Structure and Calcium Binding Properties of a Neuronal Calcium-Myristoyl Switch Protein, Visinin-Like Protein 3

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Abstract

Visinin-like protein 3 (VILIP-3) belongs to a family of Ca²⁺-myristoyl switch proteins that regulate signal transduction in the brain and retina. Here we analyze Ca²⁺ binding, characterize Ca²⁺-induced conformational changes, and determine the NMR structure of myristoylated VILIP-3. Three Ca²⁺ bind cooperatively to VILIP-3 at EF2, EF3 and EF4 (K_D = 0.52 μM and Hill slope of 1.8). NMR assignments, mutagenesis and structural analysis indicate that the covalently attached myristoyl group is solvent exposed in Ca²⁺-bound VILIP-3, whereas Ca²⁺-free VILIP-3 contains a sequestered myristoyl group that interacts with protein residues (E26, Y64, V68), which are distinct from myristate contacts seen in other Ca²⁺-myristoyl switch proteins.

The myristoyl group in VILIP-3 forms an unusual L-shaped structure that places the C14 methyl group inside a shallow protein groove, in contrast to the much deeper myristoyl binding pockets observed for recoverin, NCS-1 and GCAP1. Thus, the myristoylated VILIP-3 protein structure determined in this study is quite different from those of other known myristoyl switch proteins. We propose that myristoylation serves to fine tune the three-dimensional structures of neuronal calcium sensor proteins as a means of generating functional diversity.

Introduction

VILIP-3 is a neuronal calcium sensor (NCS) protein that belongs to the calmodulin superfamily of calcium sensors [1–4]. VILIP-3 is expressed in Purkinje cells of the cerebellum [5,6] and rat hippocampus [7,8], where it may regulate synaptic plasticity relevant for learning and memory. VILIP-3 is 94% identical in sequence to the NCS protein, hippocalcin that regulates Ca²⁺-dependent K⁺ channels involved in triggering slow afterhyperpolarization (sAHP) current important for spike frequency adaptation [9,10]. The physiological target of VILIP-3 is currently not known, but its high sequence identity to hippocalcin (94% identity) suggests that VILIP-3 may also interact with Ca²⁺-gated sAHP channels in hippocampal neurons. VILIP-3 has also been suggested to affect MAP kinase signaling [6].

VILIP-3 is structurally related to a family of Ca²⁺-myristoyl switch proteins that contain four EF-hand motifs and a covalently attached N-terminal myristoyl group (Fig 1). NMR and/or crystal
structures are known for myristoylated forms of GCAP1 [11,12], NCS-1 [13], and recoverin [14,15]. The binding of Ca$^{2+}$ to NCS proteins promotes their binding to cellular membranes [16–21]. The myristoyl group attached to recoverin is sequestered structurally inside the Ca$^{2+}$-free protein [15], whereas the myristoyl group becomes solvent exposed in Ca$^{2+}$ recoverin [14,21]. This Ca$^{2+}$-dependent extrusion of the myristoyl group, referred to as a Ca$^{2+}$-myristoyl switch, enables recoverin and VILIP-3 to bind cellular membrane targets only at high Ca$^{2+}$ levels [22].

Here, we analyze the Ca$^{2+}$ binding and folding energetics of VILIP-3, and present the NMR structure of myristoylated VILIP-3. VILIP-3 binds to 3 Ca$^{2+}$ at saturation with an apparent dissociation constant ($K_d$) of 0.52 μM and positive cooperativity (Hill slope = 1.8). NMR evidence demonstrates that the covalently attached myristoyl group is solvent exposed in Ca$^{2+}$-bound VILIP-3. By contrast, the Ca$^{2+}$-free VILIP-3 structure contains a long N-terminal loop that positions the myristoyl group inside a protein cavity that is structurally quite different from myristate binding sites seen in recoverin [15], NCS-1 [13] and GCAP1 [11,12]. The unusual structure of myristoylated VILIP-3 suggests that N-terminal myristoylation may serve to help mold each NCS protein into a unique fold [23].

Materials and Methods
Preparation and Purification of Recombinant VILIP-3
Human VILIP-3 gene was subcloned in pET3d (+). Site specific VILIP-3 mutants (Y64A and V68A) were generated by QuikChange site-directed mutagenesis kit (Stratagene). Bacterial cells for
expressing recombinant myristoylated VILIP-3 protein were generated by co-transforming BL21 (DE3) cells with both pET3d-VILIP and pBB131 vector encoding yeast N-myristoyltransferase.

The expression and purification of recombinant VILIP-3 has been described previously [24]. Expression of recombinant VILIP-3 protein and yeast N-myristoyltransferase were both induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cell culture at a final concentration of 0.5 mM (when the cell density reached OD$_{600}$ = 0.5) and the cells were then grown at 25°C for 12–16 hr. Myristic acid (10 mg/L) was added exogeneously 1 hr before induction. Bacterial cells harvested by centrifugation from a 1-L culture typically contained ~10 mg of expressed myristoylated VILIP-3. The isolation and purification of myristoylated VILIP-3 was described previously [24]. The final purified myristoylated VILIP-3 protein was more than 95% pure as determined by SDS-PAGE. Final purified myristoylated VILIP-3 samples contained less than 5% of unmyristoylated protein as judged by reverse-phase HPLC.

Isothermal Titration Calorimetry
A VP-ITC calorimeter (Micro-Cal) was used to measure Ca$^{2+}$ binding data as described previously [25]. VILIP-3 protein (50 μM) used for ITC studies was dissolved in 20 mM Tris buffer (pH 7.5), 50mM NaCl, 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP). The precise protein concentration was determined by measuring optical density at 280-nm as described previously [24]. For each ITC titration, a total of 50 injections (5-μL each) of 2.0 mM CaCl$_2$ were added to the protein sample during the titration. All titrations were performed at 30°C.

Differential Scanning Calorimetry
A VP-DSC calorimeter from MicroCal was used for all DSC measurements as described previously [25]. Each DSC scan used a temperature range of 10–110°C at a scan rate of 60°C/h. A buffer baseline was subtracted from each scan. Protein samples for DSC experiments consisted of either myristoylated and unmyristoylated VILIP-3 proteins (50 μM) dissolved in 20 mM Tris buffer (pH 7.5) containing 100 mM NaCl and 1 mM β-mercaptoethanol with 2 mM CaCl$_2$ (Ca$^{2+}$-bound state) or 2 mM EDTA (Ca$^{2+}$-free state).

NMR Spectroscopy
NMR experiments were performed using Bruker Avance III 600 or 800 MHz spectrometers equipped with a triple-resonance TCI-cryoprobe probe. VILIP-3 samples for NMR were dissolved in 0.3 ml of 90% H$_2$O, 10% [${}^2$H]H$_2$O containing 10mM [${}^{15}$H$_{11}$]Tris, pH 7.4, and 2 mM EDTA (apo-) or 5mM CaCl$_2$ (Ca$^{2+}$-bound). Two-dimensional $^1$H-$^15$N HSQC spectra of VILIP-3 were recorded at 30°C as described previously [24]. Two dimensional $^1$H-$^{13}$C HMQC and $^{13}$C(F1)-edited, $^{13}$C(F3)-filtered NOESY-HMQC experiments were recorded on VILIP-3 samples that contained a 99% $^{13}$C labeled myristoyl group as described previously [24]. All triple-resonance and $^{13}$C,$^{15}$N-edited NOESY experiments were performed and analyzed as described by Clore et al. [26] on a sample of Ca$^{2+}$-free $^{13}$C/$^{15}$N-labeled myristoylated VILIP-3 (in 95% H$_2$O, 5% $^2$H$_2$O). All NMR data sets were processed and analyzed using NMRPipe [27] and Sparky. Sequence specific NMR assignments were described by [26].

Structure Calculation
Multi-dimensional $^{15}$N-edited NOESY-HSQC and $^{13}$C-edited NOESY-HSQC spectra of Ca$^{2+}$-free myristoylated VILIP-3 were acquired and analyzed as described previously [13]. A total of 1738 NOE distance restraints were derived from NOESY spectra. In addition to the NOE-derived
distances, 156 distance constraints for 78 hydrogen bonds and 189 dihedral angle constraints (ϕ and ψ) were calculated using TALOS+ [28] and were used as restraints in the structure calculation. Fifty independent structures were calculated by XPLOR-NIH software [29] using the YASAP protocol [30,31] as described previously [32]. The 15 lowest energy structures were selected and overlaid with RMSD of 0.9 Å.

Results

Three Ca\textsuperscript{2+} Bind Cooperatively to VILIP-3

Calcium binding to myristoylated VILIP-3 and mutants (E26A, F64A and V68A) were monitored by ITC (Fig 2A) and flow dialysis (Fig 2B). Optimal Ca\textsuperscript{2+} binding parameters are listed in Table 1. The ITC Ca\textsuperscript{2+}-binding isotherm for wild type VILIP-3 exhibited exothermic binding of three Ca\textsuperscript{2+} with a steep Ca\textsuperscript{2+} dependence (K\textsubscript{D} = 0.3 μM, ΔH = -6.9 kcal/mol). The fractional

Fig 2. Ca\textsuperscript{2+} binding to VILIP-3. (A) Ca\textsuperscript{2+} binding ITC isotherm for myristoylated VILIP-3. The overall Ca\textsuperscript{2+} binding stoichiometry is 3:1, consistent with Ca\textsuperscript{2+} bound at EF2, EF3 and EF4. The average dissociation constant (K\textsubscript{D}) and binding enthalpy (ΔH) for the three sites are 0.3 μM and -6.4 kcal/mol, respectively. (B) Ca\textsuperscript{2+}-binding data measured by flow dialysis [33]. Representative Ca\textsuperscript{2+} binding data for VILIP-3 wildtype (black squares) and Y64A (open circles) are shown. Fitted curves (dashed lines) were calculated using the Hill model. Fitting parameters for wild type and mutants are listed in Table 1.

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saturation ($Y = [\text{bound Ca}^{2+}] / [\text{Protein}]$) was measured as a function of free Ca$^{2+}$ concentration using flow-dialysis Ca$^{2+}$ binding experiments (Fig 2B) as described by [33]. The fractional saturation ($Y$) was fit by the Hill equation:

$$Y = \frac{[\text{Ca}^{2+}]^\alpha}{[\text{Ca}^{2+}]} + K_D$$

Wild type VILIP-3 binds to Ca$^{2+}$ with Hill coefficient ($\alpha$) of 1.8 and apparent dissociation constant ($K_D$) equal to 0.52 μM. The VILIP-3 mutants (E26A and F64A) each bind to Ca$^{2+}$ with higher apparent affinity compared to wild type (Table 1), consistent with each mutant forming weaker myristate contacts in Ca$^{2+}$-free VILIP-3. These mutants increase the Ca$^{2+}$-binding affinity by destabilizing the Ca$^{2+}$-free VILIP-3 structure (with sequestered myristoyl group) more so than the Ca$^{2+}$-bound state (extruded myristate), which makes the free energy of Ca$^{2+}$ binding more negative and hence more favorable. By contrast, the corresponding mutants in recoverin (E27A and Y65A) did not affect Ca$^{2+}$ binding affinity, which is consistent with both E26 and Y65 not making any contact with the myristate in the Ca$^{2+}$-free and Ca$^{2+}$-bound recoverin structures [14,15]. In summary, E26 and F64 of VILIP-3 make important contacts with the myristate (see below) and these contacts are not seen in recoverin.

### Myristoylation Increases Folding Stability of VILIP-3

Differential scanning calorimetry (DSC) experiments were performed on VILIP-3 to measure the effect of myristoylation on protein folding stability. Representative DSC scans of wild type VILIP-3 are shown in Fig 3. The unfolding temperature of unmyristoylated Ca$^{2+}$-free VILIP-3 (transition temperature, $T_m = 53^\circ$C) is lower than the unfolding temperature of myristoylated VILIP-3 ($T_m = 57^\circ$C), consistent with a stabilization caused by sequestration of the covalently attached myristoyl group inside Ca$^{2+}$-free VILIP-3. The myristoylated VILIP-3 mutants (E26A and F64A) exhibited a detectably lower folding stability ($T_m = 54^\circ$C, Table 1) compared to wild type, whereas the unmyristoylated mutants had the same folding stability as unmyristoylated wild type. The lower folding stability of myristoylated E26A and F64A is consistent with the side-chains of E26 and F64 both making important contacts with the myristoyl group in VILIP-3 as seen in the structure below. By comparison, the corresponding mutants in

### Table 1. Ca$^{2+}$ Binding and Folding Stability of VILIP-3, Recoverin and Mutants.

|                | Hill Coefficent ($\alpha$) | $K_D$ (μM) | $T_M$ (˚C) |
|----------------|---------------------------|------------|------------|
| VILIP-3 WT     | 1.8 ±0.2                  | 0.52 ±0.05 | 57 ±1      |
| VILIP-3 WT, unmyr | ND                       | ND         | 53 ±1      |
| VILIP-3 E26A   | 1.8 ±0.2                  | 0.34 ±0.05 | 54 ±1      |
| VILIP-3 E26A, unmyr | ND                       | ND         | 54 ±1      |
| VILIP-3 F64A   | 1.8 ±0.2                  | 0.23 ±0.05 | 53 ±1      |
| VILIP-3 F64A, unmyr | ND                       | ND         | 54 ±1      |
| Recoverin WT   | 1.5 ±0.2                  | 17 ±2      | 65 ±1      |
| Recoverin E27A | 1.5 ±0.2                  | 20 ±2      | 64 ±1      |
| Recoverin Y65A | 1.5 ±0.2                  | 19 ±2      | 63 ±1      |

Ca$^{2+}$-binding data for myristoylated VILIP-3 were fit to the Hill model with Hill coefficient ($\alpha$) and apparent dissociation constant ($K_D$) in units of micromolar at 30°C. Ca$^{2+}$-binding parameters were not determined (ND) for unmyristoylated VILIP-3. The unfolding temperatures ($T_m$) of Ca$^{2+}$-free forms of myristoylated VILIP-3, unmyristoylated VILIP-3 (unmyr), and myristoylated recoverin were determined by DSC in units of ˚C. VILIP-3 and Recoverin mutants are designated in the superscript.

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myristoylated recoverin (E27A and Y65A) did not affect the melting temperature (Table 1), which is consistent with E27 and Y65 both not making contact with the myristate in the recoverin structure [14].

For Ca$^{2+}$ saturated myristoylated VILIP-3, the protein started to aggregate at around 42°C and the precise unfolding temperature could not be accurately measured by DSC (blue trace in Fig 3). The Ca$^{2+}$-induced aggregation of VILIP-3 was most likely caused by Ca$^{2+}$-induced exposure of the myristoyl group like that observed for recoverin [34].

### NMR Structure of Ca$^{2+}$-free VILIP-3

$^1$H-$^{15}$N HSQC NMR spectra of Ca$^{2+}$-free VILIP-3 (Fig 4A) exhibited a total of 223 highly dispersed peaks with uniform intensities, indicating that Ca$^{2+}$-free VILIP-3 is monomeric under...
NMR conditions and stably folded. Sequence-specific NMR assignments of Ca$^{2+}$-free myristoylated VILIP-3 were analyzed and described previously [35] (BMRB no. 18627). The assigned NMR resonances were used to analyze multi-dimensional NOESY spectra in order to obtain NOE distance restraints that defined the VILIP-3 structure. Secondary structure derived from the NMR data is illustrated in Fig 1. Three-dimensional protein structures of VILIP-3 were calculated based on NOE distance restraints and chemical shift analysis (see Methods). The final NMR-derived structures of Ca$^{2+}$-free myristoylated VILIP-3 are illustrated in Fig 5 (PDB ID: 5T7C). Table 2 summarizes the structural statistics calculated for the 15 lowest-energy conformers.

Fig 4. NMR spectroscopy of myristoylated VILIP-3. Two-dimensional ($^1$H-$^{15}$N HSQC) NMR spectra are shown for $^{15}$N-labeled VILIP-3 in the Ca$^{2+}$-free (A) and Ca$^{2+}$-bound (B) states. Spectra were recorded at 600 MHz at 30˚C.

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Fig 5. NMR-derived structures of myristoylated VILIP-3 (PDB ID: 5T7C). Ribbon diagrams show an overlay of the 10 lowest energy structures (A), and average main chain structure of Ca$^{2+}$-free VILIP-3 (B) and rotated by 180˚ (C) with N-terminal myristoyl group highlighted magenta. The unstructured N-terminal loop region is depicted by a dashed line. Secondary structure elements (helices and strands) and EF-hand motifs are drawn in the same colors as in Fig 1 (EF1 green, EF2 red, EF3 cyan and EF4 yellow). Side-chain atoms of hydrophobic residues at the domain interface (A) and that contact the myristoyl group (B) are highlighted yellow.

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The entire polypeptide chain of Ca\(^{2+}\)-free myristoylated VILIP-3 was defined by the NMR data, except for residues 4–20 and the last six residues at the C-terminus whose resonances were overlapped and/or exchange broadened. VILIP-3 contains a total of nine α-helices and four β-strands: α1 (residues 25–35), α2 (residues 46–56), α3 (residues 62–72), α4 (residues 82–91), α5 (residues 101–108), α6 (residues 118–129), α7 (residues 146–156), α8 (residues 166–176), α9 (residues 179–187), β1 (residues 42–44), β2 (residues 79–81), β3 (residues 115–117) and β4 (residues 163–165) (Fig 1A). The covalently attached myristoyl group at G2 is connected to a long unstructured loop (residues G2 – D24 depicted by the dashed line in Fig 5) that places the fatty acyl chain inside a shallow protein cavity formed by hydrophobic residues in EF1 and EF2 (residues L27, W30, F48, I51, Y52, Y64, V68, F82, F85, I86, L89). The myristoyl group interaction with protein residues (E26, F64, V68 and I86, highlighted yellow in Fig 5B) is unique to VILIP-3 and these contacts are not seen in structures of myristoylated recoverin [15], NCS-1 [13], or GCAP1 [11,12]. VILIP-3 contains two domains formed by the four EF-hands: EF1 (residues 26–55) and EF2 (residues 62–91) are connected together and form the N-domain; likewise, EF3 (residues 101–130) and EF4 (residues 146–175) form the C-domain. The domain interface is stabilized by residue contacts between EF2 (I87 and A88 in helix α4) and EF3 (W103, A104 and M107 in helix α5; and A127 and M131 in helix α6) that connect the two domains (see side-chains highlighted yellow in Fig 5A). Each EF-hand in VILIP-3 consists of a helix-turn-helix structure that is similar to the closed structure of Ca\(^{2+}\)-free EF-hands seen previously in Ca\(^{2+}\)-free recoverin [15] and apo-CaM [36]. The interhelical angles for the EF-hands of VILIP-3 are 142.8° (EF1), 123.7° (EF2), 120.1° (EF3) and 109.7° (EF4). The overall main chain structure of Ca\(^{2+}\)-free myristoylated VILIP-3 (Fig 5) is quite different from the myristoylated forms of recoverin [15], GCAP1 [11], and NCS-1 [13]. A quantitative comparison of the main chain atoms of Ca\(^{2+}\)-free VILIP-3 with those of recoverin, NCS-1 and GCAP1, indicates root-mean-squared deviations of 3.1, 3.6, and 3.9 Å, respectively.
The HSQC NMR spectrum of Ca\(^{2+}\)-bound myristoylated VILIP-3 (Fig 4B) looks quite different from that of Ca\(^{2+}\)-free VILIP-3 (Fig 4A), consistent with a Ca\(^{2+}\)-induced structural change. Fewer NMR peaks are detected for Ca\(^{2+}\)-bound VILIP-3, perhaps due to spectral broadening caused by Ca\(^{2+}\)-induced aggregation of the Ca\(^{2+}\)-bound protein. The aggregation of Ca\(^{2+}\)-bound myristoylated VILIP-3 is most likely caused by a Ca\(^{2+}\)-induced extrusion of the covalently attached myristoyl group like that seen previously for recoverin [21,34].

Myristoyl Binding Site in VILIP-3

The structure of the covalently attached myristoyl group in VILIP-3 was probed by NMR experiments (3-D ([\(^{13}\)C/F]_1)-edited and ([\(^{13}\)C/F]_3)-filtered NOESY-HSQC) performed on Ca\(^{2+}\)-free VILIP-3 samples that contained a \(^{13}\)C-labeled myristoyl group (Fig 6). These NMR spectra probed atoms in VILIP-3 located less than 5 Å away from the \(^{13}\)C-labeled fatty acyl chain. Representative Nuclear Overhauser effect (NOE) dipolar interactions are shown for the C\(^{14}\)methyl of the myristoyl group (\(^{13}\)C\(^{14}\): F\(_2\) = 16.88 ppm, Fig 6A), C\(^{12}\)methylene (\(^{13}\)C\(^{12}\): F\(_2\) = 34.31 ppm, Fig 6B), and the C\(^{2}\)methylene (\(^{13}\)C\(^{2}\): F\(_2\) = 37.94 ppm, Fig 6C) of the myristoyl chain. The spectrum that probes the C\(_{14}\)methyl group (Fig 6A) reveals off-diagonal NMR resonances assigned to protein residues with aromatic ring protons (F48, F82 and F85) and aliphatic side-chains (I51, A65, V68 and I86). These NMR data imply that the C\(_{14}\)methyl group is surrounded by hydrophobic side-chains from residues in a protein pocket formed by the exiting helix of EF1 (F48, I51, Y52) and both helices of EF2 (F64, A65, V68, F82 and F85). The spectrum that probes the C\(_{12}\)position of the myristoyl moiety (Fig 6B) shows off-diagonal resonances assigned to protein residues in the exiting helix of EF2 (F85, I86 and I89). The spectrum that probes the C\(_{2}\)position of the myristoyl moiety (Fig 6C) shows off-diagonal resonances assigned to residues in EF1 (E26, L27 and W30). These NMR data reveal that the myristoyl group in VILIP-3 forms an unusual L-shaped structure with a 90° bend at C\(_{7}\) (Fig 6E) that positions the terminal C\(_{14}\)-methyl group inside a protein cavity located in the N-terminal domain (see residues F48, I51, Y52, F82 and F85 in Fig 6D and 6E) that is quite different from the myristoyl group binding site in recoverin [15], GCAP1 [12] and NCS-1 [13]. The myristoyl group attached to VILIP-3 is about 40% buried inside the protein (Fig 6D and 6E). The C\(_{14}\)methyl group of the myristate makes close contacts with hydrophobic side-chains from F48, I51, Y52, F82, F85, I86 located inside the hydrophobic core (Fig 6D and 6E). The middle of the fatty acyl chain makes hydrophobic contacts with side-chains of Y52, F64, F85, I86 and I89. The carbonyl end of the myristate contacts the side-chains of E26 and W30 on the protein surface (Fig 6D). The environment around the myristoyl group in VILIP-3 consists of three amino acids (E26, F64, and V69) that do not make any myristate contacts in recoverin, GCAP1 or NCS-1, demonstrating that the myristate is located in a unique protein environment in VILIP-3. Indeed, alanine mutations of these myristate binding site amino acids in VILIP-3 (E26A, F64A and V69A) each affect Ca\(^{2+}\)-binding affinity and folding stability of VILIP-3 (Table 1). The corresponding mutations in recoverin (E27A, Y65A and V69A) do not affect Ca\(^{2+}\)-binding affinity or folding stability (Table 1), consistent with a lack of myristate contact by these residues in recoverin.

Ca\(^{2+}\)-induced Extrusion of the Myristoyl Group

To probe Ca\(^{2+}\)-induced structural changes to the attached myristoyl group, two-dimensional \(^1\)H-\(^{13}\)C HMQC experiments were performed on a VILIP-3 sample that contained a \(^{13}\)C-labeled myristoyl group attached to unlabeled VILIP-3 (Fig 7). The \(^1\)H-\(^{13}\)C HMQC experiment detects protons of myristate that are covalently attached to \(^{13}\)C and therefore only NMR resonances of the myristoyl group appear in the spectra. The HMQC spectrum of the \(^{13}\)C-labeled myristoyl group attached to Ca\(^{2+}\)-free VILIP-3 exhibited the expected number of well resolved resonances (see chemical shift assignments in Table 3). The myristate resonances at positions 2, 3,
Fig 6. NMR spectroscopy of myristoyl binding site in VILIP-3. Three-dimensional $^{13}$C(F1)-edited $^{13}$C(F3)-filtered HMQC NOESY spectra of unlabeled VILIP-3 containing a $^{13}$C-labeled myristoyl group. (A-C) Two-dimensional slices of $^{13}$C(F1)-edited $^{13}$C(F3)-filtered HMQC NOESY spectra of $^{13}$C-labeled myristoyl group attached to Ca$^{2+}$-free myristoylated VILIP-3 edited at $^{13}$C frequencies 16.89 (A), 34.31 (B) and 37.94 (C) ppm. NOE crosspeaks represent protons of myristate that are less than 5 Å away from aliphatic and aromatic resonances of the Ca$^{2+}$-free protein (marked by residue labels). Schematic view of protein contacts along fatty acyl chain (D) and same view rotated by 180° (E). Side-chain atoms of hydrophobic residues near fatty acyl chain are highlighted yellow.

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12, 13 and 14 were unambiguously assigned based on characteristic $^{13}$C chemical shifts and these resonances formed dipolar interactions with nearby protein residues in Ca$^{2+}$-free VILIP-3 (Fig 6). The upfield shifted proton chemical shifts observed for H12, H13 and H14 of the myristate are consistent with the close proximity of these atoms to aromatic side chains (F48, Y52, F55, F85) inside the VILIP-3 hydrophobic core. The myristate NMR data are therefore consistent with the sequestration of the attached myristoyl group inside Ca$^{2+}$-free VILIP-3 (Fig 6). The $^1$H-$^{13}$C HMQC spectrum of Ca$^{2+}$-bound VILIP-3 reveals significant chemical shift changes to the myristate resonances (Fig 7 and Table 3). The methylene resonances at positions C$_4$-C$_{11}$ all collapse into a single peak, suggesting that the covalently attached myristate becomes located in a more solvent exposed environment in the Ca$^{2+}$-bound protein. The chemical shifts of the methylene resonances from the myristoyl group attached to Ca$^{2+}$-bound VILIP-3 are all quite similar to those of free myristic acid in solution [34]. The NMR data demonstrate that the myristoyl group attached to Ca$^{2+}$-bound VILIP-3 is most likely solvent exposed.

**Discussion**

In this study, we determined the energetics of Ca$^{2+}$ binding (Fig 2) and folding (Fig 3) of VILIP-3 as well as the NMR structure of Ca$^{2+}$-free VILIP-3 (Fig 5). Ca$^{2+}$ binds cooperatively (Hill slope of 1.8) to myristoylated VILIP-3 in the sub-micromolar range ($\Delta$H = -6.4 kcal/mol and K$_D$ = 0.52 μM). A Hill coefficient of 1.8 is consistent with 3 Ca$^{2+}$ binding sites in VILIP-3 having positive cooperativity, which resembles the cooperative Ca$^{2+}$ binding observed for myristoylated recoverin [33]. Ca$^{2+}$-free myristoylated VILIP-3 has a higher unfolding temperature than the Ca$^{2+}$-free unmyristoylated protein, consistent with protein stabilization caused by the covalently attached myristoyl group. The fatty acyl group is sequestered inside a unique hydrophobic core of Ca$^{2+}$-free VILIP-3 that involves myristate contacts to E26, Y64 and V68 (Fig 5) that are not seen in recoverin [15], NCS-1 [13] and GCAP-1 [12]. The sequestered myristoyl group in VILIP-3 forms an unusual L-shaped conformation with a 90° bend at C$_7$ (Fig 6E) and the bent fatty acyl chain makes contact with a shallow protein cavity lined by residues solely in the N-terminal domain (EF1 and EF2). By contrast, the myristoyl group in recoverin is buried in a deeper protein cavity and makes more extensive contact with the protein. The shallower myristate binding pocket in VILIP-3 and fewer protein-myristate contacts may explain its 30-fold higher Ca$^{2+}$-binding affinity and lower folding stability compared to myristoylated recoverin (Table 1).

The distinctive structure of Ca$^{2+}$-free VILIP-3 (Fig 5) is consistent with the idea that N-terminal myristoylation helps to forge each NCS protein into a unique three-dimensional fold [23]. The different structures of the Ca$^{2+}$-free forms of recoverin [15], NCS-1 [13], GCAP1 [11] and VILIP-3 (this study) imply that the Ca$^{2+}$-free states of NCS proteins may have diverse functional activity. Indeed, the Ca$^{2+}$-free state of GCAP1 binds and activates retinal guanylyl cyclases [37,38], Ca$^{2+}$-free DREAM binds to specific DNA sequences [39–43] and blocks transcription [44]. And Ca$^{2+}$-free calmodulin binds to IQ motifs in numerous target proteins [45–47]. Accordingly, we suggest that the Ca$^{2+}$-free state of VILIP-3 and the other NCS proteins may also bind to specific target proteins and possess distinct biological functions. Future studies are needed to look for target proteins that bind to Ca$^{2+}$-free VILIP-3 and the other NCS proteins. We propose that N-terminal myristoylation plays an important role in creating unique Ca$^{2+}$-free structures of NCS proteins that could provide a means of generating functional diversity.

The physiological target proteins that bind to VILIP-3 are currently not known. Hippocalcin, a close homolog of VILIP-3 (94% identity), binds and regulates Ca$^{2+}$-gated sAHP channels in hippocampal neurons that are important for learning and memory [10]. The very high sequence identity between hippocalcin and VILIP-3 suggests that VILIP-3 could also bind to sAHP channels. Similar to hippocalcin, VILIP-3 may also serve as a Ca$^{2+}$ sensor important for regulating
Fig 7. Ca^2+--induced extrusion of myristate. Overlay of two dimensional $^1$H-$^{13}$C HMQC NMR spectra of the $^{13}$C-labeled myristoyl group attached to unlabeled Ca^2+-free VILIP-3 (black peaks) and Ca^2+--bound VILIP-3 (red peaks). The spectral changes reflect Ca^2+--induced environmental changes around the myristoyl group, indicative of Ca^2+--induced extrusion of the myristate. Chemical shift assignments are provided in Table 3.

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Table 3. \(^1\)H and \(^{13}\)C (in parentheses) chemical shift assignments of myristoyl resonances.

| Position | Ca\(^{2+}\)-free VILIP-3 (ppm) | Ca\(^{2+}\)-bound VILIP-3 (ppm) |
|----------|-------------------------------|-------------------------------|
| C2       | 2.12, 2.06 (38.0)             | 2.1 (38.2)                    |
| C3       | 1.17 (27.7)                   | 1.4 (28.1)                    |
| C4–C11   | 0.26–1.00 (30.5–31.2)         | 1.0 (31.5)                    |
| C12      | 0.98 (34.0)                   | 1.2 (34.0)                    |
| C13      | 1.26 (25.3)                   | 1.1 (24.5)                    |
| C14      | 0.66 (16.9)                   | 0.65 (16.5)                   |

The NMR sample conditions were 0.2 mM VILIP-3 in 10 mM Tris at pH 7.4 containing either 5 mM EDTA \(^a\) or 5 mM Ca\(^{2+}\) \(^b\).

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long-term depression (LTD) and hippocampal synaptic plasticity in learning and memory. The Ca\(^{2+}\)-dependent regulation of sAHP channels mediated by hippocalcin, therefore, might be similar to Ca\(^{2+}\)-dependent regulation of voltage-gated Ca\(^{2+}\) channels mediated by CaM [48,49]. The Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of CaM each bind to separate sites on the CaV1.3 channel [49]. Apo-CaM binds to the C-terminal regulatory region of CaV1.3, which promotes channel activation [48]. By contrast, Ca\(^{2+}\)-bound CaM binds to an N-terminal site (called NSCaTE), which is responsible for inhibiting channel activity [50,51]. Hippocalcin and VILIP-3 might bind and regulate sAHP channels in a similar fashion. Future studies are needed to test whether VILIP-3 binds directly to sAHP channels and find out whether the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of VILIP-3 both bind to distinct regulatory sites on channel targets.

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