



Molecular structure and target recognition of neuronal calcium sensor proteins

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Neuronal calcium sensor (NCS) proteins, a sub-branch of the EF-hand superfamily, are expressed in the brain and retina where they transduce calcium signals and are genetically linked to degenerative diseases. The amino acid sequences of NCS proteins are highly conserved but their physiological functions are quite distinct. Retinal recoverin and guanylate cyclase activating proteins (GCAPs) both serve as calcium sensors in retinal rod cells, neuronal frequenin (NCS1) modulates synaptic activity and neuronal secretion, K⁺ channel interacting proteins (KChIPs) regulate ion channels to control neuronal excitability, and DREAM (KChIP3) is a transcriptional repressor that regulates neuronal gene expression. Here we review the molecular structures of myristoylated forms of NCS1, recoverin, and GCAP1 that all look very different, suggesting that the sequestered myristoyl group helps to refold these highly homologous proteins into very different structures. The molecular structure of NCS target complexes have been solved for recoverin bound to rhodopsin kinase (RK), NCS-1 bound to phosphatidylinositol 4-kinase, and KChIP1 bound to A-type K⁺ channels. We propose that N-terminal myristoylation is critical for shaping each NCS family member into a different structure, which upon Ca²⁺-induced extrusion of the myristoyl group exposes a unique set of previously masked residues that interact with a particular physiological target.

Keywords: calcium, EF-hand, Ca²⁺-myristoyl switch, NCS-1, recoverin, GCAP1, NCS proteins, NMR

INTRODUCTION

Intracellular calcium (Ca²⁺) regulates a variety of neuronal signal transduction processes in the brain and retina (Berridge et al., 2000; Augustine et al., 2003). The effects of changes in neuronal Ca²⁺ are mediated primarily by a subclass of neuronal calcium sensor (NCS) proteins (Ames et al., 1996; Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001; Burgoyne et al., 2004; Weiss et al., 2010) that belong to the EF-hand superfamily (Moncrief et al., 1990; Ikura, 1996; Ikura and Ames, 2006). The human genome encodes 14 members of the NCS family (Weiss and Burgoyne, 2002). The amino acid sequences of NCS proteins are highly conserved from yeast to humans (Figure 1). Recoverin, the first NCS protein to be discovered, and the guanylate cyclase activating proteins (GCAPs) are expressed exclusively in the retina where they serve as Ca²⁺ sensors in vision (Dizhoor et al., 1991, 1994; Palczewski et al., 1994, 2000; Stephen et al., 2008). Other NCS proteins are expressed in the brain and spinal cord such as neurocalcin (Hidaka and Okazaki, 1993), frequenin (NCS1) (Pongs et al., 1993; McFerran et al., 1998), visinin-like proteins (Bernstein et al., 1999; Braunewell and Klein-Szanto, 2009), K⁺ channel interacting proteins (KChIPs) (An et al., 2000), DREAM/calsenilin (Buxbaum et al., 1998; Carrion et al., 1999), and hippocalcin (Kobayashi et al., 1992, 1993; Tzingounis et al., 2007). Frequenin is also expressed outside of the central nervous system (Kapp et al., 2003) as well as in invertebrates including flies (Pongs et al., 1993), worms (Gomez et al., 2001), and yeast (Frq1) (Hendricks et al., 1999; Huttner et al., 2003; Hamasaki-Katagiri et al., 2004).

The common features of these proteins are an approximately 200-residue chain containing four EF-hand motifs, the sequence CPXG in the first EF-hand that markedly impairs its capacity to bind Ca²⁺, and an amino-terminal myristoylation consensus sequence.

The amino acid sequences of the NCS proteins are all quite similar and their sequence identities range from 35% to 60% (Figure 1). Residues in the EF-hand regions are the most highly conserved, particularly in the Ca²⁺-binding loops and exposed hydrophobic residues in EF1 and EF2 (W30, F35, C39, F49, I52, Y53, F69, F82, L89). The sequence in the fourth EF-hand is somewhat variable and may explain why Ca²⁺ binds to EF4 in some NCS proteins [frequenin (Cox et al., 1994; Ames et al., 2000) and GCAPs (Peshenko and Dizhoor, 2007; Stephen et al., 2007)] but not in others [recoverin (Ames et al., 1995), and VILIPs (Cox et al., 1994; Li et al., 2011)]. Non-conserved residues are also found near the C-terminus and linker between EF3 and EF4 that both interact with target proteins/membranes and may play a role in target specificity.

The structurally similar NCS proteins have remarkably different physiologic functions (Table 1). Perhaps the best characterized NCS protein is recoverin that serves as a calcium sensor in retinal rod cells. Recoverin prolongs the lifetime of light-excited rhodopsin (Kawamura, 1993; Erickson et al., 1998; Makino et al., 2004) by inhibiting rhodopsin kinase (RK) only at high Ca²⁺ levels (Calvert et al., 1995; Chen et al., 1995; Klenchin et al., 1995; Komolov et al., 2009). Hence, recoverin makes receptor

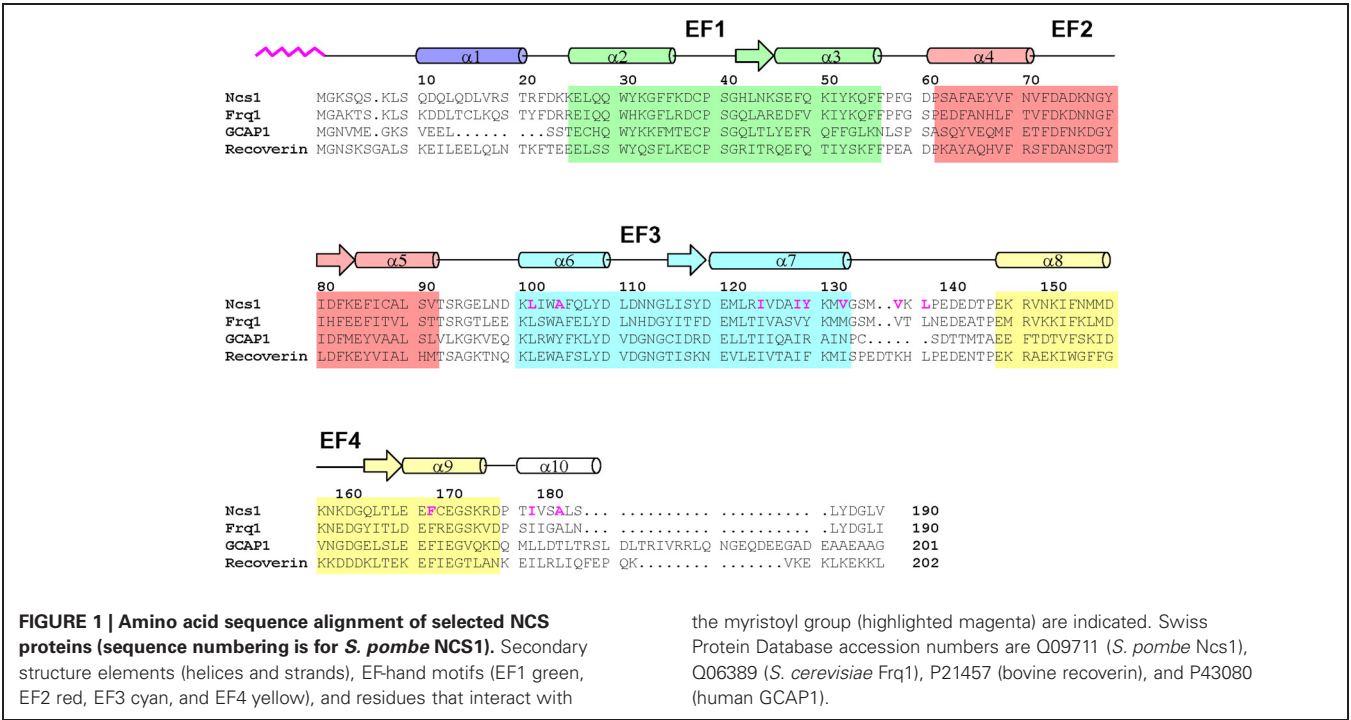


Table 1 | Function of NCS proteins.

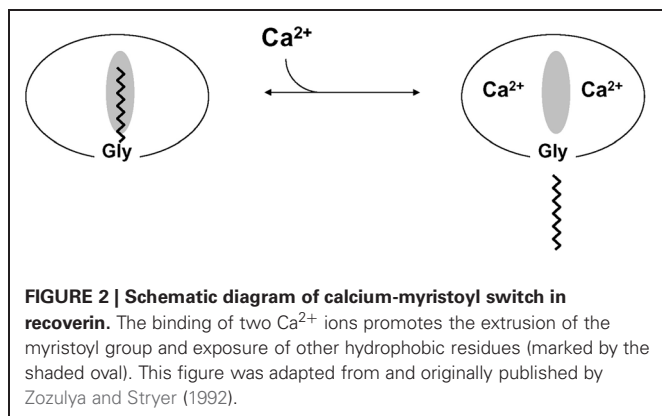
NCS protein	Function
Recoverin	Inhibit rhodopsin kinase in retinal rods.
GCAP1	Activate guanylate cyclase in retinal cones.
GCAP2	Activate guanylate cyclase in retinal rods.
GCIP	Inhibit guanylate cyclase in frog photoreceptors.
KChIP1	Regulate K ⁺ channel gating kinetics in brain.
KChIP2	Regulate K ⁺ channel gating kinetics in cardiac cells.
Calsenilin/DREAM	Repress transcription of numerous genes. [prodynorphin, c-fos and many others Mellstrom et al. (2008)].
NCS1	Activate PI(4) kinase; regulate Ca ²⁺ and K ⁺ channels.
Neurocalcin δ	Activate membrane guanylate cyclase
Hippocalcin	Activate phospholipase D; MAP kinase signaling.
VILIP-1	Activate guanylate cyclase; traffic nicotinic receptors.

desensitization Ca²⁺-dependent, and the resulting shortened life-time of rhodopsin at low Ca²⁺ levels may promote visual recovery and contribute to the adaptation to background light. Recoverin may also function in the rod inner segment (Strissel et al., 2005) and was identified as the antigen in cancer-associated retinopathy, an autoimmune disease of the retina caused by a primary tumor in another tissue (Polans et al., 1991; Subramanian and Polans, 2004). Other NCS proteins in retinal rods include the GCAP1 and GCAP2 that activate retinal guanylate cyclase only at low Ca²⁺ levels and inhibit the cyclase at high Ca²⁺ (Dizhoor et al., 1994; Palczewski et al., 1994, 2004). GCAPs also bind functionally to Mg²⁺ at low Ca²⁺ levels in light-adapted photoreceptors (Peshenko and Dizhoor, 2004, 2006), and Mg²⁺ binding to the second and third EF-hands stabilizes a conformational form of

the myristoyl group (highlighted magenta) are indicated. Swiss Protein Database accession numbers are Q09711 (*S. pombe* Ncs1), Q06389 (*S. cerevisiae* Frq1), P21457 (bovine recoverin), and P43080 (human GCAP1).

GCAPs needed to activate the cyclase (Peshenko and Dizhoor, 2006; Lim et al., 2009). GCAPs are important for regulating the recovery phase of visual excitation and particular mutants are linked to various forms of retinal degeneration (Semple-Rowland et al., 1996; Sokal et al., 1998; Baehr and Palczewski, 2007; Bondarenko et al., 2010; Jiang and Baehr, 2010). Yeast and mammalian frequenins bind and activate a particular PtdIns 4-OH kinase isoform (Pik1 gene in yeast) (Hendricks et al., 1999; Kapp et al., 2003; Strahl et al., 2003, 2007) required for vesicular trafficking in the late secretory pathway (Hama et al., 1999; Walch-Solimena and Novick, 1999). Mammalian frequenin (NCS1) also regulates voltage-gated Ca²⁺ and K⁺ channels (Weiss et al., 2000; Nakamura et al., 2001). The KChIPs regulate the gating kinetics of voltage-gated, A-type K⁺ channels (An et al., 2000). The DREAM/calsenilin/KChIP3 protein binds to specific DNA sequences in many genes, including prodynorphin and c-fos (Carrion et al., 1999; Mellstrom et al., 2008). DREAM forms a tetramer that binds to DNA only in the absence of Ca²⁺ (Carrion et al., 1998, 1999) and serves as a calcium sensor and transcriptional repressor for pain modulation (Cheng et al., 2002; Lilliehook et al., 2003). Hence, the functions of the NCS proteins all appear to be quite diverse and non-overlapping.

Mass spectrometric analysis of retinal recoverin and some of the other NCS proteins revealed that they are myristoylated at the amino terminus (Dizhoor et al., 1992; Kobayashi et al., 1993; Ladant, 1995). Recoverin contains an N-terminal myristoyl (14:0) or related fatty acyl group (12:0, 14:1, 14:2). Retinal recoverin and myristoylated recombinant recoverin, but not unmyristoylated recoverin, bind to membranes in a Ca²⁺-dependent manner (Zozulya and Stryer, 1992; Dizhoor et al., 1993). Likewise, bovine neurocalcin and hippocalcin contain an N-terminal myristoyl group and both exhibit Ca²⁺-induced

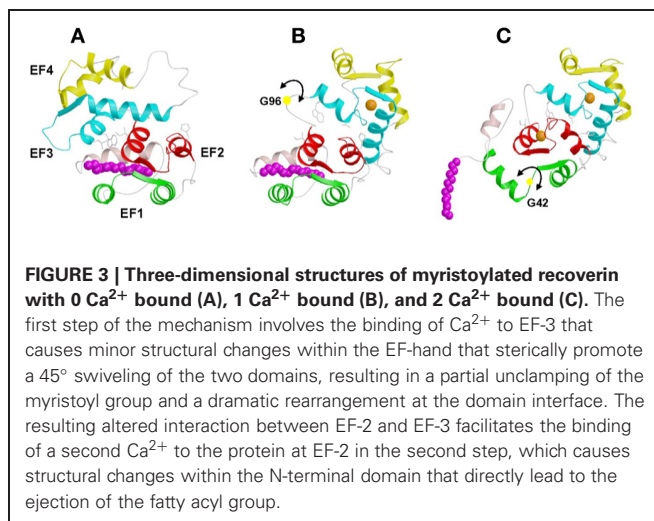


membrane binding (Ladant, 1995). These findings led to the proposal that NCS proteins possess a Ca^{2+} -myristoyl switch (Figure 2). The covalently attached fatty acid is highly sequestered in recoverin in the calcium-free state. The binding of calcium to recoverin leads to the extrusion of the fatty acid, making it available to interact with lipid bilayer membranes or other hydrophobic sites. The Ca^{2+} -myristoyl switch function by recoverin also enables its light-dependent protein translocation in retinal rods (Strissel et al., 2005).

In this review, the atomic-level structures of various NCS proteins and their target complexes will be discussed and compared with that of calmodulin. We begin by examining the large effect of N-terminal myristoylation on the structures of recoverin, GCAP1, and NCS1. Ca^{2+} -induced extrusion of the myristoyl group exposes unique hydrophobic binding sites in each protein that in turn interact with distinct target proteins. An emerging theme is that N-terminal myristoylation is critical for shaping each NCS family member into a unique structure, which upon Ca^{2+} -induced extrusion of the myristoyl group exposes a unique set of previously masked residues, thereby exposing a distinctive ensemble of hydrophobic residues to associate specifically with a particular physiological target.

STRUCTURE OF RECOVERIN'S CALCIUM-MYRISTOYL SWITCH

The x-ray crystal structure of recombinant unmyristoylated recoverin (Flaherty et al., 1993; Weiergraber et al., 2003) showed it to contain a compact array of EF-hand motifs, in contrast to the dumbbell shape of calmodulin (Babu et al., 1988) and troponin C (Herzberg and James, 1988). The four EF-hands are organized into two domains: the first EF-hand, EF-1 (residues 27–56, colored green in (Figures 1 and 3)), interacts with EF-2 (residues 63–92, red) to form the N-terminal domain, and EF-3 (residues 101–130, cyan), and EF-4 (residues 148–177, yellow) form the C-terminal domain. The linker between the two domains is U-shaped rather than α -helical. Ca^{2+} is bound to EF-3 and Sm^{3+} (used to derive phases) is bound to EF-2. The other two EF hands possess novel features that prevent ion binding. EF-1 is disrupted by a Cys-Pro sequence in the binding loop. EF-4 contains an internal salt bridge in the binding loop that competes with Ca^{2+} binding. Myristoylated recoverin, the physiologically active form has thus far eluded crystallization.

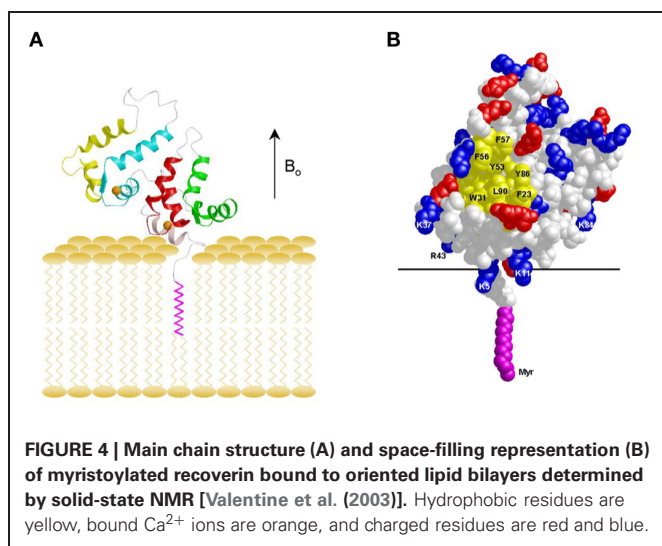


The structures of myristoylated recoverin in solution with 0, 1, and 2 Ca^{2+} bound have been determined by nuclear magnetic resonance (NMR) spectroscopy (Tanaka et al., 1995; Ames et al., 2002) (Figure 3). In the Ca^{2+} -Stateplacefree state, the myristoyl group is sequestered in a deep hydrophobic cavity in the N-terminal domain. The cavity is formed by five α -helices. The two helices of EF-1 (residues 26–36 and 46–56), the exiting helix of EF-2 (residues 83–93), and entering helix of EF-3 (residues 100–109) lie perpendicular to the fatty acyl chain and form a box-like arrangement that surrounds the myristoyl group laterally. A long, amphipathic α -helix near the N-terminus (residues 4–16) packs closely against and runs antiparallel to the fatty acyl group, and serves as a lid on top of the four-helix box. The N-terminal residues Gly 2 and Asn 3 form a tight hairpin turn that connects the myristoyl group to the N-terminal helix. This turn positions the myristoyl group inside the hydrophobic cavity and gives the impression of a cocked trigger. The bond angle strain stored in the tight hairpin turn may help eject the myristoyl group from the pocket once Ca^{2+} binds to the protein.

The structure of myristoylated recoverin with one Ca^{2+} bound at EF-3 (half saturated recoverin, Figure 3B) (Ames et al., 2002) represents a hybrid structure of the Ca^{2+} -free and Ca^{2+} -saturated states. The structure of the N-terminal domain (residues 2–92, green and red in Figure 3) of half saturated recoverin (Figure 3B) resembles that of Ca^{2+} -free state (Figure 3A) and is very different from that of the Ca^{2+} -saturated form (Figure 3C). Conversely, the structure of the C-terminal domain (residues 102–202, cyan and yellow in Figure 3) of half saturated recoverin more closely resembles that of the Ca^{2+} -saturated state. Most striking in the structure of half saturated recoverin is that the myristoyl group is flanked by a long N-terminal helix (residues 5–17) and is sequestered in a hydrophobic cavity containing many aromatic residues from EF-1 and EF-2 (F23, W31, Y53, F56, F83, and Y86). An important structural change induced by Ca^{2+} binding at EF-3 is that the carbonyl end of the fatty acyl group in the half saturated species is displaced far away from hydrophobic residues of EF-3 (W104 and L108, Figures 3A,B) and becomes somewhat solvent exposed. By contrast, the myristoyl group of Ca^{2+} -free recoverin is highly sequestered by residues of EF-3 (Tanaka et al., 1995).

The structure of myristoylated recoverin with two Ca^{2+} bound shows the amino-terminal myristoyl group to be extruded (Ames et al., 1997) (**Figure 3C**). The N-terminal eight residues are solvent exposed and highly flexible and thus serve as a mobile arm to position the myristoyl group outside the protein when Ca^{2+} is bound. The flexible arm is followed by a short α -helix (residues 9–17) that precedes the four EF-hand motifs, arranged in a tandem array as was seen in the x-ray structure. Calcium ions are bound to EF-2 and EF-3. EF-3 has the canonical “open conformation” similar to the Ca^{2+} occupied EF-hands in calmodulin and troponin C. EF-2 is somewhat unusual and the helix-packing angle of Ca^{2+} -bound EF-2 (120°) in recoverin more closely resembles that of the Ca^{2+} -free EF-hands (in the “closed conformation”) found in calmodulin and troponin C. The overall topology of Ca^{2+} -bound myristoylated recoverin is similar to the x-ray structure of unmyristoylated recoverin described above. The root-mean-square (RMS) deviation of the main chain atoms in the EF-hand motifs is 1.5 Å in comparing Ca^{2+} -bound myristoylated recoverin to unmyristoylated recoverin. Hence, in Ca^{2+} -saturated recoverin, the N-terminal myristoyl group is solvent exposed and does not influence the interior protein structure.

The Ca^{2+} -induced exposure of the myristoyl group (**Figures 2 and 3**) enables recoverin to bind to membranes only at high Ca^{2+} (Zozulya and Stryer, 1992; Lange and Koch, 1997). Recent solid-state NMR studies have determined the structure of Ca^{2+} -bound myristoylated recoverin bound to oriented lipid bilayer membranes (**Figure 4**) (Valentine et al., 2003). Membrane-bound recoverin appears to retain approximately the same overall structure as it has in solution (Valentine et al., 2010). The protein is positioned on the membrane surface such that its long molecular axis is oriented 45° with respect to the membrane normal. The N-terminal region of recoverin points toward the membrane surface, with close contacts formed by basic residues K5, K11, K22, K37, R43, and K84. This orientation of membrane-bound recoverin allows an exposed hydrophobic crevice (lined primarily by residues F23, W31, F35, I52, Y53, F56, Y86, and L90), near the membrane surface that may serve as a potential binding site for the target protein, RK (**Figure 4B**).



STRUCTURAL DIVERSITY OF NCS PROTEINS

MYRISTOYLATION RESHAPES STRUCTURE OF NCS PROTEINS

Three-dimensional structures have been determined for myristoylated NCS proteins: recoverin (Ames et al., 1997), GCAP1 (Stephen et al., 2007), and NCS1 (Lim et al., 2011) that each contain a sequestered myristoyl group (**Figure 5**). Surprisingly, the myristoylated forms of GCAP1, NCS1, and recoverin all have very distinct three-dimensional folds (**Figure 5**). The overall RMS deviations are 2.8 and 3.4 Å when comparing the main chain structures of Ca^{2+} -free NCS1 with recoverin and GCAP1, respectively. These very different structures reveal that the N-terminal myristoyl group is sequestered inside different protein cavities at different locations in each case. In NCS1, the N-terminal myristoyl group is sequestered inside a cavity near the C-terminus formed between the helices of EF3 and EF4 (**Figure 5A**). The fatty acyl chain in NCS1 is nearly parallel to the helices of EF3 and EF4 that form walls that surround the myristoyl moiety (**Figure 5D**). This arrangement in NCS1 is in stark contrast to recoverin where the myristoyl group is sequestered inside a protein cavity near the N-terminus (**Figure 5B**). The myristate in recoverin is wedged perpendicularly between the helices of EF1 and EF2 (**Figure 5E**) that contrasts with the parallel arrangement in NCS1 (**Figure 5D**). For GCAP1 (**Figure 5C**), the myristoyl group is located in between the N-terminal and C-terminal domains. In essence, the myristate bridges both domains of GCAP1 by interacting with helices at each end of the protein. The structural location and environment around the myristoyl group is very different in the various NCS proteins (**Figure 5**). We suggest that each Ca^{2+} -myristoyl switch protein may adopt a distinct structure because its N-terminal myristoyl group associates with patches of hydrophobic residues that are unique to that protein. We point out, however, that myristoylation is not required for the function of GCAP2 (Olshevskaya et al., 1997), suggesting that additional factors besides myristoylation must also play a role.

Non-conserved residues of NCS proteins interact closely with the N-terminal myristoyl group and help stabilize the novel protein structure in each case. NCS1, recoverin, and GCAP1 all have non-conserved residues near the N-terminus (called an N-terminal arm highlighted purple in **Figure 5**) that make specific contacts with the myristoyl moiety. GCAP1 also contains an extra helix at the C-terminus that contacts the N-terminal arm and myristoyl group (**Figure 5C**). Thus, non-conserved residues at the N-terminus, C-terminus, and loop between EF3 and EF4 all play a role in creating a unique environment around the myristoyl group. In NCS1, the long N-terminal arm and particular hydrophobic residues in the C-terminal helix are crucial for placing the C14 fatty acyl chain in a cavity between EF3 and EF4 (**Figure 5D**). By contrast, the much shorter N-terminal arm in both recoverin and GCAP1 prevents the myristoyl group from reaching the C-terminal cavity and instead places the fatty acyl chain between EF1 and EF2 (**Figure 5E**). We propose that non-conserved residues at the N-terminus, C-terminus, and/or loop between EF3 and EF4 may play a role in forming unique myristoyl binding environments in other NCS proteins, such as VILIPs, neurocalcins, and hippocalcins that may help explain their capacity to associate with functionally diverse target proteins.

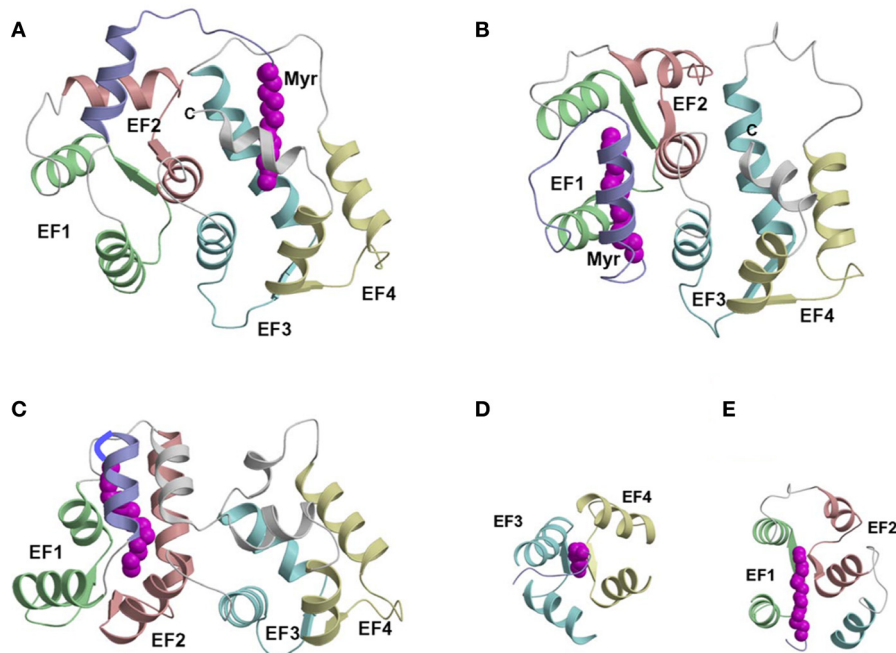


FIGURE 5 | Main chain structures of Ca^{2+} -free myristoylated NCS1 (PDB ID: 212e) (A), recoverin (PDB ID: 1iku) (B), and GCAP1 (PDB ID: 2r2i) (C). Close-up views of the myristate binding pocket in

NCS1 (D), and recoverin (E). EF-hands and myristoyl group (magenta) are colored as defined in **Figure 1**. Adapted from and originally published by Lim et al. (2011).

STRUCTURES OF Ca^{2+} -BOUND NCS PROTEINS

Three-dimensional structures have been determined for unmyristoylated forms of Ca^{2+} -bound neurocalcin (Vijay-Kumar and Kumar, 1999), frequenin (Bourne et al., 2001), KChIP1 (Scannevin et al., 2004; Zhou et al., 2004), Frq1 (Ames et al., 2000), and GCAP2 (Ames et al., 1999). The first eight residues from the N-terminus are unstructured and solvent exposed in each case, consistent with an extruded myristoyl group that causes Ca^{2+} -induced membrane localization of NCS proteins (Zozulya and Stryer, 1992; Bourne et al., 2001; Spilker et al., 2002). The overall main chain structures of the Ca^{2+} -bound NCS proteins are very similar in each case, which is not too surprising given their sequence relatedness. However, if the main chain structures are so similar, then how can one explain their ability to bind unique target proteins? One distinguishing structural property is the number and location of bound Ca^{2+} . Recoverin has Ca^{2+} bound at EF-2 and EF-3; KChIP1 has Ca^{2+} bound at EF-3 and EF-4; and frequenin, neurocalcin, and GCAP2 have Ca^{2+} bound at EF-2, EF-3, and EF-4. Another important structural property is the distribution of charged and hydrophobic residues on the protein surface. Surface representations of hydrophobicity and charge density of the various NCS structures are shown in **Figure 6**. All NCS structures exhibit a similar exposed hydrophobic surface located on the N-terminal half of the protein, formed primarily by residues in EF-1 and EF-2 (F35, W31, F56, F57, Y86, and L90 for recoverin in **Figure 6A**). The exposed hydrophobic residues in this region are highly conserved (labeled and colored yellow in **Figure 6**) and correspond to residues of recoverin that interact with the myristoyl group in the Ca^{2+} -free

state (**Figure 3A**). A similar hydrophobic patch is also seen in membrane-bound recoverin (**Figure 4B**). These exposed residues in the hydrophobic patch have been implicated in target recognition from mutagenesis studies [Emilov et al. (2001); Krylov et al. (1999); Olshevskaya et al. (1999a); Tachibanaki et al. (2000)], and these residues very likely form intermolecular contacts with target proteins as has been demonstrated in the recent crystal structure of KChIP1 (see below).

The distribution of charged (red and blue) and hydrophobic (yellow) residues on the surface of the C-terminal half of the NCS proteins is highly variable (**Figure 6**). Frequenin exhibits exposed hydrophobic residues in the C-terminal domain that fuse together with the exposed hydrophobic crevice in the N-terminal domain, forming one continuous and elongated patch (**Figure 6B**). By contrast, recoverin (**Figure 6A**) has mostly charged residues on the surface of the C-terminal half, whereas neurocalcin (**Figure 6C**) and KChIP1 (**Figure 6D**) have mostly neutral residues shown in white. The different patterns of charge distribution on the C-terminal surface of NCS proteins might be important for conferring target specificity.

Ca^{2+} sensitive dimerization of NCS proteins is another structural characteristic that could influence target recognition. Neurocalcin (Vijay-Kumar and Kumar, 1999), recoverin (Flaherty et al., 1993), VILIP-1 (Li et al., 2011), and KChIP1 (Zhou et al., 2004) exist as dimers in their x-ray crystal structures. Hydrodynamic studies have confirmed that neurocalcin and DREAM form dimers in solution at high Ca^{2+} and are monomeric in the Ca^{2+} -free state (Olshevskaya et al., 1999b; Osawa et al., 2005). Indeed, the NMR structure of Ca^{2+} -bound DREAM forms a dimer in solution with

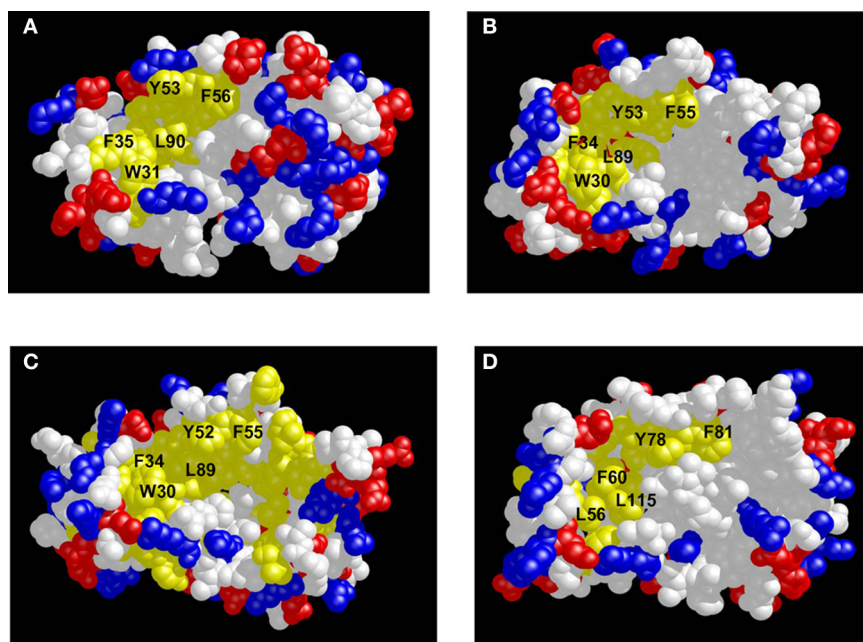


FIGURE 6 | Space-filling representations of the Ca^{2+} -bound structures of recoverin (A), frequenin (B), neurocalcin (C), and KChIP1 (D). Exposed hydrophobic residues are yellow, neutral residues are white, and charge residues are red and blue.

intermolecular contacts involving Leu residues near the C-terminus (Lusin et al., 2008). By contrast, GCAP2 forms a dimer only in the Ca^{2+} -free state and is monomeric at high Ca^{2+} (Olshevskaya et al., 1999b). Ca^{2+} sensitive dimerization of GCAP-2 has been demonstrated to control its ability to activate retinal guanylate cyclase. Ca^{2+} -sensitive protein oligomerization is also important physiologically for DREAM: the Ca^{2+} -free DREAM protein serves as a transcriptional repressor by binding to DNA response elements as a protein tetramer (Carrion et al., 1999). Ca^{2+} -induced dimerization of DREAM appears to disrupt DNA binding and may activate transcription of prodynorphin and c-fos genes (Carrion et al., 1999). In a related fashion, Ca^{2+} -bound KChIP1 forms a dimer in solution and in complex with an N-terminal fragment of the Kv4.2 K^+ channel (Zhou et al., 2004). By contrast, the full-length Kv4.2 channel tetramer binds to KChIP1 with a 4:4 stoichiometry (Kim et al., 2004), suggesting that KChIP1 dimers may assemble as a protein tetramer to recognize the channel. Such a protein tetramerization of KChIP1 may be Ca^{2+} sensitive like it is for DREAM. The dimerization of VILIP-1 has been implicated in the trafficking of the dimeric α -subunit of the $\alpha_4\beta_2$ nicotinic acetylcholine receptor (nAChR) (Lin et al., 2002; Zhao et al., 2009; Li et al., 2011). In short, the oligomerization properties of some NCS proteins appear to be Ca^{2+} sensitive and may play a role in target recognition.

TARGET RECOGNITION BY NCS PROTEINS

RECOVERIN BOUND TO RHODOPSIN KINASE FRAGMENT (RK25)

The structure of Ca^{2+} -bound recoverin bound to the N-terminal region of rhodopsin kinase (residues 1–25, hereafter referred to as RK25) was the first atomic-resolution structure of a Ca^{2+} -myristoyl switch protein bound to a functional target protein

(Ames et al., 2006) (**Figure 7A**). The structure of this complex revealed that RK25 forms a long amphipathic α -helix, whose hydrophobic surface interacts with the N-terminal hydrophobic groove of recoverin described above (**Figure 6**). The structure of recoverin in the complex is quite similar to that of Ca^{2+} -bound recoverin alone in solution (RMS deviation = 1.8 Å). The structure of RK25 in the complex consists of an amphipathic α -helix (residues 4–16). The hydrophobic surface of the RK25 helix (L6, V9, V10, A11, F15) interacts with the exposed hydrophobic groove on recoverin (W31, F35, F49, I52, Y53, F56, F57, Y86, and L90). Previous mutagenesis studies on recoverin (Tachibanaki et al., 2000) and RK (Higgins et al., 2006; Komolov et al., 2009) have shown that many of the hydrophobic residues at the binding interface are essential for the high affinity interaction. These hydrophobic contacts are supplemented by a π -cation interaction involving F3 (RK25) and K192 from recoverin (Zernii et al., 2011). Dipolar residues on the opposite face of the RK25 helix (S5, T8, N12, I16) are solvent exposed. The helical structure of RK25 in the complex is stabilized mostly by hydrophobic intermolecular interactions with recoverin, as free RK25 in solution is completely unstructured.

The Ca^{2+} -myristoyl switch mechanism of recoverin (i.e., Ca^{2+} -induced extrusion of the N-terminal myristoyl group, **Figure 2**) is structurally coupled to Ca^{2+} -induced inhibition of RK (Calvert et al., 1995; Chen et al., 1995; Klenchin et al., 1995). The exposed hydrophobic residues of recoverin that interact with RK correspond to the same residues that contact the N-terminal myristoyl group in the structure of Ca^{2+} -free recoverin (Ames et al., 1997). The size of the myristoyl group is similar to the length and width of the RK25 helix in the complex, which explains why both effectively compete for binding to the exposed

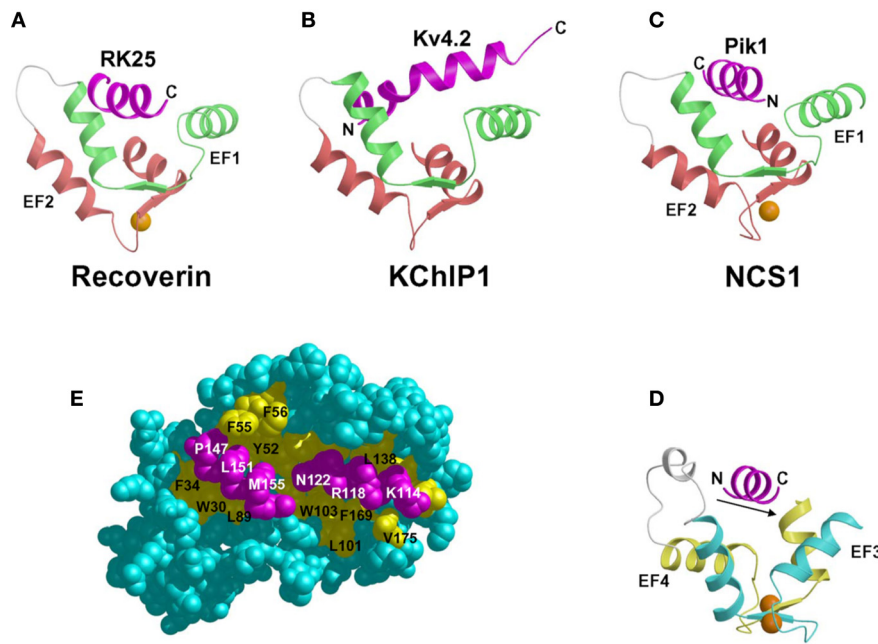


FIGURE 7 | Ribbon diagrams illustrating intermolecular interactions for recoverin bound to RK25 (A), KChIP1 bound to Kv4.32 (B), NCS1 (N-domain) bound to Pik1(111–151) (C), NCS1 (C-domain) bound to Pik1(111–151) (D), and space-filling view of NCS1

bound to Pik1(111–151) (E). In each case, a target helix (magenta) is inserted in groove formed by the helices of the EF-hands. The intermolecular interactions are mostly hydrophobic as described in the text.

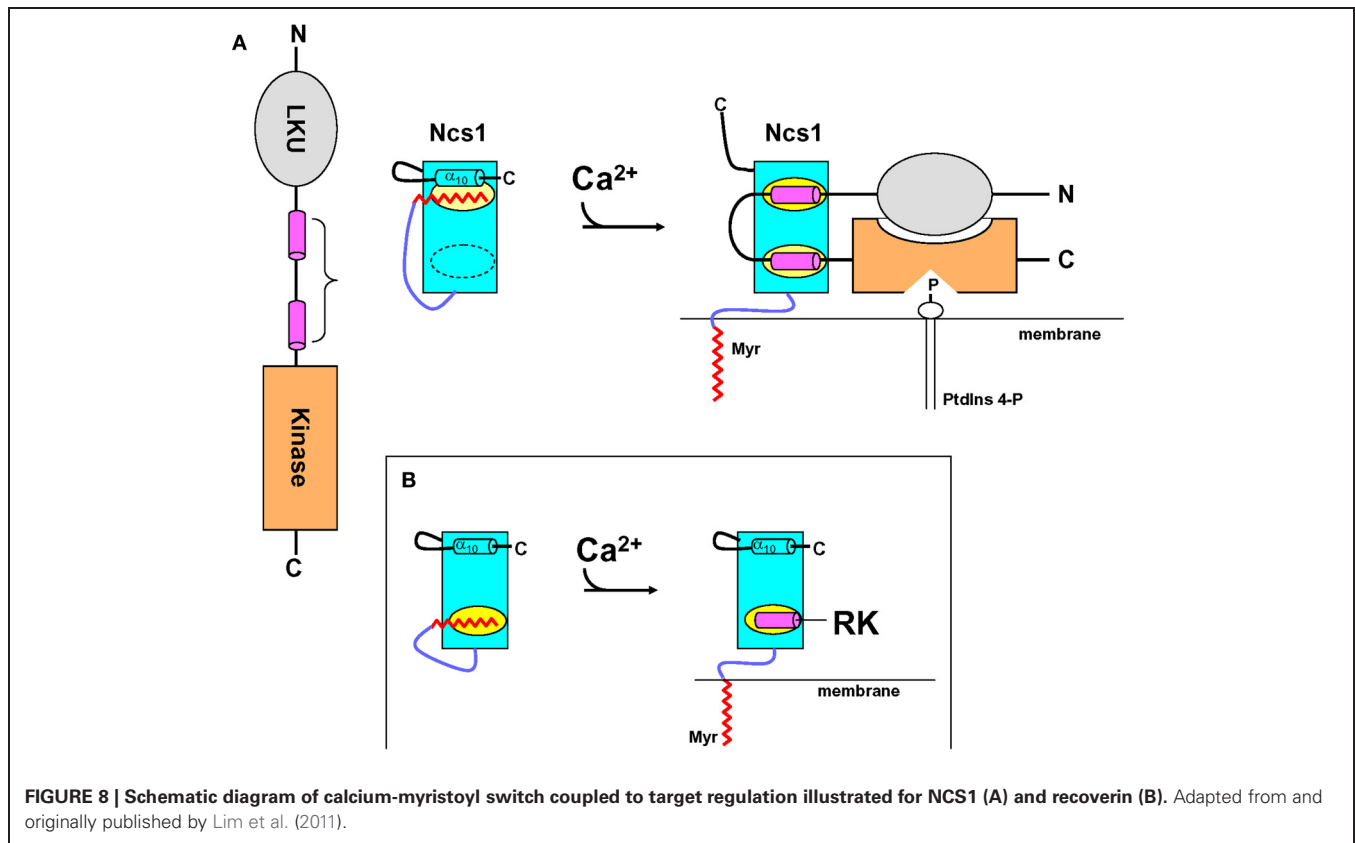
hydrophobic groove (**Figure 4B**). The Ca^{2+} -induced exposure of the N-terminal hydrophobic groove, therefore, explains why recoverin binds to RK only at high Ca^{2+} levels. In the Ca^{2+} -free state, the covalently attached myristoyl group sequesters the N-terminal hydrophobic groove and covers up the target-binding site. Ca^{2+} -induced extrusion of the myristoyl group of recoverin causes exposure of residues that bind and inhibit RK (**Figure 8**). This mechanism elegantly explains how recoverin controls both the localization and activity of RK in response to light. In the dark (high Ca^{2+}), Ca^{2+} -bound recoverin binds to RK [thereby inhibiting it (Calvert et al., 1995; Chen et al., 1995; Klenchin et al., 1995; Komolov et al., 2009)] and delivers RK to the membrane via a Ca^{2+} -myristoyl switch that pre-positions RK near rhodopsin. Upon light activation (low Ca^{2+}), recoverin rapidly dissociates from both RK and the membrane, allowing RK to bind efficiently to its nearby substrate, rhodopsin, and cause rapid desensitization.

NCS1 BOUND TO PHOSPHATIDYLINOSITOL 4-KINASE FRAGMENT (PIK1)

The structure of Ca^{2+} -bound NCS1 [or yeast Frq1 (Strahl et al., 2007)] bound to a functional fragment of Pik1 [residues 111–159, hereafter referred to as Pik1(111–159)] was determined by NMR (Lim et al., 2011) (**Figures 7C,D**). The structure of NCS1 in the complex is very similar to the crystal structure in the absence of target (Bourne et al., 2001) with a concave solvent-exposed groove lined by two separate hydrophobic patches (highlighted yellow in **Figure 6**). These two hydrophobic surfaces represent bipartite binding sites on NCS1 that interact with two helical segments

in Pik1(111–159) (**Figure 7E**). The structure of Pik1(111–159) in the complex adopts a conformation that contains two α -helices (residues 114–127 and 143–156) connected by a disordered loop. The N-terminal helix contains hydrophobic residues (I115, C116, L119, and I123) that contact C-terminal residues of NCS1 (L101, W103, V125, V128, L138, I152, L155, and F169). Interestingly, these same hydrophobic residues in Ca^{2+} -free NCS1 make close contacts with the myristoyl group. Therefore, Ca^{2+} -induced extrusion of the myristoyl group causes exposure of hydrophobic residues in NCS1 that forms part of the Pik1 binding site (**Figures 7E** and **8**). The C-terminal helix of Pik1(111–159) contains many hydrophobic residues (V145, A148, I150, and I154) that contact the exposed N-terminal hydrophobic groove of NCS1 (W30, F34, F48, I51, Y52, F55, F85, and L89), very similar to the exposed hydrophobic groove seen in all NCS proteins (**Figure 6**). The two helices of Pik1(111–159) do not interact with one another or with the unstructured connecting loop and are highly stabilized by interactions with NCS1.

Non-conserved residues in NCS1 at the C-terminus and immediately following EF3 may be structurally important for explaining target specificity. The non-conserved C-terminal region of NCS1 (residues, 180–190) is structurally disordered in the target complex, in contrast to a well-defined C-terminal helix seen in Ca^{2+} -free NCS1 in the absence of target (Lim et al., 2011). The C-terminal helix in Ca^{2+} -free NCS1 (target free state) makes contact with the myristoyl group and residues in EF3 and EF4 (L101, A104, M121, I152, F169, and S173). These same residues in Ca^{2+} -bound NCS1 make contact with Pik1 in the complex (**Figure 7E**). Therefore, the N-terminal Pik1 helix



appears to substitute for and perhaps displace the C-terminal helix of Frq1, likely leading to the observed C-terminal destabilization in the complex (**Figure 8**). The corresponding C-terminal helix of Kv4.3 channel (Pioletti et al., 2006; Wang et al., 2007) but not upon its binding to Kv4.2 (Zhou et al., 2004). The C-terminal helix in recoverin forms a stable interaction with EF3 and EF4, enabling the C-terminal helix to perhaps serve as a built-in competitive inhibitor that would presumably block its ability to bind to targets like Pik1 and Kv4.3. This role for the C-terminus may explain why the C-terminal sequences of NCS proteins are not well conserved (**Figure 1**). Another non-conserved region of NCS1 implicated in target specificity is the stretch between EF3 and EF4 (residues 134–146). This region of NCS1 adopts a short α -helix in the complex that contacts the N-terminal helix of Pik1. By contrast, the region between EF3 and EF4 is unstructured in many other NCS proteins (Ames et al., 1999; Bourne et al., 2001; Scannevin et al., 2004; Zhou et al., 2004; Lusin et al., 2008).

The structure of the NCS1-Pik1 complex (**Figures 5A and 7C,D**) suggest how a Ca^{2+} -myristoyl switch might promote activation of PtdIns 4-kinase (**Figure 8**). Under resting basal conditions, 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 **Figure 8B**) 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. 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 (Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001; Haeseleer et al., 2002; Zheng et al., 2005). 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 (**Figure 8**, inset) that interacts with a single target helix in RK (Ames et al., 2006). 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 9). The Ca^{2+} -induced binding of NCS1 to PtdIns 4-kinase may promote a structural changes that cause increased 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.

MECHANISMS OF TARGET RECOGNITION

NCS proteins bind to helical target proteins analogous to the target binding seen for CaM (Hoeflich and Ikura, 2002) (**Figure 7**).

Helical segments of target proteins bind to an exposed hydrophobic crevice formed by the two EF-hands in either the N-terminal or C-terminal domain in NCS proteins. In recoverin, the two N-terminal EF-hands form an exposed hydrophobic groove that interacts with a hydrophobic target helix from rhodopsin kinase (RK25) (Ames et al., 2006) (**Figure 7A**). The N-terminal EF-hands of KChIP1 interact with a target-helix derived from the T1 domain of Kv4.2 channels (**Figure 6B**) (Zhou et al., 2004) and Kv4.3 channels (Pioletti et al., 2006). The orientation of the target helices bound to recoverin and KChIP1 are somewhat similar: the C-terminal end of the target helix is spatially close to the N-terminal helix of EF-1. By contrast, the Pik1 target helix binds to NCS1 in almost the exact opposite orientation (**Figure 6C**). The N-terminal end of the Pik1 helix is closest to EF1 (green) in NCS1, whereas the C-terminal end of the RK25 target helix is closest to the corresponding region of recoverin. Non-conserved residues in NCS1 (G33 and D37) make important contacts with the Pik1 target helix and presumably assist in imposing the observed orientation of the helix. Thus, the requirement that the helix (in this case, from Pik1) must bind to NCS1 with a polarity opposite to that observed for the helices in other target-NCS family member complexes could clearly contribute to dictating the substrate specificity of frequenins, as compared to other NCS sub-types. Another important structural feature seen in the NCS1-Pik1 interaction is that two helical segments of the target are captured in the complex, whereas in the target complexes characterized for recoverin and KChIP1, only one helix is bound. Therefore, selective substrate recognition by NCS proteins may be explained by both by the orientation of the bound target helix; and, the number of target helices bound.

CONCLUSIONS

We reviewed the molecular structures of NCS proteins and examined structural determinants important for target recognition. N-terminal myristoylation has a profound effect on the structures of Ca^{2+} -free recoverin, GCAP1 and NCS1. Surprisingly, the sequestered myristoyl group interacts with quite different protein residues in each case and, therefore, is able to reshape these homologous NCS proteins into very different structures. The structures of the Ca^{2+} -bound NCS proteins all contain an extruded N-terminus with an exposed hydrophobic crevice implicated in target binding. We propose that N-terminal myristoylation is critical for shaping each NCS family member into a unique structure, which upon Ca^{2+} -induced extrusion of the myristoyl group exposes a unique set of previously masked residues, thereby exposing a distinctive ensemble of hydrophobic residues to associate specifically with a particular physiological target. Differences in their surface charge density and protein dimerization properties may also help to explain NCS target specificity and functional diversity. In the future, atomic resolution structures of additional NCS proteins both with a sequestered myristoyl group and in their extruded forms bound to their respective target proteins are needed to improve our understanding of how this structurally conserved family of proteins can uniquely recognize their diverse biological targets.

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