Identification of Residues That Determine the Absence of a Ca\textsuperscript{2+}/Myristoyl Switch in Neuronal Calcium Sensor-1*

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The neuronal calcium sensor (NCS) family of Ca\textsuperscript{2+}-binding proteins regulates a number of different processes in neurons and photoreceptor cells. The first of these proteins to be characterized, recoverin, was shown to exhibit a Ca\textsuperscript{2+}/myristoyl switch whereby its N-terminal myristoyl group is sequestered in the Ca\textsuperscript{2+}-free form and is exposed on Ca\textsuperscript{2+} binding to allow the protein to become membrane-associated. It has subsequently been shown that certain other family members also exhibit this mechanism in living cells. In contrast, NCS-1 does not show the Ca\textsuperscript{2+}/myristoyl switch and is membrane-associated even at low Ca\textsuperscript{2+} concentrations. We have used sequence comparison combined with information from structural analyses to attempt to identify candidate residues within the NCS proteins that determine whether or not the Ca\textsuperscript{2+}/myristoyl switch operates in cells and have tested their functional significance by mutagenesis. The results show that NCS-1 possesses residues within its N terminus that lock the myristoyl group in an exposed conformation. In addition, other structural aspects within the C-terminal domains are required to allow the switch to operate. We have determined a key role for residues within the motif EELTRK in NCS-1 in keeping the myristoyl group exposed and allowing the protein to be constitutively membrane-associated.

The neuronal calcium sensor (NCS) proteins are a family of closely related Ca\textsuperscript{2+}-binding proteins (1, 2). They each contain four EF-hands, but only two or three of these are functional in Ca\textsuperscript{2+} binding. There are 14 NCS proteins encoded in the human genome including recoverin, GCAP1–3, KChIP1–4, VILIP1–3, neurocalcin δ, hippocalcin, and NCS-1 (2). Orthologues of these proteins appeared at various stages of evolution, but NCS-1 (also known as frequentin) is the earliest member of this family being represented in yeast (3, 4) with other members of the family appearing later in evolution. They are found predominantly in neurons and neuroendocrine cells and have a wide range of identified functions including the modulation of neurotransmitter release (5, 6), the regulation of Ca\textsuperscript{2+} (7–9) and potassium (10, 11) channels, regulation of phosphoinositide metabolism (3, 12), pain perception (13), phototransduction (14), and learning (15).

With the exception of KChIP2–4 (11, 16), all of the NCS proteins are N-terminally myristoylated. The significance of myristoylation was initially studied for recoverin through analysis of structures of recoverin in the Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound forms (17–21). The myristoyl group is sequestered in the Ca\textsuperscript{2+}-free state within a hydrophobic pocket consisting in part of the first EF-hand, which cannot bind Ca\textsuperscript{2+}. On binding of Ca\textsuperscript{2+} to EF-hands 2 and 3, a considerable conformational change is triggered in recoverin that flips out the myristoyl group. This so-called Ca\textsuperscript{2+}/myristoyl switch would allow recoverin to become reversibly associated with membranes via the myristoyl group in response to [Ca\textsuperscript{2+}] elevation. The hydrophobic residues that contact the myristoyl group in the recoverin structure are conserved in all of the family members, but surprisingly not all NCS proteins show the Ca\textsuperscript{2+}/myristoyl switch mechanism. This property has been demonstrated for neurocalcin δ (22–24), VILIP1 and VILIP9 (25, 26), and hippocalcin (23, 27) by biochemical approaches and also in living cells. In contrast, NCS-1 binds to membranes even in the nominal absence of Ca\textsuperscript{2+} (28) and is membrane-associated in cells even at low Ca\textsuperscript{2+} levels (29, 30). From structural studies using nuclear magnetic resonance, the yeast orthologue of NCS-1, frequentin, appears to have its myristoyl group exposed even in the Ca\textsuperscript{2+}-free state (4). As NCS-1/frequentin is the member of the family that appeared first during evolution, this suggests that the Ca\textsuperscript{2+}/myristoyl switch was a later evolutionary development in this family of proteins. Other NCS proteins, the KChIPs (11), that appeared only in fish also do not have a Ca\textsuperscript{2+}/myristoyl switch. The only KChIP to be myristoylated, KChIP1, is also membrane-associated at low [Ca\textsuperscript{2+}] (31). GCAP2 was suggested to have a reversed Ca\textsuperscript{2+}/myristoyl switch being membrane-bound at low [Ca\textsuperscript{2+}] and dissociating as [Ca\textsuperscript{2+}] is elevated (32), although more recently it has been shown that both GCAP1 and GCAP2 lack the Ca\textsuperscript{2+}/myristoyl switch (33, 34). NCS-1 does undergo a conformational change on Ca\textsuperscript{2+} binding (6, 35), but the exact nature of this is unknown as the only detailed structures solved so far are for the Ca\textsuperscript{2+}-bound forms of myristoylated yeast frequentin (4) and non-myristoylated human NCS-1 (36). Nuclear magnetic resonance analysis of the Ca\textsuperscript{2+}-free form of yeast frequentin did not reveal an unambiguous structure. The structures of the Ca\textsuperscript{2+}-bound forms of NCS-1/frequentin are very similar to those of Ca\textsuperscript{2+}-bound recoverin (17) and neurocalcin δ (37) and do not provide any immediate clues about the basis for the lack of a Ca\textsuperscript{2+}/myristoyl switch in NCS-1.

Using hippocalcin and NCS-1 as green fluorescent protein variant-tagged proteins we have characterized their behavior in living cells (23, 31). These two proteins have 60% identity and associate with the same intracellular membranes, but only hippocalcin does so in a Ca\textsuperscript{2+}-dependent manner. The mem-

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1 The abbreviations used are: NCS, neuronal calcium sensor; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; GCAP, guanylyl cyclase-activating protein; KChIP, potassium channel-interacting protein; VILIP, visinin-like protein.
brane association of hippocalcin and NCS-1 both in cells (23) and in vitro (27) is dependent on their myristoylation, and the myristoylated N terminus alone is sufficient for membrane association and targeting (21). We have now set out to determine which residues within their sequences determine whether or not the Ca\(^{2+}\)/myristoyl switch can operate in living cells. These studies identify structural features in the N-terminal domain of NCS-1 that prevent sequestration of the myristoyl group in the Ca\(^{2+}\)-free form of the proteins. The asterisks indicate positions selected for subsequent mutagenesis. The GenBank\textsuperscript{TM}/EBI accession numbers for the sequences are as follows: NCS-1, AF186409; S. cerevisiae frequenin, NM 002143; and recoverin proteins are compared with other proteins. Residues outlined in yellow are conserved in human and yeast NCS-1/frequenin but are different in all other proteins. Residues outlined in red are conserved in proteins with the Ca\(^{2+}\)/myristoyl switch but differ in NCS-1/frequenin. The filled circles underneath the alignment indicate residues in recoverin that contact the myristoyl group in the Ca\(^{2+}\)-free form of the proteins. The asterisks indicate positions selected for subsequent mutagenesis. The GenBank\textsuperscript{TM}/EBI accession numbers for the sequences are as follows: NCS-1, AF186409; S. cerevisiae frequenin, NM 002143; and recoverin, NM 002143; and recoverin.

FIG. 1. Alignments of sequences of human NCS-1, yeast frequenin, and members of the NCS protein family. The human NCS-1 and S. cerevisiae frequenin proteins are compared with other members of the NCS protein family that possess the Ca\(^{2+}\)/myristoyl switch, and the human sequences are shown. Residues outlined in blue are identical in all of the proteins. Residues outlined in yellow are conserved in human and yeast NCS-1/frequenin but are different in all other proteins. Residues outlined in red are conserved in proteins with the Ca\(^{2+}\)/myristoyl switch but differ in NCS-1/frequenin. The filled circles underneath the alignment indicate residues in recoverin that contact the myristoyl group in the Ca\(^{2+}\)-free form of the proteins. The asterisks indicate positions selected for subsequent mutagenesis. The GenBank\textsuperscript{TM}/EBI accession numbers for the sequences are as follows: NCS-1, AF186409; S. cerevisiae frequenin, NM 002143; and recoverin, NM 002143; and recoverin.

Materials and Methods

Plasmids—The hippocalcin-EYFP fusion construct (pHippo-EYFP) and the NCS-1-ECFP fusion construct (pNCS-1-ECFP) were as described previously (23) and were used as the basis for all other constructs. Specific mutations described under "Results" were made using the QuikChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands).

The hippocalcin-NCS-1 chimeric fusion constructs pHippo(1,2)-NCS-1(3,4)-ECFP and pHippo(1)-NCS-1(2,3,4)-EYFP were made in two stages. The first stage used site-directed mutagenesis to introduce a SpeI site at 273 bp after the start of translation of both NCS-1, in pHippo SpeI-EYFP to create pHippo(1,2)NCS-1(3,4)-ECFP and the NCS-1-ECFP fusion construct (pNCS-1-ECFP) were as described previously (23) and were used as the basis for all other constructs. Specific mutations described under "Results" were made using the QuikChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands).
were plated onto glass coverslips in a 24-well plate at 40,000 cells/well or in a 6-well plate at 200,000 cells/well as required. The cells were allowed 4–24 h to adhere before they were transfected. The transfection reaction mixture contained 93 µl of Dulbecco’s modified Eagle’s medium (Invitrogen), 3 µl of FuGENETM (Roche Applied Science), and 4 µl of plasmid DNA (250 µg/µl). This was incubated at room temperature for 30 min before being added dropwise to HeLa cells in a 24-well plate. The cells were maintained for 16–96 h before being used in experiments.

Confocal Laser Scanning Microscopy of Live and Fixed Cells—For confocal laser scanning microscopy, live or fixed (23) transfected HeLa cells were examined with a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany) using a 22-µm pinhole and a ×63 water immersion objective with a 1.2 numerical aperture. For optimal imaging of the spatial distribution of EFPP constructs, the cells were excited at 514 nm, and light was collected at 525–575 nm. For ECFP, the cells were excited at 430 nm, and light was collected at 450–500 nm. The excitation laser was set at 10% power. For imaging the effects of ionomycin treatment, transfected HeLa cells were washed three times in Krebs-Ringer buffer (145 mM NaCl, 5 mM KCl, 1.3 mM MgCl2, 1.2 mM NaH2PO4, 10 mM glucose, 20 mM HEPES, pH 7.4). The cells were then mounted in a bath containing 450 µl of Krebs-Ringer buffer with 3 mM CaCl2 and 30 µM ionomycin were added to the bath giving a final ionomycin concentration in the bath of 3 µM. Images were captured at 1.86 s/frame, exported as Tiff files, and compiled in CorelDraw.

**Sequence and Structure Analysis—**Sequences of NCS protein family members from GenBank™ were aligned using the ClustalW alignment procedure within BioEdit version 5.0.9 (www.mbio.ncsu.edu/BioEdit/bioedit.html) (38). The structure of human NCS-1 derived from the procedure within BioEdit version 5.0.9 (www.mbio.ncsu.edu/BioEdit/bioedit.html) (38). The structure of human NCS-1 derived from the Protein Data Bank file was rendered inViewerLite™ 4.2 (Accelrys Inc.).

**RESULTS**

The existence of the Ca2+/myristoyl switch in hippocalbin but not in NCS-1 could be due either to the presence of structural features in NCS-1 that inhibit sequestration of the myristoyl group in the Ca2+-free form or features that appeared in later NCS proteins that are needed for myristoyl group sequestration. Additional features could also be important to drive the Ca2+-dependent conformational change, due to Ca2+ binding to EF-hands 2–4, through to the N terminus. To begin to define the requirements for the existence of a Ca2+/myristoyl switch, we examined sequence alignments of mammalian NCS-1, yeast frequenin, and six of the human NCS proteins that have been shown to have a Ca2+/myristoyl switch, VILIP1–3, neurocalcin δ, hippocalbin, and recoverin. Amino acids were highlighted that are conserved in yeast and human NCS-1 but not in the other NCS proteins (shown in yellow in Fig. 1) or alternatively that are conserved in the NCS proteins with a Ca2+/myristoyl switch but not in NCS-1 or yeast frequenin (shown in red in Fig. 1). Residues that are conserved in all of these NCS proteins are outlined in blue. Fig. 1 also indicates the position of the hydrophobic residues that are required to contact the myristoyl group as shown from the structure of Ca2+-free myristoylated recoverin (18). These 14 residues are either fully conserved or replaced with other hydrophobic residues in all of the proteins including NCS-1, and so the presence or absence of these cannot contribute to the lack of the Ca2+/myristoyl switch in NCS-1. As all of the proteins must have evolved from an NCS-1-like protein it was hypothesized that all NCS proteins that have the switch must share the residues that are required for it to operate and that these would be different in NCS-1. Alternatively, residues conserved in only NCS-1 and yeast frequenin might be ones that prevent the operation of the switch. These two possibilities were tested by mutagenesis of hippocalbin-EYFP and NCS-1-ECFP and examination of the effects of these mutations on their localization and Ca2+-dependent translocation to membranes in transfected HeLa cells, aspects of their function that are dependent on their myristoyl groups.

Likely significant residues as targets for mutagenesis were selected from those identified in Fig. 1 by examining the conserved residues for significant changes in charge, hydrophobicity, or size (residues marked by asterisks). Interestingly, all of the residues selected in this way were found in the crystal structure of NCS-1 to lie across the boundary between the N- and C-terminal parts of the molecule (Fig. 2) where considerable conformational change occurs in recoverin on Ca2+ binding. Differences between NCS-1/frequenin and other NCS proteins at the extreme C terminus (Fig. 1) seemed unlikely to be significant as this region is not involved in conformational changes that underlie the Ca2+/myristoyl switch in recoverin (17). The first set of mutations were introduced into hippocal-
cin-EYFP to see whether the corresponding NCS-1 residues could inhibit the Ca\(^{2+}\)/myristoyl switch. Two pairs of selected mutations (A53K plus N54Q and R97T plus Q99E) were introduced both individually and in combination. Wild-type hippocalcin-EYFP expressed in HeLa cells is cytosolic at resting Ca\(^{2+}\) concentration and translocates to membrane structures identified as the trans-Golgi network and the plasma membrane when [Ca\(^{2+}\)] is elevated by ionomycin treatment (Fig. 3, A and B). This requires myristoylation as mutation of the glycine at position 2 to alanine to prevent myristoylation abolishes the translocation (Fig. 3, C and D). The three constructs bearing the mutations separately or together behaved the same as the wild-type protein (Table I and Fig. 3, E and F) indicating that these NCS-1 residues could not prevent the switch mechanism from operating.

The second approach used was to examine whether residues conserved in the NCS proteins that have the switch would allow a switch to operate when introduced into NCS-1-ECFP. In transfected HeLa cells, NCS-1-ECFP is already fully membrane-associated at resting [Ca\(^{2+}\)] (Fig. 3G) or even when [Ca\(^{2+}\)] is depleted (23). Again this requires myristoylation as the G2A mutant of NCS-1 shows a cytoplasmic localization at both resting and elevation Ca\(^{2+}\) levels (Fig. 3, I and J). A series of eight residues selected from the alignments in Fig. 1 based on the properties of the amino acids were mutated alone or in combination into NCS-1-ECFP (Table I). The localization of the constructs in HeLa cells was examined to see whether the mutations affected the membrane association of NCS-1 due to changes in the exposure of the myristoyl group. Even when all eight mutations were introduced the behavior of NCS-1-ECFP was unaffected, and the ECFP fusion proteins remained on the trans-Golgi network and the plasma membrane in resting conditions (Fig. 3H). This shows that mutation of these residues to those conserved in NCS proteins with the Ca\(^{2+}\)/myristoyl switch did not allow sequestration of the myristoyl group by NCS-1 in the Ca\(^{2+}\)-free state.

Despite using a logical approach through analysis of conserved and divergent residues no evidence was obtained that any of the residues highlighted in this way had any role in determining the existence or otherwise of the Ca\(^{2+}\)/myristoyl switch. An alternative strategy was used, therefore, based on swapping large domains between hippocalcin and NCS-1 (Fig. 4). The proteins were divided by introduction of silent restriction endonuclease sites in their fusion constructs. The fusion constructs were then restriction-digested, and the domains

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**Table I**

| Construct | Localization at resting [Ca\(^{2+}\)] | Localization at elevated [Ca\(^{2+}\)] |
|-----------|---------------------------------------|--------------------------------------|
| Wild-type hippocalcin-EYFP | Cytosolic | Membrane |
| Hippo(A53K,N54Q)-EYFP | Cytosolic | Membrane |
| Hippo(R97T,Q99E)-EYFP | Cytosolic | Membrane |
| Wild-type NCS-1-ECFP | Membrane | Membrane |
| NCS-1(F67H,N70R)-ECFP | Membrane | Membrane |
| NCS-1(R79T,E81D)-ECFP | Membrane | Membrane |
| NCS-1(E99Q)-ECFP | Membrane | Membrane |
| NCS-1(N112G)-ECFP | Membrane | Membrane |
| NCS-1(A182L,S184Q)-ECFP | Membrane | Membrane |
| NCS-1(F67H,N70R,E99Q)-ECFP | Membrane | Membrane |
| NCS-1(F67H,N70R,E99Q,A182L,S184Q)-ECFP | Membrane | Membrane |
| Hippo(1,2)NCS-1(3,4)-ECFP | Cytosolic | Cytosolic |
| Hippo(1)NCS-1(2,3,4)-EYFP | Cytosolic | Cytosolic |
| NCS-1(1,2)Hippo(2,3,4)-ECFP | Membrane | Membrane |
| NCS-1(1)Hippo(2,3,4)-ECFP | Membrane | Membrane |
| Hippo(1,2)NCS-1(3,4)-ECFP | Membrane | Membrane |
| Hippo(1,2)NCS-1(3,4)-EYFP | Membrane | Membrane |
| NCS-1(1)Hippo(2,3,4)-ECFP | Membrane | Membrane |
| NCS-1(1)Hippo(2,3,4)(Q41L,D43N,A44V)-ECFP | Membrane | Membrane |
| NCS-1(1)Hippo(2,3,4)(Q41L,D43N,A44V)-ECFP | Membrane | Membrane |

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**Fig. 4.** Localization and behavior of NCS-1-hippocalcin chimeric constructs in transfected HeLa cells. Images show the localization of the chimeric constructs in control (A, C, E, and G) and in ionomycin-stimulated (B, D, F, and H) cells as indicated. The description of the constructs is based on which EF-hands are included. The scale bar represents 10 \(\mu\)m.
were swapped around to create tagged chimeric hippocalcin-NCS-1 constructs. These were expressed in HeLa cells to see which domains were required for cytosolic distribution at resting \([\text{Ca}^{2+}]\) and for \([\text{Ca}^{2+}]\)-dependent translocation (Fig. 4). Only wild-type hippocalcin could undergo \([\text{Ca}^{2+}]\)-dependent translocation, but the chimeric proteins had two distinct localizations. The proteins with the N terminus and EF-hand 1 of hippocalcin were all cytosolic at resting \([\text{Ca}^{2+}]\) (Fig. 4, A and C), whereas those with the N terminus of NCS-1 were localized to membranes in unstimulated cells (Fig. 4, E and G). The construct containing half of each parent protein showed a modification of its localization with it being entirely plasma membrane-associated (Fig. 4E). Constructs that possessed the N terminus and EF-hand 1 of hippocalcin and either two or three of the C-terminal EF-hands of NCS-1 were unable to translocate from the cytosol to membranes in response to \([\text{Ca}^{2+}]\) elevation (Fig. 4, B and D). These findings suggest that two aspects contribute to the \([\text{Ca}^{2+}]\)/myristoyl switch. First, differences in the N terminus and EF-hand 1 domain of hippocalcin and NCS-1 determine the ability of the proteins to sequester the myristoyl group in the \([\text{Ca}^{2+}]\)-free state and thereby determine whether the protein is cytosolic or membrane-bound at

Fig. 5. Effect of mutation in the EELTRK motif on localization and behavior of hippocalcin and NCS-1. An alignment of the sequences of the N-terminal domains of NCS-1 and hippocalcin is shown at the top of the figure with identical residues outlined in blue and the two most divergent regions indicated as 1 and 2. HeLa cells were transfected, and the constructs were visualized in control conditions and after \([\text{Ca}^{2+}]\) elevation with ionomycin treatment. A and B, hippocalcin-EYFP bearing the mutation QDLREN to EELTRK, C and D, hippocalcin-EYFP with the mutations QDLREN to EELTRK and G2A, E and F, NCS-1-ECFP bearing the mutation TRK to REN, G and H, NCS-1-hippocalcin chimera (NCS-1(1)Hippo-(2,3,4)-ECFP) bearing the mutation TRK to REN. I and J, the NCS-1-hippocalcin chimera also bearing the G2A mutation. The scale bar represents 10 \(\mu\text{m}\).
resting [Ca^{2+}]. Second, other residues in the C-terminal part of hippocalcin must be required to allow transmission of the conformational change on Ca^{2+} binding to allow the myristoyl group to be ejected and for the switch to operate. The second aspect may be determined by residues spread throughout the C-terminal half of the proteins, but candidate residues in this region were not apparent from our mutagenic analysis of conserved and divergent residues. We therefore concentrated on determining which residues within the N-terminal domain contribute to the locking of the myristoyl group in an open conformation in NCS-1.

Comparison of the N-terminal domains that were swapped in the chimeric proteins indicated that the residues differ in 24 positions (Fig. 5). Two areas within the N terminus covering residues 12–19 and 44–49 differed substantially between NCS-1 and hippocalcin (Fig. 5). Of particular interest appeared to be the two acid (Glu-14 and Glu-15) and two basic (Arg-18 and Lys-19) residues (in the EELTRK motif of region 1) situated close to each other. Examination of the crystal structure of NCS-1 indicated that these residues in helix A, which joins the myristoyl tail to EF-hand 1, would allow significant structural interactions in NCS-1 that would not occur in hippocalcin (Fig. 6). For this reason the residues in this motif were chosen for mutagenesis. A hippocalcin-EYFP construct was made with mutations (hippocalcin (Q14E, D15E, R17T, E18R, and N19K)) to convert QDLRE to EELTRK. When expressed in HeLa cells this construct differed from wild-type hippocalcin as it was membrane-associated (on the trans-Golgi network and plasma membrane) even at resting Ca^{2+} concentration, and its localization was not affected by Ca^{2+} elevation (Fig. 5, A and B). This localization was dependent on its myristoylation as the introduction of the G2A mutation in this construct resulted in the protein remaining cytosolic (Fig. 5). This did not, however, reconstruct the Ca^{2+}-myristoyl switch in this protein (Fig. 5). This allowed it to translocate from the cytosol to membranes in response to Ca^{2+} elevation (Fig. 5, G and H). The translocation to membranes following Ca^{2+} elevation was dependent on its myristoylation as the introduction of the G2A mutation in this construct resulted in the protein remaining cytosolic (Fig. 5, I and J). Mutation of residues in divergent region 2 of the N terminus (QLDA to ILNV) in the same chimera had no effect (Table I, bottom line). This confirms that the presence of the TRK residues is sufficient to lock open the myristoyl group in an open conformation and that additional features within the C terminus of hippocalcin allow the Ca^{2+}-myristoyl switch to operate.

**DISCUSSION**

The extensive investigation of the NCS protein recoverin (17, 18) lead to the concept of the Ca^{2+}/myristoyl switch for reversible exposure of the N-terminal myristoyl group following Ca^{2+} binding and subsequent membrane attachment. Originally it was assumed that this mechanism would also operate in all other NCS proteins as they all possess the hydrophobic pocket required for myristoyl group sequestration. Some other NCS proteins do indeed show the Ca^{2+}/myristoyl switch in vitro and in living cells, but others do not, the best characterized being NCS-1. We have now established that the lack of the switch in NCS-1 is determined in part by key residues within its N terminus that must lock the myristoyl group in an open conformation.

The experimental approach we have used to examine the Ca^{2+}/myristoyl switch and thereby myristoyl group exposure has been to examine the localization in resting and high Ca^{2+} conditions of NCS-1-ECFP and hippocalcin-EYFP constructs in living cells. The evidence available indicates that this is a good indicator of myristoyl-dependent membrane interactions. Previous work on the NCS protein S-modulin suggested that its membrane binding in vitro was dependent on a series of C-terminal basic residues (39). This does not apply to NCS-1 or hippocalcin as they do not possess this charged domain. In any case, the cellular role of the basic residues in S-modulin is unclear as membrane binding via this mechanism is inhibited at physiological ionic strength (39). This highlights the need to examine membrane association in intact living cells. Furthermore, membrane binding by hippocalcin in vitro (27) and in vivo (23) has an essential requirement for its myristoylation, and short N-terminal myristoylation motifs (11–14 residues) of the NCS proteins are sufficient not only for membrane binding but also for specific targeting to distinct organelles (31). In addition, we have provided data showing that mutation of glycine at position 2 to alanine, which will prevent myristoylation of the expressed proteins, results in both NCS-1 and hippocalcin being cytosolic at resting and elevated Ca^{2+} concentrations. The essential requirements for myristoylation in membrane association were also confirmed by analysis of G2A mutations in key mutant constructs.

NCS-1 appeared first during evolution, and an orthologue is present in yeast (3). At some stage new members of the family evolved from an ancestral form that allowed expression of the Ca^{2+}/myristoyl switch mechanism. We suggest that this would require changes in amino acids within the N-terminal domain to prevent the locking of this domain in an open conformation with the myristoyl group exposed in both the Ca^{2+}-bound and Ca^{2+}-free forms. In addition, our data suggest that other structural changes would be required to allow the conformational change following Ca^{2+} binding in EF-hands 2–4 to be transmitted to the N terminus of the protein to allow the myristoyl group to flip out. The latter suggestion stems from the inability of the chimeric constructs with the hippocalcin N terminus and

**FIG. 6.** The structure of NCS-1 indicating the residues within the EELTRK motif. Left, ribbon representation of the crystal structure of human NCS-1 is shown with residues Glu-14, Glu-15, Arg-18, and Lys-19 shown as a ball-and-stick representation in the boxed region. Right, expansion of the boxed region (left) showing hydrogen-bonding interactions between the indicated residues.
C-terminal domains of NCS-1 to show the Ca\(^{2+}\)/myristoyl switch. Even though these proteins were cytosolic at resting [Ca\(^{2+}\)] they could not translocate to membranes when Ca\(^{2+}\) was elevated. This was not due to some structural problem derived from the spacing together of domains from the two proteins; the NCS-1/Hippo(2,3,4)-ECFP chimera became cytosolic and the Ca\(^{2+}\)/myristoyl switch was reconstituted when the key TRK residues were mutated in the N terminus.

Our logical approach to studying the amino acids that would determine the existence of the Ca\(^{2+}\)/myristoyl switch by analysis of conserved and divergent residues in human NCS-1, *Saccharomyces cerevisiae* frequenin, and other NCS proteins followed by mutagenesis failed to identify key amino acids based on their mutagenesis. This type of approach has been advocated previously for the identification of function-specific residues (40, 41) but also failed to identify key residues in effector interactions in a recent study of small GTP-binding proteins (42). Examination of conserved and differing residues in human NCS-1 and *S. cerevisiae* frequenin may have been misleading; both are likely to have diverged from the putative ancestral form of the protein, and it is unclear what would be the exact nature of the ancestral protein of the NCS family. Indeed, around the key motif that was identified (EELTRK) in the N terminus of NCS-1 to show the Ca\(^{2+}\)/myristoyl switch, GCAP2 has been established not to determine the Ca\(^{2+}\)/myristoyl switch. GCAP2 has been established not to allow the Ca\(^{2+}\)–myristoyl switch. Interestingly, a potentially similar mechanism to that we postulate for NCS-1 could be envisaged in GCAP2 through the interaction of Glu-26 and -33 and Lys-29 and -30 within its N-terminal helix. It seems likely, therefore, that changes in the N terminus are among those that occurred during the evolution of the NCS protein family to allow the Ca\(^{2+}\)/myristoyl switch to operate. These findings help explain, therefore, why NCS-1 does not demonstrate the Ca\(^{2+}\)/myristoyl switch. The yeast orthologue is also unlikely to possess the Ca\(^{2+}\)/myristoyl switch, but the residues in positions 12–19 differ from those in NCS-1. It is possible that as NCS proteins with the Ca\(^{2+}\)/myristoyl switch appeared in higher organisms the sequence of NCS-1 became inevitable to maintain its distinct properties, being membrane-associated at resting [Ca\(^{2+}\)], that contribute to its function as a Ca\(^{2+}\) sensor.

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