Flexible-body motions of calmodulin and the farnesylated hypervariable region yield a high-affinity interaction enabling K-Ras4B membrane extraction

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In calmodulin (CaM)-rich environments, oncogenic KRAS plays a critical role in adenocarcinomas by promoting PI3K/Akt signaling. We previously proposed that at elevated calcium levels in cancer, CaM recruits PI3Kα to the membrane and extracts K-Ras4B from the membrane, organizing a K-Ras4B–CaM–PI3Kα ternary complex. CaM can thereby replace a missing receptor-tyrosine kinase signal to fully activate PI3K. Recent experimental data show that CaM selectively promotes K-Ras signaling but not of N-Ras or H-Ras. How CaM specifically targets K-Ras and how it extracts it from the membrane in KRAS-driven cancer is unclear. Obtaining detailed structural information for a CaM–K-Ras complex is still challenging. Here, using molecular dynamics simulations and fluorescence experiments, we observed that CaM preferentially binds unfolded K-Ras4B hypervariable regions (HVRs) and not α-helical HVRs. The interaction involved all three CaM domains including the central linker and both lobes. CaM specifically targeted the highly polybasic anchor region of the K-Ras4B HVR that stably wraps around CaM’s acidic linker. The docking of the farnesyl group to the hydrophobic pockets located at both CaM lobes further enhanced CaM–HVR complex stability. Both CaM and K-Ras4B HVR are highly flexible molecules, suggesting that their interactions permit highly dynamic flexible-body motions. We, therefore, anticipate that the flexible-body interaction is required to extract K-Ras4B from the membrane, as conformational plasticity enables CaM to orient efficiently to the polybasic HVR anchor, which is partially diffused into the liquid-phase membrane. Our structural model of the CaM–K-Ras4B HVR association provides plausible clues to CaM’s regulatory action in PI3Kα activation involving the ternary complex in cell proliferation–signaling by oncogenic K-Ras.

Calmodulin (CaM) is a calcium-binding protein. It is 148 amino acids long (16.8 KDa). CaM consists of two symmetric (approximately) globular domains, the N-lobe and the C-lobe, connected by a flexible linker. Each lobe contains a pair of EF-hand motifs, serving as Ca2+-binding sites. Up to four Ca2+ can be loaded with four EF-hand motifs. CaM is abundantly expressed in all eukaryotic cells, mediating intracellular Ca2+ signaling (1). Many proteins with a deficiency in Ca2+-binding ability utilize CaM as a Ca2+ sensor and signal transducer via direct association with CaM (2). There are >500 crystal structures currently available for CaM. These crystal structures illustrate that CaM adopts an extended dumbbell-like shape (3). Ca2+-loaded CaM (holo-CaM) has a rigid central linker helix, but the linker becomes flexible in Ca2+-free CaM (apo-CaM) (4). CaM can yield a compact (collapsed) conformation depending on its binding partner. The flexibility of the linker region allows CaM to wrap around its binding partner such as an α-helical peptide (5). The linker region is mainly composed of acidic residues, indicating that CaM may target proteins rich in basic residues via electrostatic attraction. The interior of each CaM lobe is packed with several hydrophobic residues that form a hydrophobic pocket (Fig. 1), harboring hydrophobic residues from target proteins (6, 7).

CaM mediates many physiological processes such as growth, proliferation, inflammation, metabolism, apoptosis, smooth muscle contraction, intracellular movement, and autophagy (8, 9). Of particular interest is the role of CaM in cancer. Recent data suggested that CaM exclusively interacts with K-Ras but
experiments revealed that CaM interacts directly with PI3K/phoinositide-3-kinase (PI3K)/Akt signaling (22–25). Early K-Ras4B plays a key role in adenocarcinomas promoting phosphorylation of two symmetric globular domains, N-lobe and C-lobe, connected by a flexible linker. Each lobe of CaM contains a hydrophobic pocket. In the protein structures, hydrophobic, polar/glycine, positively charged, and negatively charged residues are colored white, green, blue, and red, respectively.

not with the other Ras isoforms including H-Ras or N-Ras (10–16). K-Ras4B is the most abundant oncogenic isoform, extensively promoting cell proliferation (17, 18), particularly in pancreatic (95%), colorectal (45%), and lung (35%) cancers (19–21). In Ca²⁺/CaM-rich environments such as ductal tissues, K-Ras4B plays a key role in adenocarcinomas promoting phosphoinositide-3-kinase (PI3K)/Akt signaling (22–25). Early experiments revealed that CaM interacts directly with PI3Kα through the Src homology 2 (SH2) domain of its p85 subunit, implicating the contribution of CaM to the regulation of K-Ras4B in the PI3K/Akt pathway (26). Recent studies supported the early observations that CaM recruits PI3Kα to the membrane organizing a K-Ras4B–CaM–PI3K complex, which substitutes the missing receptor-tyrosine kinase signal (23, 24). Increasing evidence shows that CaM can extract K-Ras from the membrane (14–16). Isoform-specific interaction with CaM is in line with farnesylation and the polybasic nature of the hypervariable region (HVR) of K-Ras4B (11–13). It is apparent that the acidic central helical linker of CaM favorably binds to the basic K-Ras4B HVR.

Although a number of experimental studies show that farnesylation and the polybasic region of the HVR are essential for CaM–K-Ras4B interactions (11–13, 27, 28), obtaining experimental structures has been elusive due to the multiple modes of interactions of CaM with K-Ras4B (12, 13). To elucidate the mode of CaM’s interaction, we modeled the CaM–K-Ras4B HVR complex using molecular dynamics (MD) simulation. Because, unlike the Ras dimerization involving the catalytic domain (29, 30), the interactions of CaM with K-Ras4B mainly involved the HVR, the model complex was constructed in the absence of the catalytic domain. Because CaM is highly flexible and the K-Ras4B HVR is unstructured, their interactions are thought to be highly dynamic, yielding various conformational ensembles. All three domains of CaM, including the central linker and both lobes, involved in the interaction with the unfolded HVR suggest that CaM yields a collapsed compact globular conformation. Their dynamic assembly by the flexible-body motion exhibits high-binding affinity, efficiently extracting K-Ras4B from the membrane. Compared with the interaction of the HVR with the anionic membrane, the HVR interacts more strongly with CaM, supporting the observations that CaM could extract K-Ras4B from the membrane (14–16). With increased Ca²⁺, CaM easily competes with anionic lipids and sequesters the polybasic HVR anchor and the farnesyl group. However, it was suggested that phosphorylation at Ser-181 of K-Ras4B or its HVR by protein kinase C (PKC) prohibits the interaction of CaM with K-Ras4B (31, 32). Our structural data provide valuable information about how CaM binds to the highly flexible K-Ras4B HVR, suggesting possible conformational ensembles of the complex in atomic detail.

**Results**

*Does CaM prefer to bind the HVR as a helical structure?*

The crystal structure of CaM with the α-chain domain of myosin light chain kinase (MYLK; PDB code 1CDL, hereafter referred to as MYLK peptide) provided a hint for how CaM interacts with its targets. CaM is a highly acidic molecule. With −24e, it intrinsically attracted basic molecules and subsequently sequesters a target by wrapping around it. The linker region connecting both lobes was highly flexible, assisting in the wrapping mechanism. The MYLK peptide is a good candidate for the CaM target as the α-chain domain was highly positively charged with +6e (Fig. 2A). Compared with the MYLK peptide, the K-Ras4B HVR was also highly positively charged with +9e and had the same length (19 amino acids; Fig. 2B). This prompted us to suggest that the K-Ras4B HVR might adopt the same α-helical topology as the MYLK peptide when it was sequestered by CaM (Fig. 2C). To corroborate the α-helical motif for the K-Ras4B HVR as a possible target of CaM, we
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modeled the CaM–HVR complex with the α-helical K-Ras4B HVR. In the model construction we directly applied the α-helical conformation from the MYLK peptide to the K-Ras4B HVR, yielding the CaM–α-HVR complex (Fig. 2D). To capture another possible mode of the CaM interaction with the α-helical HVR, we inverted the direction of α-helix insertion at the CaM-binding site, generating the CaM–α-HVR \textsuperscript{in} complex (Fig. 2E).

Atomistic MD simulations were performed on the two modeled CaM–K-Ras4B HVR complexes with the α-helical HVR motif and the crystal CaM–MYLK peptide complex. During the simulations, we observed that the crystal CaM–MYLK peptide complex is very stable, preserving the initial compact globular conformation (Fig. 3A). In contrast, both CaM–α-HVR and CaM–α-HVR \textsuperscript{in} complexes exhibited fluctuations resulting from large conformational changes in CaM (Fig. 3, B and C). Opened CaM conformations emerging from the relaxed process largely deviated from their starting conformations (Fig. 3D). In particular, CaM with the inverted α-helical HVR underwent a large conformational transition at a later simulation time frame as the farnesyl group of the K-Ras4B HVR tried to dock into the hydrophobic pocket in the N-lobe of CaM. To observe the mode of the α-helical HVR interactions with CaM at the binding site, we calculated the cross-correlation of the atomic fluctuations over the simulation trajectories and presented it in a dynamical cross-correlations map. Positively correlated residues, which move in the same direction with a correlation coefficient C(i,j) → 1, represent direct interacting pairs at the dimeric interface. For the CaM–MYLK peptide complex, highly positively correlated residue motions spanned entire domains of CaM, indicating that the α-helical peptide is completely encompassed by the flexible central linker and both lobes of CaM (Fig. 3E). However, in the CaM–α-HVR complex the α-helical HVR dominantly interacted with the C-lobe of CaM (Fig. 3F), and the inverted α-helical HVR mainly interacted with the N-lobe of CaM in the CaM–α-HVR \textsuperscript{in} complex (Fig. 3G). These lopsided interactions of the α-helical HVR suggest that CaM unwraps itself around the α-helix, tending to release it. Moreover, we observed that the α-helix of the HVR proceeds to unfold during the farnesyl docking. Taken together, we suggest that CaM does not favor α-helical HRVs as binding partners.

CaM favors sequestering unfolded HRVs allowing the farnesyl to dock toward the hydrophobic pockets located at both lobes

To elucidate the interaction of CaM with the K-Ras4B HVR, we traced chemical shift perturbations (CSPs) on the CaM residues caused by the HVR from previous studies (12, 13). The highly perturbed residues are mapped on the crystal structures of CaM (Fig. 4, A and B). The nuclear magnetic resonance (NMR) data indicate that without the post-translational modification in the HVR, significant CSPs are mainly observed in the linker region and the C-lobe of CaM, whereas CSPs can be observed in both lobes of CaM when the HVR is modified with the prenyl group. The NMR CSPs provided broad ensembles of molecular interaction for how the farnesylated HVR is sequestered by CaM. To render all possible modes of CaM–HVR interactions, we constructed a number of different initial configurations based on the NMR observations representing the models of CaM–HVR association (Fig. 4, C and D). In the initial model constructions, because the NMR CSPs did not provide an exact CaM structure when bound to the HVR, we adopted two different CaM topologies from the crystal structures presenting the stretched and collapsed central linkers. Four independent initial configurations were generated for each topology of CaM. We ensured that in the initial configurations the farnesyl moiety can be docked to either the hydrophobic pocket of both lobes of CaM, and the unfolded HVR backbone can reside at either side of the linker of CaM. A total of eight initial configurations, configurations 1–4 containing the stretched CaM and configurations 5–8 retaining the collapsed CaM, were generated for the MD simulations.

During the simulations, we observed that CaMs with the stretched linker underwent large conformational changes and fluctuations, yielding relatively large values of root-mean-square deviations (r.m.s.d.) with respect to their starting points, which were 6.2, 5.3, 4.0, and 8.1 Å for configurations 1–4, respectively. In contrast, CaMs with the collapsed topology yielded relatively small values of r.m.s.d., which were 3.7, 3.2, 3.1, and 4.3 Å for configurations 5–8, respectively. Unexpectedly, despite the unfolded nature, the HVRs showed fairly small values of r.m.s.d. comparable with the CaM’s r.m.s.d., which were 4.0, 3.8, 4.9, 4.4, 5.3, 5.8, 5.5, and 3.7 Å for configurations 1–8, respectively. The HVR stably wrapped around the acidic linker domain with the farnesyl docking to the hydrophobic pocket of CaM (Fig. 5). Immediate drifting away of the peptide from the complex was prevented due to strong salt-bridge interactions between the HVR and CaM. Almost all Lys residues at the polybasic HVR anchor (residues 175–180) participated in salt-bridge interactions.

The hydrophobic docking of the farnesyl suppressed large fluctuations in the C-terminal region of HVR. Both lobes provided favorable environments for farnesyl with several hydrophobic residues in the pockets (Fig. 6). The farnesyl group formed a hydrophobic cluster with residues Phe-19, Ile-27, Leu-32, Met-36, Leu-39, Leu-48, Met-51, Val-55, Ile-63, Phe-68, Met-71, and Met-72 at the N-lobe, and at the C-lobe, the residues Phe-92, Ile-100, Leu-105, Met-109, Met-124, Ile-125, Ala-128, Val-136, Phe-141, Met-144, and Met-145 are involved in the hydrophobic cluster with the farnesyl.

Strong electrostatic interactions drive CaM to wrap around the HVR, forming a high affinity CaM–K-Ras4B HVR complex

The salt-bridge interactions are a major driving force in stabilizing the CaM–K-Ras4B HVR complex. For CaM, the salt bridges occurred with high probability for the anionic residues at the N-lobe (Glu-11) and linker (Asp-78 and Glu-84) regions (Fig. 7A). For the HVR, those Lys residues in the polybasic region (residues 175–180) exhibited high probability in forming salt bridges. The HVR appeared to engage in electrostatic interactions with the N-lobe more than with the C-lobe of CaM. The residue pairs between CaM and the HVR that form a salt bridge with high probability were Glu-11–Lys-184, Glu-14–Lys-184, Asp-50–Lys-175, Glu-54–Lys-179, Asp-78–Lys-182, Glu-84–Lys-179, Glu-84–Lys-180, and Glu-87–Lys-180 (Fig. 7B), where the former is the CaM residue and the latter is...
the HVR residue. The total interaction energy of the HVR with CaM averaged over all 8 configurations was $-1002.2 \pm 152.7$ kcal/mol, where the electrostatic interaction is a large contribution to the total interaction energy. The averaged interaction energy of the farnesyl group with CaM was $-33.7 \pm 4.1$ kcal/mol, where the van der Waals interaction between farnesyl and the CaM hydrophobic pocket was the main contributor to the total energy. To compare the HVR–CaM interaction with the HVR–membrane interaction, we extracted the total interaction energy of the HVR domain of GTP-bound K-Ras4B with the
anionic membrane and averaged over all four configurations from our previous studies (33). The averaged interaction energies were $706.6 \pm 176.5$ kcal/mol and $36.2 \pm 5.2$ kcal/mol for the HVR–membrane and farnesyl–membrane interactions, respectively. Although the farnesyl interactions are very similar, the HVR–CaM interaction was highly compatible with the HVR–membrane interaction, suggesting that CaM provides favorable environments for the farnesylated K-Ras4B HVR.

To grade the conformations of CaM–K-Ras4B HVR complex, we calculated the binding free energy of the complex formation using molecular mechanics energies combined with the Poisson-Boltzmann surface area continuum solvation (MM-PBSA) method (34, 35). The average binding free energy was calculated as a sum of the gas-phase contribution, the solvation energy contribution, and the entropic contribution,

$$\langle \Delta G_b \rangle = \langle \Delta G_{\text{gas}} \rangle + \langle \Delta G_{\text{sol}} \rangle - T \Delta S$$  (Eq. 1)

where $\langle \rangle$ denotes an average along the MD trajectory. The change in binding free energy due to the complex formation was calculated by using equation 2,

$$\Delta G_b = G_b^{\text{complex}} - (G_b^{\text{CaM}} + G_b^{\text{HVR}})$$  (Eq. 2)

where we closely followed the protocol reported in our previous studies (30). The details of the decomposition of each term show very similar patterns for all CaM–HVR configurations (Fig. 8). Among them, we observed that the lowest value of the binding free energy occurs for configuration 5 with the collapsed compact CaM topology. This can be designated as the most stable complex. However, the highest value of the binding free energy also occurs for the collapsed CaM (configuration 6), suggesting that a precise wrapping is required to securely sequester the HVR backbone. On the other hand, the values of the binding free energy are rather similar for all extended CaMs, allowing multiple modes of the CaM–HVR associations. For all configurations, it is common that the N-terminal linker portion of the HVR (residues 167–174) produced the fluctuations that contributed to the variations in the binding free energy. The fluctuations can be reduced by attachment of the catalytic domain to the HVR linker, which may also shift the free energy landscape. It has been known that the MM-PBSA method practically exaggerates the value of absolute $\Delta G_b$ and poorly predicts the experimental measurements. The MM-PBSA calculations are affected by the choices of the solute dielectric constant, parameters for the nonpolar energy, and the radii used for the PB, which greatly influence the calculated results (36, 37). Nevertheless, the method has been used successfully in determining relative differences in binding free energies (38–40). Our results show that the method is useful for grading CaM–HVR complexes.

Figure 4. Shown are NMR CSPs of residues by the K-Ras4B HVR mapped onto the crystal structures of CaM with a stretched central linker (A) and a collapsed linker (B). In the structures, residues with high CSPs induced by non-farnesylated and farnesylated HVRs are marked by red and blue letters, respectively. Initial configurations of CaM–K-Ras4B HVR complex are constructed using the crystal structures of CaM with a stretched central linker (configurations 1–4) (C) and a collapsed linker (configurations 5–8) (D). Unfolded HVRs are modeled in the complex, ensuring that the initial contacts of the HVR peptides on the CaM residues are determined by the NMR CSPs.
for calculating the relative strengths of $\Delta G_p$, being able to deduce the favorable complex conformation among several configurations.

**All three domains of CaM are involved in binding to farnesylated K-Ras4B HVR peptide**

To determine the domains of CaM involved in binding to the modified K-Ras4B HVR peptide, tryptophan fluorescence experiments were performed using three tryptophan mutants of CaM, namely T27W, S82W, and Q137W. The three mutations were created in the three domains of CaM that included the N-lobe, central linker, and C-lobe regions. Upon titration, the fluorescence spectra data showed that a shift in the absorbance can be seen in all three mutants (Fig. 9A). Using non-linear regression analysis, we obtained the dissociation constants, $K_D = 0.14 \pm 0.02 \mu M$, $0.19 \pm 0.03 \mu M$, and $0.08 \pm 0.04 \mu M$ for the N-lobe, central linker, and C-lobe regions, respectively (Fig. 9B). The results suggest that both lobes of CaM along with the linker region are involved in binding to the farnesylated HVR.

**CaM can recruit farnesylated K-Ras4B HVR peptide from the membranes**

The difference in the cooperative-binding mode with different phospholipid types can have implications for how CaM can...
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Figure 6. Highlights of the hydrophobic interactions of the farnesyl moiety (white stick) with the CaM residues (yellow sticks) at the hydrophobic pockets in the C-lobe of configuration 5 (upper panel) and the N-lobe of configuration 7 (lower panel) in a stereo representation.

bind and/or remove the farnesylated K-Ras4B HVR peptide, which bound to dipalmitoylphosphatidylcholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) phospholipids. We performed surface plasmon resonance (experiments and compared the sensograms for binding of 10 μM farnesylated HVR peptide followed by application of Tris citrate buffer and injection of 400 nM CaM to DPPC and DOPC phospholipids. When CaM at low concentrations was applied to farnesylated HVR peptide bound to DPPC phospholipids, the response units decreased, indicating that the farnesylated HVR peptide is efficiently removed by CaM (Fig. 10.A). When the same low concentration of CaM was applied to farnesylated HVR peptide bound to DOPC or anionic dipalmitoylphosphatidylserine (DPPS) or 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) phospholipids, there was a noticeable increase in the response units (Fig. 10, B–D). This suggested that when the farnesylated HVR peptide is bound to more loosely packed DOPC, DPPS, or DOPS phospholipids it allows CaM to bind to it and stay on the membrane. Such a distinct response of the farnesylated HVR peptide to CaM based on the type of phospholipids may have important implications for how K-Ras4B is differentially regulated in different membrane microdomains enriched in distinct phospholipid types. For controls, 10 μM unmodified HVR peptide was injected onto DPPC, DOPC, DPPS, or DOPS phospholipids followed by buffer and CaM (right panels of Fig. 10). In the control experiments there was no effect of CaM on HVR dissociation from the phospholipid bilayers, implicating the critical role of farnesyl in the observed differential interactions between CaM and HVR assembled on saturated and unsaturated lipids.

To corroborate the CaM’s recruitment of the K-Ras4B HVR from the membranes, we further performed all-atom MD simulations on the CaM–K-Ras4B HVR complex at the membranes (Fig. 11). The initial configurations for the HVR peptide that were placed on the surface of the lipid bilayer were obtained from our previous studies (41). Three different types of the lipid bilayers, the zwitterionic DPPC and DOPC bilayers and the anionic DOPC:DOPS (mole ratio 4:1) bilayer, were employed in the simulations. At the starting point, CaM was merely located next to the HVR. It was observed that in the absence of CaM, the farnesyl group inserted spontaneously into the hydrophobic cores of both DOPC and DOPC:DOPS lipid bilayers, whereas it failed to insert in the DPPC bilayer (41). The spontaneous farnesyl insertion exhibited fast kinetics, occurring on the scale of <1 ns (30, 41). However, in the presence of CaM, there were competing interactions of the HVR with CaM and the membranes. In the DPPC bilayer CaM completely removed the HVR, preventing the farnesyl insertion. In the DOPC bilayer, CaM extracted the HVR linker portion but failed to remove the farnesyl from the hydrophobic core of the bilayer. Large fluctuations of the CaM–HVR complex at the bilayer could be observed during the simulation. We suspect that the farnesyl was eventually removed from the bilayer due to the reversible nature caused by the intrinsic cis conformation in the unsaturated carbon chain (42). In the anionic DOPC:DOPS bilayer, CaM successfully removed the farnesyl group and extracted the HVR from the anionic bilayer. Our MD results were consistent with the surface plasmon resonance observations, suggesting that CaM comparatively extracts K-Ras4B HVR from the membranes with different microdomains.

Discussion

Here we provide possible modes of the interaction of CaM with the K-Ras4B HVR in atomic detail using explicit MD simulations based on structural data from the NMR experiments (12, 13). We observed that CaM stably bound to the unfolded K-Ras4B HVR with high-binding affinity, but the binding interface in the complex appeared to be highly dynamic. Unlike well-defined domain-domain interaction between two rigid-body proteins, CaM accommodated multiple binding modes in the interaction with the HVR. Variable conformations of CaM due to the flexible linker and unfolded nature of the HVR resulted in several conformational ensembles for the CaM–K-Ras4B HVR complex. The lack of crystal data for the conformation of CaM–K-Ras4B complex, despite many efforts to obtain it, suggests that the interaction of CaM with K-Ras4B accommodated multiple states; thus, the difficulties in crystallization. By providing atomic level conformations of the CaM–K-Ras4B HVR complex we verified experimental postulates that both the farnesylated and polybasic regions of the HVR are essential for complex formation (11–13, 27, 28). Both CaM and HVR are highly flexible, indicating that the CaM–HVR complex can be assem-
bled by flexible-body motion. We observed that the HVR interaction with CaM is very strong when compared with the HVR interaction with the anionic membrane (33).

CaM can exist in two distinct states, extended and collapsed, depending on the target molecule. With an α-chain domain of MYLK, CaM retained the collapsed topology wrapping around the α-helical peptide with high-binding affinity. However, when replacing the MYLK peptide with α-helical K-Ras4B HVR, with the same length and ~60% sequence similarity, CaM abandoned the collapsed topology, trying to release both the α-HVR and the inverted α-helix, α-HVRin. Large conformational changes in CaM due to instability from the unconstrained farnesyl group of the HVR that persists in docking toward the hydrophobic pocket results in conformational evolution into the opened structure. In the relaxed complex conformations, the α-helical HVRs resided in either side of the CaM lobe. However, this observation is inconsistent with the fluorescence experiments that all three domains of CaM, including the central linker and both lobes, interact with the farnesylated K-Ras4B HVR. The one-sided interaction suggests that CaM does not favor the α-helix of HVR as a binding partner, as it is unlikely to grasp the farnesyl when the HVR folds into an α-helix. It was observed that a lipidated protein identified as a specific binding partner for CaM is unstructured, not possessing the α-helical motif (43).

With the unfolded HVRs, we observed that CaM involves all three domains in the interaction with the HVR. Whether CaM is extended or collapsed, there are two major forces that drive the formation of CaM–HVR complex. It is common for all configurations that the strong electrostatic interaction between the negatively charged central linker of CaM and the polybasic region at the anchor portion of HVR stabilizes the complex conformation. The hydrophobic interaction via docking of the farnesyl group into a hydrophobic pocket in CaM imparts stabilization of the complex. The farnesyl tail can dock into either lobe of CaM because both lobes provide a similar hydrophobic bath. The farnesyl docking reduces the fluctuations of the C terminus, stabilizing the interaction of polybasic backbone of HVR with CaM. Note that apo-CaM is also negatively charged and that with Ca2+ loaded, CaM opens its hydrophobic pockets and allows interactions with binding partners. This suggests that for the interaction to be sensitive to calcium, the hydrophobic component has to be dominant. We previously investigated the energetics of HVR binding to CaM by isothermal titration calorimetry (13). We found that electrostatic attraction alone is sufficient for this interaction. The HVR peptide without the farnesyl group exhibited endothermic entropically driven binding to CaM. This suggests the existence of structured regions in the HVR that do not change their conformation upon association with CaM. For example, a type I β-turn structure has been described in the polybasic region of K-Ras4B in complex with farnesyltransferase (44). In this case the enthalpy of binding is dominated by the positive enthalpy of dehydration of the interaction interface. The introduction of farnesyl at the C-terminal cysteine most likely disrupts the structured elements in the HVR, altering the binding mechanism to CaM. Binding of farnesylated HVR is exothermic and

Figure 7. A, shown are the probabilities of salt-bridge formation for residues in CaM (left panel) and K-Ras4B HVR (right panel). B, probabilities of salt-bridge formation for the residue pair between CaM and K-Ras4B HVR. In the three-dimensional contour plot, those residues with high probability are selectively presented.
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Figure 8. Binding free energy for the CaM interaction with the K-Ras4B HVR. In the calculation, gas-phase contribution, $\Delta G_{\text{gas}}$, the solvation energy contribution, $\Delta G_{\text{sol}}$, and the entropic contribution, $-T\Delta S$, combines the average binding free energy, $\Delta G_b$. In the box graphs, the blue and black horizontal lines denote the mean and median values, respectively.

enthalpically driven, which suggests a conformational transition in the peptide upon encountering CaM.

Our free energy calculations showed that the collapsed compact globular conformation of CaM exhibits the highest binding affinity in the interaction with the unfolded HVR, which can serve as the best candidate. CaM also adopts the extended conformation in the HVR interaction with high-binding affinity. The extended topology of CaM can be populated in the event of the initial contact of K-Ras4B and the membrane. Efficient extraction of K-Ras4B from the membrane requires CaM; CaM’s exposure of its acidic residues at the central linker facilitates the electrostatic attraction of the polybasic region of K-Ras4B HVR. The dynamic interactions by the flexible-body motion enhance the contact between these two flexible molecules, promoting the extraction of the partially diffused HVR tail from the liquid-phase lipid substrate. After the extraction, the populations can be shifted toward a more stable and compact CaM conformation depending on the dynamics and orientation of the HVR (Fig. 12).

The farnesyl group plays an important role in binding to CaM. The NMR studies show that non-farnesylated K-Ras4B HVR predominantly binds to the central linker and the C-lobe of CaM with the dissociation constant $K_D = 11.2 \pm 1.6 \mu M$ (12). However, for the interaction of CaM with the farnesylated HVR, the binding affinity increases with $K_D = 0.35 \pm 0.06 \mu M$, engaging all three domains of CaM in the interaction (13). This suggests that the absence of farnesyl or the one liberated from the pocket results in the unilateral interaction of the HVR with CaM as observed in the CaM–α-HVR complex. Along similar lines, the δ subunit of the cGMP phosphodiesterase from retinal rod cells (PDEδ) extracts prenylated proteins, such as Ras, from the membrane (45, 46). The nucleotide-independent and isoform-nonspecific extractions of Ras indicate that PDEδ specifically targets the C terminus prenyl moiety (47), which is common for all Ras isoforms. Recent studies show that PDEδ preferentially targets the farnesyl group of K-Ras4B as well as K-Ras4A and N-Ras in the depalmitoylated state (48). In contrast, CaM exclusively extracts K-Ras4B from the membrane (10–16), suggesting that CaM specifically targets the polybasic region of K-Ras4B HVR as well as the farnesyl moiety.

CaM selectively binds to the GTP-bound K-Ras4B (10–13), suggesting that the CaM interaction with K-Ras4B is nucleotide-dependent. However, it was also reported that the CaM interaction with K-Ras4B is nucleotide-independent, extracting the GDP-bound K-Ras4B from the membrane (14–16). Our previous studies of K-Ras4B in the membrane can reconcile the discrepancy that the active or inactive state of K-Ras4B can be determined by the composite effects of the GDP/GTP exchange, HVR sequestration, farnesyl insertion, and orientation/localization of the catalytic domain at the membrane (33) (Fig. 12). This indicates that the GDP-bound state, which is highly populated in the inactive state, can be active, and similarly, the GTP-bound state, which is highly populated in the active state, can be inactive. The environments, solution, or membrane conditions can also determine the functional state. Thus, it can be expected that CaM is less likely to bind K-Ras4B in the inactive state regardless of the nucleotide binding due to inaccessibility of the HVR that is in the autoinhibition state (33, 49, 50). In the CaM–K-Ras4B interaction, CaM, a negatively charged protein, predominantly targets the lysine-rich, polybasic region of the K-Ras4B HVR. The splice variant K-Ras4A also contains the polybasic region but not to the same extent as K-Ras4B. K-Ras4A can exist in two states: K-Ras4B-like with depalmitoylation and N-Ras-like with palmitoylation due to the reversible nature of the palmitoyl (42, 51). We expect that CaM might also interact with K-Ras4A, and this interaction could be regulated by the palmitoylation/depalmitoylation cycle (52).

To summarize, the challenging and to date insurmountable hurdle of CaM–K-Ras4B crystallization can now be understood. Here, we combine structural experimental data with comprehensive simulations to obtain realistic conformations and their dynamics. Our structural data provide insights into the mechanism of how CaM targets the polybasic region of HVR and how it can facilitate the K-Ras4B extraction from the
membrane in a Ca\textsuperscript{2+}-dependent manner. CaM plays a critical role in the connection between K-Ras4B and PI3K in cell proliferation (Fig. 12). Obtaining the CaM-bound K-Ras4B structure is crucial for understanding the role of CaM in KRAS-driven adenocarcinomas, providing a putative target in drug discovery.

**Experimental procedures**

**Generating initial configurations of CaM–K-Ras4B HVR complex**

Two Ca\textsuperscript{2+}-loaded CaM crystal structures (PDB codes 1CLL and 1CDL) were used to model the CaM–K-Ras4B HVR complex.
Calmodulin binding of K-Ras4B HVR

A

B

C

D

DPPC

DOPC

DPPS

DOPS

Buffer

400 nM CaM

Buffer

Buffer

400 nM CaM

Buffer

Buffer

400 nM CaM

Buffer

Buffer

400 nM CaM

Buffer

Buffer

400 nM CaM

Buffer

Normalized Response Units (%)

Time (s)
plex (Fig. 1). The former is an isolated CaM with an extended linker. The latter is a compact globular CaM with a flexible linker that wraps around an α-helical peptide known as a CaM-binding α-chain domain (residues 1731–1749) from MYLK, smooth muscle.

The K-Ras4B HVR (residues 167–185) with the post-translational modification was extracted from previous simulations (30, 33, 41). In the HVR peptide, the N terminus was capped with NH4+ group, whereas the Cys-185 residue at the C terminus was modified with both farnesylation and methylation. Preliminary simulations were performed on the HVR peptide in solution, generating relaxed conformations of the disordered HVR chain. These disordered ensembles of the HVR peptide were used in the initial construction of the complex. Based on the NMR CSP results (12, 13), the HVR peptide was relocated on to the CaM-binding sites using interactive molecular dynamics (IMD) simulation (53) on a visual molecular dynamics (VMD) (54) window with the NAMD (55) code. The tail of the farnesyl moiety was initially headed to either hydrophobic pockets in both lobes of CaM. To render all possible ensembles from the NMR observations (12, 13), four different initial configurations were generated for each CaM conformation. For comparison, we also constructed CaM–HVR complex with the collapsed CaM embedding an α-helical HVR peptide. The helical conformation was adopted from the crystal MYLK peptide structure.

Atomistic MD simulations

A total of 11 initial configurations were subject to the MD simulations in an aqueous environment; four extended CaMs interacting with unfolded HVR, four compact globular CaMs interacting with unfolded HVR, two compact globular CaMs wrapping around α-helical HVR, and a crystal structure of CaM embedding the MYLK peptide. The modified TIP3P water model was used to create the isometric unit cell box containing the protein complex. In addition to counter ions to neutralize the system, additional Na+ and Cl− were added to satisfy a total ion concentration near 100 mM. The updated CHARMM all-atom additive force field (56) (version C36) was used to construct the set of starting points and to relax the systems to a production-ready stage, closely following the same protocol as in our previous works (30, 33, 41, 49–51, 57).

A series of minimization cycles were performed for the solvents around the harmonically restrained complex. In the pre-equilibrium stages, 2-ns dynamics was performed on each configuration system with the restrained CaM–HVR until the solvent reached 310 K. At the final pre-equilibrium stage, CaM–HVRs were gradually relaxed by removing the harmonic restraints through dynamic cycles with the full Ewald electrostatics calculation, adapting to the surrounding heat bath. In the production runs, the Langevin temperature control maintained the constant temperature at 310 K, and the Nosé-Hoover Langevin piston pressure control sustained the pressure at 1 atm. A total of 5.5-μs simulations, each with 500 ns, were performed using the NAMD parallel-computing code (55) on a Biowulf cluster at the National Institutes of Health (Bethesda, MD). To exempt the initial transients, the first 50-ns trajectories were removed, and thus averages were taken afterward. The simulated trajectories were analyzed using the same CHARMM programming package (56) as used in the initial construction.

Farnesylation and methylation of the HVR peptide

The modification of the HVR peptide was carried out according to our previously published protocol (13). The modified version of the HVR peptide with the sequence KEEKSMKDGGK-KKKKSKC was purchased from the Protein Research Laboratory at the University of Illinois at Chicago. 68 μl of 73.6 mM S-farnesyl-l-cysteine methyl ester dissolved in 100% dimethyl sulfoxide (DMSO) was added to 2 mg of no-weigh sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific) and mixed thoroughly until all of the sulfo-SMCC had dissolved. 50 μl of 50% (w/v) N-octyl-β-D-glucopyranoside (Sigma) was added to the above solution followed by 381 μl of 1× phosphate buffer, pH 7.4 (137 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4) and 1 μl of 50 mM di-t-ert-amyl peroxide (DTAP). The reaction mix was incubated for 1.5 h and thereafter diluted to a final volume of 8 ml with 1× phosphate buffer. The solution was centrifuged at 4500 rpm for 30 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in 150 μl of 100% ethanol followed by the addition of 100 μl of 50% (w/v) N-octyl-β-D-glucopyranoside. The above solution was added to 2.09 mg of HVR peptide dissolved in 750 μl of 1× phosphate buffer and incubated at room temperature overnight. The reaction mix was then applied to a C-18 column and purified by reversed phase high performance liquid chromatography (RP-HPLC). Briefly, the column was equilibrated with 90% Buffer A (0.1% trifluoroacetic acid) and 10% Buffer B (90% acetonitrile) before applying the sample. Thereafter, a linear gradient up to 80% Buffer B was applied. The fractions eluted at 66–68% Buffer B were collected and subjected to mass spectrometric analysis for purity. The pure fractions were pooled and dried using an Eppendorf Microcentrifuge. Because the peptide does not have aromatic residues, the yield was estimated using a previously described protocol that takes advantage of the absorbance difference at 215 nm and 225 nm (58).

Expression and purification of CaM

We expressed and purified CaM using our previously published protocol (12, 13). The plasmids were transformed into...
BL21A1 cells and grown in LB media. Incubation was carried out at 37 °C for 15 h and shaken at a 250 rpm with the use of kanamycin for antibiotic selection. Then the cells were transferred to M9 media with the dilution of 1:20 and grown at 37 °C and 250 rpm until the optical density reached 0.6–0.8 at 600 nm. Induction was carried out by adding 0.2% arabinose, 2% ethanol, and 200 µM isopropyl 1-thio-β-D-galactopyranoside. Harvesting was done after 4 h of induction at 8000 rpm. The pellets were lysed using 10 mM Tris-HCl, 1 mM CaCl$_2$, and 10 mM β-mercaptoethanol and 60 °C heat for 1 h. The lysate was
centrifuged again at 18,000 rpm for 30 min. The pellets from this centrifugation were discarded, and the supernatant was loaded on a phenyl-Sepharose column pre-equilibrated with 10 mM Tris-HCl, 1 mM CaCl$_2$, and 10 mM \(-\) mercaptoethanol. The same buffer was used for washing the column loaded with supernatant until no more protein was detected by the Coomassie Plus Assay. The elution of the protein was done using 10 mM Tris-HCl, 5 mM EDTA, and 10 mM \(-\) mercaptoethanol. Eluted fractions were assessed for purity using an SDS-PAGE gel. Pure fractions were dialyzed in 50 mM Tris citrate, pH 6.5, 50 mM NaCl, 5 mM MgCl$_2$, 10 mM \(-\) mercaptoethanol, and 10 mM CaCl$_2$.

Fluorescence experiments

Tryptophan fluorescence experiments were performed by titrating the farnesylated and non-farnesylated hypervariable peptide against tryptophan CaM mutant proteins. For this, three tryptophan mutants (T27W, S81W, and Q137W) were prepared that have been reported to be isofunctional and isostructural with native CaM (59). The synthesis of these CaM mutant proteins was done using the same expression and purification procedure as explained above. For fluorescence measurements, a PTI Quantamaster spectrofluorimeter was used that contained monochromators at the emission and excitation sides. The emission spectra were recorded at 290 nm. The slit width for emission and excitation was 4 nm and 0.5 nm, respectively. Reference data were obtained by titrating the buffer into CaM, and these data were subtracted from the peptide titration data. The change in intensity was plotted against concentration, and fitting was done using the “one set of sites” model to obtain the related parameters.

Nanodiscs

Nanodiscs for surface plasmon resonance experiments were prepared as previously described (41). Membrane scaffold protein MSP1D1 was expressed in BL21DE3-Star Escherichia coli cells, purified on Ni$^{2+}$ beads, and concentrated to 40 M in 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.5 mM EDTA, and 0.1% NaN$_3$. DPPC, DOPC, DPPS, and DOPS purchased from Avanti Lipids were used for nanodisc construction. Phosphatidylethanolamine lipids 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and dipalmitoylphosphatidylethanolamine (DPPE) were added to the phospholipid mixture to increase the efficiency of nanodisc immobilization on surface plasmon resonance sensor chips. Either phosphatidylcholine- or phosphatidylserine-based lipids were dissolved in chloroform, mixed with either DPPE or DOPE in a 95:5 molar ratio, and dried in an Eppendorf Vacufuge. The mixture was dissolved in the storage buffer containing a 2-fold molar excess of sodium cholate. MSP1D1 was added to achieve the molar ratio of 1:5:95 of the protein to either DPPE:DPPC or DPPE:DPPS or DOPE:DOPC or DOPE:DOPS. The mixture of protein and phospho
The sensor chips were equilibrated in 50 mM Tris citrate, pH 7.6, and 0.1 M NaCl were immobilized on CM5 sensor chips (Biacore) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to achieve a maximum 6000 response units. Ethanolamine was immobilized on the control flow cell of the same sensor chip for referencing. The sensor chips were equilibrated in 50 mM Tris citrate, pH 6.5, 50 mM NaCl, 5 mM MgCl2, 10 mM CaM was injected and allowed to interact with the sensor chip. During the peptide dissociation phase, 750 s after the start of the experiment 400 nM Ca2+ was injected and allowed to interact with the immobilized nanodisks for 300 s, at which point buffer was applied to the sensor chip. After a 200-s equilibration, the peptide solution was injected and allowed to interact with the immobilized nanodisks for 300 s, at which time point buffer was applied to the sensor chip. During the peptide dissociation phase, 750 s after the start of the experiment 400 nM CaM was injected and allowed to interact with the sensor chip for 300 s followed by application of buffer. All experiments were performed at 25 °C with a flow rate of 10 μl/min and a contact time of 240 s. The regeneration time and dissociation time were set to 30 s and 240 s, respectively. The sensor chips were regenerated by 2 M NaCl. Experiments were repeated to assess reproducibility. The control experiments were performed on the same nanodisks but with unmodified HVR.

Author contributions—H. J. and R. N. conceived and designed the study. H. J. conducted most of the simulations, analyzed the results, and wrote most of the paper. A. B., T. C., and V. G. conducted most of the experiments, analyzed the results, and wrote the paper. All authors edited and approved the manuscript.

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