Differential Use of Myristoyl Groups on Neuronal Calcium Sensor Proteins as a Determinant of Spatio-temporal Aspects of Ca\(^{2+}\) Signal Transduction*

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Dermott W. O’Callaghan‡, Lenka Ivings‡, Jamie L. Weiss, Michael C. Ashby‡, Alexei V. Tepikin, and Robert D. Burgoyne§

From the Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, United Kingdom

The localizations of three members of the neuronal calcium sensor (NCS) family were studied in HeLa cells. Using hippocalcin-EYFP and NCS-1-ECFP, it was found that their localization differed dramatically in resting cells. NCS-1 had a distinct predominantly perinuclear localization (similar to trans-Golgi markers), whereas hippocalcin was present diffusely throughout the cell. Upon the elevation of intracellular Ca\(^{2+}\), hippocalcin rapidly translocated to the same perinuclear compartment as NCS-1. Another member of the family, the neuronal calcium δ, also translocated to this region after a rise in Ca\(^{2+}\) concentration. Permeabilization of transfected cells using digitonin caused loss of hippocalcin and neuronal calcium δ in the absence of calcium, but in the presence of 10 μM Ca\(^{2+}\), both proteins translocated to and were retained in the perinuclear region. NCS-1 localization was unchanged in permeabilized cells regardless of calcium concentration. The localization of NCS-1 was unaffected by mutations in all functional EF hands, indicating that its localization was independent of Ca\(^{2+}\). A minimal myristoylation motif (hippocalcin-(1–14)) fused to EGFP resulted in similar perinuclear targeting, showing that localization of these proteins is because of the exposure of the myristoyl group. This was confirmed by mutation of the myristoyl motif of NCS-1 and hippocalcin that resulted in both proteins remaining cytosolic, even at elevated Ca\(^{2+}\) concentration. Dual imaging of hippocalycin-EYFP and cytosolic Ca\(^{2+}\) concentration in Fura Red-loaded cells demonstrated the kinetics of the Ca\(^{2+}\)/myristoyl switch in living cells and showed that hippocalycin rapidly translocated with a half-time of ~12 s after a short lag period when Ca\(^{2+}\) was elevated. These results demonstrate that closely related Ca\(^{2+}\) sensor proteins use their myristoyl groups in distinct ways in vivo in a manner that will determine the time course of Ca\(^{2+}\) signal transduction.

Calcium is a widely used intracellular signal that regulates many different cellular processes in a very specific manner. An aspect that has received considerable attention in recent years is the varied use of Ca\(^{2+}\) signal mechanisms that determine spatio-temporal aspects of the changes in Ca\(^{2+}\) concentration (1). The existence of highly localized changes in Ca\(^{2+}\) concentration in addition to global Ca\(^{2+}\) changes has been increasingly highlighted, and these local Ca\(^{2+}\) signals are likely to contribute to the specificity of Ca\(^{2+}\) actions (2). The specificity of Ca\(^{2+}\) signaling is also the result, in part, of the existence of many different Ca\(^{2+}\)-binding proteins, which act as Ca\(^{2+}\) sensors in the transduction of Ca\(^{2+}\) signals. Localization of Ca\(^{2+}\) sensors could be a significant factor, and the effect of localization of Ca\(^{2+}\) sensors on signal transduction has been examined for two such proteins. Calmodulin responds to global Ca\(^{2+}\) changes by binding Ca\(^{2+}\) and then subsequently to target proteins; it can also translocate into the nucleus (3–5). Protein kinase C isoforms also show specific patterns of translocation to the plasma membrane (6). Analysis of the factors that determine the localization of other Ca\(^{2+}\) sensors is likely to be crucial for further understanding of Ca\(^{2+}\) signal transduction, but has so far been little studied.

The neuronal calcium sensor (NCS)1 proteins are a family of high affinity Ca\(^{2+}\)-binding proteins that can sense Ca\(^{2+}\) elevations above resting Ca\(^{2+}\) concentration in the range of 0.1–1.0 μM (7). The NCS family includes proteins expressed only in retinal photoreceptors (recoverin (Ref. 8) and the GCAPs (Ref. 9)) or in neurons, in some cases most highly in particular cell types (hippocalcin (Ref. 10), neurocalcin/VILIPs (Ref. 11)) or with more widespread expression (NCS-L/frequenin (Refs. 12 and 13)). All the NCS family members share four characteristic EF hands and a myristoylated N terminus. In the NCS proteins, three (or only two in some family members) of the four EF hands are capable of binding calcium and the EF hand nearest the N terminus (EF1) is altered so that it cannot bind Ca\(^{2+}\) (7). The myristoyl tail is a fatty acid post-translationally added to the NCS protein. Its function has been extensively studied in recoverin (14). In recoverin, the myristoyl group is buried in a nonpolar pocket formed by EF1 and the surrounding region in the Ca\(^{2+}\)-free state (15). When recoverin binds calcium, there is a large conformational change that ejects the myristoyl group from its pocket (16). This exposed hydrophobic tail is then free to interact with the nonpolar cell membranes or hydrophobic protein domains. This exposure of the myristoyl tail on calcium binding is referred to as a “calcium/myristoyl switch” and considered to be primarily a mechanism by which the protein can translocate from the cytosol to intracellular membranes (14, 16). The structural features required for the Ca\(^{2+}\)/myristoyl switch are apparently conserved in all NCS proteins. The presence of this Ca\(^{2+}\)-mediated membrane-association process was, therefore, suggested for the other members

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§ To whom correspondence should be addressed. Tel.: 44-151-794-5305; Fax: 44-151-794-5337; E-mail: burgoyne@liverpool.ac.uk.

1 The abbreviations used are: NCS, neuronal calcium sensor; GCAP, guanylyl cyclase-activating protein; VILIP, visinin-like protein; PI, phosphatidylinositol; GFP, green fluorescent protein; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline.
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of the neuronal calcium sensor protein family (16). There are biochemical data from disrupted cells for some of the family members in support of this mechanism (17, 18). A clear understanding of the localization and translocation of these proteins in response to calcium will allow the generation of models of how these proteins modulate and integrate calcium signals. There has, however, been little in vivo work to demonstrate the Ca\(^{2+}\)/myristoyl switch during elevation of Ca\(^{2+}\) concentration within live cells, and so kinetic aspects of the proposed membrane translocation are unknown.

In this study we have examined the potential Ca\(^{2+}\)/myristoyl switch in three NCS proteins. Hippocalcin is expressed most highly in hippocampal neurons (19) and the closely related neurocalcin \(\delta\) (VILIP-3) in cerebellar Purkinje cells (20, 21). Biochemical studies with these proteins suggest Ca\(^{2+}\)-dependent membrane interactions (17, 18), but this has not been examined within intact cells. The functions of these two proteins are unknown. In contrast, NCS-1 has been shown to play examined within intact cells. The functions of these two proteins to compare their Ca\(^{2+}\)/myristoyl switch mechanisms. We demonstrate different uses of N-terminal myristoylation, which would generate distinct spatio-temporal Ca\(^{2+}\) sensing by members of the NCS family and have assessed the kinetics of the Ca\(^{2+}\)/myristoyl switch-dependent membrane translocation in living cells.

MATERIALS AND METHODS

Plasmids—The EYFP-tagged hippocalcin fusion construct (pHippo-EYFP) was made by the in-frame insertion of a hippocalcin sequence, amplified from rat brain cDNA, into the pEYFP-N1 vector (CLONTECH). The primers contained endonuclease sites (underlined) to facilitate this cloning. The primers were based on the rat nucleotide sequence (GenBank accession no. NM017122). The sense primer was designed to the 5'-untranslated region from –165 to –135 (5'-GGCGGG- GCTAGCTCTTTTTGGGTCAAATGAG-3'; Nhel) and the antisense primer with a mutated stop codon to the region +735 to +765 (5'- CCTCTACAATCGAGAGCAGCTGCTAGG-3'; XhoI). The amplified sequence digested with the endonucleases Nhel/XhoI was cloned into the vector pEYFP-N1 digested with Nhel and XhoI using standard methods. The ECFP-tagged NCS-1 fusion construct (pNCS-1-ECFP) was made by the in-frame insertion of a NCS-1 sequence, amplified from the pNCS-1 plasmid (23), into the pECFP-N1 vector (CLONTECH). The primers contained endonuclease sites (underlined) that were used to generate the NCS-1 EF2 mutant construct, pNCS-1 EF2 (sense) and 5'-GACGCTAGGAACTGGCTAGCCTAGAAGG-3' (antisense).

The neurocalcin \(\delta\) plasmid (pNeurocalcin \(\delta\)) was made by the insertion of the bovine neurocalcin \(\delta\) sequence, amplified from the pGEX-\(\delta\) (kindly provided by Dr. Michael Ladant, Laboratoire de Physiologie des Neurotransmetteurs, Paris, France), into the pcDNA 3.1(-) vector (Invitrogen, Groningen, The Netherlands). The primers contained endonuclease sites (underlined) to facilitate this cloning. The sense primer was 5'-CCAGGATCCATT GGGCAAGCAAGCAGCAA-3' (BamHI), and the antisense primer was 5'-CCGAGATCTTACGGAGATCTGACAGCT-3' (HindIII). The HindIII/EcoRI- digested construct, pNCS-1-ECFP was made by using pNCS-1 as a template and the QuikChange™ site-directed mutagenesis kit (Stratagene Europe). Three pairs of primers were used sequentially, one pair for each EF hand. The primers used for EF2 were 5'-CCGAGATCTTACGGAGATCTGACAGCT-3' and 5'-GAGCTCGTGAATGTTGGAGAGAAGTCGATCTGC-3' (antisense). The primer pair used for EF3 were 5'-CCAGGATCCATT GGGCAAGCAAGCAGCAA-3' (BamHI), and 5'-CGGACGCTAGGAACTGGCTAGCCTAGAAGG-3' (antisense). The primer pair used for EF4 were 5'-AATCTTTTCAGGAGTTCCAGAGGAGAAGTCGATCTGC-3' and 5'-CGGACGCTAGGAACTGGCTAGCCTAGAAGG-3' (antisense).

The sequence of all these constructs were confirmed by automated sequencing by Osvel (Southampton, United Kingdom (UK)).

Culture and Transfection of HeLa Cells—HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing 5% fetal calf serum (Invitrogen) and 1% nonessential amino acids (Invitrogen), incubated at 37 °C in an atmosphere of 5% CO\(_2\) and plated onto glass coverslips in a 24-well plate at 40,000 cells/well. 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 PuGENSE™ (Roche), 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 8–96 h before being used in experiments.

Immunofluorescence Staining—Anti-NCS-1 was prepared in rabbits as described previously (22). Anti-neurocalcin \(\delta\) was prepared as a rabbit antiserum using a similar protocol in which rabbits were immunized with purified recombinant neurocalcin \(\delta\) expressed in Escherichia coli and affinity-purified on immobilized recombinant neurocalcin \(\delta\). Anti-γ-adaptin was from Sigma (Poole, UK), anti-GFP from CLONTECH (Basingstoke, UK), and anti-transferrin receptor from Roche (East Sussex, UK). Cells attached to coverglasses were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 2 mM NaH\(_2\)PO\(_4\)) and fixed in PBS containing 4% formaldehyde for 30 min. The cells were washed twice in PBS and incubated in blocking buffer (0.3% bovine serum albumin, 0.25% Triton X-100 in PBS) for 1 h. The blocking buffer was removed and the primary antibody was added at the appropriate dilution (anti-γ-adaptin 1 in 100, anti-GFP 1 in 400, anti-transferrin receptor 1 in 400, anti-NCS-1 1 in 1,000, anti-neurocalcin \(\delta\) 1 in 1,000). The primary antibody was incubated for 1–2 h and removed, and the cells were washed three times in blocking buffer. The cells were then incubated for an additional 1 h with the appropriate biotinylated secondary antibody diluted to 1 in 100, (anti-rabbit for anti-NCS-1 and anti-neurocalcin \(\delta\) primary antibodies and anti-mouse for the other primary antibodies; Amersham Biosciences, Buckinghamshire, UK). The cells were washed three times and then incubated in streptavidin-Texas Red (Amersham Biosciences) diluted 1 in 50 for 30 min. The cells were washed three times, and the coverslips were dried and mounted on antibody glycerol (glycerol/PBS 565; 0.25% diazabicyclo(2-2-2)octane, 0.002% p-phenyldiamine). The sections were examined on a Zeiss Universal microscope and fluorescence imaged using the following standard Zeiss filter sets: ECFP and EYFP (BG39 LP480, PT460, LP540; ECFP, BP410–490, PT510, BP515–585, Texas Red, BP546/12, FT580, LP590).

Polyacrylamide Gel Electrophoresis and Western Blotting—The cells of each well in a 24-well plate were lysed using 100 μl of Laemmli buffer (Sigma, Dorset, UK)/well and boiled for 10 min. The samples were loaded onto and separated using a 15% SDS-polyacrylamide gel and then blotted onto nitrocellulose by transfer for 1 h at 100 V in Tris-glycine/PBS 9:1, 0.25% dextran powder, 0.002% p-phenyldiamine). The sections were examined on a Zeiss Universal microscope and fluorescence imaged using the following standard Zeiss filter sets: ECFP and EYFP (BG39 LP480, PT460, LP540; ECFP, BP410–490, PT510, BP515–585, Texas Red, BP546/12, FT580, LP590).
absence of 3 mM CaCl2 at 37 °C. For optimal imaging of the
objective with a 1.2 numerical aperture. For optimal imaging of the
Germany) using a 166.27-

FIG. 1. Demonstration of fusion protein expression in trans-
fected HeLa cells. Western blots used an anti-GFP antibody (A and
C), an anti-NCS-1 antibody (B), or an anti-neurocalcin δ antibody with
cross-reactivity for hippocalcin (D). The samples were lysates from cells
transfected with pNCS-1-ECFP, pHippo-EYFP, pECFP-N1, and
pEYFP-N1. A polypeptide was detected with both the anti-GFP and
anti-NCS-1 antibodies at the expected size (60 kDa) in cells transfected
with pNCS-1-ECFP. A polypeptide was also detected with both the
anti-GFP and anti-neurocalcin δ antibodies at the expected size (60
kDa) in cells transfected with pHippo-CFP. This demonstrated that
both fusion proteins were being expressed correctly. For both the con-
trols, pEYFP-N1 and pECFP-N1, the only band detected with any of the
antibodies was a 27-kDa band, corresponding to EYFP or ECFP, using
the anti-GFP antibody.

bated for 1–2 h and removed, and the membranes were washed three
times. The membranes were then incubated for an additional 1 h with
the appropriate horseradish peroxidase-conjugated secondary antibody
diluted to 1 in 400 with PBT, (anti-rabbit for anti-NCS-1 and anti-
neurocalcin δ primary antibodies and anti-mouse for anti-GFP primary
antibodies; Sigma). The membranes were washed once in PBT, three
times in PBS, and once with distilled water. The signal was detected
using enhanced chemiluminescence (Amersham Biosciences).

Stimulation of Cells with Ionomycin and Imaging of Fixed Cells—
Transfected HeLa cells were transfected three times in Krebs-Ringer buffer
(145 mM NaCl, 5 mM KCl, 1.3 mM MgCl2, 1.2 mM NaHPO4, 10 mM
glucose, 20 mM HEPES, pH 7.4). The cells were then incubated for 10
min in 1 μM ionomycin in Krebs-Ringer buffer in the presence or
absence of 3 mM CaCl2 at 37 °C. The Krebs-Ringer buffer was removed,
and the cells were fixed in 4% formaldehyde for immunofluorescence
staining and imaged using a Zeiss Universal microscope fitted with a
Nikon Coolpix 995 digital camera.

Confocal Laser Scanning Microscopy on Living Cells—For confocal
laser scanning microscopy, live transfected HeLa cells were examined
with a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg,
Germany) using a 166.27-μm pin-hole and a 63× water immersion
objective with a 1.2 numerical aperture. For optimal imaging of the
spatial distribution of hippocalcin-EYFP, the cells were excited at 514
nm and light collected at 545–625 nm and fluorescence images were
collected every 1.86 s with alternate phase-contrast images to check for
changes in cell morphology. For dual imaging of hippocalcin-EYFP and
NCS-1-ECFP, the cells were excited at 514 nm with light collected at
560–600 nm for EYFP or excited at 458 nm for and light collected at
465–500 nm for ECFP detection. In experiments on the kinetics of
Ca2+-dependent translocation, cells were loaded with Fura Red (Molecu-
lar Probes) by incubation in 5 μM Fura Red in growth medium for 30
min. The cells were then excited at 488 nm and light collected at
625–725 nm for Fura Red emission and at 525–590 for hippocalcin-
EYFP emission and images collected every 137 ms. Ionomycin was
added to the bath solution a final concentration of 3 μM.

Permeabilization Experiments—Transfected HeLa cells were washed
three times in Krebs-Ringer buffer and incubated for 15 min in 10 μM
digitonin in 300 μl of permeabilization buffer (139 mM potassium glu-
tamate, 20 mM PIPES, 5 mM EGTA, pH 6.5) in the presence or absence
of 10 μM free calcium at 37 °C. If the cells were to be fixed, the buffer
was removed and 500 μl of 4% formaldehyde was added per well. For
the leakage experiments, the permeabilization buffer was removed and
centrifuged briefly at 12,000 rpm to sediment any detached cells. The
proteins were concentrated by precipitation with cold methanol (30)
and resuspended in 100 μl of Laemmli buffer (Sigma). The cells remaining
in the wells were solubilized in Laemmli buffer. All samples were boiled
for 10 min and then used for polyacrylamide gel electrophoresis and
Western blotting.

RESULTS

Expression and Localization of NCS Proteins in HeLa Cells—To determine the localization of the neuronal calcium
sensing proteins NCS-1 and hippocalcin in living cells, con-
structs were made that fused the C terminus of these proteins
to the fluorescent proteins ECFP and EYFP to produce the
plasmids pNCS-1-ECFP and pHippo-EYFP, respectively. Vec-
tor-derived sequence produced a 10- or 13-amino acid linker between the NCS-1 protein and hippocalcin, respectively, and the fluorescent protein. These constructs were transduced into HeLa cells, which do not express detectable levels of the proteins endogenously, and the expressed proteins observed in living and fixed cells. HeLa cells were chosen as a convenient cell type to allow examination of the basic localization and translocation mechanism of these proteins within a cellular environment. To demonstrate their correct expression, Western blots were carried out on samples from transfected cells. Fig. 1 shows that the expressed fusion proteins were of the expected size (~60 kDa) and recognized by both anti-GFP and specific antibodies. Anti-NCS-1 was used for cells transfected with pNCS-1-ECFP. Expression of Hippo-EYFP could be demonstrated using an antiserum prepared against the closely related protein neurocalcin δ (96% identical (Ref. 7)). The 60-kDa band representing the fusion proteins was not seen in samples from cells transfected with the control vectors pEYFP-N1 and pECFP-N1 but instead a polypeptide band corresponding to the free fluorescent proteins.

Fig. 2 shows the localization of proteins encoded by pNCS-1-ECFP, pHippo-EYFP, and constructs expressing the free fluorescent proteins ECFP and EYFP. The location patterns were not dependent upon expression levels of the proteins and were indistinguishable in cells transfected for between 8 and 96 h and with varying levels of fluorescence intensity at each time point. The free fluorescent proteins were found in the cytoplasm but were particularly concentrated in the nucleus (Fig. 2, A and B). The hippocalcin-EYFP fusion protein was present in the cytoplasm and also in the nucleus but to a lesser extent than ECFP or EYFP (Fig. 2C). The NCS-1 fusion protein, in contrast, had a radically different distribution. The fluorescence of NCS-1-ECFP was at its most intense in a perinuclear region to one side of the nucleus, whereas the nucleus was devoid of fluorescence (Fig. 2D). Additionally, NCS-1 ECFP was also associated with plasma membrane, usually in a patchy distribution. The presence of the ECFP and EYFP tags did not influence the localization of the NCS proteins, as similar localization patterns were seen with wild-type protein detected by immunofluorescence (shown below). In addition, similar localization patterns were seen in living and fixed cells. Previous work has shown that endogenous NCS-1 in COS cells is also localized to a perinuclear compartment that overlaps partially with the trans-Golgi network marker γ-adaptin (28). Fig. 3 (A–C) shows that ECFP-NCS-1 also partially co-localizes with γ-adaptin. In contrast, ECFP-NCS-1 did not significantly co-localize with the transferrin receptor used as a marker of endosomal compartments (Fig. 3, D–F).

Effect of Ca\(^{2+}\) Elevation on Localization of NCS Proteins—The effect of a global elevation in intracellular Ca\(^{2+}\) concentration on the localization of the NCS fusion proteins was assessed by treating cells with 1 μM ionomycin in the presence of external Ca\(^{2+}\). The NCS-1 fusion protein did not change its localization in response to an increase in intracellular Ca\(^{2+}\) levels (Fig. 4, A and B). This suggests that, at both resting and raised levels of Ca\(^{2+}\), NCS-1 has its myristoyl tail exposed allowing membrane association. In contrast, following Ca\(^{2+}\) elevation, the hippocalcin fusion protein translocated from its apparent diffuse cytosolic localization to a predominantly perinuclear pattern (Fig. 4, C and D). This perinuclear localization appeared to be very similar to that seen with the NCS-1 fusion protein at resting Ca\(^{2+}\) levels. A more diffuse localization was also present, spreading out to the cell periphery consistent with an additional patchy plasma membrane localization. The Ca\(^{2+}\)-dependent translocation of hippocalcin-EYFP was rapidly reversed when the external buffer was exchanged for a buffer without added Ca\(^{2+}\) (data not shown). In cells transfected with a construct expressing an untagged version of neurocalcin δ, another member of the NCS family that is closely related to hippocalcin (7), a similar diffuse localization was seen in unstimulated cells using immunofluorescence to detect the protein in fixed cells (Fig. 4E). Translocation of neurocalcin δ to the perinuclear region plasma membrane structures was also observed following an increase in intracellular Ca\(^{2+}\) by ionomycin treatment (Fig. 4F). The apparent translocation of hippocalcin and neurocalcin δ from the cytosol to membranes is consistent with their known role in Ca\(^{2+}\) handling.
The data from intact cells on the effects of raising Ca\(^{2+}\) with ionomycin are consistent with membrane association of NCS-1-ECFP even at low Ca\(^{2+}\) concentration and Ca\(^{2+}\)-dependent translocation of cytosolic hippocalcin-EYFP and neurocalcin \(\delta\) to membranes. To confirm this interpretation, the localization of the protein was examined after digitonin permeabilization to allow soluble proteins to leak out of the cells. Transfected cells were permeabilized with 10 \(\mu\)M digitonin in a buffer containing either 0 or 10 \(\mu\)M free Ca\(^{2+}\). At 10 \(\mu\)M free Ca\(^{2+}\) concentration, the hippocalcin fusion protein translocated to the perinuclear compartment as seen with ionomycin treatment (Fig. 6). Cells transfected with the pNCS-1-ECFP also retained their perinuclear fluorescence after permeabilization in 10 \(\mu\)M Ca\(^{2+}\). These results showed that the perinuclear localization was because of the fusion proteins associating with intracellular structures. When transfected cells were permeabilized in a buffer containing 0 Ca\(^{2+}\), essentially all fluorescence was rapidly lost from pHippo-EYFP-transfected cells except a small amount that remained trapped in the nucleus. The hippocalcin fusion protein was, therefore, essentially completely cytosolic at 0 Ca\(^{2+}\) levels and free to diffuse from the permeabilized cells. Permeabilization of pNCS-1-ECFP-transfected cells in 0 Ca\(^{2+}\) buffer did not affect the fluorescence pattern. The NCS-1 fusion protein remained attached to the perinuclear membrane construct regardless of the presence or absence of Ca\(^{2+}\) (Fig. 6). The data from examination of fluorescence were confirmed by biochemical analysis using Western blotting of cells and medium containing leaked proteins (Fig. 7). The majority of the NCS-1-ECFP was retained in the permeabilized cells irrespective of Ca\(^{2+}\) concentration (Fig. 7). In contrast, the majority of hippocalcin-YFP leaked into the medium at 0 Ca\(^{2+}\) but the protein was fully retained in cells at 10 \(\mu\)M free Ca\(^{2+}\) (Fig. 7). Retention was half-maximal at \(\approx 0.2-0.3 \mu\)M free Ca\(^{2+}\) (data not shown). Similar Ca\(^{2+}\)-dependent translocation to the perinuclear region (Fig. 6, E and F) and Ca\(^{2+}\)-dependent retention in permeabilized cells (Fig. 7) was seen in cells transfected with untagged neurocalcin \(\delta\) with expression detected using anti-neurocalcin \(\delta\) antiserum. The extent of retention of the NCS proteins in the presence of Ca\(^{2+}\) (Fig. 7) suggested that the majority of the expressed proteins were myristoylated.

**Requirement for Ca\(^{2+}\) Binding and Myristoylation for Perinuclear Localization of NCS Proteins**—The data so far showed that localization of NCS-1-ECFP to the perinuclear compartment was independent of Ca\(^{2+}\) concentration in intact and permeabilized cells. To confirm that Ca\(^{2+}\) binding was not required for the localization of NCS-1, use was made of constructs encoding untagged wild-type NCS-1 and a version with all three functional EF hands (EF2-4) mutated to eliminate Ca\(^{2+}\) binding. These mutations (E84Q, E120Q, and E168Q) replaced conserved acidic residues in the EF hands required for Ca\(^{2+}\) coordination (18). Cells were fixed after permeabilization, and NCS-1 localization was determined by immunofluorescence staining. The untagged wild-type protein was found to be localized to the perinuclear compartment in the presence or absence of Ca\(^{2+}\) (Fig. 8, A and B) as previously seen for NCS-1-ECFP. The same localization pattern was seen with the NCS-1(EF2-4) mutant, indicating a complete lack of dependence on Ca\(^{2+}\) binding for the localization of NCS-1 (Fig. 8, C and D).

To determine whether myristoylation of the NCS proteins was required for their localization and translocation, constructs were made with the second glycine residue, essential for myristoylation (31), mutated to an alanine in both the NCS-1 and hippocalcin fusion proteins. Fig. 9 shows that intact cells with the proposed Ca\(^{2+}/\)myristoyl switch mechanism and suggests that these proteins only become membrane-associated at cytosolic Ca\(^{2+}\) concentrations above those in resting cells.

In fixed cells the localization of hippocalcin-EYFP after ionomycin treatment was similar to the perinuclear localization of NCS-1-ECFP. The extent of co-localization of the two proteins in living cells was examined directly using laser scanning confocal microscopy of cells transfected to express both hippocalcin-EYFP and NCS-1-ECFP. In resting cells, hippocalcin-EYFP showed a diffuse localization (Fig. 5A). In contrast, NCS-1-ECFP was associated with the perinuclear compartment and also had a patchy distribution on the plasma membrane (Fig. 5B). Following treatment with ionomycin for 2 min, hippocalcin-EFYP was observed to have translocated (Fig. 5D). A portion of hippocalcin-EYFP remained in the nucleus but in the rest of the cell showed almost complete co-localization with NCS-1-CFP (Fig. 5, D–F).
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To determine the degree to which the exposure of the myristoyl tail was sufficient for perinuclear membrane localization, a construct was made that fused the myristoylation sequence taken from hippocalcin (hippocalcin-(1-14)) to EGFP. This was expressed in HeLa cells, and its localization is shown in Fig. 9E. The distribution of the hippocalcin-(1-14)-EGFP protein appeared to be much the same as the perinuclear localization of the NCS proteins. There was strong perinuclear fluorescence together with some possible plasma membrane localization. This indicates that the myristoyl tail can be the sole determinant for intracellular targeting of these proteins. Using the permeabilization assay in 0 Ca\(^{2+}\) solution, it was found that the hippocalcin-(1-14)-EGFP remained in the cells, concentrated in the perinuclear region (Fig. 9F). This demonstrated that the myristoyl group accounted for all the intracellular targeting seen with the NCS-1 and hippocalcin fusion proteins but not the Ca\(^{2+}\) dependence of hippocalcin localization.

Kinetics of the Ca\(^{2+}\)/Myristoyl Switch in Living Cells—To examine the kinetics of the Ca\(^{2+}\)-dependent translocation of hippocalcin-EYFP, live cells were imaged using confocal laser scanning microscopy and fluorescence was monitored following addition of ionomycin (Fig. 10). Cells initially showed a diffuse fluorescence indicative of cytosolic and nuclear localization. Within a few seconds of ionomycin addition, translocation of hippocalcin-EYFP occurred with the appearance of a bright punctate spots with a perinuclear localization. In addition, hippocalcin-EYFP also appeared to a lesser extent associated with the plasma membrane in distinct patches (arrows in the last frame of Fig. 10), confirming the earlier interpretation from epifluorescence microscopy. The time course of translocation was similar to that previously seen for the Ca\(^{2+}\)-dependent C\(_2\) domain of PKC\(\gamma\) (6) or the pH domain of PLC\(\delta\) (32). Translocation of hippocalcin-EYFP was also observed transected either with pHippo(G2A)-EYFP or pNCS-1(G2A)-ECFP showed a diffuse fluorescence pattern compatible with cytosolic localization of the proteins (Fig. 9, A and C). No indication of translocation was seen in response to elevated intracellular Ca\(^{2+}\) levels. In addition, when the cells were permeabilized, even in the presence of 10 \(\mu\)M free Ca\(^{2+}\), the unmyristoylated fusion proteins rapidly leaked out of the cells with only low levels of fluorescence remaining in the nuclei (Fig. 9, B and D). These data indicate that myristoylation was essential for the perinuclear localization of NCS-1 and for Ca\(^{2+}\)-dependent translocation of hippocalcin.

Within a few seconds of ionomycin addition, translocation of hippocalcin-EYFP occurred with the appearance of a bright punctate spots with a perinuclear localization. In addition, hippocalcin-EYFP also appeared to a lesser extent associated with the plasma membrane in distinct patches (arrows in the last frame of Fig. 10), confirming the earlier interpretation from epifluorescence microscopy. The time course of translocation was similar to that previously seen for the Ca\(^{2+}\)-dependent C\(_2\) domain of PKC\(\gamma\) (6) or the pH domain of PLC\(\delta\) (32). Translocation of hippocalcin-EYFP was also observed...
under more physiological conditions following stimulation with histamine and was reversible several minutes after the initial translocation.2

The relationship of kinetics of the Ca$^{2+}$/myristoyl switch for membrane translocation to changes in cytosolic Ca$^{2+}$ concentration was examined in cells expressing hippocalcin-EYFP and loaded with the fluorescence Ca$^{2+}$ indicator Fura Red. Dual imaging using confocal microscopy indicated that changes in hippocalcin-EYFP localization could be measured in parallel with an expected decrease in Fura Red emission because of Ca$^{2+}$ elevation following ionomycin addition. Measurement at high time resolution (1 frame/137 ms) allowed a comparison of the time course of hippocalcin-EYFP translocation and changes in cytosolic Ca$^{2+}$ concentration (Fig. 11). Cytosolic Ca$^{2+}$ concentration was monitored over the whole cell. Translocation was assessed by monitoring the fluorescence of 9 spots/cell where translocation occurred and averaging the time courses for each cell. Cytosolic Ca$^{2+}$ concentration began to increase in a homogeneous manner over the whole cell immediately upon ionomycin addition. In contrast, hippocalcin-EYFP translocation began only after a lag period of 2 s (2.17 ± 0.13 s from 4 cells). The time course of translocation, which then followed, could be fitted with a single hyperbolic function, indicating a first order reaction, with a half-time of ~12 s.

2 D. W. O’Callaghan, L. Ivings, J. L. Weiss, M. C. Ashby, A. V. Tepikin, and R. D. Burgoyne, unpublished observations.
It has become increasingly apparent that the control of various cellular activities involves proteins that either are tightly co-localized in signaling complexes or can translocate in a dynamic way into such complexes (33, 34). Studies on protein dynamics have been complemented by increasing information on the differential use of local versus global Ca\(^{2+}\) signals in the regulation of cell function (2). In contrast, relatively little is known about the dynamics and localization of Ca\(^{2+}\) sensor proteins apart from calmodulin and protein kinase C (3–6). Here we have examined the localization of three members of the NCS family of Ca\(^{2+}\) sensors and demonstrate that, under the same conditions in the same cellular context, their N-terminal myristoyl groups are used in quite different ways to determine their localization. We also provide data on the kinetics of the Ca\(^{2+}\)-myristoyl switch mechanism in living cells. The classic example of the NCS proteins has been the well characterized retinal protein recoverin, which is believed to show Ca\(^{2+}\)-dependent translocation to membranes via a Ca\(^{2+}\)-myristoyl switch mechanism based on biochemical and structural analyses (14–16), although this has not been confirmed in living cells. The differences in behavior we observed for other NCS proteins are surprising, as the features within the sequence of recoverin that determine the Ca\(^{2+}\)/myristoyl switch mechanism including the inactivated first EF hand and hydrophobic residues that stabilize the myristoyl group are highly conserved in all of the NCS proteins (15). It has recently been shown, however, that residues within EF1 of GCAP-2 are crucial for binding to guanylyl cyclase (35). The inactivated EF hand may therefore be more generally important in NCS proteins for target protein interactions.

In HeLa cells, NCS-1 was constitutively associated with, and hippocalcin and neurocalcin \(\delta\) could become associated with, membranes of a perinuclear compartment overlapping the trans-Golgi network (shown using \(\gamma\) adaptin as the marker). All three proteins also appeared to associate with the plasma membrane. This localization was not dependent on the presence of the fluorescent tags and is likely to be the result of the preference of the myristoyl group for cholesterol- and sphingolipid-rich domains (36). Although hippocalcin and neurocalcin \(\delta\) are expressed only in neurons, NCS-1 is expressed in many non-neuronal cell types (23, 28, 37). Association with a perinuclear compartment overlapping the trans-Golgi was reported for endogenous NCS-1 in COS-7 cells (28). Importantly, studies on NCS-1 in fixed brain slices indicated by the use of electron microscopy that NCS-1 was present on cisternae of the trans-Golgi complex, in addition to its presence in both pre- and post-synaptic structures (38). The Ca\(^{2+}\) dependence of the localization of NCS-1 to the trans-Golgi was not determined in these studies, and so it remained possible that resting cytosolic Ca\(^{2+}\) concentration was sufficient to activate a Ca\(^{2+}\)/myristoyl switch in NCS-1 as it has a high affinity for Ca\(^{2+}\) (24, 39). We have demonstrated here that NCS-1 association with the perinuclear compartment is independent of free Ca\(^{2+}\) and that NCS-1 does not need functional Ca\(^{2+}\)-binding motifs for this localization. The presence of NCS-1 on the trans-Golgi network may be related to its regulation of PI 4-kinase \(\beta\) (28, 29), which...
has the same localization. The ability of other NCS proteins such as hippocalcin and neurocalcin δ to interact with PI 4-kinase has not been examined, and their localization in neurons has only been investigated after fixation without elevation of intracellular Ca$^{2+}$. The presence of NCS-1 on the plasma membrane that we have observed would be consistent with the known involvement of NCS-1 in Ca$^{2+}$ channel regulation (25, 26). The membrane association of NCS-1 and hippocalcin in HeLa cells was entirely dependent upon myristoylation. In addition, a minimal myristoylation sequence (hippocalcin-(1–14)) was sufficient for localization to the perinuclear compartment. The latter result is compatible with previous work on the targeting of constructs bearing the myristoylation sequence of Src family kinases (40). The difference between the NCS proteins that we investigated, however, was that NCS-1 was membrane-associated even at nominally 0 Ca$^{2+}$, whereas hippocalcin and neurocalcin δ required Ca$^{2+}$ elevation above resting levels to translocate from the cytosol to a membrane-associated pool. The membrane association of NCS-1 additionally did not require the presence of any functional EF hand motifs. These data suggest that, in NCS-1, the myristoyl group is exposed even in the Ca$^{2+}$-free form but that hippocalcin and neurocalcin δ have a classic recoverin-like Ca$^{2+}$/myristoyl switch. Their myristoyl group would, therefore, be sequestered in the Ca$^{2+}$-free form and then exposed in the Ca$^{2+}$-bound form to allow the proteins to become membrane-associated. The findings on NCS-1 are consistent with biochemical data showing that recombinant

**FIG. 11. Kinetics of changes in cytosolic Ca$^{2+}$ concentration and hippocalcin-EYFP translocation.** HeLa cells transfected to express hippocalcin-EYFP were loaded with Fura Red and imaged by confocal microscopy. To provide optimal time resolution, frames were taken every 137 ms. The decrease in Fura Red fluorescence (top) began as soon as ionomycin was added. The lower panel shows averaged data from 9 regions of translocation in the same cell. The standard errors of the means are not shown for clarity but did not exceed ±4% of the mean values. Translocation began after a 2-s lag and then followed first order kinetics (as shown by the fitted curve) with a half-time of 11.6 s.
myristoylated NCS-1 became bound to membranes in the absence of Ca\(^{2+}\) (24). In addition, analysis of the yeast orthologue FRQ1 by NMR suggested that the myristoyl group might always be solvent-exposed (41). This idea could be directly confirmed by x-ray crystallography, but so far the only structure solved is for nonmyristoylated NCS-1 in its Ca\(^{2+}\)-bound form (28). The study of yeast FRQ also showed, from a biochemical analysis of cell fractions, that some Ca\(^{2+}\)-dependent membrane association occurred even in the absence of myristoylation (41). In contrast, in intact or permeabilized HeLa cells, NCS-1 localization to the perinuclear membrane compartment was completely dependent upon its ability to associate already at Ca\(^{2+}\). The most similar to neurocalcin that membrane association of hippocalcin-EYFP does require a diffusion-limited translocation to membranes. The time course of this association is 20 ms when Ca\(^{2+}\) elevation above resting levels, and that this occurs with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)). In contrast, hippocalcin and neurocalcin δ would respond to more global Ca\(^{2+}\) changes in a time course of no more than milliseconds and result in its binding with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)). In contrast, hippocalcin and neurocalcin δ would respond to more global Ca\(^{2+}\) changes in a time course of no more than milliseconds and result in its binding with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)).

The different usage of the myristoyl group in the NCS proteins is likely to be important for differential aspects of Ca\(^{2+}\) signal transduction. EF hand motifs in Ca\(^{2+}\) sensors bind Ca\(^{2+}\) very rapidly with binding limited only by the rate of Ca\(^{2+}\) diffusion (42). The off-rate is also rapid in Ca\(^{2+}\) sensors but can be slowed in Ca\(^{2+}\)-sensor and Ca\(^{2+}\)-buffer proteins by changes in the residue at position 9 of the EF hand (42). This allows fine-tuning of the activation and time scale of activity of EF hand proteins. The NCS proteins show the properties of fast Ca\(^{2+}\) sensors with rapid on- and off-rates of Ca\(^{2+}\) binding and inactivation occurring on a millisecond time scale (43, 44). It would be predicted that NCS-1, with an affinity of 0.3 μM, would be able to react to Ca\(^{2+}\) concentration changes in a time course of no more than milliseconds and result in its binding with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)). In contrast, hippocalcin and neurocalcin δ would respond to more global Ca\(^{2+}\) changes in a time course of no more than milliseconds and result in its binding with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)). In contrast, hippocalcin and neurocalcin δ would respond to more global Ca\(^{2+}\) changes in a time course of no more than milliseconds and result in its binding with a time constant of ~20 ms when Ca\(^{2+}\) concentration falls (see similar calculations for GCAP1 (Ref. 44)). 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Differential Use of Myristoyl Groups on Neuronal Calcium Sensor Proteins as a Determinant of Spatio-temporal Aspects of Ca^{2+} Signal Transduction
Dermott W. O'Callaghan, Lenka Ivings, Jamie L. Weiss, Michael C. Ashby, Alexei V. Tepikin and Robert D. Burgoyne

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