Neuronal calcium sensor (NCS) proteins transduce Ca$^{2+}$ signals and are highly conserved from yeast to humans. We determined NMR structures of the NCS-1 homolog from fission yeast (Ncs1), which activates a phosphatidylinositol 4-kinase. Ncs1 contains an α-NH$_2$-linked myristoyl group on a long N-terminal arm and four EF-hand motifs, three of which bind Ca$^{2+}$, assembled into a compact structure. In Ca$^{2+}$-free Ncs1, the N-terminal arm positions the fatty acyl chain inside a cavity near the C terminus. The C14 end of the myristate is surrounded by residues in the protein core, whereas its amide-linked (C1) end is flanked by residues at the protein surface. In Ca$^{2+}$-bound Ncs1, the myristoyl group is extruded (Ca$^{2+}$-myristoyl switch), exposing a prominent patch of hydrophobic residues that specifically contact phosphatidylinositol 4-kinase. The location of the buried myristate and structure of Ca$^{2+}$-free Ncs1 are quite different from those in other NCS proteins. Thus, a unique remodeling of each NCS protein by its myristoyl group, and Ca$^{2+}$-dependent unmasking of different residues, may explain how each family member recognizes distinct target proteins.

Brain NCS proteins have diverse functions. Neurocalcins and visinin-like proteins regulate guanylate cyclase and nicotinamide acetylcholine receptors implicated in synaptic plasticity (16). KChIPs (17), hippocalcin (19), and NCS-1 (20) bind to various ion channels and thus control neuronal excitability.

Remarkably, even the genomes of yeasts (Saccharomyces cerevisiae and Schizosaccharomyces pombe) encode a protein that is more than 60% identical to mammalian NCS-1 (Fig. 1A). The budding yeast (S. cerevisiae) homolog (Frq1) is essential for cell growth (21) and activates a PtdIns 4-kinase (Pik1) (22, 23). The fission yeast (S. pombe) homolog (Ncs1) regulates sporulation (24) and confers Ca$^{2+}$ tolerance (25). Sporulation defects in Ncs1 knock-out fission yeast are rescued by overexpressing S. cerevisiae Frq1 or Pik1, suggesting that Ncs1 activates the homologous S. pombe PtdIns 4-kinase. Indeed, the Frq1-binding site in Pik1 (26) is conserved in its fission yeast ortholog (also called Pik1) (Fig. 1B) (27).

The high degree of sequence identity among NCS proteins suggests that their three-dimensional structures should be quite similar. Indeed, the overall structures of the Ca$^{2+}$-bound state of the unmyristoylated forms of several NCS family members look rather similar, as determined by x-ray crystallography (28–32) or nuclear magnetic resonance (NMR) spectroscopy (33–35). The four EF-hands are packed in a tandem array, in contrast to the dumbbell-shaped arrangement seen in CaM (36) and troponin C (37). However, much less is known about the structure of the Ca$^{2+}$-free and/or myristoylated forms of NCS proteins. The fact that NCS proteins, like recoverin, GCAP1, and NCS-1, all recognize different physiological target proteins suggests that they must have some distinguishing structural characteristic that may be conferred by the interaction of the N-myristoyl moiety with the rest of the protein. Consistent with this notion, the structures of myristoylated recoverin (PDB code 1iku) and myristoylated GCAP1 (PDB code 2r2i) are quite different from each other. Moreover, although there is only one NCS protein (recoverin) whose structure has been determined for its myristoylated form in both the absence (38) and presence of Ca$^{2+}$ (39), the structure of Ca$^{2+}$-free recoverin is quite different from the structure of Ca$^{2+}$-bound recoverin. In its Ca$^{2+}$-free state, the N-terminal myristoyl group of recoverin is sequestered inside a deep hydrophobic cavity in the N-domain. In its Ca$^{2+}$-bound state, the N-myristoyl group is extruded, permitting the now solvent-exposed fatty acyl chain to interact with membranes (40, 41), allowing recoverin (42) and other NCS proteins (30, 43, 44) to bind to target membranes when the Ca$^{2+}$ level is high. The
Ca\(^{2+}\)-induced conformational change that exposes the myristoyl group has been dubbed a Ca\(^{2+}\)-myristoyl switch. Frq1 in budding yeast both activates the PtdIns 4-kinase Pik1 (21, 23) and its N-terminal myristoyl group enhances its membrane binding. When bound to the enzyme, Frq1 occupies residues 121–174 of Pik1, which forms a U-shaped structure that lies upstream of the catalytic domain (residues 792–1066) (26). Mammalian NCS-1 can interact with yeast Pik1 (45) and reportedly regulates PtdIns 4-kinase activity in animal cells (46, 47). Ca\(^{2+}\)-dependent activation of PtdIns 4-kinase by NCS-1 may be especially important in neurons because modulation of phosphoinositide synthesis by intracellular Ca\(^{2+}\) controls synaptic vesicle exocytosis (48) and is involved in synaptic plasticity (49). However, previously, we were unable to obtain structural information for myristoylated Frq1.

Here, we report the NMR structures of Ca\(^{2+}\)-free myristoylated Ncs1 in solution and the Ca\(^{2+}\)-loaded form of the same protein bound to its target site (residues 111–159) in fission yeast Pik1, hereafter referred to as Pik1(111–159). Strikingly, the location of the myristoyl-binding site in Ca\(^{2+}\)-free Ncs1 and the resulting structure of Ncs1 are quite different from that of either recoverin (38) or GCAP1 (50). Our data support the conclusion that myristoylation shapes each NCS protein into a distinct structure. Moreover, we find that Ncs1 undergoes large Ca\(^{2+}\)-induced conformational changes that lead to extrusion of the myristoyl group, causing hydrophobic residues in the C-domain that sequester the fatty acyl chain in Ca\(^{2+}\)-free Ncs1 to become solvent-exposed in Ca\(^{2+}\)-bound Ncs1 and thus available to interact with Pik1(111–159). In vivo, this Ca\(^{2+}\)-myristoyl switch presumably promotes membrane localization of Ncs1 and its association with Pik1. Furthermore, based on the structure of Pik1-bound Ncs1, we propose a mechanism for simultaneous Ca\(^{2+}\)-induced membrane localization and activation of the enzyme. Finally, given the profound structural differences between the Ca\(^{2+}\)-free states of myristoylated Ncs1 (this study), myristoylated recoverin (38), and myristoylated...
protein. The dilute preparation was then concentrated 20-fold in 8 M urea buffer and purified using Ni²⁺-NTA column chromatography. The purified protein was used in NMR experiments.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Expression and purification of recombinant myristoylated *S. pombe* Ncs1 (and *S. cerevisiae* Frq1) was described previously (24). Briefly, Ncs1 and Frq1 (without any affinity tags) were expressed using pET11 vector harboring the *ncl1* (or *frq1*) coding sequence and was co-expressed with yeast N-myristoyl-CoA transferase using pBB131 vector harboring the N-myristoyl-CoA transferase coding sequence in *Escherichia coli* strain BL21(DE3). Ncs1 was labeled with ¹⁵N or ¹⁵N/¹³C isotopes by growing cells at 37 °C in M9 minimal media supplemented with ¹⁵N-labeled ammonium chloride and ¹³C-labeled glucose as described previously (26). Unlabeled or ¹³C-labeled myristic acid was added to the culture 20 min prior to induction that is needed for N-terminal myristoylation. Cells were harvested, lysed, and spun down to collect supernatant. Protein was then purified from the supernatant using hydrophobic interaction (butyl-Sepharose), anion exchange (DEAE-Sepharose), and size exclusion (Superdex 200) columns. The final protein purity is ~95% judged by SDS-PAGE, and more than 90% of the protein was myristoylated determined by mass spectrometry analysis.

A functional polypeptide fragment of *S. pombe* Pik1 (residues 111–159, named Pik1(111–159)) uniformly labeled with nitrogen-15 and/or carbon-13 and tagged with a C-terminal His₆ and Ura to ensure maintenance of the plasmid, and extracts were prepared by glass bead breakage and clarified by brief centrifugation at 500 x g to remove beads, unbroken cells, and large debris. Samples were assayed under conditions specific for Pik1 activity (51).

**NMR Spectroscopy**—NMR samples of Ca²⁺-free myristoylated or unmyristoylated Ncs1 (~0.7 mM) or Frq1 (0.5 mM) were prepared in 90:10% H₂O/D₂O or 100% D₂O with 5 mM Tris-d₄ (pH 7.4) buffer, 4 mM DTT-d₄, and 0.3 mM EDTA-d₁₂. NMR samples of Ca²⁺-bound Ncs1 bound to Pik1(111–159) consisted of ¹⁵N-labeled or ¹³C/¹⁵N-labeled Ncs1 bound to 1 eq of unlabeled Pik1(111–159) (1.0 mM) in 0.3 ml of a 95% H₂O, 5% [²H]H₂O solution containing 5 mM sodium acetate and 2 mM CaCl₂ (pH 5.0). Reverse labeled samples (i.e. ¹⁵N- or ¹³C/¹⁵N-labeled Pik1(111–174) bound to 1 eq of unlabeled Ncs1) were also prepared for some of the NMR experiments. All NMR experiments were performed at 37 °C on Bruker Avance 500 or 800 spectrometers with an Ultrashield Bruker magnet equipped with a four-channel interface, triple resonance probe, and cryo-probe with z axis pulsed field gradients. ¹⁵N-H HSQC spectra were recorded on samples of ¹⁵N-labeled Ncs1 in the presence or absence of unlabeled Pik1(111–159) in 95% H₂O, 5% [²H]H₂O. The number of complex points and acquisition times were as follows: 256, 180 ms (¹⁵N(F₁)) and 512, 64 ms (¹H(F₃)). The ¹³C(F₁)-edited and ¹³C(F₃)-filtered NOESY-HSQC spectra (see Figs. 4A and 5A) were recorded on a sample of unlabeled Ca²⁺-free Ncs1 protein attached to a ¹³C-labeled myristoyl group (Fig. 4A) or unlabeled Ncs1 bound to ¹³C-labeled Pik1(111–159) (Fig. 5A) as well as ¹³C-labeled Ncs1 bound to unlabeled Pik1(111–159) (data not shown). Intermolecular NOESY experiments were performed as described previously (52). Stereospecific assignments of chiral methyl groups of valine and leucine were obtained by analyzing ¹H-¹³C HSQC experiments performed on a sample that contained 10% ¹³C-labeled in either Ncs1 or Pik1(111–159). All triple resonance experiments were performed, processed, and analyzed as described previously (53) on a sample of ¹³C/¹⁵N-labeled Ncs1 in the presence or absence of unlabeled Pik1(111–159) in 95% H₂O, 5% [²H]H₂O with the following number of complex points and acquisition times: HNCO (¹⁵N(F₂) 32, 23.7 ms; ¹³C(F₂) 64, 22.7 ms; ¹H(F₃) 512, 64 ms); HNCA( C(F₁) 32, 23.7 ms; ¹³C(F₂) 48, 6.3 ms; ¹H(F₃) 512, 64 ms); CBCACONNH (¹⁵N(F₂) 32, 23.7 ms; ¹H(F₃) 48, 6.3 ms; ¹H(F₃) 512, 64 ms); CBCACOCAH (¹³C(F₁) 52, 6.8 ms, ¹³C(F₂) 64, 42 ms, ¹H(F₃) 384, 64 ms); and HBHACONHH (¹¹N(F₁) 32, 23.7 ms, ¹H(F₃) 64 21 ms, ¹H(F₃) 512, 64 ms).

The triple resonance and NOESY spectra measured above were analyzed to determine secondary and tertiary structure in Ca²⁺-free myristoylated Ncs1 and Ca²⁺-bound unmyristoylated Ncs1-Pik1(111–159) complex. The chemical shift index (see Ref. 54 for detailed description) ¹³N/H₂O coupling constants, and NOE connectivity patterns for each residue were analyzed.
and provided a measure of the overall secondary structure. Small $^3$JHNH coupling constants (<5 Hz), strong NOE connectivities (NN(i,i + 1) and αNN(i,i + 3)), and positive chemical shift index are characteristic of residues in an α-helix. Conversely, large $^3$JHNH coupling constants (>8 Hz), strong αNN(i,i + 1), and weak NN(i,i + 1) NOE connectivities and negative chemical shift index are characteristic of residues in a β-strand. The results of the secondary structure analysis of Ncs1 and Pik1(111–159) are summarized schematically in Fig. 1.

**Structure Calculation**—Three-dimensional 15N-NOESY-HSQC and 13C-NOESY-HSQC and two-dimensional homonuclear NOESY spectra of Ca$^{2+}$-free myristoylated Ncs1 and Ca$^{2+}$-bound Ncs1 (bound to Pik1(111–159)) were analyzed to obtain 1553 and 1225 NOE distance restraints used in the structure calculations, respectively. In addition to the NOE-derived distance constraints, the following additional constraints were included in the structure calculation: 18 distance constraints involving Ca$^{2+}$ bound to loop residues 1, 3, 5, 7, and 12 in each EF-hand motif (EF-2, EF-3, and EF-4); 124 distance constraints for 80 hydrogen bonds; and 200 dihedral angle constraints (φ and ψ) derived from TALOS (55). Fifty independent structures were calculated by XPLOR-NIH software (56) implemented with YASAP protocol (57), and the 15 structures of lowest energy were selected and overlaid with r.m.s.d. of 0.69 Å (Ca$^{2+}$-free) and 0.9 Å (Ca$^{2+}$-bound). Structures of Ca$^{2+}$-free unmyristoylated Ncs1 were also calculated in a similar fashion. Figures of NMR structures in this paper were prepared with PyMOL or VMD (University of Illinois at Urbana-Champaign).

**RESULTS**

**Structure of Ca$^{2+}$-free Myristoylated Ncs1—**15N-1H HSQC NMR spectra of Ca$^{2+}$-free Ncs1 (both myristoylated and unmyristoylated forms) exhibited the expected number of highly dispersed peaks with uniform intensities, indicating that Ca$^{2+}$-free Ncs1 adopts a stable three-dimensional fold. Analysis of 15N relaxation parameters ($T_1$ and $T_2$) indicates an average rotational correlation time of 9.65 ± 0.5 ns, suggesting that Ca$^{2+}$-free Ncs1 is monomeric in solution under NMR conditions. The spectral similarity for both myristoylated and unmyristoylated forms of Ca$^{2+}$-free Ncs1 suggests their overall protein structures are similar, and thus we were able to determine the NMR structures of both myristoylated and unmyristoylated forms of Ca$^{2+}$-free Ncs1. The sequence-specific NMR assignments of Ca$^{2+}$-free myristoylated Ncs1 were analyzed and described previously (BMRB 16446) (58). The assigned resonances in the HSQC spectrum represent main chain and side chain amide groups that serve as fingerprints of the overall conformation. Three-dimensional protein structures derived from the NMR assignments were calculated on the basis of NOE data, chemical shift analysis, and $^3$JHNH spin-spin coupling constants (see “Experimental Procedures”). The final NMR-derived structures of Ca$^{2+}$-free myristoylated Ncs1 are illustrated in Fig. 2, A and B (atomic coordinates have been deposited in the RCSB Protein Data bank, code 2lze). Table 1 summarizes the structural statistics calculated for the 15 lowest energy conformers.

Aside from the last two residues at the C terminus, the entire polypeptide chain of Ca$^{2+}$-free myristoylated Ncs1 could be determined from the NMR data. Ncs1 contains a total of 10 α-helices and 4 β-stands: α1 (residues 10–20), α2 (residues 25–35), α3 (residues 46–56), α4 (residues 61–71), α5 (residues 84–91), α6 (100–109), α7 (residues 120–133), α8 (residues 146–157), α9 (residues 167–176), and α10 (residues 178–184) and 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 Gly$^2$ is connected to a long extended structure (residues Gly$^{2+}$–Arg$^{21}$) called an N-terminal arm (highlighted purple in Fig. 2B) that reaches more than 15 Å to insert the fatty acyl chain inside a protein cavity near the C terminus. Ncs1 contains two domains comprising four EF-hands (Fig. 2, A and B) as follows: EF1 (green, residues 26–55) and EF2 (red, residues 62–91) are linked and form the N-domain; likewise, EF3 (cyan, residues 101–130) and EF4 (yellow, residues 146–175) form the C-domain. The interface between the two domains is established by interactions between EF2 (Asp$^{84}$, Cys$^{87}$, Ala$^{88}$, and Ser$^{90}$ in helix α5) and EF3 (Lys$^{100}$, Trp$^{103}$, Ala$^{104}$, and Leu$^{107}$ in helix α6; and Arg$^{123}$, Ala$^{127}$, Ile$^{138}$, and Met$^{131}$ in helix α7) that connect the two domains. The domain interface in Ncs1 is similar to that in Ca$^{2+}$-free myristoylated recoverin (Fig. 2C) but very different from that of myristoylated GCAP1 (Fig. 2D) (50). Each EF-hand in Ncs1 consists of a helix-turn-helix structure similar to the closed structure of Ca$^{2+}$-free EF-hands seen in previous structures of Ca$^{2+}$-free recoverin (38) and apo-CaM (59). The interhelical angles for the EF-hands of Ncs1 are 146.9° (EF1), 121.7° (EF2), 119.3° (EF3), and 107.8° (EF4). The four EF-hands of Ncs1 are grouped in a compact globular topology (Fig. 2, A and B) only somewhat similar to that found in Ca$^{2+}$-free recoverin (Fig. 2C) and very different from the tandem EF-hand arrangement seen in GCAP1 (Fig. 2D). The C-terminal helix (α10) of Ncs1 is displaced somewhat from the protein core to make room for the N-terminal myristoyl group that inserts inside a cavity formed between EF3 and EF4 (Fig. 2B). The fatty acyl chain in the cavity is nearly parallel to the helices of EF3 and EF4 that form walls that surround the myristoyl moiety (Fig. 2E). This arrangement is in stark contrast to recoverin where the myristate is wedged perpendicularly between the helices of EF1 and EF2 (Fig. 2F). Indeed, the overall main chain structure of Ca$^{2+}$-free myristoylated Ncs1 (Fig. 2B) is different from the myristoylated forms of recoverin (Fig. 2C) and GCAP1 (Fig. 2D). When comparing the main chain atoms of Ca$^{2+}$-free Ncs1 with those of recoverin and GCAP1, the root mean square deviations are 2.3 and 3.4 Å, respectively.

**Structure of Ca$^{2+}$-free Unmyristoylated Ncs1—**The NMR chemical shift assignments and structure of Ca$^{2+}$-free myristoylated Ncs1 are very similar to those of Ca$^{2+}$-free unmyristoylated Ncs1 (see overlaid structures in Fig. 3A). The root mean square deviation between the two structures is 1.18 Å. The main structural differences are detected in the N-terminal arm (residues Gly$^{2+}$–Arg$^{21}$) and C-terminal helix, both of which are somewhat destabilized and shortened by the absence of the myristoyl group (e.g. helix α1, residues 10–18, and helix α10, residues 178–186). The 15N chemical shift differences between myristoylated and unmyristoylated Ncs1 are plotted as a function of residue number in Fig. 3B. The largest chemical shift differences are observed for the residues that contact the myristoyl group.
The overall main chain structures for myristoylated and unmyristoylated Ncs1 are nearly identical in the EF-hand regions (r.m.s.d. = 1.04 Å). Thus, the myristoyl group does not alter the main chain structure of the EF-hands in Ca\(^{2+}\)-free Ncs1, but instead the myristoyl chain penetrates inside the protein by displacing hydrophobic side chains. Indeed, the Ca\(^{2+}\)-free myristoylated Ncs1 has a slightly higher melting temperature compared with unmyristoylated Ncs1 (measured by differential scanning calorimetry, data not shown), consistent with stabilization of the hydrophobic core by the sequestered myristoyl group.

Myristoyl-binding Site in Ncs1—The structural environment around the covalently attached myristoyl group in Ncs1 was determined by analyzing NMR experiments (three-dimensional (\(^{13}\)C/F\(_1\)) edited and (\(^{13}\)C/F\(_3\)) filtered NOESY-HSQC) performed on unlabeled Ca\(^{2+}\)-free Ncs1 samples that contained a \(^{13}\)C-labeled myristoyl group (Fig. 4A). These NMR spectra selectively probed atoms in Ncs1 that lie within 5 Å of the \(^{13}\)C-labeled fatty acyl chain. We analyzed nuclear Overhauser effect (NOE) dipolar interactions between the C14 methyl of the myristoyl group (\(^{13}\)C\(_{14}\), F\(_2\) = 16.62 ppm) and the protein (Fig. 4A, upper panel), and between the C2 methylene of the myristoyl chain (\(^{13}\)C\(_{2}\), F\(_2\) = 38.05 ppm) and the protein (Fig. 4A, lower panel). The spectra probing the C\(_{14}\) methyl group (Fig. 4A, upper panel) exhibit many off-diagonal peaks, which could be assigned to residues with aromatic ring protons.
NMR Structure of NCS-1 Homolog in Fission Yeast

### Table 1

| Structural statistics for Ca^{2+}-free Ncs1 | Myristoylated | Unmyristoylated |
|------------------------------------------|---------------|-----------------|
| NMR restraints                            |               |                 |
| Short range NOE \((i \leftrightarrow j, | 1010 | 506             |
| Long range NOE \((i \leftrightarrow j, | 446 | 268             |
| Hydrogen bonds                            | 124 | 124             |
| Dihedral angle restraints                 | 200 | 200             |
| Protein-myristate                         | 18 |                 |
| r.m.s.d. to the mean coordinates          |               |                 |
| Backbone of structured regions*           | 0.69 ± 0.05 Å | 0.85 ± 0.09 Å   |
| Heavy atoms of structured regions         | 1.17 ± 0.08 Å | 1.34 ± 0.1 Å    |
| r.m.s.d. from idealized geometry          |               |                 |
| Bond lengths                              | 0.0064 ± 0.0001 Å | 0.0059 ± 0.0001 Å |
| Bond angles                               | 2.00 ± 0.0014 Å | 2.00 ± 0.0014 Å |
| Improper                                  | 0.9 ± 0.005*   | 0.9 ± 0.005*    |

* Pairwise r.m.s.d. was calculated among 15 refined structures: residues in regions of regular secondary structure (10–20, 25–37, 42–55, 62–72, 79–89, 98–108, 115–130, 146–156, 163–174, 178–183).

Ca^{2+}-induced Activation of Fission Yeast PtdIns 4-Kinase by Ncs1—In budding yeast, Frq1 activates a PtdIns 4-kinase isoform called Pik1 (21). Both Frq1 and Pik1 are highly conserved in fission yeast, and therefore we set out to verify whether S. pombe Pik1 is similarly activated by Ncs1. The effects of Ca^{2+} and Ncs1 on the catalytic activity of Pik1-4-kinase in fission yeast were monitored using an in vitro enzyme assay described previously (see supplemental Fig. 1) (27). Although S. pombe Pik1 displayed detectable basal activity in the absence of exogenously added Ncs1 and Ca^{2+}, the enzyme was stimulated close to 5-fold in the presence of a saturating concentration of Ca^{2+} and myristoylated Ncs1 (supplemental Fig. 1). Thus, Ca^{2+-bound Ncs1 activates S. pombe Pik1 similar to Frq1 activation of S. cerevisiae Pik1 (21). In the absence of added Ca^{2+}, addition of myristoylated Ncs1 had a negligible stimulatory effect (supplemental Fig. 1). The most likely explanation for this observation is that sequestration of the myristoyl group in Ca^{2+}-free Ncs1 (Fig. 2) blocks the residues in Ncs1 that are necessary to contact its binding site on Pik1. In agreement with this view, unmyristoylated Ncs1 yielded modest, but detectable, stimulation of the lipid kinase even in the absence of added Ca^{2+} (supplemental Fig. 1). Furthermore, as we demonstrate below, Ncs1 undergoes a Ca^{2+}-myristoyl switch in which Ca^{2+}-induced extrusion of the myristoyl group exposes critical residues involved in interacting with the PtdIns 4-kinase (see under “Discussion”).

Structure of Ca^{2+}-bound Ncs1 Bound to PtdIns 4-Kinase—Previous studies showed that budding yeast Frq1 interacts with a localized region of Pik1 (residues 121–174, called Pik1(121–174) (22, 26). Fission yeast Pik1 contains a very similar sequence at a very similar location (Fig. 1B). We therefore tested whether a peptide fragment of this region of S. pombe Pik1 (residues 111–159), hereafter Pik1(111–159), might interact with Ncs1. Isothermal titration calorimetry data (supplemental Fig. 2) show that S. pombe Pik1(111–159) does indeed bind to Ca^{2+}-bound Ncs1 \((K_a = 0.5 ± 0.3 \mu M) and \Delta H = 2.4 \text{ kcal/mol}) very similar to the binding of Frq1 to Pik1(121–174) in budding yeast (22). The high affinity and endothermic reaction of Ca^{2+}-bound Ncs1 with Pik(111–159) indicates that Ca^{2+}-bound Ncs1 must bind to the PtdIns 4-kinase in a largely entropy-driven manner, suggesting primarily a hydrophobic interaction, as was described previously for S. cerevisiae Pik1-Frq1 association (22).

Next, we set out to determine the NMR structure of Ca^{2+}-bound Ncs1 bound to S. pombe Pik1(111–159), as was done previously for Frq1 bound to Pik1(121–174) (26). The NMR spectra and assignments of Ca^{2+}-bound Ncs1-Pik1(111–159) complex are shown in supplemental Fig. 5. More than 85% of the backbone assignments for Ncs1 and Pik1(111–159) were obtained as described under “Experimental Procedures.” The unassigned residues were located in unstructured regions as follows: loop residues 134–139 for Pik1(111–159) and the last eight residues at the C terminus for Ncs1. The NMR assignments in supplemental Fig. 5 then served as the basis for analyzing both intramolecular and intermolecular NOE spectra as described for the Frq1-Pik1(121–174) complex (26). This analysis of the NOE spectra provides distance constraints for determining the overall protein fold and probing contacts from key residues at the protein interface (Fig. 5A). The NMR-derived structure of Ca^{2+}-bound Ncs1-Pik1(111–159) complex is shown in Fig. 5, B–D, and structural statistics are given in Table 2.

The four EF-hands in Ncs1 are arranged in a tandem array and, overall, form a globular structure with a concave solvent-exposed groove lined by two separate hydrophobic patches (highlighted yellow in Fig. 5C). These two hydrophobic surfaces represent bipartite binding sites on Ncs1 that interact with Pik1(111–159). The structure of Pik1(111–159) in the complex adopts a conformation that contains two \(\alpha\)-helices (residues (Tyr^{129} and Phe^{169}) and to protons in aliphatic side chains (Leu^{101}, Ile^{124}, Val^{125}, Met^{121}, Ile^{179}, and Leu^{185}). Thus, the C_{14} methyl group is surrounded by hydrophobic side chains from residues in EF3 and EF4 and the C-terminal helix. The spectra probing the C_{2} position of the myristoyl moiety (Fig. 4A, lower panel) exhibit off-diagonal peaks assigned to residues in the loop between EF3 and EF4 (Val^{132}, Val^{136}, and Pro^{139}) and the C-terminal helix (Thr^{178}, Ala^{182}, and Leu^{185}). On the basis of these NMR data, the N-terminal myristoyl group in Ncs1 resides inside a protein cavity located in the C-terminal domain; in marked contrast, the myristoyl group in recoverin (38) and in GCAP1 (50) is housed in an N-terminal cavity in both proteins. The myristoyl group attached to Ncs1 adopts an extended conformation (Fig. 4B) that is about 75% buried inside the protein (Fig. 4C). The C_{14} methyl group of the myristate makes close contacts with hydrophobic side chains from Val^{122}, Phe^{169}, and Ile^{179} located inside the hydrophobic core (Fig. 4, B and C). Thus, the C_{14} methyl end of the myristoyl group protru-
FIGURE 3. A, superposition of Ca\(^{2+}\)-free myristoylated Ncs1 (light gray) onto Ca\(^{2+}\)-free unmyristoylated Ncs1 (dark gray). Overall, the two structures are superimposed with a main chain r.m.s.d. of 1.18 Å. B, chemical shift difference (amide $^{15}$N) between myristoylated and unmyristoylated Ncs1 plotted versus residue number showing structural differences at the N and C termini and loop region between EF3 and EF4, where the myristate interacts. C, ribbon diagram of Ca\(^{2+}\)-free Ncs1 highlighting the residues in dark gray that contact the myristoyl group and show the largest $^{15}$N chemical shift differences from B.
114–127 and 143–156) connected by a disordered loop. The N-terminal helix contains hydrophobic residues (Ile\textsuperscript{115}, Cys\textsuperscript{116}, Leu\textsuperscript{119}, and Ile\textsuperscript{123}) that contact C-terminal residues of Ncs1 (Leu\textsuperscript{101}, Trp\textsuperscript{103}, Val\textsuperscript{125}, Val\textsuperscript{128}, Leu\textsuperscript{138}, Ile\textsuperscript{152}, Leu\textsuperscript{155}, and Phe\textsuperscript{169}) (see Fig. 5D). Interestingly, these same hydrophobic residues in Ca\textsuperscript{2+}-free Ncs1 make close contacts with the myristoyl group.

**FIGURE 4. Myristoyl-binding site environment in Ncs1.** A, selected slices of \textsuperscript{13}C(F1)-edited and \textsuperscript{13}C(F3)-filtered NOESY-HSQC spectra that selectively probe resonances of Ncs1 less than 5 Å away from C\textsubscript{14}-methyl (top panel) and C\textsubscript{2} carbonyl group (bottom panel) of myristate. B, ball-and-stick model of myristoyl group and C-terminal hydrophobic side chain atoms located less than 5 Å away. C, space-filling model of myristate and hydrophobic side chain atoms with same view as in B.
FIGURE 5. NMR-derived structure of Ca$^{2+}$-bound Ncs1 bound to Pik(111–159). A, selected slices of $^{13}$C(F1)-edited/$^{13}$C(F3)-filtered NOESY-heteronuclear multiple quantum coherence spectra of $^{13}$C-labeled Pik1(111–159) bound to unlabeled Ncs1. B, ribbon diagram of main chain structure of Ca$^{2+}$-bound Ncs1 bound to Pik(111–159). EF-hands are colored as in Fig. 2. Pik(111–159) is highlighted magenta. C, space-filling representation of Ca$^{2+}$-bound Ncs1 with same view as in B. D, close-up view of Pik1(111–159) (magenta) with hydrophobic side chains in Ncs1.
NMR Structure of NCS-1 Homolog in Fission Yeast

**TABLE 2**

Structural statistics for the Ca\(^{2+}\)-bound Ncs1-Pik1 complex

|                      | Ncs1                  | Pik1(111–159) |
|----------------------|-----------------------|---------------|
| NMR restraints       | Short range NOE       | 715           | 112           |
|                      | Long range NOE        | 250           | 62            |
|                      | Hydrogen bonds        | 124           | 38            |
|                     | Calcium bound at EF2, EF3, and EF4 | 18 | 18 |
|                     | Dihedral angle restraints | 200     | 54            |
|                     | Intermolecular distance restraints | 66 | 66 |
| r.m.s.d. to the mean coordinates | Backbone of structured regions | 0.9 ± 0.07 Å | 0.9 ± 0.09 Å |
|                      | Heavy atoms of structured regions | 1.4 ± 0.08 Å | 1.4 ± 0.1 Å |
| r.m.s.d. from idealized geometry | Bond lengths | 0.007 ± 0.0001 Å | 0.009 ± 0.0001 Å |
|                      | Bond angles | 2.07 ± 0.04° | 2.09 ± 0.04° |
|                     | Improper errors | 0.9 ± 0.05° | 0.95 ± 0.05° |
| Ramachandran statistics of 15 structures | Most favored regions | 76% | 80% |
|                      | Additional allowed regions | 17% | 15% |
|                     | Generously allowed regions | 6% | 4% |
|                     | Disallowed regions | 1% | 1% |

\* Residues in regions of regular secondary structure: Ncs1, residues 9–16, 24–37, 42–55, 62–72, 79–92, 99–108, 115–129, 145–156, 163–175; Pik1, residues 113–125, 144–156.

***DISCUSSION***

In this study, we determined NMR structures for Ca\(^{2+}\)-free myristoylated Ncs1 and Ca\(^{2+}\)-bound Ncs1 complexed to a fragment (residues 111–159) of a fission yeast PtdIns 4-kinase. Ca\(^{2+}\)-free Ncs1 adopts a novel structure where the N-terminal myristoyl group is sequestered inside a protein cavity located near the C terminus (Figs. 2B and 4C). The structural location and environment around the myristoyl group in Ncs1 is very different from that in either recoverin (38) or GCAP1 (Fig. 2) (50). We suggest that each NCS protein adopts a distinct structure because its N-terminal myristoyl group associates with patches of hydrophobic residues that are unique to that protein; thus, upon Ca\(^{2+}\)-evoked extrusion of the myristoyl group, a distinctive ensemble of hydrophobic residues is unmasked, exposing surface residues that allow each class of NCS protein to associate specifically with a particular physiological target. This scenario explains how a Ca\(^{2+}\) signal can cause each NCS family member to engage a different physiological target, despite the high degree of sequence similarity among NCS family members (1).

As we have documented here, Ca\(^{2+}\)-induced extrusion of Ncs1 causes very large protein structural changes that causes extrusion of the myristoyl group, quite analogous to the Ca\(^{2+}\)-myristoyl switch described previously for recoverin (39). The Ca\(^{2+}\)-induced exposure of the myristoyl group for Ncs1 explains why myristoylated (but not unmyristoylated) Ncs1 binds to <i>S. pombe</i> cell membranes only at high Ca\(^{2+}\) levels (24). The Ca\(^{2+}\)-induced extrusion of the myristoyl moiety also exposes two hydrophobic patches on a concave surface of the Ca\(^{2+}\)-bound protein that provide sites for making important contacts with PtdIns 4-kinase (Fig. 5). Therefore, the Ca\(^{2+}\)-myristoyl switch promotes both the capacity of Ncs1 to bind and activate the lipid kinase and controls the delivery of the Ncs1-Pik1 complex to the membrane where the substrate for this enzyme resides.

Previous studies have shown that various frequenins (mammalian NCS-1 (30) and yeast Frq1 (61)) appear localized to membranes even at low Ca\(^{2+}\) levels, suggesting that NCS-1 and Frq1 may not possess a functional myristoyl switch (62). Indeed, NMR structural studies on Frq1 suggested the Ca\(^{2+}\)-free myristoylated Frq1 protein is in a partially unfolded molten-globule state, and the myristoyl group remains solvent-exposed regardless of Ca\(^{2+}\) level (63). These observations of a constitutively exposed myristoyl group are in stark contrast with the Ca\(^{2+}\)-induced extrusion of the myristoyl group in recoverin (39) and Ncs1 (this study). Mutagenesis studies have suggested that particular residues in NCS-1 (62) might be responsible for preventing a Ca\(^{2+}\)-myristoyl switch. However,
these residues are somewhat conserved in both *S. pombe* Ncs1 and mammalian NCS-1 and do NOT prevent the Ca\(^{2+}\)-myristoyl switch in this case. The very high sequence identity (\(\geq\)60\%) among NCS-1, Frq1, and Ncs1, would imply that the three-dimensional structures of Ncs1, NCS-1, and Frq1 must all be very similar. We considered the possibility that the persistent exposure of the myristoyl group in Frq1 and NCS-1 observed in prior work might be an artifact caused by protein misfolding due to the tags (His6 or GFP) attached to the C terminus in those previous studies because our structure of Ncs1 shows that the myristoyl group makes important contacts with residues close to the C terminus (Ile179, Leu183, and Leu185). Indeed, NMR spectra of Ca\(^{2+}\)-free myristoylated Frq1 prepared without a C-terminal His6 tag exhibit methyl resonances below 0 ppm (due to aromatic ring currents in the hydrophobic core), which are similar to those observed for Ncs1 and characteristic of a folded protein (supplemental Fig. 4). By stark contrast, NMR spectra of Ca\(^{2+}\)-free myristoylated Frq1 prepared without a C-terminal His6 tag lack any resonances below 0 ppm and above 9 ppm, indicative of a molten-globule (unfolded) state. Based on these findings and our structure of untagged myristoylated Ncs1 in the absence and presence of Ca\(^{2+}\), we feel that it is highly likely that all frequentins (from mammalian NCS-1 to yeast Frq1) will have structures very similar to that of Ncs1 (Figs. 2 and 5) and will undergo a Ca\(^{2+}\)-myristoyl switch that is critical for its function. Thus, it seems clear from our findings that some aspects of studies of the subcellular localization and cellular roles of NCS family members might have been compromised by the use of tagged derivatives that may have caused significant structural perturbations.

Nonconserved residues of NCS proteins at the C terminus (\(\leq\)10) and immediately following EF3 (Fig. 1A) interact closely with the N-terminal myristoyl group in Ncs1 and thus help stabilize the novel structure of Ca\(^{2+}\)-free Ncs1 (Fig. 2B). The corresponding residues in recoverin and GCAP1 do not contact the myristoyl moiety. GCAP1 also contains an extra helix at the C terminus that contacts the N-terminal arm and myristoyl group (Fig. 2D). Thus, nonconserved residues at the N and C termini and/or loop between EF3 and EF4 may play a role in forming unique myristoyl binding environments in other NCS proteins, such as visinin-like proteins, neurocalcins, and hippocalcins that may explain their capacity to associate with functionally diverse targets once they have undergone a Ca\(^{2+}\)-induced conformational change.

**FIGURE 6. Model of Ca\(^{2+}\)-induced activation or PtdIns 4-kinase by Ncs1.** Left panel shows schematic diagrams of Pik1 and Ncs1 at low Ca\(^{2+}\). LKU motif (residues 23–98) is in gray; Ncs1-binding region (111–159) is in magenta, and catalytic (kinase) domain (residues 642–916) is in orange. In Ca\(^{2+}\)-free Ncs1, an N-terminal arm (purple) places the myristoyl group (red) in a hydrophobic cavity (yellow) flanked by a C-terminal helix (\(\alpha\)_\(d\)). Right panel shows Ca\(^{2+}\)-induced conformational changes in Ncs1 that cause exposure of myristate and two hydrophobic patches (yellow), followed by structural rearrangement in Pik1 induced by its binding to Ca\(^{2+}\)-bound Ncs1. Ncs1 binding to Pik1 imposes a U-turn in the main chain of Pik1 that is necessary to allow the LKU domain (gray) to interact with the catalytic domain (orange). In addition, N-myristoylation of Ncs1 (red) helps deliver the complex to membranes where it can bind to substrate (PtdIns). Insert shows schematic diagram of a somewhat different Ca\(^{2+}\)-myristoyl switch mechanism for recoverin.
Aside from NCS family members, N-terminal myristoylation confers important structural effects in many other classes of proteins. The structures of myristoylated forms of ARF (64), Bcr-Abl (65), c-Abl (66), and the HIV-1 matrix protein (67) all reveal intimate contacts between the protein and the fatty acyl chain that help mold these proteins into biologically active structures. Interaction of the myristoyl group with oncogenic Bcr-Abl protein is critical for activating tyrosine kinase activity, and drugs (e.g. imatinib) important for treating human leukemias prevent access to the active conformation by occluding the myristoyl binding pocket (65). In the ARF protein, the myristoyl moiety interacts with important switch residues that control GTPase activity involved in regulating vesicular trafficking. Finally, interactions of the myristoyl group with other parts of the HIV-1 matrix protein control its oligomerization, an important step in how this virus targets a host cell. These highly diverse structural interactions demonstrate that N-terminal myristoylation is an important tool for shaping protein structures into distinct and physiologically active conformations.

The structures of Ca\(^{2+}\)-free Ncs1 and Ncs1-Pik1 complex (Figs. 2 and 5) suggest how a Ca\(^{2+}\)-myristoyl switch might promote activation of PtdIns 4-kinase (Fig. 6). Under resting basal conditions, cytosolic Ca\(^{2+}\) levels are presumably maintained below 100 nM and Ncs1 exists in its Ca\(^{2+}\)-free state with a sequestered myristoyl group buried in the C-domain that covers part of its binding site for PtdIns 4-kinase (highlighted yellow in Fig. 6) and prevents binding of Ncs1 to Pik1. The fatty acyl chain has the same molecular dimensions (length and width) as the N-terminal helix of Pik1(111–159), which explains why the myristoyl group and Pik1 helix can effectively compete for the same binding site in Ncs1. A rise in cytosolic Ca\(^{2+}\) will cause Ca\(^{2+}\)-induced conformational changes in Ncs1, resulting in extrusion of the N-terminal myristoyl group (see supplemental Movie 1). Ca\(^{2+}\)-induced extrusion of the myristoyl group exposes a hydrophobic crevice in the C-terminal domain of Ncs1, and concomitantly, Ca\(^{2+}\)-induced structural changes in its N-domain result in formation of a second exposed hydrophobic crevice, also seen in all other Ca\(^{2+}\)-bound NCS proteins examined to date (26, 28–31). These two separate hydrophobic sites on the surface of Ca\(^{2+}\)-bound Ncs1 are different from Ca\(^{2+}\)-bound recoverin that contains only one exposed hydrophobic patch (Fig. 6, inset) that interacts with a single target helix in rhodopsin kinase (68). The two exposed hydrophobic sites on Ncs1 bind to the hydrophobic faces of the two antiparallel amphipathic α-helices in Pik1(111–159) (colored magenta in Figs. 5B and 6), akin to the mechanism proposed previously for S. cerevisiae Frc1 and Pik1(121–174) (26). The Ca\(^{2+}\)-induced binding of Ncs1 to PtdIns 4-kinase may promote a long range structural interaction between the LKU and catalytic domains, which are conserved in both S. cerevisiae and S. pombe Pik1 (26), leading to acquisition of the conformational optimal for lipid kinase activity. Simultaneously, Ncs1 binding to PtdIns 4-kinase will also promote membrane localization of the lipid kinase, because Ca\(^{2+}\)-bound Ncs1 contains an extruded myristoyl group that serves as a membrane anchor. Thus, Ncs1 controls both delivery of PtdIns 4-kinase to the membrane where its substrates are located and formation of the optimally active state of the enzyme. We propose that a corresponding Ca\(^{2+}\)-induced membrane localization and activation of PtdIns 4-kinase-β by NCS-1 may take place in neurons and may serve to couple phosphoinositide cascades with calcium signaling pathways, which is thought to be important in synaptic plasticity (49).

REFERENCES

1. Burgoyne, R. D., O’Callaghan, D. W., Hadsemir, B., Haynes, L. P., and Tepkin, A. V. (2004) Trends Neurosci. 27, 203–209
2. Burgoyne, R. D., and Weiss, J. L. (2001) Biochem. J. 353, 1–12
3. Brauneewell, K. H., and Gundelfinger, E. D. (1999) Cell Tissue Res. 295, 1–12
4. Ikura, M., and Ames, J. B. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 1159–1164
5. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1996) Curr. Opin. Struct. Biol. 6, 432–438
6. Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philippov, P. P., Hurley, J. B., and Stryer, L. (1991) Science 251, 915–918
7. Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995) J. Biol. Chem. 270, 18060–18066
8. Kawamura, S. (1993) Nature 362, 855–857
9. Erickson, M. A., Lagnado, L., Zozulya, S., Neubert, T. A., Stryer, L., and Baylor, D. A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6474–6479
10. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekari, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., and Johnson, R. S. (1994) Neuron 13, 395–404
11. Dizhoor, A. M., Olshevskaia, E. V., Henzel, W. J., Wong, S. C., Stults, I. T., Ankoudinova, I., and Hurley, J. B. (1995) J. Biol. Chem. 270, 25202–25206
12. Payne, A. M., Downes, S. M., Bessant, D. A., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattacharya, S. S. (1998) Hum. Mol. Genet. 7, 273–277
13. Sokal, I., Li, N., Surgucheva, I., Warren, M. J., Payne, A. M., Bhattacharya, S. S., Baehr, W., and Palczewski, K. (1998) Mol. Cell 2, 129–133
14. Hidaka, H., and Okazaki, K. (1993) Neurosci. Res. 16, 73–77
15. Kobayashi, M., Takamatsu, K., Saitoh, S., and Noguchi, T. (1993) J. Biol. Chem. 268, 18898–18904
16. Brauneewell, K. H., Klein-Szanto, A. J., and Diagram, A. J. (2009) Cell Tissue Res. 335, 301–316
17. An, W. F., Bowfly, M. R., Betty, M., Cao, J., Ling, H. P., Mendoza, G., Hisson, J. W., Mattsson, K. I., Strassle, B. W., Trimmer, J. S., and Rhodes, K. J. (2000) Nature 403, 535–556
18. Porta, O. R. C., Helle, L., and Schwiger, C., and Rivas, R. C. (1999) J. Biol. Chem. 270, 25202–25206
19. Tzingounis, A. V., Kobayashi, M., Takamatsu, K., and Nicoll, R. A. (2007) Neuron 53, 487–493
20. Tsujimoto, T., Jerom, A., Saitoh, N., Roder, J. C., and Takahashi, T. (2002) Science 295, 1272–1277
21. Hendriks, K. B., Wang, B. Q., Schnieders, E. A., and Thorner, J. (1999) Nat. Cell Biol. 1, 234–241
22. Huttner, I. G., Strahl, T., Osawa, M., King, D. S., Ames, J. B., and Thorner, J. (2003) J. Biol. Chem. 278, 4862–4874
23. Strahl, T., Hama, H., DeWald, D. B., and Thorner, J. (2005) J. Cell Biol. 171, 967–979
24. Hamasaki-Katagiri, N., Molchanova, T., Takeda, K., and Ames, J. B. (2004) J. Biol. Chem. 279, 12744–12754
25. Hamasaki-Katagiri, N., and Ames, J. B. (2010) J. Biol. Chem. 285, 4405–4414
