Residues within the myristoylation motif determine intracellular targeting of the neuronal Ca^{2+} sensor protein KChIP1 to post-ER transport vesicles and traffic of Kv4 K^+ channels

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Accepted 28 July 2003

Journal of Cell Science 116, 4833-4845 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00803

Summary

KChIPs (K^+ channel interacting proteins) regulate the function of A-type Kv4 potassium channels by modifying channel properties and by increasing their cell surface expression. We have explored factors affecting the localisation of Kv4.2 and the targeting of KChIP1 and other NCS proteins by using GFP-variant fusion proteins expressed in HeLa cells. ECFP-Kv4.2 expressed alone was not retained in the ER but reached the Golgi complex. In cells co-expressing ECFP-Kv4.2 and KChIP1-EYFP, the two proteins were co-localised and were mainly present on the plasma membrane. When KChIP1-EYFP was expressed alone it was instead targeted to punctate structures. This was distinct from the localisation of the NCS proteins NCS-1 and hippocalcin, which were targeted to the trans-Golgi network (TGN) and plasma membrane. The membrane localisation of each NCS protein required myristoylation and minimal myristoylation motifs of hippocalcin or KChIP1 were sufficient to target fusion proteins to either TGN/plasma membrane or to punctate structures. The existence of targeting information within the N-terminal motifs was confirmed by mutagenesis of residues corresponding to three conserved basic amino acids in hippocalcin and NCS-1 at positions 3, 7 and 9. Residues at these positions determined intracellular targeting to the different organelles. Myristoylation and correct targeting of KChIP1 was required for the efficient traffic of ECFP-Kv4.2 to the plasma membrane. Expression of KChIP1(1-11)-EYFP resulted in the formation of enlarged structures that were positive for ERGIC-53 and β-COP. ECFP-Kv4.2 was also accumulated in these structures suggesting that KChIP1(1-11)-EYFP inhibited traffic out of the ERGIC. We suggest that KChIP1 is targeted by its myristoylation motif to post-ER transport vesicles where it could interact with and regulate the traffic of Kv4 channels to the plasma membrane under the influence of localised Ca^{2+} signals.

Key words: Calcium-binding proteins, NCS-1, KChIP, Potassium channels

Introduction

The neuronal calcium sensor (NCS) proteins are a family of EF-hand containing Ca^{2+} binding proteins. They include the proteins NCS-1 (frequenin), recoverin, the VILIPs/neurocalcins, GCAPs and KChIPs (Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001) that have a diverse array of functions including the regulation of phototransduction (Dizhoor et al., 1995; Ray et al., 1992), neurotransmitter release (McFerran et al., 1998; Pongs et al., 1993), learning (Gomez et al., 2001) dopamine receptor internalisation (Kabbani et al., 2002), channel function (An et al., 2000; Weiss et al., 2000; Weiss and Burgoyne, 2002) and activation of phosphatidylinositol 4-kinase (Hendricks et al., 1999) leading to changes in Ca^{2+} signalling (Koizumi et al., 2002). With the exception of KChIPs 2-4, all of the NCS proteins are predicted to be N-terminally myristoylated. Numerous cellular proteins involved in signal transduction are targeted to discrete membrane microdomains such as cholesterol- and sphingolipid-enriched rafts (Simons and Toomre, 2000). Many such proteins are acylated and N-terminal myristoylation (Farazi et al., 2001) of these proteins can be essential but not necessarily sufficient for stable membrane association (Resh, 1999; Zacharias et al., 2002). Myristoylation is determined by a 10-17 residue motif at the N terminus of the protein (Farazi et al., 2001; Maurer-Stroh et al., 2002). Several plasma membrane proteins are known to be targeted by a combination of myristoylation with palmitoylation of a nearby cysteine (Alland et al., 1994; Carreno et al., 2000) or the presence of a polybasic domain that can be some distance from the N terminus (Kwong and Lublin, 1995; Silverman and Resh, 1992). Much less is known about the targeting of myristoylated proteins localised to intracellular organelles. Despite the weak affinity of myristoylated peptides for lipid bilayers (Peitzsch and McLaughlin, 1993), certain myristoylation motifs are sufficient to stably target green fluorescent protein (GFP) variants to membranes within cells

(McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001) whereas other myristoylation motifs attached to GFP-fusion proteins remain cytosolic (Zlatkine et al., 1997). The myristoylation motif derived from the NCS protein hippocalcin is sufficient for its intracellular targeting (O'Callaghan et al., 2002), suggesting that targeting information must reside within the 10 or so amino acids of the motifs but nothing is known about the additional targeting information in myristoylation motifs that determines intracellular localisation.

Recoverin, the first of the NCS proteins to be characterised at a structural level, possesses a reversible Ca^{2+}/myristoyl switch mechanism whereby Ca^{2+}-binding leads to exposure of the myristoyl group and subsequent membrane association (Ames et al., 1997). The Ca^{2+}/myristoyl switch is present in certain other but not all NCS proteins despite them being myristoylated (Hwang and Koch, 2002a; Hwang and Koch, 2002b; Ivings et al., 2002; O'Callaghan et al., 2002; Oleshevskaya et al., 1997). Biochemical analysis suggested that the myristoyl group of NCS-1 is exposed even in the absence of Ca^{2+} (Ames et al., 2000; McFerran et al., 1999). In addition, analysis of full-length proteins fused to GFP variants in live cells (O'Callaghan et al., 2002) or native protein in fixed cells (Bourne et al., 2001; Martone et al., 1999) showed that NCS-1 was already associated with the TGN and the plasma membrane at resting or lowered Ca^{2+} levels. In contrast, the closely related protein hippocalcin was cytosolic but translocated to the same sites as NCS-1 following Ca^{2+} elevation (O'Callaghan et al., 2002). Similar Ca^{2+}-dependent translocation to the TGN and plasma membrane was also observed for untagged neurocalcin δ (Ivings et al., 2002; O'Callaghan et al., 2002) and VILIP-1 was shown to possess the Ca^{2+}/myristoyl switch in hippocampal neurons where it also translocated to the plasma membrane and TGN following an increase in cytosolic Ca^{2+} concentration (Spilker et al., 2002). In all cases, membrane association required N-terminal myristoylation and the minimal myristoylation sequence from hippocalcin was sufficient for localisation (O'Callaghan et al., 2002).

Other members of the NCS protein family are the KChIPs (An et al., 2000), a subfamily that directly interact with the pore-forming α-subunits of Kv4 K^+ channels (Baldwin et al., 1991; Jan and Jan, 1997; Pak et al., 1991; Serodio et al., 1994). The rapidly inactivating A-type K^+ channels are important in controlling the excitability of neurons and cardiac myocytes (Serodio et al., 1994). KChIPs regulate the function of Kv4 channels by directly interacting and modifying channel properties, implying that they are channel subunits. In addition, KChIPs increased the cell surface channel density in co-expressing cells (An et al., 2000; Bahring et al., 2001; Hatano et al., 2002; Holmqvist et al., 2002; Morohashi et al., 2002; Takimoto et al., 2002). KChIP2 and 3 are targeted to the plasma membrane by palmitoylation and their plasma membrane localisation is enhanced by co-expression of Kv4.3 (Takimoto et al., 2002). In contrast, KChIP1 is not palmitoylated but predicted instead to be myristoylated at its N terminus (An et al., 2000). Ca^{2+}-binding to KChIP1 is required for its effect on plasma membrane expression (An et al., 2000). It is not known whether KChIP1 shows Ca^{2+}-dependent association with membranes (a Ca^{2+}/myristoyl switch) and little is known about the factors determining its localisation or how this could contribute to the trafficking of Kv4 channels. It is beginning to become apparent that several NCS proteins control the membrane traffic of channels and receptors (An et al., 2000; Kabbani et al., 2002; Lin et al., 2002) but the underlying mechanisms are not known. One issue to be resolved is the cellular compartments within which NCS proteins such as KChIPs interact with their target proteins to affect their traffic. We have, therefore, characterised the intrinsic targeting of Kv4.2 and KChIP1, and compared KChIP1 to the targeting of other members of the NCS family of Ca^{2+} binding proteins. We have determined key residues within the myristoylation motif that determines the localisation of these proteins to either TGN/plasma membrane or to structures involved in ER to Golgi transport. Unlike other K^+ channels that have been examined (Ma and Jan, 2002), Kv4.2 is not retained in the ER but can traffic to the Golgi complex when expressed alone. We suggest that the regulation of Kv4 channel traffic by KChIP1 could occur through interaction of these proteins during ER to Golgi traffic of the channels.

Materials and Methods

Plasmids

The hippocalcin-EYFP fusion construct (pHippo-EYFP), hippocalcin(14)-EYFP and the NCS-1-ECFP fusion construct (pNCS-1-CFP) were as described previously (O'Callaghan et al., 2002). The NCS-1-EYFP construct (pNCS-1-YFP) was made by replacing the CFP tag of the NCS-1-ECFP fusion construct (pNCS-1-CFP) described previously (O'Callaghan et al., 2002) with the YFP tag of the pEYFP-N1 vector (Clontech, California, USA) using the endonucleases *Ba*mHI and *Not*I. The KChIP1-EYFP construct (pKChIP1-EYFP) was made by inserting a KChIP1 sequence, amplified from the IMAGE 2500491 vector (Image consortium, Cambridge, UK) by PCR, into the pEYFP-N1 vector (Clontech, California, USA). The primers contained endonucleases sites (underlined) to facilitate this cloning. The sense primer used was 5′-CCGCTCGAGATGGGGGC-CGTCATGGGCACC-3′ (*Sac*I) and the antisense primer was 5′-ATATGAATTCCCATGACATTTTGAAACAGC-3′ (*Eco*RI). The pcDNA3-KChIP1 construct was made by inserting the KChIP1 sequence, amplified from the pKChIP1-EYFP plasmid by PCR, into the pcDNA3.1(–) vector (Invitrogen). The primers contained endonucleases sites (underlined) to facilitate this cloning. The sense primer used was 5′-CCGGGATCCATGGGGGCCGTCATGGGCACC-3′ (*Bam*HI) and the antisense primer was 5′-ATATAAGCTTACATGA-CATTTTGAAACAGC-3′ (*Hin*dIII). The KChIP1-ECFP construct was made by replacing EYFP with ECFP. The KChIP-1(1-11)-EYFP construct was made by introducting a *Bam*HI endonuclease site into pKChIP-1-EYFP with the primers 5′-TGGGCACCTTCTCAT-CTCTGGATCCCAAACAAAGGCGACCCTCG-3′ and 5′-CGAGG-GTCGCCTTTGTTTGGGATCCAGAGATGAGAAGGTGCCCA-3′ using the QuikChange™ site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands). The *Bam*HI-mutated pKChIP-1-EYFP was digested by the endonuclease *Bam*HI, the digestion fragment removed and the vector backbone religated using standard methods. The ECFP-Kv4.2 construct (pECFP-Kv4.2) was made by inserting the human Kv4.2 sequence (NM_012281), amplified from the AB1701_A07 plasmid (Origene Technologies, Inc., Maryland, USA) by PCR, into the pECFP-C1 vector (Clontech, California, USA). The primers contained endonucleases sites (underlined) to facilitate this cloning. The sense primer used was 5′-CTTCAGAATTCCATGGCGGCGGGGGTGGCA-3′ (*Eco*RI) and the antisense primer was 5′-ATTCCAGGTACCTTACAAAGCA-GAAACTC-3′ (*Kpn*I). The non-myristoylated hippocalcin pKChIP-1(G2A)-EYFP and other mutations were made using the QuikChange™ site-directed mutagenesis kit. The sequences of all the constructs were confirmed using automated DNA sequencing by

Oswel, Southampton, UK. The plasmid pEYFP-Mem was obtained from Clontech (Palo Alto, California, USA).

Transfection

The transfection reaction mixture contained 93 μl Dulbecco's modified Eagle's medium (Gibco-BRL, Paisley, UK), 3 μl Fugene™ (Roche, UK) and 4 μl plasmid DNA (250 μg/μl). This was incubated at room temperature for 30 minutes before being added drop-wise to cells in a 24-well plate. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 24-72 hours before being used in experiments.

Permeabilisation experiments

Transfected HeLa cells were washed three times in Krebs-Ringer buffer and incubated for 15 minutes in 10 μM digitonin in 300 μl permeabilisation buffer (potassium glutamate 139 mM, Pipes 20 mM, EGTA 5 mM; 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 protein leakage assay, the permeabilisation buffer was removed and added to 1.5 ml tubes and centrifuged at 12,000 rpm for 10 minutes. The cells remaining in the wells were solubilised in Laemmli buffer (Sigma, Dorset, UK). The supernatant was carefully removed and added to 1.5 ml tubes containing 300 μl of cold methanol and placed at –20°C for 30 minutes. The tubes were then centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded, the pellet air-dried and resuspended in 100 μl Laemmli buffer (Sigma, Dorset, UK). All samples were boiled for 10 minutes and then used for polyacrylamide gel electrophoresis and western blotting.

Transferrin uptake

Transfected HeLa cells on coverslips in a 24-well plate were washed three times in Krebs-Ringer buffer and incubated with 2.5 μg/ml of biotinylated-transferrin (Sigma, Dorset, UK) at 37°C for 30 or 60 minutes. The cells were then fixed with 500 μl of 4% formaldehyde. The cells were washed three times in Krebs-Ringer buffer and incubated with streptavidin-Texas Red, diluted 1 in 50 in Krebs-Ringer buffer, for 30 minutes. The cells were then finally washed three times in Krebs-Ringer buffer, air dried and mounted.

Immunocytochemistry

Cells attached to coverslips to be immunostained were washed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4) and fixed in PBS containing 4% formaldehyde for 30 minutes. The cells were incubated in PBT (PBS, 0.1% Triton X-100, 0.3% bovine serum albumin) for 30 minutes. The PBT was removed and the primary antibody added at the appropriate dilution in PBT. The primary antibody was incubated for 1 hour, removed and the cells washed three times in PBT. The cells were incubated for a further hour with the appropriate biotinylated secondary antibody (Amersham, Buckinghamshire, UK), diluted to 1 in 100 with PBT. The cells were washed three times in PBT and then incubated in streptavidin-Texas Red (Amersham, Buckinghamshire, UK) diluted 1 in 50 with PBT for 30 minutes. The cells were washed three times with PBT. The coverslips were dried and mounted in antifade glycerol.

Confocal laser scanning microscopy

For confocal laser scanning microscopy, live and or fixed transfected cells were examined with a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany) using a 22 μm pin-hole and a 63× water immersion objective with a 1.2 numerical aperture. For optimal imaging of the spatial distribution of KChIP1-EYFP, the cells were excited at 514 nm and light collected at 545-625 nm. GFP was imaged with excitation at 488 nm and light collected at 500-550 nm. For dual imaging of EYFP and ECFP, the cells were excited at 514 nm and light collected at 560-600 nm for EYFP or excited at 458 nm and light collected at 465-500 nm for ECFP detection. Texas Red was imaged using excitation at 543 nm and light collection at 600-650 nm. Images were exported as TIFF files and compiled in CorelDraw.

Results

Intracellular localisation of Kv4.2 K^+ channels and effect of co-expression of NCS proteins

Previous work has shown that co-expression with KChIP1 increased the current density of heterologously expressed Kv4 K^+ channels and this has been attributed to increased cell surface expression of the channels and changes in localisation to the plasma membrane of both KChIP1 and Kv4 channel α-subunits (An et al., 2000; Hatano et al., 2002). It is not known, however, how traffic of the Kv4 channels is regulated or where Kv4 and KChIP1 interact in the secretory pathway. Therefore, we set out to examine the effect of co-expression of NCS proteins and Kv4.2 channels on their localisation and also to determine which intracellular compartments the individual proteins are associated with, using GFP-variant fusion proteins. A fusion of ECFP to the N terminus of Kv4.2 was constructed because such a fusion protein for Kv4.3 has been shown to have similar electrophysiological properties to wild-type channels and to respond normally to KChIP1 (Hatano et al., 2002). Kv4.2 expressed alone in COS-1 or HEK293 cells has been shown to remain in an intracellular perinuclear compartment (An et al., 2000; Hatano et al., 2002) but the nature of this compartment has not be examined. ECFP-Kv4.2 also had a perinuclear localisation in transfected HeLa cells and did not co-localise with the ER markers calnexin (not shown) or protein disulphide isomerase (PDI). Partial colocalisation with ERGIC-53, a marker for the ER-Golgi intermediate compartment, was seen (Fig. 1) but ECFP-Kv4.2 showed clearer co-localisation with the cis-Golgi marker β-COP and to a lesser extent with the TGN marker γ-adaptin (Fig. 1). In contrast to many other channel types that have been examined (Ma and Jan, 2002; Margeta-Mitrovic et al., 2000; O'Kelly et al., 2002; Standley et al., 2000; Zerangue et al., 1999) Kv4.2 is, therefore, not retained within the ER when expressed alone but can traffic at least as far as the Golgi complex.

Initially we aimed to establish that we could demonstrate increased plasma membrane association of ECFP-Kv4.2 by co-expression of wild-type untagged KChIP1 by examining co-localisation with a plasma membrane-targeted EYFP (EYFP-Mem). In double transfections, ECFP-Kv4.2 showed an intracellular localisation with no overlap with EYFP-Mem (Fig. 2A-C). In contrast, in triple transfected cells with pcDNA3-KChIP1, ECFP-Kv4.2 was now detected on regions of the plasma membrane although some intracellular ECFP-Kv4.2 remained (Fig. 2D-F). Having established that we can demonstrate re-location of ECFP-Kv4.2 to the plasma membrane by co-expression of KChIP1, we next examined the effects on ECFP-Kv4.2 localisation of co-expression of EYFP fusion proteins of NCS-1, hippocalcin or KChIP1. In cells expressing NCS-1-EYFP or hippocalcin-EYFP, ECFP-Kv4.2

was localised intracellularly and did not extensively overlap with either of these NCS proteins (Fig. 3A-F). In contrast, co-expression of KChIP1-EYFP resulted in increased plasma membrane localisation of both proteins with a high level of co-localisation (Fig. 3, G-I). Based on this assay the EYFP fusion protein of KChIP1 retains the ability to interact with and

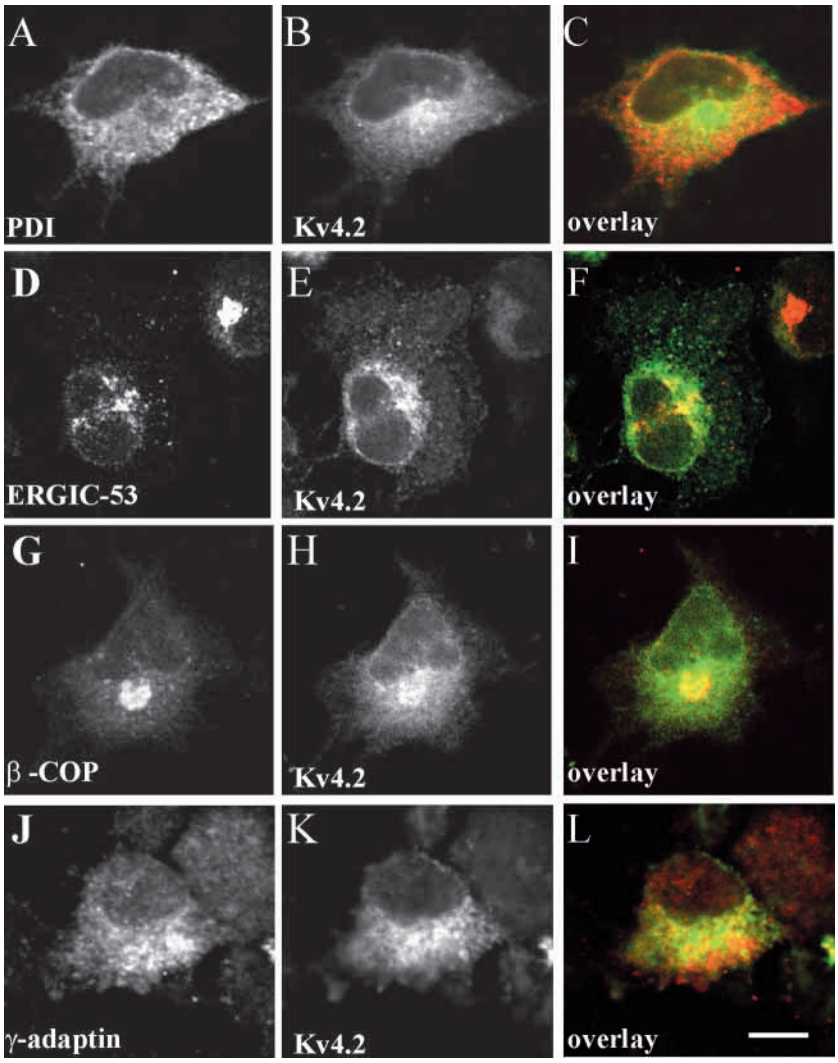


Fig. 1. Localisation of ECFP-KV4.2 within the secretory pathway. HeLa cells were transfected with plasmid encoding ECFP-Kv4.2. After fixation the cells were immunostained with anti-PDI (A-C), anti-ERGIC-53 (D-F), anti-β-COP (G-I) or anti-γ-adaptin (J-L) visualised with Texas Red-streptavidin and imaged by laser scanning confocal microscopy. Black and white images are shown in each case for ECFP-Kv4.2 and antibody staining as indicated in the overlaid images shown in colour (Kv4.2 in green and antibody staining in red with overlap seen in yellow). Scale bar: 10 μm.

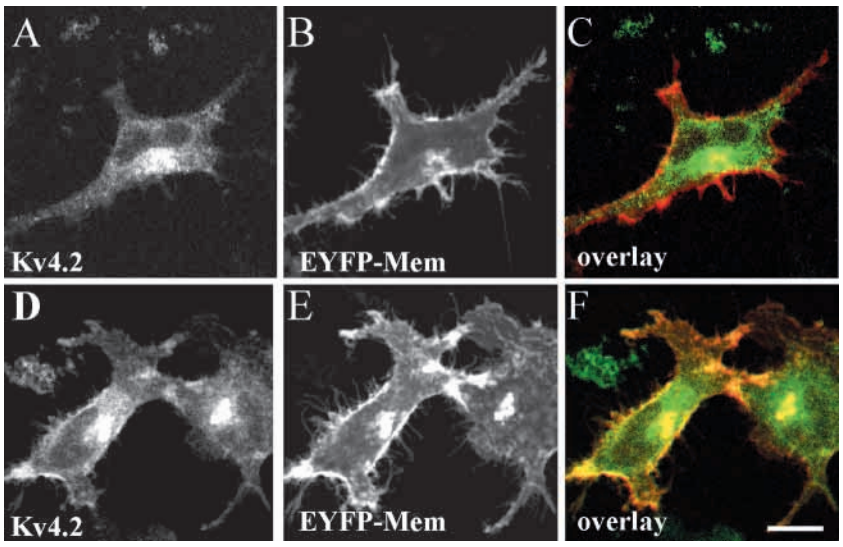


Fig. 2. Effect of KChIP1 on the localisation of ECFP-Kv4.2. HeLa cells were transfected with plasmids encoding ECFP-Kv4.2 and EYFP-Mem together (A-C) or in a triple transfection in combination with pcDNA3-KChIP1 (D-F). The localisation of ECFP-Kv4.2 and EYFP-Mem are shown individually and in a combined colour image (overlay; ECFP-Kv4.2 in green, EYFP-Mem in red) with co-localisation shown in yellow. Triple transfection resulted in translocation of ECFP-Kv4.2 to regions of the plasma membrane. Scale bar: 10 μm.

regulate the traffic of Kv4.2 as reported for wild-type protein (An et al., 2000; Hatano et al., 2002).

Comparison of the localisation of KChIP1 and NCS-1 in HeLa cells

To examine the basic factors determining the subcellular localisation of KChIP1 and where it is likely to initially interact with Kv4 channels in the secretory pathway, the EYFP fusion protein of KChIP1 was expressed alone in HeLa cells without Kv4.2. These cells were chosen as they do not normally express KChIPs, Kv4 K^+ channels or other NCS proteins and so allow analysis of the intrinsic targeting mechanisms of the NCS proteins. Previous work on NCS-1 has shown that this protein associates with the same organelles in HeLa cells as the endogenous protein does in neurons. HeLa cells transfected to express both NCS-1-ECFP and KChIP1-EYFP showed distinct distributions of the proteins (Fig. 4A). While both proteins have some perinuclear localisation, KChIP1 has a more obvious punctate distribution unlike that for NCS-1 that is associated with the TGN, both in HeLa cells (O'Callaghan et al., 2002) and neurons (Martone et al., 1999). A more diffuse distribution of NCS-1 was also visible because of its association with the plasma membrane. The association of NCS-1 with the plasma membrane was confirmed by examination of serial confocal sections which showed most intense fluorescence close to the substratum. Little of the NCS-1 is cytosolic in these cells (O'Callaghan et al., 2002). The distribution of the two proteins was unaffected by expression levels or time of expression over 16-72 hours and was similar in living and fixed cells. KChIP1 appeared to be largely membrane associated as little leakage was seen from cells permeabilised in either the absence or presence of Ca^{2+} (Fig. 4B). In contrast, hippocalcin and neurocalcin δ, which exhibit the Ca^{2+}/myristoyl switch, are retained in permeabilised cells only when Ca^{2+} is present (O'Callaghan et al., 2002). In addition, no change in distribution of KChIP1-EYFP was seen following Ca^{2+} elevation in living cells using the Ca^{2+} ionophore ionomycin (Fig. 4C). KChIP1, therefore, does not display a Ca^{2+}-myristoyl switch mechanism but is constitutively membrane associated. This is similar to the situation previously observed for NCS-1 but with targeting to distinct organelles and is in contrast with the Ca^{2+}-dependent translocation of other NCS proteins in HeLa cells.

Membrane-association of KChIP1 requires N-terminal myristoylation

In order to determine whether the punctate targeting of KChIP1-EYFP requires N-terminal myristoylation the glycine at position 2 was mutated to alanine. This non-myristoylated mutant showed a diffuse pattern consistent with cytoplasmic localisation (Fig. 5A,B). Following digitonin-permeabilisation KChIP1-EYFP was retained within the cells on punctate structures (Fig. 5C) but all of the KChIP1(G2A)-EYFP was lost from the cells apart from a residual amount in the nucleus (Fig. 5D) confirming that the non-myristoylated protein was not membrane bound. To determine if the myristoylation motif of KChIP1 is not only necessary but also sufficient for membrane localisation, the N-terminal 11 amino acids of KChIP1 were fused to EYFP. Initially after a 24 hours transfection, this construct showed a similar punctate localisation to that seen for full-length KChIP1-EYFP (Fig. 5E). After more prolonged transfection times (up to 3 days), however, the minimal myristoylation motif of KChIP1 resulted in a progressive enlargement of the associated structures which were tightly localised to the perinuclear region (Fig. 5F). This

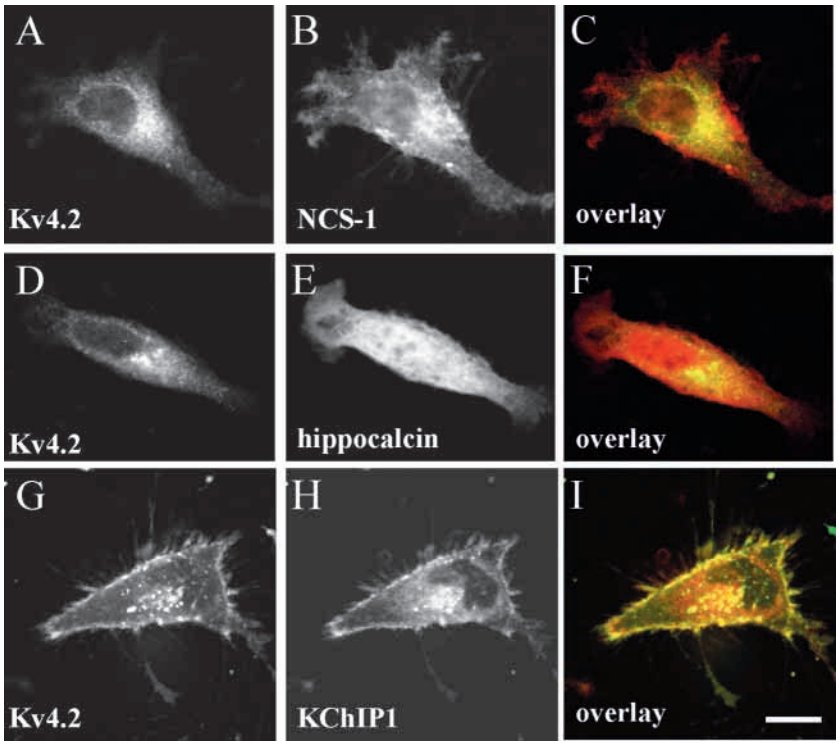


Fig. 3. Localisation of ECFP-Kv4.2 in HeLa cells co-expressing NCS-1 (A-C), hippocalcin (D-F), KChIP1 (G-I) EYFP fusion proteins. HeLa cells were co-transfected with ECFP-Kv4.2 and the indicated EYFP fusion protein and single and combined colour images (overlay; Kv4.2 in green and the EYFP in red) are shown. Scale bar: 10 μm.

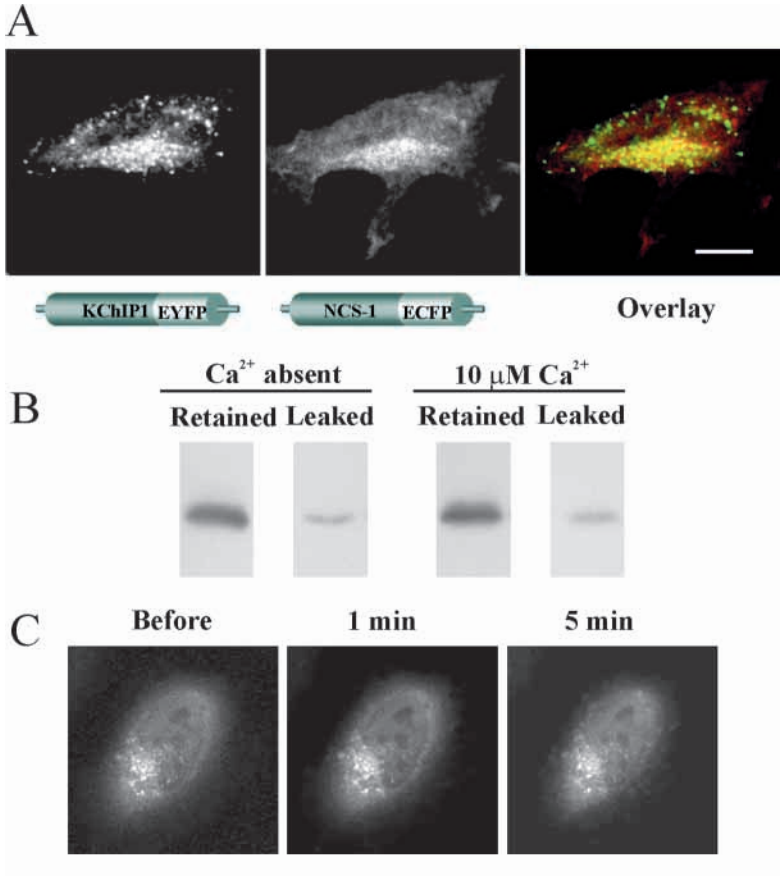


Fig. 4. Differential localisation of KChIP1 and NCS-1 expressed in HeLa cells. In dual-transfected cells (A), NCS-1-ECFP showed a perinuclear localisation because of association with the TGN and a diffuse distribution because of plasma membrane association. In contrast, KChIP1-EYFP had a predominantly punctate localisation. The overlaid colour image shows KChIP1-EYFP in green and NCS-1-ECFP in red. (B) Following permeabilisation with digitonin KChIP1-EYFP remained mainly cell associated in the absence and presence of Ca^{2+}. (C) KChIP1-EYFP localisation in HeLa cells during live cell imaging before and after 1- and 5-minute treatment with 3 μM ionomycin. Scale bar: 10 μm.

was not due to a different targeting of this construct compared to the full-length protein as both KChIP1-ECFP (Fig. 5G) and KChIP1(1-11)-EYFP (Fig. 5H) were localised to these same structures following co-transfection. We also examined targeting by a minimal myristoylation motif (residues 1-14) from hippocalcin that is closely similar to that of NCS-1. In dual transfected cells, the myristoylation motif from hippocalcin targeted EGFP to exactly the same sites (plasma membrane and TGN) as NCS-1 (Fig. 6A-C). In contrast, KChIP1(1-11)-EYFP showed a distinct localisation to that seen for NCS-1 even after organelle enlargement (Fig. 6D-F). These results show that the N-terminal myristoylation motifs of KChIP1 and hippocalcin contain all the information that determines the distinct intracellular localisation of these proteins.

In order to assess whether or not the targeting of NCS-1, hippocalcin and KChIP1 to distinct organelles was peculiar to HeLa cells, we also examined their localisation in PC12 cells. These cells were chosen as they are a widely used neuronal model and also they express endogenous NCS-1 localised to the plasma membrane and perinuclear organelles (Rajebhosale et al., 2003). NCS-1-ECFP and hippocalcin (1-14)-EGFP showed a distinct plasma membrane and perinuclear localisation (Fig. 7A,B) whereas KChIP1-EYFP and KChIP1(1-11)-EYFP were localised to punctate structures (Fig. 7C,D). In addition, ECFP-Kv4.2 had a perinuclear localisation when expressed alone but was redistributed to the plasma membrane in cells co-expressing KChIP1-EYFP (Fig. 7E,F). It appears, therefore, that the distinct targeting of the NCS proteins that we have studied in HeLa cells is likely to represent the situation in cells that endogenously express NCS proteins.

Characterisation of residues within N-terminal myristoylation sequences that determine intracellular targeting

Comparison of the N-terminal sequences of NCS-1, hippocalcin and KChIP1 showed that these were very similar in NCS-1 and hippocalcin but that of KChIP1 was completely different beyond gly2. We noted the presence of three basic residues conserved in NCS-1 and hippocalcin. These are also conserved in neurocalcin δ, another family member that translocates to the plasma membrane and TGN in response to Ca^{2+} (O'Callaghan et al., 2002), but these basic residues are absent in KChIP1. Basic residues in some proteins are involved in interactions with acidic phospholipids such as phosphoinositides (Ford et al., 2001; Lemmon and Ferguson, 2000) and contribute to targeting of certain myristoylated Src-family members to the plasma membrane (Kwong and Lublin, 1995; Silverman and Resh, 1992). These were, therefore, chosen for mutagenesis as indicated in Fig. 8. All of the mutations were made in constructs containing only the myristoylation motifs. Since these mutations would be within the recognition motif for N-myristoyl transferase we initially checked that the constructs would be substrates for myristoylation using a newly refined algorithm (Maurer-Stroh et al., 2002). With one exception, noted below, all of the constructs were predicted to be good substrates for myristoylation by eukaryotic N-myristoyl transferase and in

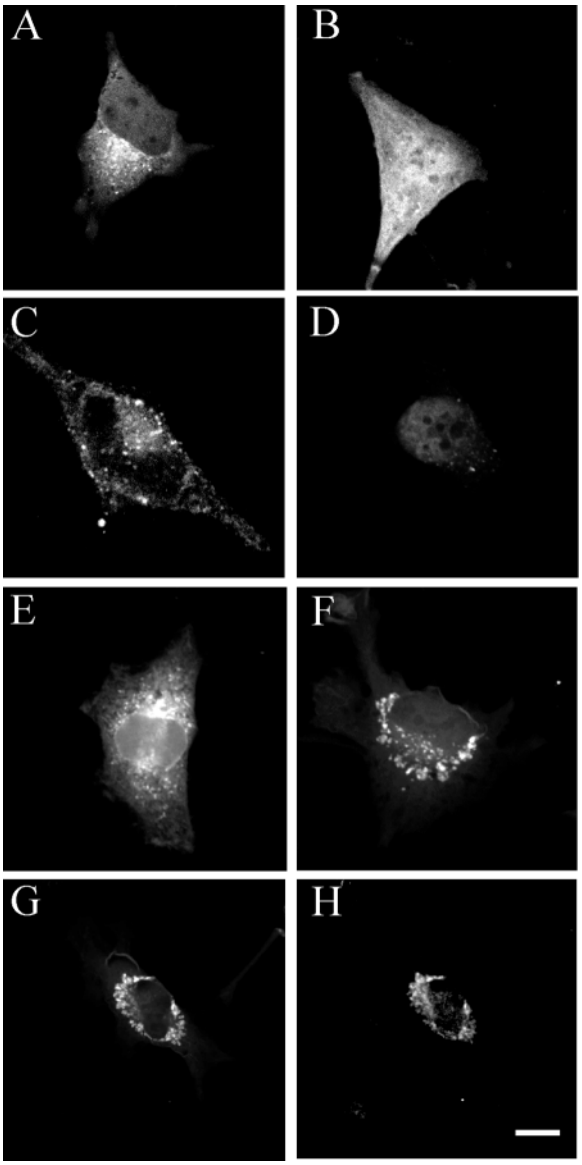


Fig. 5. Localisation of KChIP1 requires myristoylation and a minimal myristoylation motif is sufficient. HeLa cells were transfected to express KChIP1-EYFP (A,C), KChIP1(G2A)-EYFP (B,D), KChIP1(1-11)-EYFP (E,F) or KChIP1-ECFP and KChIP1(1-11)-EYFP (G,H) and examined after 3 days (A-D, F-G) or 1 day (E). The cells in C and D were fixed and examined after permeabilisation with 1 μM digitonin for 15 minutes in the absence of added Ca^{2+}. The images in G and H are from the same co-transfected cell after 3 days. Scale bar: 10 μm.

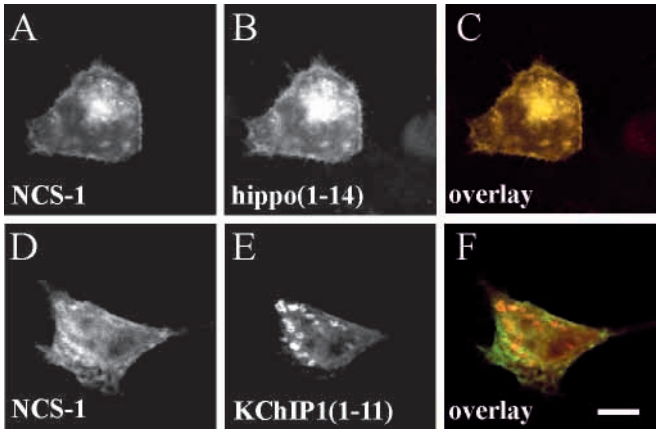


Fig. 6. Hippocalcin(1-14) co-localises with NCS-1 but KChIP1(1-11) does not. Cells were transfected to express hippocalcin(1-14)-EGFP or KChIP1-EYFP along with NCS-1 either as a wild-type or ECFP-tagged construct. In these dual transfected cells the minimal myristoylation sequence from hippocalcin (hippocalcin (1-14)) targeted EGFP (B) to the same sites as NCS-1 detected by immunofluorescence (A) with complete overlap of localisation as shown in yellow in the overlaid image (C). In contrast, KChIP1(1-11)-EYFP (E) showed a distinct localisation to that of NCS-1-ECFP (D,F). The colour overlays show NCS-1 in green and the other constructs in red. Scale bar: 10 μm.

fact were found to be membrane-associated. Mutation of all three basic residues in hippocalcin(1-14)-GFP to the non-charged residues found in KChIP1 was sufficient to convert the targeting from TGN (Fig. 8A) to a punctate pattern (Fig. 8D) similar to that seen with KChIP1(1-11)-EYFP (Fig. 8E). Mutation of only the lysine at position 3 or of lysines at positions 3 and 7 was insufficient to fully prevent localisation to the TGN, but more punctate fluorescence was visible (Fig. 8B,C) suggesting that all three basic amino acids contributed to the localisation to the TGN and plasma membrane. The corresponding positions in KChIP1 were mutated to basic residues as found in hippocalcin. Mutation to basic residues at position 7 and 9 of KChIP1(1-11)-EYFP did not prevent the punctate localisation (Fig. 8F). A construct with mutations in all three residues 3, 7 and 9 showed a diffuse cytoplasmic distribution (Fig. 8G). This was expected as a lysine at position 3 is predicted to prevent myristoylation unless position 6 is a serine. We, therefore, made further mutations in positions 3, 7 and 9 in a construct bearing serine at position 6 to ensure efficient myristoylation. Mutation to basic amino acids at position 3, or 3 and 7 did not modify the localisation from the punctate pattern (Fig. 8H,I). In contrast, a marked change in localisation was seen with the construct A3K, G6S, T7K, S9R with all three positions mutated to basic amino acids. This now showed a clear plasma membrane and perinuclear TGN-like pattern (Fig. 8J) with exact co-localisation with NCS-1-ECFP (Fig. 8K) as indicated in the merged image in Fig. 8L. These data show that the three basic residues can switch targeting from punctate structures to the TGN and that within the KChIP1 sequence all three positions must be mutated to change the targeting.

Role of the myristoylation of KChIP1 in the traffic of Kv4.2 K^+ channels to the plasma membrane

In order to test the requirement for myristoylation of KChIP1 for the traffic of Kv4.2 K^+ channels, ECFP-Kv4.2 was co-expressed with wild-type KChIP1-EYFP or with its G2A mutant. Some expression of Kv4.2 at the cell surface was observed even with the G2A mutant. A quantitative analysis of the percentage of total ECFP-Kv4.2 at the plasma membrane revealed that this was markedly increased by co-expression with KChIP1-EYFP (Fig. 9A,B). Co-expression with KChIP1(G2A)-EYFP was significantly less effective in increasing ECFP-Kv4.2 at the cell surface as the increase over

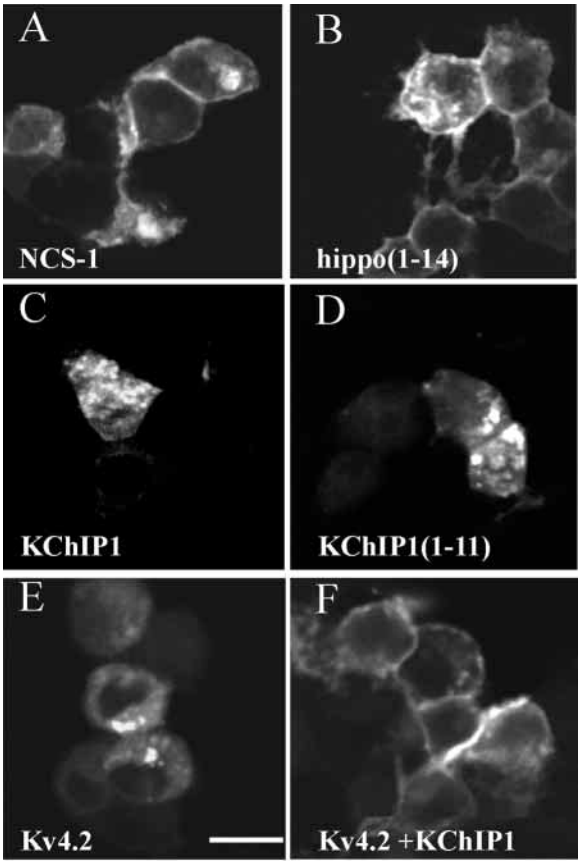


Fig. 7. Localisation of NCS-1, KChIP1 and Kv4.2 in PC12 cells. PC12 cells were transfected to express NCS-1-ECFP (A), hippocalcin(1-14)-EGFP (B), KChIP1-EYFP (C), KChIP1(1-11)-EYFP (D), ECFP-Kv4.2 (E) or ECFP-Kv4.2 along with KChIP1-EYFP (F). KChIP1-EYFP and KChIP1(1-11)-EYFP showed more punctate intracellular staining than NCS-1-ECFP and hippocalcin(1-14)-EGFP and the latter constructs that were also localised to the plasma membrane. For the cells co-expressing ECFP-Kv4.2 and KChIP1-EYFP only the image for ECFP-Kv4.2 is shown in F but essentially complete overlap of the two constructs was observed. Scale bar: 10 μm.

that seen with ECFP-Kv4.2 alone was reduced by 60%. These data suggest that myristoylation of KChIP1 increased its efficiency in stimulating traffic of Kv4.2 K^+ channels to the cell surface. Some effect of the G2A mutant on Kv4.2 traffic, as observed, would be expected as this soluble non-myristoylatable form of KChIP1 would still be able to interact with Kv4.2. As a further test of the significance of the targeting of KChIP1, the N terminus of full-length KChIP1-EYFP was mutated to incorporate those residues that allow targeting to the TGN. Co-expression of KChIP1(A3K,G6S,T7K,S9R)-EYFP with ECFP-Kv4.2 did not stimulate any detectable traffic of the K^+ channels to the cell surface and both proteins were concentrated in the perinuclear Golgi region (Fig. 9C).

Characterisation of the intracellular compartment with which KChIP1 is associated

The punctate distribution of KChIP1 when expressed alone differed from that of the ER lumenal protein PDI, or markers for mitochondria, the endosomal pathway (transferrin uptake) or early endosomes (EEA1). Antisera for a range of rab proteins were used as markers of various endosomal compartments including rab4, rab5 (early endosomes), rab11 (recycling endosomes) and rab7 (late endosomes) (Zerial and McBride, 2001). None of these showed convincing co-localisation with KChIP-EYFP (see Fig. S1, http://jcs.biologists.org/supplemental/). We therefore examined markers for compartments and vesicles involved in traffic between the ER and the Golgi complex. Anti-ERGIC53 (Schweizer et al., 1988) was used as a marker for the ER/Golgi intermediate compartment (Hauri et al., 2000; Klumperman et al., 1998). Sec13 was examined as a marker of COPII-coated vesicles at ER exit sites and the transitional ER (Hammond and Glick, 2000) and β-COP for cis-Golgi. KChIP1-EYFP did not co-localise with Sec13 or β-COP nor did it exactly co-localise with ERGIC-53. The perinuclear KChIP1-EYPF labelled structures were, however, closely associated with ERGIC-53-positive structures (Fig. 10).

We made use of the finding that expression of KChIP1(1-11)-EYFP leads to progressive enlargement of the compartment on which KChIP1-EYFP is also expressed. In cells expressing KChIP(1-11)-EYFP, the enlarged structures showed essentially complete overlap for KChIP(1-11)-EYFP and ERGIC-53. In addition, as they increased in size KChIP1(1-11)-EYFP also overlapped with β-COP. These enlarged organelles were not labelled for Sec13 (Fig. 10), Rabs4, 5, 7 or 11 nor did they accumulate transferrin (data not shown) suggesting that they represent a specifically enlarged ERGIC. This could result from blockade of the traffic from this compartment that is mediated by COPI (β-COP-containing) vesicles as suggested by the accumulation of β-COP on the larger structures. In support of this interpretation, ECFP-Kv4 in cells co-expressing KChIP(1-11) was also partially localised to the enlarged compartment (Fig. 10) indicating that traffic from the ERGIC to the cis-Golgi of the newly synthesised channels had been disrupted.

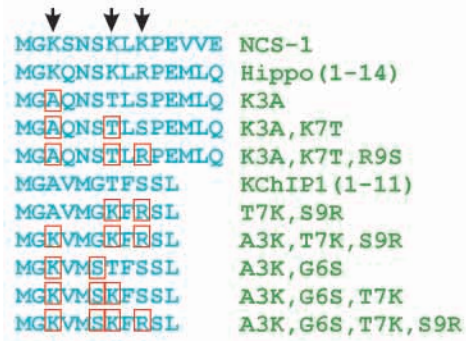
Discussion

We have made a number of observations in this study. First, Kv4.2 K^+ channels when expressed alone in heterologous cells are not impeded in their traffic to the plasma membrane by retention in the ER but can traffic at least as far as the cis-Golgi complex. Second, KChIP1, NCS-1 and hippocalcin contain information within their myristoylation motifs which is both necessary and sufficient for their specific intracellular targeting. Third, KChIP1 when expressed alone is localised to punctate structures which appear to be post-ER pre-Golgi transport vesicles. Fourth, targeting of KChIP1 to these vesicles by myristoylation is necessary for efficient traffic of Kv4.2 K^+ channels to the cell surface. The finding that Kv4.2 can traffic to the cis-Golgi is surprising as several characterised channels and receptors that do not reach the cell surface when expressed alone are regulated by retention within the ER. They need the association of other escort proteins to shield an ER retrieval/retention signal to allow them to traffic beyond the ER (Ma and Jan, 2002; Margeta-Mitrovic et al., 2000; O'Kelly et al., 2002; Standley et al., 2000; Zerangue et al., 1999). Our finding that expressed Kv4.2 co-localises, at least in part, with the cis-Golgi marker β-COP is consistent with the lack of a

conserved recognised ER retention motif in the N-terminal 40 amino acids of Kv4 channels with which KChIPs interact and which determine the intracellular retention of the Kv4 subunits when they are expressed alone (Bahring et al., 2001). Another recent study has concluded that Kv4.2 is retained in the ER when expressed alone (Shibata et al., 2003). Close examination of the data presented indicate, however, that Kv4.2 did not co-localise with the ER marker calnexin.

NCS proteins are either cytosolic or associated with the cytosolic faces of intracellular membranes in resting cells at basal Ca^{2+} concentrations. Previous work has established that NCS-1 is localised to the TGN in neuronal and non-neuronal cells based on co-localisation with γ-adaptin (Bourne et al., 2001; O'Callaghan et al., 2002) and by electron microscopy (Martone et al., 1999) and that hippocalcin, neurocalcin δ and VILIP-1 translocate to the same sites following elevation of cytosolic Ca^{2+} concentration (Ivings et al., 2002; O'Callaghan et al., 2002; Spilker et al., 2002). In previous work (Ivings et al., 2002; O'Callaghan et al., 2002) and in the present study we have examined localisation in transfected HeLa cells that do not express the NCS proteins. The rationale behind the use of HeLa cells rather than neuronal cells was that this would allow analysis of the intrinsic targeting of these proteins independent of interactions with neuronal-specific interacting proteins. It does appear that localisation of NCS proteins in HeLa cells faithfully represents the situation in neurons as the same organelle localisation for NCS-1 has been reported for endogenous and expressed protein in HeLa cells, adrenal chromaffin cells, PC12 cells and neurons in vivo (Martone et al., 1999; O'Callaghan et al., 2002; Pan et al., 2002). Here we report a punctate localisation of KChIP1 that was observed not only in HeLa cells but also in PC12 cells. We have now demonstrated that KChIP1, like NCS-1, does not have a Ca^{2+}/myristol switch mechanism, being already membrane-associated at resting Ca^{2+} concentration. NCS-1 and KChIP1 are targeted, however, to distinct organelles. In each case, membrane-association requires N-terminal myristoylation. In addition, we have shown that the NCS proteins possess intrinsic targeting information within their N-terminal myristoylation motifs that determines their association with distinct intracellular organelles.

The N-terminal eight amino acids of myristoylated proteins are required for recognition by N-myristoyl transferase but are diverse in sequence (Farazi et al., 2001; Resh, 1999). The data presented here demonstrate a crucial role for basic residues at positions 3, 7 and 9 in the N-terminal myristoylation motif for targeting of NCS proteins to the TGN and plasma membrane and non-charged residues in these positions for targeting to the punctate structures. The importance of the N-terminal residues is supported by the finding that N-terminal basic residues of membrane-associated myristoylated recoverin appear to interact directly with the lipid bilayer (Valentine et al., 2003). The organelles that we have investigated are not a default



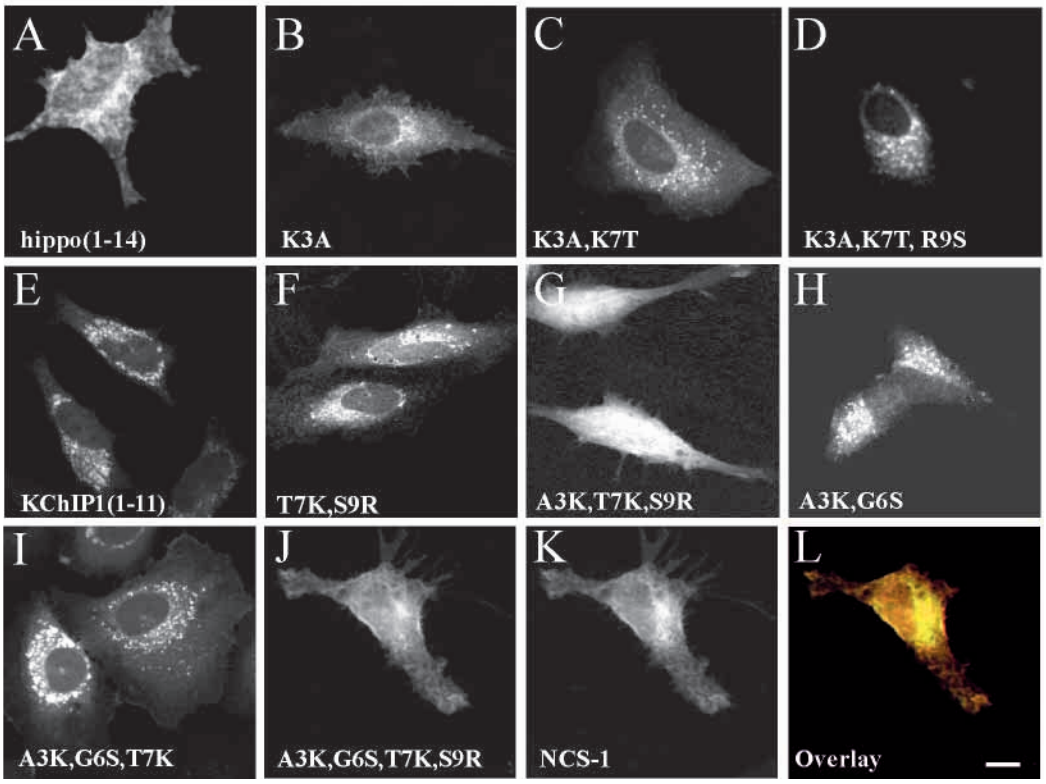


Fig. 8. Effect of mutations within the myristoylation motifs of hippocalcin and KChIP1 on intracellular targeting. (Top) N-terminal sequences of hippocalcin and KChIP1 showing the mutations introduced and the positions of the conserved basic residues in NCS-1 and hippocalcin (arrows). (A-H) Localisation of minimal myristoylation sequences with the indicated mutations is shown after expression of EGFP (hippocalcin) or EYFP (KChIP1) fusion proteins in HeLa cells. The localisation of KChIP1(A3K,G6S, T7K, S9R)-EYFP was examined in cells co-transfected to also express NCS-1-CFP and the localisation of each fusion protein is shown individually and in an overlaid colour image where co-localisation is seen in yellow. Scale bar: 10 μm.

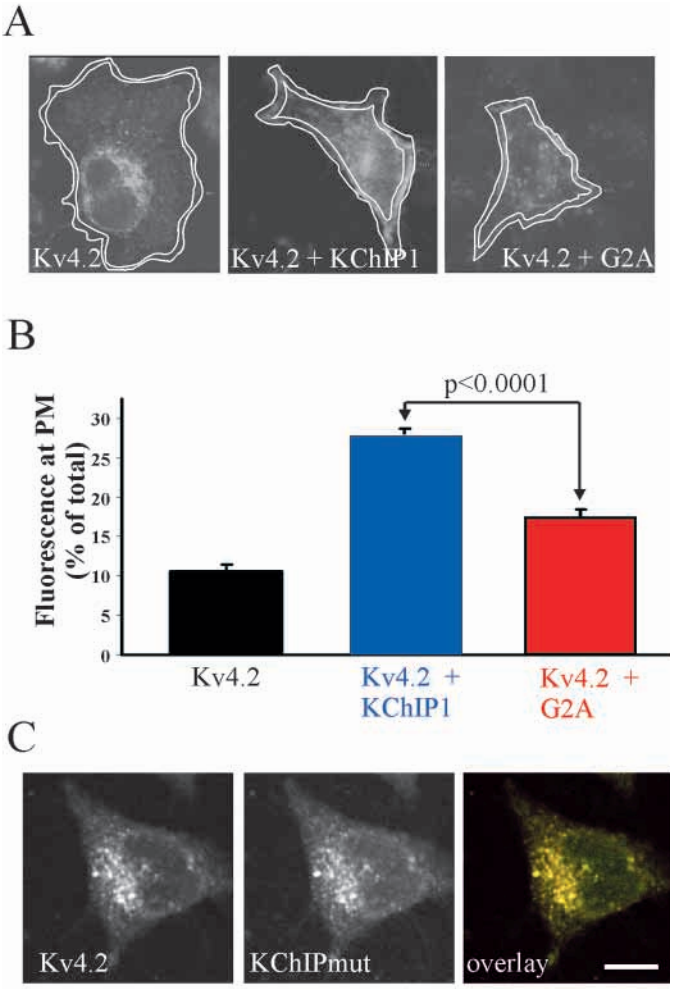


Fig. 9. Quantification of the effect of KChIP1 on ECFP-Kv4.2 traffic to the plasma membrane and requirement for correct targeting of KChIP1. (A) HeLa cells were transfected to express ECFP-Kv4.2 alone or in combination with KChIP1-EYFP or KChIP1(G2A)-EYFP as indicated. ECFP-Kv4.2 fluorescence was imaged and quantified by drawing around regions of interest on the outside and the inside of the plasma membrane to allow determination of the percentage of total fluorescence at the plasma membrane. (B) Mean data±s.e.m. derived from 25 cells expressing ECFP-Kv4.2 alone and 47 and 24 co-expressing KChIP1-EYFP or KChIP1(G2A)-EYFP, respectively. (C) HeLa cells transfected to co-express KChIP1(A3K,G6S,T7K,S9R)-EYFP (KChIPmut) and ECFP-Kv4.2 did not show cell surface expression of Kv4.2. The overlaid colour image shows Kv4.2 in red and KChIP1(A3K,G6S,T7K,S9R)-EYFP in green with co-localisation in yellow. Scale bar: 10 μm.

localisation for myristoylated proteins as GFP with other myristoyl motifs remain cytosolic (Zlatkine et al., 1997). Certain Src family members that do not have an N-terminal cysteine for palmitoylation, such as Src itself, are targeted to the plasma membrane because of the presence of multiple basic residues both within the myristoylation motif and also at positions 14-16 that are required for most efficient plasma membrane localisation (Kwong and Lublin, 1995; McCabe and Berthiaume, 1999; Silverman and Resh, 1992). These proteins do not have a basic residue at position 3 demonstrating the importance of a lysine in this position for targeting to the TGN.

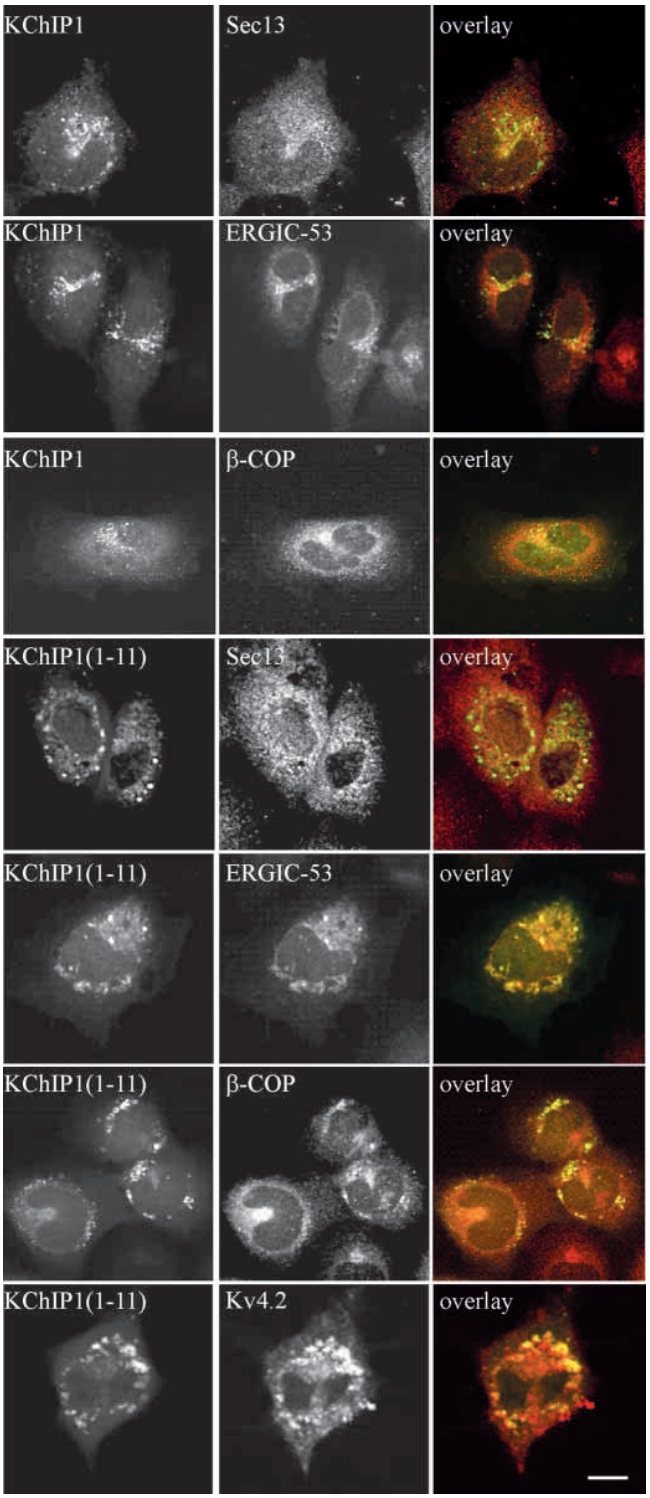


Fig. 10. Comparison of the localisation of KChIP1 and markers for the transitional ER and ERGIC compartment. HeLa cells were transfected to express KChIP1-EYFP or KChIP1(1-11)-EYFP, fixed, immunostained with the indicated antibodies visualised with Texas Red-streptavidin and imaged by laser scanning confocal microscopy. HeLa cells in the bottom row were transfected with KChIP1(1-11)-EYFP and ECFP-Kv4.2. Black and white images are shown in each case as indicated with overlaid images shown in colour with KCHIP-EYFP or KChIP1(1-11)-EYFP in green and antibody staining or ECFP-Kv4.2 in red and overlap seen in yellow. Scale bar: 10 μm.

Interestingly, a recently discovered myristoylated protein that is localised to the Golgi, GARP-1 also has lysines at position 3 and 7 (Eberle et al., 2002). However, there was no examination of whether its minimal myristoylation motif was sufficient for Golgi targeting of this protein. The targeting of myristoylated proteins to distinct organelles could potentially be mediated either by specific protein interactions or lipid preference coupled with differences in lipid composition of membranes. If protein-protein interactions are responsible then this must involve binding of the myristoylation motifs to proteins that are not neuronal specific.

When Kv4 channels are expressed alone in heterologous cell types some channel expression at the cell surface can be detected by electrophysiolgical recording. The current density of these channels can be substantially increased by 15- to 60-fold by co-expression with KChIPs (An et al., 2000; Bahring et al., 2001). It has been suggested that the increased current density of Kv4 K^+ channels due to KChIP co-expression is a result of an increase in channels at the cell surface (An et al., 2000; Hatano et al., 2002). We have demonstrated that increased localisation of ECFP-Kv4.2 on the plasma membrane can be observed in HeLa cells co-transfected to express KChIP1 or KChIP1-EYFP. The specificity of this effect was shown by the lack of obvious plasma membrane targeting or co-localisation of Kv4.2 with co-expression of either NCS-1-EYFP or hippocalcin-EYFP under our conditions. NCS-1 has previously been suggested to increase the channel density in Kv4-expressing cells although the effect was markedly smaller than that due to KChIPs, being only about a 70% increase (Guo et al., 2002; Nakamura et al., 2001). More recently, it was shown that both KChIP and NCS-1 modified the properties of Kv4 channels expressed in lobster neurons but only KChIP increased the current density (Zhang et al., 2003). In another study, direct interaction of NCS-1 with Kv4 α-subunits was apparently not observed (Ren and Takamoto, 2002) and so the extent of interaction between NCS-1 and Kv4 channels remains to be resolved but cannot be ruled out by the observations reported here.

We suggest that the punctate structures identified by KChIP1-EYFP are likely to be post-ER transport vesicles or components of the vesiculo-tubular complexes (VTCs) involved in traffic to the Golgi complex (Bannykh and Balch, 1997). In fact, in live cell imaging experiments the KChIP1-EYFP-labelled structures were mobile in a microtubule-dependent (nocadozole-sensitive) manner (B.H. and R.D.B., unpublished observations) as previously described for such structures (Presley et al., 1997). The reasons for the conclusion that they are post-ER vesicles are as follows. The strongest argument for the identification of these vesicles comes from the analysis of the KChIP1(1-11)-EYFP construct. This initially localises with the full-length KChIP1-EYFP in the same punctate structures which then enlarge over time and become progressively ERGIC-53 and β-COP positive. This is consistent with the KChIP1-EYFP and KChIP1(1-11)-EYFP being present on vesicles trafficking to the ERGIC and then transport out of this compartment by COPI vesicles being blocked by KChIP1(1-11)-EYFP. A similar enlargement of the ERGIC has been seen following blockade of outward traffic at low temperature (Klumperman et al., 1998). The mechanisms by which such a small motif from KChIP1 could do this is unclear but our interpretation is supported by the observation that when KChIP1(1-11)-EYFP and Kv4.2 are co-expressed much of the Kv4.2 accumulated in the enlarged ERGIC-53-positive structures, indicating that transport of newly synthesised channels to the Golgi was blocked. Kv4 α-subunits do not interact directly with the N terminus of KChIP1 (An et al., 2000) and so this localisation is likely to be due to inhibition of the traffic of Kv4.2 out of the ERGIC by KChIP1(1-11)-EYFP by an indirect mechanism. The peripheral KChIP-labelled vesicles are not labelled by ERGIC-53 but those in the perinuclear region are closely associated with ERGIC-53-positive structures. The distribution of the KChIP1 vesicles throughout the cell with a concentration in the perinuclear region is as expected for such transport vesicles. Labelling of post-ER transport vesicles by antiserum against Sec13, a CopII coat component, is not expected as these vesicles are rapidly uncoated following budding from the ER (Stephens et al., 2000).

We have demonstrated that targeting of KChIP1 to post ER vesicles contributes to the traffic of Kv4.2 K^+ channels to the cell surface as shown by the reduced plasma membrane expression of ECFP-Kv4.2 following co-expression with the non-myristoylatable G2A mutant of KChIP1. This protein was distributed throughout the cell, explaining why it could still interact with Kv4.2 and stimulate a low level of traffic. Significantly, no evidence for increased traffic of Kv4.2 to the cell surface was observed in cells expressing a form of KChIP1-EYFP that was mis-targeted to the TGN. We suggest that in cells normally expressing both Kv4.2 and KChIP1, these proteins would meet and interact with each other at the level of post-ER transport vesicles so that their interaction would prevent subsequent retention of Kv4.2 in the Golgi and thereby allow efficient forward transport of the channels to the plasma membrane.

The distinct organelle localisation of NCS proteins generated by the intrinsic N-terminal targeting information that we have described may be functionally important in Ca^{2+} sensing by the NCS proteins and in how they contribute to the regulation of membrane traffic. Cells can generate not only global but also very localised Ca^{2+} signals (Berridge et al., 2000; Bootman et al., 2001). The findings reported here indicate that different NCS proteins will react to distinct Ca^{2+} signals because of their targeting. NCS proteins such as hippocalcin, neurocalcin δ and VILIP-1 that possess a Ca^{2+} myristoyl switch mechanism (Ivings et al., 2002; O'Callaghan et al., 2002; Spilker et al., 2002) would require a global and prolonged Ca^{2+} elevation, from Ca^{2+} entry or release from intracellular stores, to allow translocation from the cytosol to membranes. In contrast, NCS-1 will be able to respond to brief Ca^{2+} transients close to the TGN/plasma membrane but KChIP1 would respond to distinct signals. Local Ca^{2+} release is functionally important for membrane traffic from a pre-Golgi compartment to the Golgi complex (Chen et al., 2002) and within the Golgi complex (Porat and Elazar, 2000). The increase in Kv4 channel density by co-expression with KChIP1 is dependent on the presence of functional EF-hands (An et al., 2000). NCS proteins such as KChIP1 may, therefore, act as Ca^{2+} sensors with specific roles in post-ER and post-Golgi membrane traffic.

This work was supported by grants from the Wellcome Trust to R.D.B. D.W.O'C. and B.H. were supported by Wellcome Trust Prize

Studentships. Antisera were kindly provided by M. Clague (University of Liverpool; EEA1), W. Balch (Scripps Institute, SanDiego; Sec13), H-P. Hauri (University of Basel; ERGIC-53) and M. Zerial (Max-Planck Insitute, Dresden; Rab7). We would like to thank Professor Alexei Tepikin (University of Liverpool) for assistance with confocal microscopy.

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