare CaM interacting motif and, together with Munc13-1, suggest a new class of CaM complexes adopting a modular architecture.

Previous studies utilizing small-angle neutron scattering methods have suggested that CaM adopts an extended open-clamp conformation when bound to MA (60). Tryptophan fluorescence data additionally suggested that interactions are possibly mediated by insertion of Trp16 and Trp36 in hydrophobic pockets of CaM (60). Although these findings agree with most of our previous (59,61) and current data, the structure of CaM-MA(8–43) clearly shows that Trp36 is exposed to solvent and is not involved in any interaction with CaM; the orientation of the helix positions the indole group of Trp36 away from the binding pocket (Fig. 6). Typically, changes observed in tryptophan fluorescence signal are induced when the indole ring is involved in binding or upon occurrence of conformational changes in the protein, which involve a change in the environment of the tryptophan residue (e.g., protein unfolding) (83). Indeed, CaM-induced MA unfolding leads to exposure of both Trp16 and Trp36 rings and is likely to contribute to the observed changes in the fluorescence signal.

An alternative interpretation of the fluorescence data is that Trp36 is involved in the binding of full-length MA but not MA(8–43). Besides the structural evidence, this possibility is also ruled out based on the striking similarity of chemical shift changes observed for CaM residues upon binding to full-length MA and MA(8–43). As shown in Figure 2, chemical shift changes are almost identical for CaM residues located in the hydrophobic pocket (residues surrounding Phe19, Val35, Val55 and Met72) in proximity to the MA Trp36 ring. If the indole group of Trp36 were inserted in the pocket, the ring current effect would have caused significantly different chemical shift changes in some of these CaM residues. Thus, we conclude that Trp36 is not involved in the binding of CaM to MA and that the CaM-MA(8–43) structure is a true representative model of the CaM-MA complex.
Unfolding of the MA protein appears to be one of the main requirements for the formation of CaM-MA complex (57,59-61). The vast majority of the structural studies on CaM-protein complexes were conducted with short peptides that constitute the minimal binding domain of target proteins. It is not clear how these helical peptides that are often part of the overall fold of the protein become accessible to CaM. CaM-induced unfolding of protein targets has been shown to be important for the biological function of target proteins like in enzymatically driven cleavage reactions (84). Although the consequences of MA unfolding upon binding to CaM are not known, it is reasonable to hypothesize that CaM-Gag interaction is regulated intracellularly and is dependent on calcium signaling events during virus replication. Among the multiple functions of CaM is regulation of activity of myristoylated and phosphoinositide-binding proteins to regulate their membrane localization (34-47).

The N-terminal region of MA is critical for diverse Gag functions including Gag-membrane interactions, regulation of the myr switch mechanism, and binding to several cellular constituents implicated in Gag trafficking and/or gp160 incorporation (8,9,11,16,62,85-88). Several residues in helix $\alpha_1$ of MA (Leu13, Trp16, Glu17 and Lys18) are important for gp160 incorporation (89-91), which led to the suggestion that the MA domain of Gag interacts directly with the gp160 protein via helix $\alpha_1$ (89,92). Previous studies have shown that CaM concentration in cells infected with HIV-1 is markedly increased upon expression of the HIV-1 gp160 protein (26). The CaM-binding region was identified as a helical peptide in the gp41 protein and deletion of this region led to diminished virus infectivity (26,52). Thus, the interplay between gp160, CaM and Gag could be an important and underestimated event in the virus replication cycle. We believe that characterization of the interactions between MA and CaM will facilitate future studies and help to identify the exact role of CaM in HIV-1 replication.

ACKNOWLEDGMENTS

Thanks to Madeline Shea (University of Iowa) for providing the CaM molecular clone.

REFERENCES

1. Ganser-Pornillos, B. K., Yeager, M., and Sundquist, W. I. (2008) The structural biology of HIV assembly. *Curr. Opin. Struct. Biol.* **18**, 203-217
2. Jouvenet, N., Simon, S. M., and Bieniasz, P. D. (2009) Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. *Proc. Natl. Acad. Sci. USA* **106**, 19114-19119
3. Jouvenet, N., Bieniasz, P. D., and Simon, S. M. (2008) Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* **454**, 236-240
4. Jouvenet, N., Neil, S. J. D., Bess, C., Johnson, M. C., Virgen, C. A., Simon, S. M., and Bieniasz, P. D. (2006) Plasma membrane is the site of productive HIV-1 particle assembly. *PLoS Biol.* **4**, e435
5. Ono, A. (2009) HIV-1 assembly at the plasma membrane: Gag trafficking and localization. *Future Virol.* **4**, 241-257
6. Reil, H., Bukovsky, A. A., Gelderblom, H. R., and Gottlinger, H. G. (1998) Efficient HIV-1 replication can occur in the absence of the viral matrix protein. *EMBO J.* **17**, 2699-2708
7. Ono, A., Ablan, S. D., Lockett, S. J., Nagashima, K., and Freed, E. O. (2004) Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc. Natl. Acad. Sci.* **101**, 14889-14894
8. Chukkapalli, V., Hogue, I. B., Boyko, V., Hu, W.-S., and Ono, A. (2008) Interaction between HIV-1 Gag matrix domain and phosphatidylinositol-(4,5)-bisphosphate is essential for efficient Gag-membrane binding. *J. Virol.* **82**, 2405-2417

9. Chukkapalli, V., Oh, S. J., and Ono, A. (2010) Opposing mechanisms involving RNA and lipids regulate HIV-1 Gag membrane binding through the highly basic region of the matrix domain. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 1600-1605

10. Dick, R. A., Goh, S. L., Feigenson, G. W., and Vogt, V. M. (2012) HIV-1 Gag protein can sense the cholesterol and acyl chain environment in model membranes. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4490-4494

11. Saad, J. S., Miller, J., Tai, J., Kim, A., Ghanam, R. H., and Summers, M. F. (2006) Structural basis for targeting HIV-1 Gag to virus assembly sites on the plasma membrane. *Proc. Natl. Acad. Sci.* **103**, 11364-11369

12. Vlach, J., and Saad, J. S. (2013) Trio engagement via plasma membrane phospholipids and the myristoyl moiety governs HIV-1 matrix binding to bilayers. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 3525-3530

13. Camus, G., Segura-Morales, C., Molle, D., Lopez-Vergès, S., Begon-Pescia, C., Cazevieille, C., Schu, P., Bertrand, E., Berlioz-Torrent, C., and Basyuk, E. (2007) The clathrin adaptor complex AP-1 binds HIV-1 and MLV Gag and facilitates their budding. *Mol. Biol Cell** **18**, 3193-3203

14. Batonick, M., Favre, M., Boge, M., Spearman, P., Hönig, S., and Thali, M. (2005) Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2. *Virology* **342**, 190-200

15. Dong, X., Li, H., Derdowski, A., Ding, L., Burnett, A., Chen, X., Peters, T. R., Dermody, T. S., Woodruff, E., Wang, J.-j., and Spearman, P. (2005) AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. *Cell** **120**, 663-674

16. Lopez-Vergès, S., Camus, G., Blot, G., Beauvoir, R., Benarous, R., and Berlioz-Torrent, C. (2006) Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. *Proc. Natl. Acad. Sci. USA** **103**, 14947-14952

17. Joshi, A., Garg, H., Nagashima, K., Bonifacino, J. S., and Freed, E. O. (2008) GGA and Arf proteins modulate retrovirus assembly and release. *Mol. Cell** **30**, 227-238

18. Joshi, A., Nagashima, K., and Freed, E. O. (2009) Defects in cellular sorting and retroviral assembly induced by GGA overexpression. *BMC Cell. Biol.** **10**, 72

19. Ryo, A., Tsuturani, N., Obha, K., Kimura, R., Komano, J., Nishi, M., Soeda, H., Hattori, S., Perrem, K., Yamamoto, M., Chiba, J., Mimaya, J.-i., Yoshimura, K., Matsushita, S., Honda, M., Yoshimura, A., Sawasaki, T., Aoki, I., Morikawa, Y., and Yamamoto, N. (2008) SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag. *Proc. Natl. Acad. Sci. USA** **105**, 294-299

20. Nishi, M., Ryo, A., Tsuturani, N., Obha, K., Sawasaki, T., Morishita, R., Perrem, K., Aoki, I., Morikawa, Y., and Yamamoto, N. (2009) Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag. *FEBS Lett.** **583**, 1243-1250

21. Strasner, A. B., Natarajan, M., Doman, T., Key, D., August, A., and Henderson, A. J. (2008) The Src kinase Lck facilitates assembly of HIV-1 at the plasma membrane. *J. Immunol** **181**, 3706-3713

22. Joshi, A., Garg, H., Ablan, S. D., and Freed, E. O. (2011) Evidence of a role for soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery in HIV-1 assembly and release. *J. Biol. Chem.** **286**, 29861-29871

23. Cooper, J., Liu, L., Woodruff, E. A., Taylor, H. E., Goodwin, J. S., D’Aquila, R. T., Spearman, P., Hildreth, J. E. K., and Dong, X. (2011) Filamin A Protein Interacts with Human Immunodeficiency Virus Type 1 Gag Protein and Contributes to Productive Particle Assembly. *J. Biol. Chem.** **286**, 28498-28510

24. Tomita, Y., Noda, T., Fujii, K., Watanabe, T., Morikawa, Y., and Kawaoka, Y. (2011) The cellular factors Vps18 and Mon2 are required for efficient production of infectious HIV-1 particles. *J. Virol.** **85**, 5618-5627
25. Engeland, C. E., Oberwinkler, H., Schümann, M., Krause, E., Müller, G. A., and Kräusslich, H.-G. (2011) The Cellular Protein Lyric Interacts with HIV-1 Gag. *J. Virol.* 85, 13322-13332

26. Radding, W., Pan, Z. Q., Hunter, E., Johnston, P., Williams, J. P., and McDonald, J. M. (1996) Expression of HIV-1 Envelope Glycoprotein Alters Cellular Calmodulin *Biochem. Biophys. Res. Commun.* 218, 192-197

27. Radding, W., Williams, J. P., McKenna, M. A., Tummala, R., Hunter, E., Tytler, E. M., and McDonald, J. M. (2000) Calmodulin and HIV Type 1: Interactions with Gag and Gag Products. *AIDS Res. Hum. Retroviruses* 16, 1519-1525

28. Chin, D., and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* 10, 322-328

29. Hoeflich, K. P., and Ikura, M. (2002) Calmodulin in action: diversity in target recognition and activation mechanisms. *Cell* 108, 739-742

30. Ishida, H., and Vogel, H. J. (2006) Protein-peptide interaction studies demonstrate the versatility of calmodulin target protein binding. *Protein Pept. Lett.* 13, 455-465

31. Osawa, M., Tokumitsu, H., Swindells, M. B., Kurihara, H., Orita, M., Shibanuma, T., Furuya, T., and Ikura, M. (1999) A novel target recognition revealed by calmodulin in complex with Ca\(^2+\)-calmodulin-dependent kinase kinase. *Nat. Struct. Biol.* 6, 819-824

32. Vetter, S. W., and Leclerc, E. (2003) Novel aspects of calmodulin target recognition and activation. *Eur. J. Biochem.* 270, 404-414

33. Yamniuk, A. P., and Vogel, H. J. (2004) Calmodulin's flexibility allows for promiscuity in its interactions with target proteins and peptides. *Mol. Biotechnol.* 27, 33-57

34. Matsubara, M., Jing, T., Kawamura, K., Shimojo, N., Titani, K., Hashimoto, K., and Hayashi, N. (2005) Myristoyl moiety of HIV Nef is involved in regulation of the interaction with calmodulin in vivo. *Protein. Sci.* 14, 494-503

35. Matsubara, M., Nakatsu, T., Kato, H., and Taniguchi, H. (2004) Crystal structure of a myristoylated CAP-23/NAP-22 N-terminal domain complexed with Ca\(^2+\)/calmodulin. *EMBO J.* 23, 712-718

36. Matsubara, M., Titani, K., Taniguchi, H., and Hayashi, N. (2003) Direct Involvement of Protein Myristoylation in Myristoylated Alanine-rich C Kinase Substrate (MARCKS)-Calmodulin Interaction. *J. Biol. Chem.* 278, 48898-48902

37. Hayashi, N., Matsubara, M., Jinbo, Y., Titani, K., Izumi, Y., and Matsushima, N. (2002) Nef of HIV-1 interacts directly with calcium-bound calmodulin. *Protein. Sci.* 11, 529-537

38. Ishii, M., Fujita, S., Yamada, M., Hosaka, Y., and Kurachi, Y. (2005) Phosphatidylinositol 3,4,5-trisphosphate and Ca\(^2+\)/calmodulin competitively bind to the regulators of G-protein-signalling (RGS) domain of RGS4 and reciprocally regulate its action. *Biochem. J.* 385, 65-73

39. Popov, S. G., Krishna, U. M., Falck, J. R., and Wilkie, T. M. (2000) Ca\(^2+\)/Calmodulin reverses phosphatidylinositol 3,4, 5-trisphosphate-dependent inhibition of regulators of G protein-signaling GTPase-activating protein activity. *J. Biol. Chem.* 275, 18962-18968

40. Cao, C., Zakharian, E., Borbiro, I., and Rohacs, T. (2013) Interplay between calmodulin and phosphatidylinositol 4,5-bisphosphate in Ca\(^2+\)-induced inactivation of transient receptor potential vanilloid 6 channels. *J. Biol. Chem.* 288, 5278-5290

41. Deng, W., Putkey, J. A., and Li, R. (2013) Calmodulin adopts an extended conformation when interacting with L-selectin in membranes. *PLoS One* 8, e62861

42. Coticchia, C. M., Revankar, C. M., Deb, T. B., Dickson, R. B., and Johnson, M. D. (2009) Calmodulin modulates Akt activity in human breast cancer cell lines. *Breast Cancer. Res. Treat.* 115, 545-560

43. Dong, B., Valencia, C. A., and Liu, R. (2007) Ca(2+)/calmodulin directly interacts with the pleckstrin homology domain of AKT1. *J. Biol. Chem.* 282, 25131-25140

44. Nagasaki, N., Tomioka, R., and Maeshima, M. (2008) A hydrophilic cation-binding protein of Arabidopsis thaliana, AtPCaP1, is localized to plasma membrane via N-myristoylation and
interacts with calmodulin and the phosphatidylinositol phosphates PtdIns(3,4,5)P(3) and PtdIns(3,5)P(2). FEBS Lett. **275**, 2267-2282

45. Hokanson, D. E., Laakso, J. M., Lin, T., Sept, D., and Ostap, E. M. (2006) Myo1c binds phosphoinositides through a putative pleckstrin homology domain. Mol. Biol. Cell **17**, 4856-4865

46. Hokanson, D. E., and Ostap, E. M. (2006) Myo1c binds tightly and specifically to phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate. Proc. Natl. Acad. Sci. **103**, 3118-3123

47. Matsubara, M., Titani, K., and Taniguchi, H. (1996) Interaction of calmodulin-binding domain peptides of nitric oxide synthase with membrane phospholipids: regulation by protein phosphorylation and Ca(2+)-calmodulin. Biochemistry **35**, 14651-14658

48. Hokanson, D. E., and Ostap, E. M. (2006) Myo1c binds tightly and specifically to phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate. Proc. Natl. Acad. Sci. **103**, 3118-3123

49. Kretsinger, R. H. (1996) EF-hands reach out. Nat. Struct. Biol. **3**, 12-15

50. Moorthy, A., and Murthy, M. (2001) Conformation and structural transitions in the EF-hands of calmodulin. J. Biomol. Struct. Dyn. **19**, 47-57

51. Moyer, A., and Murphy, M. (2001) Conformation and structural transitions in the EF-hands of calmodulin. J. Biomol. Struct. Dyn. **19**, 47-57

52. Yuan, T., Tencza, S., Mietzner, T. A., Montelaro, R. C., and Vogel, H. J. (2011) Calmodulin binding properties of peptide analogues and fragments of the calmodulin-binding domain of simian immunodeficiency virus transmembrane glycoprotein. Biopolymers **58**, 50-62

53. McQueen, P., Donald, L. J., Vo, T. N., Nguyen, D. H., Griffiths, H., Shojaian, S., Standing, K. G., and O'Neil, J. D. (2011) Tat peptide-calmodulin binding studies and bioinformatics of HIV-1 protein-calmodulin interactions. Proteins **79**, 2233-2246

54. Chow, J. Y., Jeffries, C. M., Kwan, A. H., Guss, J. M., and Trewhella, J. (2010) Calmodulin disrupts the structure of the HIV-1 MA protein J. Mol. Biol. **400**, 702-714

55. Izumi, Y., Watanabe, H., Watanabe, N., Aoyama, A., Jinbo, J., and Hayashi, N. (2008) Solution X-ray Scattering Reveals a Novel Structure of Calmodulin Complexed with a Binding Domain Peptide from the HIV-1 Matrix Protein p17. Biochemistry **47**, 7158-7166

56. Ghanam, R. H., Fernandez, T. F., Fledderman, E. L., and Saad, J. S. (2010) Binding of calmodulin to the HIV-1 matrix protein triggers myristate exposure. J. Biol. Chem. **285**, 41911-41920

57. Taylor, J. E., Chow, J. Y., Jeffries, C. M., Kwan, A. H., Duff, A. P., Hamilton, W. A., and Trewhella, J. (2012) Calmodulin binds a highly extended HIV-1 MA protein that refolds upon its release. Biophys. J. **103**, 541-549

58. Samal, A. B., Ghanam, R. H., Fernandez, T. F., Monroe, E. B., and Saad, J. S. (2011) NMR, Biophysical and Biochemical Studies Reveal the Minimal Calmodulin-Binding Domain of the HIV-1 Matrix Protein. J. Biol. Chem. **286**, 33533-33543

59. Tang, C., Loeliger, E., Luncsford, P., Kinde, I., Beckett, D., and Summers, M. F. (2004) Entropic switch regulates myristate exposure in the HIV-1 matrix protein. Proc. Natl. Acad. Sci. USA **101**, 517-522
63. Hill, C. P., Worthylake, D., Bancroft, D. P., Christensen, A. M., and Sundquist, W. I. (1996) Crystal Structures of the Trimeric HIV-1 Matrix Protein: Implications for Membrane Association. *Proc. Natl. Acad. Sci.* 93, 3099-3104

64. Massiah, M. A., Starich, M. R., Paschall, C., Summers, M. F., Christensen, A. M., and Sundquist, W. I. (1994) Three dimensional structure of the human immunodeficiency virus type I matrix protein. *J. Mol. Biol.* 244, 198-223

65. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277-293

66. Johnson, B. A., and Blevins, R. A. (1994) NMRview: a Computer Program for the Visualization and Analysis of NMR Data. *J. Biomol. NMR* 4, 603-614

67. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, M., Ulrich, E. L., Markley, J. L., Ionides, J., and Laue, E. D. (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* 59, 687-696

68. Löhr, F., Hänsel, R., Rogov, V. V., and Dötsch, V. (2007) Improved pulse sequences for sequence specific assignment of aromatic proton resonances in proteins. *J. Biomol. NMR* 37, 205-224

69. Markley, J. L., Bax, A., Arata, Y., Hilbers, C. W., Kaptein, R., Sykes, B. D., Wright, P. E., and Wuthrich, K. (1998) Recommendations for the presentation of NMR structures of proteins and nucleic acids. *J. Mol. Biol.* 280, 933-952

70. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS+ : a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR* 44, 213-223

71. Wishart, D. S., and Sykes, B. D. (1994) The $^{13}$C chemical-shift index: a simple method for the identification of protein secondary structure using $^{13}$C chemical-shift data. *J. Biomol. NMR* 4, 171-180

72. Gronenborn, A. M., and Clore, G. M. (1994) Identification of N-terminal helix capping boxes by means of $^{13}$C chemical shifts. *J. Biomol. NMR* 4, 455-458

73. Biekofsky, R. R., Martin, S. R., Browne, J. P., Bayley, P. M., and Feeney, J. (1998) Ca$^{2+}$ coordination to backbone carbonyl oxygen atoms in calmodulin and other EF-hand proteins: $^{15}$N chemical shifts as probes for monitoring individual-site Ca$^{2+}$ coordination. *Biochemistry* 37, 7617-7629

74. Laskowski, R. A., Rullmann, J. A. C., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and Procheck NMR: Programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477-486

75. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.* 14, 33-38, 27-28

76. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* 32, W665-667

77. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci.* 98, 10037-10041

78. Elshorst, B., Hennig, M., Försterling, H., Diener, A., Maurer, M., Schulte, P., Schwalbe, H., Griesinger, C., Krebs, J., Schmid, H., Vorherr, T., and Carafoli, E. (1999) NMR Solution Structure of a Complex of Calmodulin with a Binding Peptide of the Ca2+ Pump. *Biochemistry* 38, 12320-12332

79. Yuan, T., Ouyang, H., and Vogel, H. J. (1999) Surface Exposure of the Methionine Side Chains of Calmodulin in Solution. A nitroxide spin label and two-dimensional NMR study. *J. Biol. Chem.* 274, 8411-8420

80. Zhang, M., and Yuan, T. (1998) Molecular mechanisms of calmodulin's functional versatility. *Biochem. Cell. Biol.* 76, 313-323
81. Rodríguez-Castañeda, F., Maestre-Martínez, M., Coudevylle, N., Dimova, K., Junge, H., Lipstein, N., Lee, D., Becker, S., Brose, N., Jahn, O., Carломagno, T., and Griesinger, C. (2010) Modular architecture of Munc13/calmodulin complexes: dual regulation by Ca2+ and possible function in short-term synaptic plasticity. *EMBO J* **29**, 680-691

82. Ishida, H., and Vogel, H. J. (2010) The solution structure of a plant calmodulin and the CaM-binding domain of the vacuolar calcium-ATPase BCA1 reveals a new binding and activation mechanism. *J. Biol. Chem.* **285**, 38502-38510

83. Alston, R. W., Urbanikova, L., Sevcik, J., Lasagna, M., Reinhart, G. D., Scholtz, J. M., and Pace, C. N. (2004) Contribution of single tryptophan residues to the fluorescence and stability of ribonuclease Sa. *Biophys. J.* **87**, 4036-4047

84. Gietzen, K., Sadorf, I., and Bader, H. (1982) A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca2+-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem. J.* **207**, 541-548

85. Bryant, M., and Ratner, L. (1990) Myristoylation-Dependent Replication and Assembly of Human Immunodeficiency Virus 1. *Proc. Natl. Acad. Sci.* **87**, 523-527

86. Zhou, W., Parent, L. J., Wills, J. W., and Resh, M. D. (1994) Identification of a Membrane-Binding Domain Within the Amino-Terminal Region of Human Immunodeficiency Virus Type 1 Gag Protein Which Interacts with Acidic Phospholipids. *J. Virol.* **68**, 2556-2569

87. Fledderman, E. L., Fujii, K., Ghanam, R. H., Waki, K., Prevelige, P. E., Freed, E. O., and Saad, J. S. (2010) Myristate exposure in the HIV-1 Matrix Protein is modulated by pH. *Biochemistry* **49**, 9551–9562

88. Saad, J. S., Loeliger, E., Lunecford, P., Liriano, M., Tai, J., Kim, A., Miller, J., Joshi, A., Freed, E. O., and Summers, M. F. (2007) Point mutations in the HIV-1 matrix protein turn off the myristyl switch *J. Mol. Biol.* **366**, 574-585

89. Davis, M. R., Jiang, J., Zhou, J., Freed, E. O., and Aiken, C. (2006) A mutation in the human immunodeficiency virus type 1 Gag protein destabilizes the interaction of the envelope protein subunits gp120 and gp41. *J. Virol.* **80**, 2405-2417

90. Dorfman, A. T., Bukovsky, A., Ohagen, A. S., Hoglund, H., and Gottlinger, G. (1994) Functional domains of the capsid protein of human immunodeficiency virus type 1. *J. Virol.* **68**, 8180-8187

91. Freed, E. O., and Martin, A. M. (1996) Domains of the Human Immunodeficiency Virus Type 1 Matrix and gp41 Cytoplasmic Tail Required for Envelope Incorporation into Virions. *J. Virol.* **70**, 341-351

92. Dorfman, T., Mammano, F., Haseltine, W. A., and Gottlinger, H. G. (1994) Role of the Matrix Protein in the Virion Association of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein. *J. Virol.* **68**, 1689-1696
FOOTNOTES

* This work was supported by the NIH (1R01AI087101 to JSS), and intramural funding from the UAB Center for AIDS Research.

To whom correspondence should be addressed: Jamil S. Saad, Ph.D., 845 19th Street South, Birmingham, AL 35294; Phone: 205-996-9282; Fax: 205-996-4008; Email: saad@uab.edu.

† Authors contributed equally to this work.

Abbreviations: MA, matrix protein; HIV-1 Gag, myristoylated HIV-1 Gag polyprotein; CaM, calmodulin; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence.

Nomenclature: For clarity, MA(8–43) and CaM are denoted by superscript M and C, respectively.

Data depositions: The atomic coordinates of CaM-MA(8–43) complex have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2MGU). Chemical shift assignments of the CaM-MA(8–43) complex have been deposited in the Biological Magnetic Resonance Data Bank and assigned accession number 19604.
FIGURE LEGENDS

Figure 1. Overlay of 2D $^{1}$H-$^{15}$N HSQC spectra obtained for a $^{15}$N-labeled CaM in the free state (black) and in complex with MA(8–43) (red). Selected peaks with significant chemical shift changes are labeled.

Figure 2. Comparison of the chemical shift changes detected for CaM residues upon binding to MA or MA(8–43). (A) A selected region of the 2D $^{1}$H-$^{15}$N HSQC spectra obtained for a $^{15}$N-labeled CaM in the free state (black) and in complex with MA (green) or MA(8–43) (red). (B) A histogram of normalized $^{1}$H-$^{15}$N chemical shift changes vs. residue number calculated from the HSQC spectra obtained for CaM:MA and CaM:MA(8–43) complexes. About 10% of the $^{1}$H-$^{15}$N signals in the HSQC spectrum of CaM:MA were not detected due to severe line broadening. The very similar shifts observed in CaM residues upon binding to MA and MA(8–43) indicates that both MA and peptide bind in a very similar manner. (C) A histogram of normalized $^{1}$H-$^{13}$C chemical shift changes vs. residue number for Ala, Ile, Leu, Val (gray) and Met (blue) methyl signals of CaM when bound to MA(8–43).

Figure 3. 3D $^{13}$C-edited/half-filtered NOE data obtained for the CaM-MA(8–43) complex showing unambiguously assigned intermolecular NOEs between MA(8–43) and CaM. (A) Data obtained on $^{13}$C-CaM/$^{12}$C-MA(8–43) complex show NOEs between Trp16 of MA (8–43) and methyl groups of hydrophobic residues located in the C-terminal lobe of CaM. (B) Data obtained on $^{12}$C-CaM/$^{13}$C-MA(8–43) complex show NOEs between hydrophobic residues in the N-terminus of CaM and the C-terminus of MA(8–43). Horizontal lines indicate assignments for the $^{13}$C component, while labels above and below signals indicate assignments for the $^{12}$C component. MA(8–43) residues are colored in red. Asterisk denotes a breakthrough cross-peak between Met71•H$^{\varepsilon}$ of CaM and Tyr29•H$^{\delta}$ of MA(8–43).

Figure 4. Stereoviews showing the best-fit backbone superposition of the 20 refined structures calculated for the CaM-MA(8–43) complex. Top panel, residues 84–146 of CaM have been superimposed. Bottom panel, residues 1–76 of CaM have been superimposed. Notice that, in the 20 structures, the N- and C-terminal domains of CaM adopt different mutual orientations and appear to be disordered. Thus, only one domain is superimposed at a time. Green spheres denote calcium ions.

Figure 5. Structure of the CaM-MA(8–43) complex. (A) A representative structure of the complex shown as blue (MA(8–43)) and red (CaM) ribbons. Calcium atoms are shown as green spheres. (B) Surface representation of the CaM-MA(8–43) complex shows the large interaction interface. (C) Surface representation of the CaM-MA(8–43) complex and MA colored according to electrostatic surface potential. The CaM-MA(8–43) complex is stabilized by favorable electrostatic interactions.

Figure 6. Close-up views of the CaM-MA(8–43) structure showing that binding is mediated via extensive hydrophobic interactions and favorable electrostatic contacts. MA(8–43) is shown as an orange ribbon while CaM is represented as a grey surface. (A and B) Side and top views showing that numerous van der Waals contacts are present between the N-terminal helix of MA(8–43) and the C-terminus of CaM. Central to these interactions is the deep insertion of the indole group of Trp16 into the hydrophobic cavity of CaM. (C and D) Side and top views show that interactions between the C-terminal helix of MA(8–43) and the N-terminal lobe of CaM are mainly hydrophobic. The indole group of Trp36 is exposed and not involved in any interaction with CaM residues. The complex is further stabilized by favorable salt bridges between lysine residues of MA(8–43) and glutamate residues of CaM (indicated by dashed lines).
Table 1. Statistics for CaM-MA(8–43) structures.

| NMR-derived restraints |        |
|------------------------|--------|
| 1H-1H distance restraints | 1326   |
| Intraresidue           | 169    |
| Sequential (|i - j| = 1)     | 195    |
| Medium range (1 < |i - j| ≤ 4)   | 501    |
| Long range (4 < |i - j|)    | 274    |
| Intermolecular         | 187    |
| Backbone H-bonds (4/H-bond) | 12    |
| Side-chain H-bonds (4/H-bond) | 7     |
| Distance restraints per refined residue | 15.4  |
| Torsion angles         | 318    |

| Restraint violations   |        |
| Ave. max. upper dist. viol. (Å) | 0.09 ± 0.01 |
| Ave. max. van der Waals viol. (Å) | 0.26 ± 0.00 |
| Ave. max. torsion angle viol. (Å) | 1.13 ± 0.49 |
| Cyana target function (Å²) | 0.41 ± 0.06 |

| Residue distribution in Ramachandran plot (%)¹ |        |
| Most favored regions                  | 93.9   |
| Additional allowed regions            | 6.0    |
| Generously allowed regions            | 0.1    |
| Disallowed regions                    | 0.0    |

| Structure convergence (Å)²   |        |
| Main chain atoms RMSD        |        |
| residues 14–20M, 84–146C     | 0.56 ± 0.09 |
| residues 31–40M, 6–74C       | 0.64 ± 0.11 |
| All heavy atoms RMSD         |        |
| residues 14–20M, 84–146C     | 1.29 ± 0.13 |
| residues 31–40M, 6–74C       | 1.25 ± 0.12 |

¹ Determined for residues 14–20M, 31–40M, 6–74C, 84–146C.
² Structures were superimposed with the representative model and pair-wise RMSDs calculated for the indicated residues within structured regions.
Figure 3
Figure 5
