High-affinity interaction of the N-terminal myristoylation motif of the neuronal calcium sensor protein hippocalcin with phosphatidylinositol 4,5-bisphosphate

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Many proteins are associated with intracellular membranes due to their N-terminal myristoylation. Not all myristoylated proteins have the same localization within cells, indicating that other factors must determine their membrane targeting. The NCS (neuronal calcium sensor) proteins are a family of Ca^{2+}-binding proteins with diverse functions. Most members of the family are N-terminally myristoylated and are either constitutively membrane-bound or have a Ca^{2+}/myristoyl switch that allows their reversible membrane association in response to Ca^{2+} signals. In the case of hippocalcin and NCS-1, or alternatively KChIP1 (K^+ channel-interacting protein 1), their N-terminal myristoylation motifs are sufficient for targeting to distinct organelles. We have shown that an N-terminal myristoylated hippocalcin peptide is able to specifically reproduce the membrane targeting of hippocalcin/NCS-1 when introduced into permeabilized cells. The peptide binds to liposomes containing phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] with high affinity (K_d 50 nM). Full-length hippocalcin also bound preferentially to liposomes supplemented with PtdIns(4,5)P_2. Co-expression of hippocalcin-(1–14)–ECFP (enhanced cyan fluorescent protein) or NCS-1–ECFP partially displaced the expressed PH (pleckstrin homology) domain of phospholipase δ1 from the plasma membrane in live cells, indicating that they have a higher affinity for PtdIns(4,5)P_2 than does this PH domain. The Golgi localization of the PH domain of FAPP1 (four-phosphate-adaptor protein 1), which binds to phosphatidylinositol 4-phosphate, was unaffected. The localization of NCS-1 and hippocalcin is likely to be determined, therefore, by their interaction with PtdIns(4,5)P_2.

Key words: Golgi complex, hippocalcin, lipid-binding, calcium-binding proteins, neuronal calcium sensor-1 (NCS-1), phosphoinositide.

INTRODUCTION

Many cellular proteins are targeted to intracellular membranes following post-translational addition of fatty acyl moieties such as palmitate or myristate [1–3], with the latter occurring co-translationally. These additions allow interaction with lipid bilayers, but proteins need additional targeting information to allow them to reach the appropriate intracellular location, and indeed myristoylated and palmitoylated proteins can have diverse localizations within cells [2,3]. The NCS (neuronal calcium sensor) family of proteins are a series of Ca^{2+}-binding proteins, many of which are expressed only in neuronal cell types [4,5]. One member of the family, NCS-1, has more widespread expression in mammalian tissues, and orthologues of NCS-1 are present in all species from yeast [6] to humans. Many of the NCS proteins are N-terminally myristoylated, and the study of recoverin, the first NCS protein to be discovered, led to the concept of the Ca^{2+}/myristoyl switch [7–9]. Structural and biochemical studies established that the myristoyl group on recoverin is sequestered into a hydrophobic pocket in the Ca^{2+}-free form, and that Ca^{2+} binding leads to a substantial conformational change that flips out the myristoyl group and allows recoverin to become reversibly membrane-associated. The existence of such a myristoyl switch has been confirmed in living cells for other NCS proteins, including hippocalcin [10,11], neurocalcin δ [12], VILIP-1 (visinin-like protein 1) [13,13a] and VILIP-3 [14]. NCS-1, in contrast, is membrane-associated in a myristoyl-dependent manner even at low Ca^{2+} concentrations, suggesting [11,15] that its myristoyl group is constitutively exposed [16,17].

Hippocalcin translocates in response to an elevation of the intracellular Ca^{2+} concentration to the same membrane localizations where NCS-1 is found when the two proteins are expressed in the same cell [11]. These appear to be the plasma membrane and the Golgi complex (particularly the *trans*-Golgi network) [11,18–20]. Neurocalcin δ, VILIP-1 and VILIP-2 also translocate to the plasma membrane and Golgi complex in response to Ca^{2+} elevation [12–14]. In contrast, KChIP1 (K^+ channel-interacting protein 1), another NCS protein that is also constitutively membrane-associated through its myristoylation, is targeted to distinct vesicular structures, and this targeting is important for its ability to stimulate the traffic of Kv4 potassium channels to the cell surface [21]. Interestingly, the distinct targeting of hippocalcin/NCS-1 compared with KChIP1 can be reproduced by attaching the minimal N-terminal myristoylation sequences (of 11–14 residues) to GFP (green fluorescent protein), showing that these N-terminal sequences contain targeting information in addition to the residues required for recognition by N-myristoyltransferase [22]. In contrast, N-terminal myristoyl sequences from certain other proteins are not even sufficient for membrane association [23]. Within the hippocalcin/NCS-1 sequences, three basic residues at positions 3, 7 and 9 are crucial for targeting to the plasma membrane and Golgi complex [21].

Abbreviations used: CES liposomes, liposomes consisting of a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine; ECFP/EGFP/EYFP, enhanced cyan/green/yellow fluorescent protein; FAPP1, four-phosphate-adaptor protein 1; GFP, green fluorescent protein; KChIP1, K^+ channel-interacting protein 1; MARCKS, myristoylated alanine-rich C-kinase substrate; myr-hip-(2–14), biotinylated, myristoylated hippocalcin-(2–14) peptide; NCS, neuronal calcium sensor; PH, pleckstrin homology; PLC, phospholipase C; VILIP, visinin-like protein.

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Consistent with these findings, structural studies on another NCS protein, recoverin, bound to a phospholipid bilayer revealed that its N-terminal basic residues are in close contact with the phospholipid head groups [24]. This led us to investigate the molecular basis of the targeting of the NCS myristoylation motifs, and whether this could be determined by specific lipid interactions. Our findings demonstrate that the N-terminal motif of hippocalcin and NCS-1 specifically binds PtdIns(4,5)P_2 with high affinity both *in vitro* and in living cells.

MATERIALS AND METHODS

Reagents

The plasmids for hippocalcin-(1–14)–EGFP, NCS-1, NCS-1–ECFP, KChIP1–ECFP, KChIP1-(1–11)–EYFP [11,21,25], GFP–PLCδ1-PH [11,21,25] and GFP–FAPP1-PH [26] [where EGFP/ECFP/EYCP are enhanced green/cyan/yellow fluorescent protein, PLC is phospholipase C, -PH denotes the PH (pleckstrin homology) domain of the protein in question, and FAPP1 is four-phosphate-adaptor protein 1] were described previously [11,21,25]. The plasmid for hippocalcin-(1–14)–ECFP was prepared by exchanging the coding sequence of ECFP for that of EGFP in a plasmid encoding hippocalcin-(1–14)–EGFP [11]. Mouse monoclonal anti-GFP antibody was obtained from Clontech.

Localization of biotinylated peptides in HeLa cells

HeLa cells transfected with NCS-1–ECFP, hippocalcin-(1–14)–EGFP or KChIP1–ECFP were washed 24 h after transfection with Krebs buffer and then permeabilized for 15 min in buffer A (20 mM Pipes, pH 6.5, 139 mM potassium glutamate, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP, 20 μM digitonin) containing 50 μg/ml myristoylated or non-myristoylated peptide. Coverslips were subsequently washed twice with 1 ml of PBS, incubated for 30 min in PBT (PBS containing 0.3 % BSA and 0.1 % Triton X-100) at room temperature and washed twice in PBT prior to incubation with streptavidin–Texas Red (1:50; Amersham Biosciences) for 30 min prior to mounting and examination by laser scanning confocal microscopy.

Binding of biotinylated peptides to immobilized lipids

PIP strips™ (Echelon, Salt Lake City, UT, U.S.A.) were blocked for 1 h at room temperature in blocking buffer [10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 % (w/v) fatty acid-free BSA, 0.5 mM $MgCl_2$, 0.05 % (v/v) Tween-20]. Membranes were subsequently incubated with peptides at 100 ng/ml in blocking buffer for 1 h at room temperature, followed by six 5 min washes in blocking buffer and subsequent incubation with streptavidin–horseradish peroxidase (1:500; Amersham Biosciences) in blocking buffer for 1 h at room temperature. Membranes were washed six times with blocking buffer, and peptide binding was detected with ECL-Plus® reagents (Amersham Biosciences).

Liposome binding assays

Lipids were added to dry, chloroform-washed borosilicate vials with Teflon™-coated caps (Fisher Scientific). The standard liposome composition consisted of 1.2 mg of phosphatidylcholine (Instruchemie, Delfzijl, The Netherlands), 1.2 mg of phosphatidylethanolamine (Instruchemie), 0.3 mg of phosphatidylserine (Instruchemie), and 0.3 mg of test lipid. The additional lipids used were phosphatidylinositol (Instruchemie), PtdIns4*P* (Instruchemie), PtdIns(4,5)P_2 (Instruchemie), PtdIns3*P* (Sigma, Poole, Dorset, U.K.), PtdIns(3,5)P_2 (Sigma), PtdIns(3,4,5)P_3 (Sigma) and phosphatidic acid (Sigma). Lipid mixtures were dried under a stream of N_2 and placed in a desiccator for 10 min, resuspended in 1.5 ml of liposome buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.5 mM EDTA) and sonicated for 1 min at 20 % amplitude using a GEX-600 sonicator fitted with a micro-tip probe (Ultrasonic Processor). Samples were split into 500 μl aliquots and liposomes were pelleted by centrifugation at 500 ***g*** for 10 min. Supernatants were discarded and liposomes resuspended in liposome buffer supplemented with peptide. Binding reactions were allowed to proceed for 90 min at room temperature, after which liposomes were collected by centrifugation, washed in liposome buffer and re-isolated by centrifugation. Supernatants were discarded and final pellets were resuspended in 20 μl of liposome buffer. Then 5 μl of each sample was spotted on to nitrocellulose filters and incubated in Ponceau-S (Sigma) for 5 min at room temperature, followed by washing to reveal bound peptide, which was quantified by densitometry. The [^{35}S]methionine-labelled full-length hippocalcin was prepared using a TNT® quick coupled transcription/translation system (Promega) according to the manufacturer's protocol and with plasmid encoding hippocalcin as template. Synthesized hippocalcin was visualized by autoradiography. After incubation with liposomes and washing, bound hippocalcin was quantified using scintillation counting.

Confocal laser scanning microscopy

For confocal laser scanning microscopy, live or fixed transfected cells were analysed using a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany) with a 22 μm pin hole and a 63× water-immersion objective with a 1.2 numerical aperture. For optimal imaging of ECFP, cells were excited at 405 nm and light was collected at 450–500 nm. FITC was imaged using excitation at 488 nm and light collection at 500–560 nm. For optimal imaging of EGFP, cells were excited at 488 nm and light was collected at 500–550 nm. For imaging of EYFP, the cells were excited at 514 nm and light was collected at 545–625 nm. For optimal imaging of Texas Red, cells were excited at 543 nm and light was collected at 600–650 nm. Images were exported as TIFF files and compiled in CorelDraw. In some cases, fluorescence was quantified by drawing regions of interest around the outside and the inside of the plasma membrane to allow determination of the percentage of total fluorescence close to the plasma membrane.

RESULTS

Targeting of N-terminal myristoylation motifs of hippocalcin, and localization of hippocalcin synthetic N-terminal peptides

The N-terminal 14 residues of the myristoylation motif of hippocalcin are sufficient to target EGFP to the same structures (plasma membrane and Golgi) as full-length NCS-1 (Figure 1). In contrast, the myristoylation motif of the related NCS protein KChIP1 led to targeting to distinct cellular structures (Figure 1). These are the same structures to which full-length KChIP1 is targeted [21]. We wondered whether the molecular basis for the distinct targeting of the myristoylated proteins could be examined using synthetic peptides as probes. Three peptides were synthesized, representing the N-terminal sequences of myristoylated hippocalcin, non-myristoylated hippocalcin or myristoylated KChIP1, each with biotin added to a C-terminal lysine residue (Figure 2A). In order to determine whether these synthetic peptides could reproduce the localization of the GFP fusion proteins, they were introduced into digitonin-permeabilized HeLa cells following transfection with NCS-1–ECFP, hippocalcin-(1–14)–EGFP or KChIP1–ECFP and their localization was detected by incubation

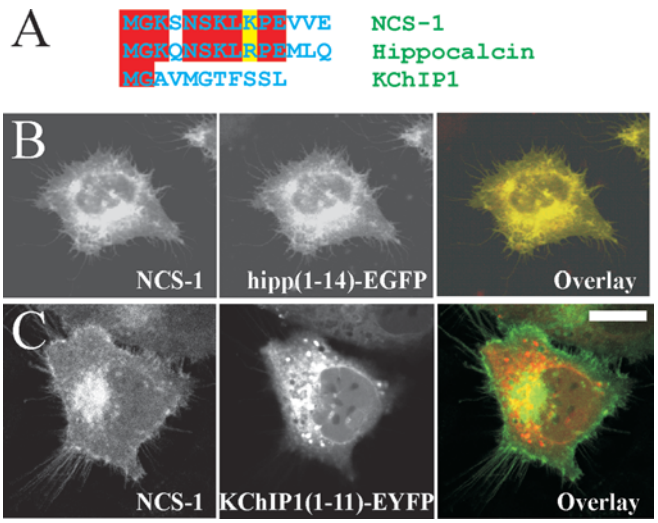

Figure 1 Targeting via the myristoylation motifs of hippocalcin and KChIP1

(**A**) Comparison of the N-terminal sequences of human NCS-1, hippocalcin and KChIP1. Identical residues are boxed in red, and similar residues in yellow. (**B**) HeLa cells were co-transfected to express NCS-1 and hippocalcin-(1–14)–EGFP, fixed, and NCS-1 was detected by immunofluorescence. The overlapping localization is seen as yellow in the overlay image [NCS-1 in red, hippocalcin-(1–14)–EGFP in green]. (**C**) HeLa cells were co-transfected to express NCS-1–ECFP and KChIP1-(1–11)–EYFP. The overlay image shows NCS-1–ECFP in green and KChIP1-(1–11)–EYFP in red. The scale bar represents 10 μm.

with streptavidin–Texas Red. The digitonin-permeabilization procedure caused the cells to round up, but it was clear that myr-hip-(2–14) [biotinylated, myristoylated hippocalcin-(2–14) peptide] became cell associated and localized to the same structures that were labelled by NCS-1–ECFP or hippocalcin-(1–14)–EGFP (Figure 2B, panels i and ii). In contrast, the non-myristoylated peptide did not become stably cell associated (Figure 2B, iii). The localization of the myr-KChIP1-(2–13) peptide was examined in cells transfected to express full-length KChIP1–ECFP, but this peptide also did not become cell-associated (Figure 2B, iv). These data suggest that the myr-hip-(2–14) peptide can specifically replicate the targeting of NCS-1 or the N-terminal sequence of hippocalcin. For reasons that are unclear, the KChIP1 peptide, unlike the sequence fused to EYFP, could not bind to cellular structures with high enough affinity. Overall, both myristoylation and N-terminally encoded targeting information within hippocalcin are required and sufficient for subcellular localization.

Binding of synthetic N-terminal peptides to lipids

Within the N-terminal myristoylation sequence of NCS-1 and hippocalcin, basic amino acids in close proximity to the myristoylation site are conserved in the two proteins, and determine their targeting to the Golgi and plasma membrane [21]. KChIP1 lacks the identified crucial basic amino acids at positions 3, 7 and 9 [21]. We wondered if these basic residues might mediate targeting by binding to specific acidic phospholipids unique to distinct organelles, such as PtdIns4*P* at the *trans*-Golgi network or PtdIns(4,5)P_2 at the plasma membrane. In these studies we used the synthetic peptides as probes. To initially examine the potential lipid-binding characteristics of the N-terminal targeting residues, we analysed the binding of both the myristoylated and non-myristoylated peptides to a series of immobilized lipids (Figure 3). The myr-hip-(2–14) peptide exhibited an apparently wide spectrum of lipid-binding activity, but binding was higher for phosphorylated lipids. Binding did not occur to all acidic phospholipids, as no binding was detectable to sphingosine 1-phosphate or phosphatidylserine. The control non-myristoylated peptide did not bind any of the lipids, and nor was any binding observed with the myristoylated peptide derived from KChIP1. These data suggest that the N-terminal hippocalcin peptide could interact specifically with particular phospholipids in a myristoylation-dependent manner.

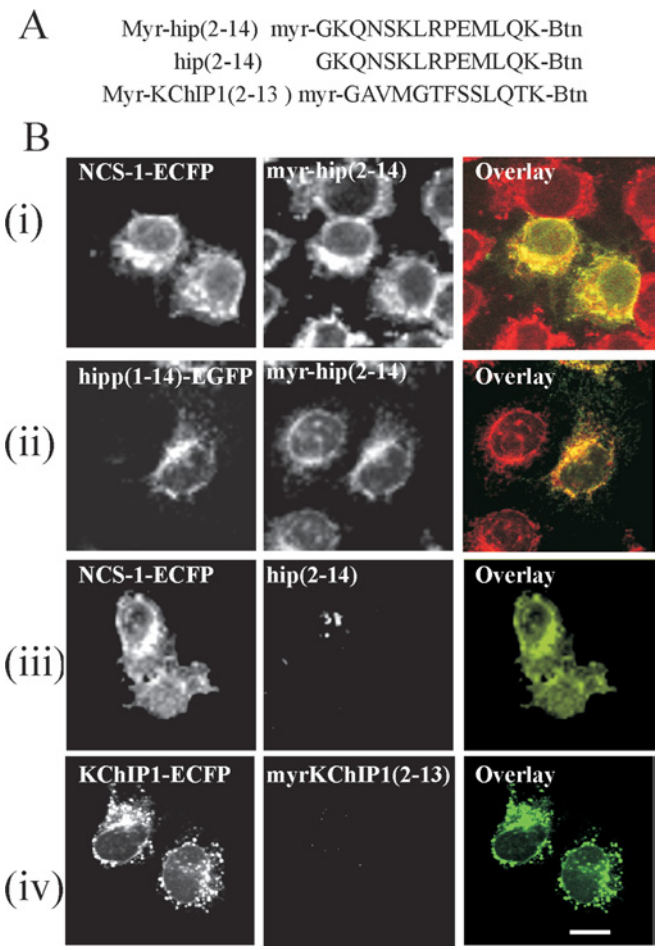

Figure 2 Intracellular localization of biotinylated peptides

(**A**) Synthetic peptides used in the study. (**B**) Myr-hip-(2–14), corresponding to the N-terminus of the hippocalcin, was incubated with digitonin-permeabilized HeLa cells transfected with NCS-1–ECFP (i) or hipp(1–14)–EGFP (ii). Non-myristoylated, biotinylated hippocalcin peptide [hippocalcin-(2–14)] was also incubated with NCS-1–ECFP-transfected cells (iii). Myristoylated KChIP1 peptide was incubated with cells transfected with KChIP-1–EYFP. The biotinylated peptides were detected using Texas Red–streptavidin. Overlay images are displayed in colour, with expressed transfected constructs in green and peptides in red, and with regions of co-localization in yellow. The scale bar represents 10 μm.

Binding of proteins to immobilized lipids does not always reproduce the correct degree of lipid specificity. Therefore we also examined the association of the myr-hip-(2–14) peptide with synthetic liposomes, a system more closely resembling membrane topology *in vivo* (Figure 4A). The standard liposome composition consisted of a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (CES liposomes), which was supplemented with various phosphoinositides. Consistent with the assay on immobilized lipids, little binding was detected to CES liposomes. The greatest level of peptide binding in these experiments (Figure 4B) was observed to liposomes supplemented with PtdIns(4,5)P_2. To delineate the likely physiological relevance of such a peptide–lipid interaction,

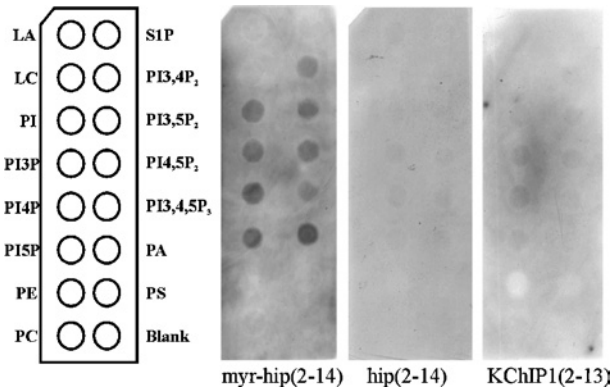

Figure 3 Determination of the binding of peptides to immobilized phospholipids

The binding of peptides to phospholipids was determined using PIP-Strip™ binding. Positions of immobilized lipid species are indicated (left panel). Myr-hip-(2–14), hip-(2–14) or myristoylated KChIP1-(2–13) was incubated with PIP-Strip™ membranes and bound peptide was detected with streptavidin–horseradish peroxidase. The myr-hip-(2–14) peptide interacted with a number of immobilized lipid species. No binding of hippocalcin-(2–14) peptide or myr-KChIP1-(2–13) peptide to lipids was observed.

we determined the binding affinity of myr-hip-(1–14) peptide for control (CES) liposomes or liposomes supplemented with PtdIns, PtdIns4*P* or PtdIns(4,5)P_2 (Figure 4C). The interaction with PtdIns(4,5)P_2 was of the highest affinity, with a K_d of $\approx$50 nM, an affinity higher than reported for isolated PH domains where binding to PtdIns(4,5)P_2 has been well characterized [27].

Binding of radiolabelled full-length hippocalcin to liposomes

In order to confirm this specific preference for PtdIns(4,5)P_2, and that this was not just a feature of the synthetic peptide, binding of full-length hippocalcin was assayed to liposomes supplemented with PtdIns, PtdIns4*P* or PtdIns(4,5)P_2. In order to do this in a quantitative manner and with myristoylated protein, radiolabelled hippocalcin was generated using *in vitro* transcription and translation (Figure 5A). This system has been shown to allow myristoylation of the newly synthesized proteins [28]. The [^{35}S]methionine-labelled hippocalcin became bound to liposomes after incubation in the presence of 1 μM free Ca^{2+}, and again did so with a preference for liposomes supplemented with PtdIns(4,5)P_2 (Figure 5B). Full-length hippocalcin has its myristoyl group sequestered in the Ca^{2+}-free state, and this is exposed on Ca^{2+} binding. Hippocalcin would be expected, therefore, to show a completely Ca^{2+}-dependent interaction with liposomes if the binding is physiologically relevant. The specificity of binding to PtdIns(4,5)P_2-supplemented liposomes was shown by the finding that this was indeed Ca^{2+}-dependent as predicted; in the absence of Ca^{2+}, binding was at the same level as to CES liposomes (Figure 5C).

Interaction of NCS-1 and hippocalcin-(1–14) with PtdIns(4,5)P_2 in living cells

Hippocalcin and its N-terminal peptide did not show absolute specificity for the phosphoinositide species in the binding assays, but showed clear preferential binding to PtdIns(4,5)P_2. This was of high affinity, and since PtdIns(4,5)P_2 is the most abundant of the phosphoinositides in cellular membranes, this suggests that the interaction with PtdIns(4,5)P_2 is likely to be physiologically relevant and contribute to the targeting of hippocalcin and NCS-1. We therefore examined the extent of co-localization of NCS-

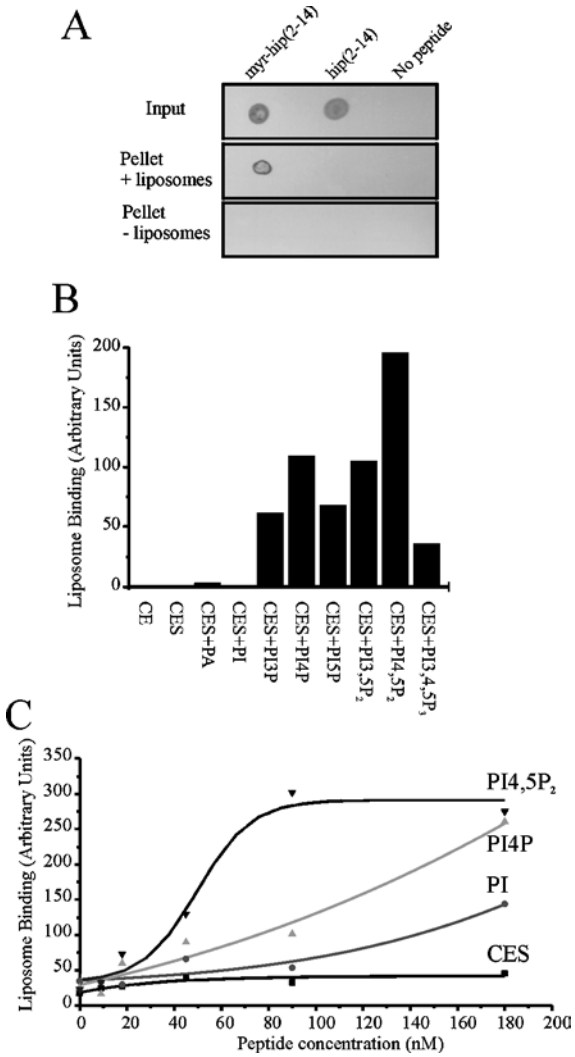

Figure 4 Binding of the myr-hip-(2–14) peptide to liposomes of various compositions

(**A**) Binding assay for analysis of peptide interaction with liposomes. The indicated peptides were incubated with or without liposomes. After centrifugation, sedimented material was spotted on to nitrocellulose filters, and bound peptide was detected by staining with Ponceau S. (**B**) Analysis of peptide binding to liposomes by quantitative densitometry. CES liposomes supplemented with 10 % of the indicated PtdIns (PI) lipid were incubated with 90 nM myr-hip-(2–14). The highest level of binding was to liposomes supplemented with PtdIns(4,5)P_2. PA, phosphatidic acid. (**C**) Determination of the affinity of myr-hip-(2–14) peptide for liposomes supplemented with PtdIns, PtdIns4*P* and PtdIns(4,5)P_2. An increasing concentration of peptide was incubated in the presence of a fixed liposome concentration, and bound peptide was quantified by densitometry and comparison with that bound to control liposomes.

1–ECFP with the well characterized PtdIns(4,5)P_2-specific PH domain of PLCδ1 linked to GFP [25] in living cells. In confocal sections it was clear that, in cells transfected to express only the PLCδ1 PH domain, the majority of the construct was targeted to the plasma membrane (Figure 6A). In contrast, in cells co-expressing NCS-1, there appeared to be less of the PH domain construct associated with the plasma membrane; instead it was more cytosolic (Figures 6C and 6D). To quantify the differences between cells with or without NCS-1–ECFP expression, areas of interest were drawn around the cells and the fluorescence at the cell periphery was determined as a percentage of the total fluorescence for each cell. This assay tends to overestimate the amount of fluorescence that is actually associated with the plasma

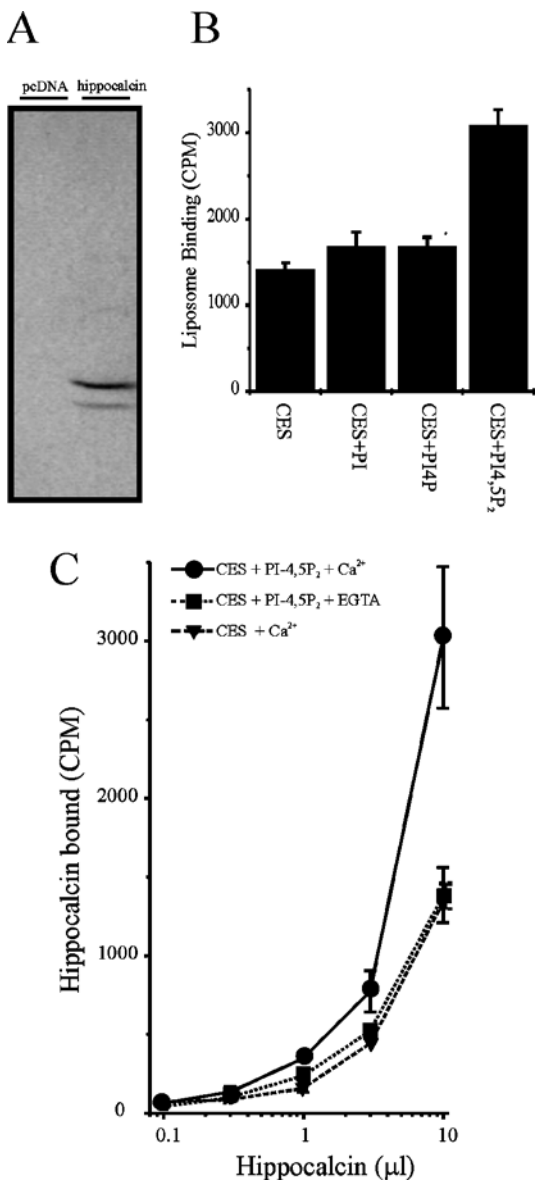

Figure 5 Binding of radiolabelled *in vitro* synthesized hippocalcin to liposomes

(**A**) Radiolabelled products from control and hippocalcin *in vitro* transcription/translation reactions. (**B**) Analysis of hippocalcin binding to liposomes. CES liposomes were supplemented with 10% of the indicated PtdIns (PI) lipid. After incubation with 10 μl of radiolabelled hippocalcin per reaction, the liposomes were washed and bound protein determined. (**C**) Binding of increasing amounts of radiolabelled hippocalcin from the *in vitro* transcription/translation mixture to CES liposomes supplemented with PtdIns(4,5)P_2 in the presence of EGTA or with 1 μM free Ca^{2+}.

membrane; nevertheless, a clear reduction in the plasma membrane association of GFP–PLCδ1-PH was observed (Figure 6K). It should also be noted that the Golgi localization of NCS-1–ECFP was not seen for the PLCδ1 PH domain.

NCS-1 is an activator of phosphatidylinositol 4-kinase IIIβ [29,30] and interacts with various other target proteins [5]. In order to rule out the possibility that NCS-1 displaced GFP–PLCδ1-PH from the plasma membrane by an indirect mechanism through regulation of a target protein, we also examined the effect on GFP–PLCδ1-PH of co-expression of hippocalcin-(1–14)–ECFP, as this minimal construct would be unlikely to interact with and activate any other proteins. Hippocalcin-(1–14)–ECFP also resulted in displacement of GFP–PLCδ1-PH from the plasma membrane (Figures 6E, 6F and 6K). To rule out the possibility that these effects on GFP–PLCδ1-PH localization were due to an increase in expression levels and saturation of binding sites, samples of transfected cells were probed with anti-GFP antibody to detect the expressed proteins. GFP–PLCδ1-PH was expressed at a slightly lower level in co-transfected cells compared with singly transfected cells (Figure 6L).

In order to assess the specificity of the effect on GFP–PLCδ1-PH localization, HeLa cells were also transfected to express the PH domain of FAPP1 linked to GFP (Figure 6B), as this binds preferentially to PtdIns4*P* and localizes to the Golgi complex [26,31]. Despite overlapping in localization with both NCS-1–ECFP and hippocalcin-(1–14)–ECFP at the Golgi complex, there was no indication of any dissociation of GFP–FAPP1-PH from the Golgi complex in co-transfected cells, as it remained in a perinuclear localization (Figures 6G–6J). These data are consistent with high-affinity binding of NCS-1–ECFP or hippocalcin-(1–14)–ECFP to PtdIns(4,5)P_2 rather than to PtdIns4*P* in live cells.

DISCUSSION

NCS-1 and the closely related protein hippocalcin are targeted to the same cellular membranes (plasma membrane and Golgi complex) through myristoylation. The N-terminal myristoylation motif (residues 1–14) is sufficient to reproduce the targeting of the full-length protein, and this requires the presence of three basic residues at positions 3, 7 and 9 [11,21]. The N-terminal myristoylation motif of KChIP, which does not possess basic residues at these positions, targets to distinct cellular structures. In order to determine the molecular basis for this specific targeting, we initially used peptides based on the N-terminal sequences, and showed that a myristoylated peptide derived from the N-terminus of hippocalcin is able to bind specifically to the same intracellular structures as NCS-1 and hippocalcin, and binds *in vitro* with high affinity to phosphoinositides, particularly PtdIns(4,5)P_2. The peptide did not bind to all acidic phospholipids, and in particular did not bind to phosphatidylserine, which has been shown previously to bind highly basic peptides [32]. The specificity of the lipid interaction was most apparent with a liposome binding assay, which has been shown to be a more reliable indicator of *in vivo* lipid interactions than the use of immobilized lipids [33]. The specificity for PtdIns(4,5)P_2 among phosphoinositides was not absolute, but the affinity was highest for this lipid, and PtdIns(4,5)P_2 is considerably more abundant than 3-phosphorylated phosphoinositides in cells [34], suggesting that PtdIns(4,5)P_2 is likely to be the physiologically relevant interaction that could target NCS-1 in resting cells. Studies with the peptides and with the proteins themselves in live cells have shown that their membrane association and binding to PtdIns(4,5)P_2 is dependent on myristoylation [11].

Myristoylated peptides themselves have very low affinities for lipid bilayers [35], with K_d values of the order of 10^{-4} M, suggesting a requirement for other motifs for stable membrane interaction. Certain proteins use a basic region for targeting in addition to myristoylation, but the characteristics of the lipid interactions are distinct from those that we have observed for hippocalcin. The MARCKS (myristoylated alanine-rich C-kinase substrate) protein, for example, has a region with 13 basic residues. Unlike in hippocalcin and NCS-1, these basic residues are situated some distance from the myristoylation motif; also, MARCKS peptides do not show preferential binding to PtdIns(4,5)P_2 over other acidic phospholipids, and in fact bind

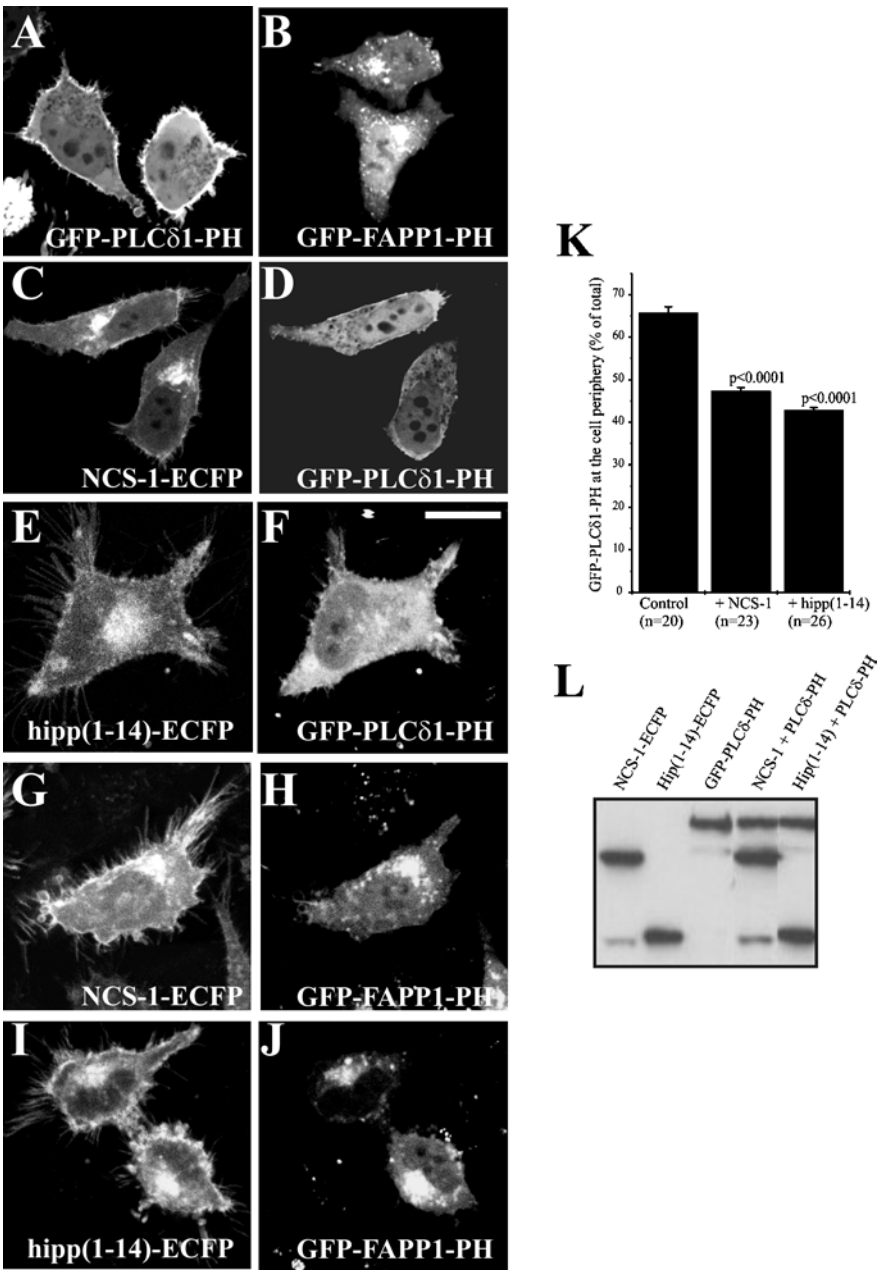

Figure 6 Effects of expression of NCS-1 or the hippocalcin myristoylation motif on the plasma membrane association of PLCδ1-PH or the Golgi complex association of FAPP1-PH

HeLa cells were transfected to express GFP–PLCδ1-PH alone (**A**) or GFP–FAPP1-PH alone (**B**), or co-transfected with GFP–PLCδ1-PH plus NCS-1–ECFP (**C**, **D**) or hippocalcin-(1–14)–ECFP (**E**, **F**). Other HeLa cells were transfected to express GFP–FAPP1-PH along with NCS-1–ECFP (**G**, **H**) or hippocalcin-(1–14)–ECFP (**I**, **J**). The percentage of fluorescence due to GFP–PLCδ1-PH at the cell periphery in cells expressing GFP–PLCδ1-PH alone (control) or in the presence of NCS-1–ECFP or hippocalcin-(1–14)–ECFP was quantified (**K**). The scale bar represents 10 μm. Samples of cells transfected to express GFP–PLCδ1-PH, NCS-1–ECFP or hippocalcin-(1–14)–ECFP, singly or in combination, were probed by Western blotting using anti-GFP (**L**).

equally well to liposomes containing phosphatidylserine, suggesting a purely electrostatic interaction [36]. Src also has multiple basic residues close to its myristoylation motif that are required for membrane targeting [37,38]. It has not been determined whether or not these sequences allow high-affinity interaction with phosphoinositides but, in contrast with the situation with hippocalcin, they do allow high-affinity binding to phosphatidylserine-containing liposomes. The high affinity for PtdIns(4,5)P_2 that we have observed for the myristoylated hippocalcin peptide may arise from the combined effects of insertion of the myristoyl group into the lipid bilayer and interaction of the nearby basic residues with lipid head groups, as suggested from structural studies for recoverin [24]. In particular, the lysine at position 3 is likely to be important, as mutation of this residue disrupts targeting of hippocalcin-(1–14)–EGFP, and this position is not occupied by a basic residue in Src.

The affinity of binding of the N-terminal hippocalcin peptide to PtdIns(4,5)P_2 was high compared with that determined for PH domains that have been classically characterized as PtdIns(4,5)P_2-binding modules, which have K_d values in the micromolar range

[27]. In addition to having a lower affinity for PtdIns(4,5)P_2, the PLCδ1 PH domain that is commonly used as a probe for PtdIns(4,5)P_2 has a high affinity for Ins(1,4,5)P_3. The PLCδ1 PH domain dissociates from the plasma membrane in response to agonist stimulation [39], most likely due to its binding to Ins(1,4,5)P_3 rather than as a consequence simply of hydrolysis of PtdIns(4,5)P_2 [39,40]. In contrast, NCS-1 remains membrane-associated in stimulated cells [11], and hippocalcin translocates in the reverse direction from the cytosolic compartment to membranes due to its Ca^{2+}/myristoyl switch [10,11], indicating that these proteins are unlikely to bind Ins(1,4,5)P_3 with high affinity.

The *in vitro* binding of the N-terminal hippocalcin peptide and *in vitro* synthesized hippocalcin to PtdIns(4,5)P_2-containing liposomes was followed up with a demonstration that NCS-1–ECFP and also hippocalcin-(1–14)–ECFP, when co-expressed with GFP–PLCδ1-PH, partially competed this PH domain off the plasma membrane, but had no effect on the localization of the FAPP1 PH domain, which binds preferentially to PtdIns4*P*. This provides strong support for the idea that these NCS proteins do indeed interact with PtdIns(4,5)P_2 in living cells. GFP–PLCδ1-PH has been widely used as a marker for the localization of PtdIns(4,5)P_2 in live cells. One important issue is that GFP–PLCδ1-PH is associated primarily with the plasma membrane, whereas NCS-1 and hippocalcin are also Golgi complex-associated, although apparent low levels of PtdIns(4,5)P_2 were detectable at the Golgi complex using the PLCδ1-PH probe in ultrastructural studies [41]. Previous work has shown that GFP–PLCδ1-PH is transiently associated with the Golgi complex following cell stimulation [39]. The lack of Golgi localization of GFP–PLCδ1-PH indicates a problem with the use of this construct as a PtdIns(4,5)P_2 probe. PtdIns(4,5)P_2 both is synthesized on and is functionally important for the Golgi complex [42–45]. Importantly, use of an anti-PtdIns(4,5)P_2 antiserum clearly detected a perinuclear Golgi compartment in fixed cells that was hardly detected in the same cells by GFP–PLCδ1-PH [46]. It has been suggested that PH domains may not report the localization of all lipids to which they could bind due to occupancy by other proteins or to interactions of the PH domain with protein targets that also determines their localization [26,44,47]. Recent work has also discovered a PtdIns(4,5)P_2-independent mode of interaction of PLCδ1-PH with lipid monolayers [48], which may explain the problems in the use of this PH domain to detect the localization of PtdIns(4,5)P_2. It is possible, therefore, that the high affinity of the minimal hippocalcin-(1–14) sequence for PtdIns(4,5)P_2 may prove to be a useful additional tool for targeting constructs for the detection of pools of PtdIns(4,5)P_2 in living cells.

This work was supported by a grant from the Wellcome Trust to R. D. B. D. W. O'C. was supported by a Wellcome Trust Prize fellowship. The plasmid encoding GFP–FAPP1-PH was a gift from Dr T. Levine (Institute of Ophthalmology, London, U.K.).

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Received 23 June 2005/29 July 2005; accepted 29 July 2005
Published as BJ Immediate Publication 29 July 2005, doi:10.1042/BJ20051001