Identification of Ca^{2+}-dependent binding partners for the neuronal calcium sensor protein neurocalcin δ: interaction with actin, clathrin and tubulin

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The neuronal calcium sensors are a family of EF-hand-containing Ca^{2+}-binding proteins expressed predominantly in retinal photoreceptors and neurons. One of the family members is neurocalcin δ, the function of which is unknown. As an approach to elucidating the protein interactions made by neurocalcin δ, we have identified brain cytosolic proteins that bind to neurocalcin δ in a Ca^{2+}-dependent manner. We used immobilized recombinant myristoylated neurocalcin δ combined with protein identification using MS. We demonstrate a specific interaction with clathrin heavy chain, α- and β-tubulin, and actin. These interactions were dependent upon myristoylation of neurocalcin δ indicating that the N-terminal myristoyl group may be important for protein–protein interactions in addition to membrane association. Direct binding of neurocalcin δ to clathrin, tubulin and actin was confirmed using an overlay assay. These interactions were also demonstrated for endogenous neurocalcin δ by co-immunoprecipitation from rat brain cytosol. When expressed in HeLa cells, neurocalcin δ was cytosolic at resting Ca^{2+} levels but translocated to membranes, including a perinuclear compartment (*trans*-Golgi network) where it co-localized with clathrin, following Ca^{2+} elevation. These data suggest the possibility that neurocalcin δ functions in the control of clathrin-coated vesicle traffic.

Key words: cytoskeleton, neurons, *trans*-Golgi network, VILIP.

INTRODUCTION

Ca^{2+} plays an important role in the regulation of numerous cell processes, including neurotransmitter release [1], changes in gene expression and many aspects of neuronal function [2,3]. The actions of Ca^{2+} are, usually, effected via Ca^{2+}-binding proteins. A superfamily of small Ca^{2+}-buffering and Ca^{2+}-sensing proteins is characterized by the possession of various numbers of a Ca^{2+}-binding motif, the so-called EF-hand [4]. The best studied of these, calmodulin, is ubiquitously expressed, whereas others are expressed in only certain tissues. The neuronal calcium sensor (NCS) family of proteins are expressed predominantly in the nervous system [5], implying key roles in the control of specific aspects of neuronal function [6]. At least twelve of these NCS proteins are expressed in mammals including frequenin (NCS-1), visinin-like proteins (VILIPs; including neurocalcins [7] and hippocalcin [8]), recoverins [9], guanylyl cyclase activating proteins [10], and the potassium channel interacting proteins [11].

NCS proteins have an N-terminal myristoyl group and four EF-hand-like domains, the first of which in the sequence is non-functional in Ca^{2+}-binding [6]. In the case of recoverin, which has been characterized in detail, it has been shown that the first EF-hand domain serves to cradle the myristoyl group in the absence of bound Ca^{2+} [12]. Ca^{2+} binding results in major changes in the conformation of recoverin, which exposes the myristoyl group [13], making it available to bind to membranes or other proteins; this has been referred to as the Ca^{2+}-myristoyl switch [14]. The assumption has been that the exposure of the myristoyl group would be sufficient for direct lipid insertion and that this is its only function [14]. It is not certain, however, whether the myristoyl group of NCS proteins in all cases binds directly to lipid membranes, or is associated with them via a protein receptor. Experiments with recoverin suggest that a protein receptor is not required [14]; however, data on hippocalcin point to an additional involvement of a protein in the interactions between the myristoylated protein and membranes [15].

Information on the structure and mechanism of function of the NCS proteins is accumulating [6], but their cellular roles have not yet been completely elucidated. Recoverin is expressed only in the retina and has been shown to directly regulate rhodopsin kinase [16]. Several other NCS proteins, including neurocalcin α (also known as VILIP-1), and NCS-1, which are expressed in the brain, can also regulate phosphorylation of rhodopsin *in vitro*, and so phosphorylation of proteins involved in signal transduction in the relevant tissues could be one of the targets of NCS proteins [17]. Frequenin overexpression in *Drosophila* leads to enhanced neurotransmitter release at neuromuscular junctions [18], and overexpression of the mammalian orthologue in PC12 cells increased evoked exocytosis [19]. It has also been shown that NCS-1 regulates non-L-type Ca^{2+} channels in bovine adrenal chromaffin cells [20,21]. Recent studies have shown that NCS-1 is necessary for associative learning and memory in *Caenorhabditis elegans* [22]. In addition, the yeast orthologue regulates the phosphatidylinositol-4-OH kinase Pik1 [23].

Neurocalcin δ (otherwise known as VILIP-3) is a high-affinity Ca^{2+} sensor (K_d of approx. 0.6 μM), which is most abundantly expressed in the Purkinje cells of the cerebellum [5,24–26]. It has been sequenced [7], and its structure has been determined by X-ray crystallography [27]. Neurocalcin δ binds three Ca^{2+} ions and this binding is co-operative [28]. Despite this information, nothing is known about the function of neurocalcin δ. There are two important questions remaining to be answered. What are the binding partners for neurocalcin δ, and what are the cellular functions of neurocalcin δ? In an attempt to begin to answer

Abbreviations used: ARF, ADP-ribosylation factor; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl β-D-thiogalactoside; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; NCS, neuronal calcium sensor; myr-ARF, myristoylated ARF; myr-NCS-1, myristoylated NCS-1; myr-neurocalcin δ, myristoylated neurocalcin δ; NMT, *N*-myristoyl transferase; TGN, *trans*-Golgi network; VILIP, visinin-like protein.

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these questions, recombinant neurocalcin δ protein was produced and used in a search for its target proteins in rat brain. Major binding partners of neurocalcin δ were identified using column chromatography with immobilized recombinant protein, combined with matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS [29]. Using this approach, major binding partners of neurocalcin δ were identified as clathrin, α- and β- tubulin, and actin. The interactions were both Ca^{2+}- and myristoylation-dependent and the interactions were confirmed using independent assays, including co-immunoprecipitation from brain cytosol. These results identify the first specific protein–protein interactions involving neurocalcin δ, and provide a framework for our understanding of its function in neurons.

MATERIALS AND METHODS

Expression and purification of recombinant proteins and preparation of anti-(neurocalcin δ) serum

The plasmid pDL1312 [28], encoding bovine neurocalcin δ, was kindly provided by Dr Daniel Ladant (Institut Pasteur, Paris, France). Myristoylated neurocalcin δ (myr-neurocalcin δ) was expressed and purified as described previously [30]. Briefly, *Escherichia coli* strain DH5α was co-transformed with pDL1312 and with pBB131, a plasmid coding for the yeast *N*-myristoyl transferase (NMT) [31] supplied by Dr J. I. Gordon (Washington University School of Medicine, St Louis, MO, U.S.A.). NMT expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG), and 2 mg/l myristic acid was added. After 30 min, neurocalcin δ expression was induced by shifting the growth temperature to 42°C. Bacteria were grown for a further 2 h, and harvested by centrifugation at 4000 ***g*** at 4 °C for 15 min. Pellets were resuspended in breaking buffer [50 mM Hepes, 100 mM KCl, 1 mM dithiothreitol (DTT) and 1 mM $MgCl_2$, pH 7.5] containing 1 mM PMSF. Following a second centrifugation at 9600 ***g*** at 4 °C for 20 min, pellets were again resuspended in breaking buffer and further protease inhibitors were added (1 mM PMSF, 10 μg/ml leupeptin and 1 mM pepstatin A). Samples were kept at −80 °C until further use. Cells were thawed and incubated on ice with 1 vol. of breaking buffer containing 0.2 mM EGTA and 1 mg/ml lysozyme for 30 min, further disrupted by sonication, incubated with 2 μg/ml DNaseI, passed through a needle several times to reduce viscosity, and centrifuged at 100000 ***g*** at 4 °C for 1 h. The supernatant was applied to a phenyl-Sepharose column in breaking buffer containing 1 mM $CaCl_2$, and neurocalcin was eluted with breaking buffer containing 5 mM EGTA, all at 4 °C. Aliquots of the fractions were boiled in SDS-dissociation buffer [4% (w/w) SDS, 2 mM EDTA, 10% (w/v) sucrose, 1% (v/v) 2-mercaptoethanol, 0.02% Bromophenol Blue, 125 mM Hepes and 10% (w/v) glycerol] separated by SDS/PAGE for analysis and fractions containing neurocalcin were pooled and stored at −80°C. Non-myr-neurocalcin δ was produced according to the protocol above, but without the addition of myristic acid and without IPTG induction of NMT. Myristoylated NCS-1 (myr-NCS-1) [32] and myristoylated ADP-ribosylation factor (myr-ARF) [33] were expressed and purified as described previously. The extent of myristoylation of the purified proteins was assessed by MS analysis and was at least 50% of the total protein. Anti-(neurocalcin δ) was prepared as a rabbit antiserum by immunization with purified recombinant neurocalcin δ using a similar protocol to that described previously for the preparation of anti-NCS-1 serum [19]. The antiserum was affinity-purified by binding to and elution from immobilized recombinant neurocalcin δ [19].

Preparation of cytosolic fractions from rat brain

Two Wistar rats were killed by cervical dislocation, and the whole brains were homogenized in 10 ml of 20 mM Tris/HCl, 1 M KCl, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF, pH 8.0, and centrifuged at 100000 ***g*** for 1 h at 4 °C. The supernatant (the cytosolic fraction) was dialysed overnight in 20 mM Tris/HCl, 50 mM KCl and 1 mM DTT, pH 7.8, clarified by centrifugation at 100000 ***g*** for 60 min at 4 °C, and stored at −80 °C until further use.

Chromatography using immobilized neurocalcin δ and NCS-1

In initial experiments, 1 g of CNBr-activated Sepharose 4B beads (Sigma) was reconstituted and washed three times in water. Recombinant neurocalcin δ (2.4 mg in 4 ml) and an equal volume of 0.5 M carbonate buffer (pH 9.0) were added to the beads. The mixture was incubated overnight at 4 °C with mixing. It was then centrifuged and washed three times with carbonate buffer. The remaining active groups were blocked with 1 M ethanolamine (pH 9.0) for 2 h at 4 °C with mixing. Three cycles of alternating washes with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, and 0.1 M Tris/HCl buffer (pH 8.0) containing 0.5 M NaCl, were carried out. Beads were then washed three times with binding buffer [25 mM Tris/HCl, 50 mM KCl, 1 mM DTT, 5 mM EGTA and 0.5% (v/v) Tween 20, pH 7.8]. The myr-NCS-1–Sepharose beads were prepared similarly. In both cases essentially complete linkage of added protein was achieved. The beads were incubated with 19 mg of rat brain cytosol and 10 ml of binding buffer containing 6 mM $CaCl_2$ (BB + Ca) overnight at 4 °C with mixing. The mixture was poured on to the column and washed through with 30 ml of BB + Ca. Fractions (12 × 1 ml) were eluted with binding buffer, and further fractions (12 × 1 ml) were eluted with binding buffer containing 500 mM NaCl [BB + HS (high salt)]. Samples of fractions were boiled in SDS-dissociation buffer, separated on an SDS/10% (w/v) polyacrylamide gel and silver stained. Control columns without bound protein were prepared as above, but neurocalcin δ was replaced with an equal volume of carbonate buffer. For large-scale columns, 3.5 g of beads was used, with 3.5-fold increased amounts of neurocalcin δ and rat brain cytosol. Following incubation, the column was washed through with 60 ml of BB + Ca. Fractions (9 × 2 ml) were eluted with binding buffer, followed by a wash with 12 ml of binding buffer, and 9 × 2 ml fractions were eluted with BB + HS, followed by a wash with 12 ml of BB + HS. When stored for longer periods, the beads were stored in BB + HS containing 0.05% sodium azide at 4 °C.

Concentration of protein fractions

Methanol (600 μl; at −20°C) was added to 600 μl aliquots of pooled column fractions. Soya-bean trypsin inhibitor (25 μl of 2.5 mg/ml) was added to each sample in methanol as a carrier protein. The mixture was left at −20 °C for 30 min and centrifuged in a micro centrifuge for 5 min at 4 °C at 15000 rev./min. The supernatant was removed, and the pellet was left to dry at approx. 24 °C for 30–60 min and subsequently boiled in SDS-dissociation buffer prior to SDS/PAGE. Overall, 8 ml of column fractions was concentrated 27-fold into a final volume of 300 μl.

MALDI–TOF MS

Protein bands were excised from Coomassie Brilliant Blue-stained gels, cut into small pieces and placed in 0.5 ml Eppendorf tubes pre-rinsed with acetonitrile and methanol. The gel pieces were washed with 100 μl of 50% (v/v) acetonitrile/25 mM

ammonium bicarbonate (pH 7.8) for 15 min with occasional gentle agitation and then dried in a SpeedVac for 15–30 min. The gel pieces were rehydrated in 4 μl of 25 mM ammonium bicarbonate containing 10 ng/μl sequencing grade trypsin (Promega) and incubated at 37 °C overnight. The resulting peptides were extracted by the addition of 4 μl of water, followed by 7 μl of 30 % (v/v) acetonitrile/0.1 % trifluoroacetic acid, mixing and brief centrifugation (this being repeated as necessary depending on the volume of the gel pieces). The supernatants were recovered and mixed 1:1 with matrix [10 mg/ml α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/50 % (v/v) ethanol/0.001 % trifluoroacetic acid] containing adrenocorticotropic hormone (50 fmol/μl), and 1 μl of the mixture was spotted on to a 96-position target. Peptide mass fingerprints were obtained semi-automatically on a MALDI mass spectrometer (M@LDI; Micromass) and resultant mass lists were searched against a non-redundant protein database (Swiss-Prot/Trembl) using ProteinLynx 3.4 (Micromass).

Biotinylated protein overlays

Actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), clathrin and tubulin (all purchased from Sigma) were boiled in SDS-dissociation buffer, and 0.4 μg of protein was loaded per lane for PAGE. Biotinylation and overlays were carried out as described previously for NCS-1 [32]. Myr-neurocalcin δ and myr-ARF were incubated with a 100 molar excess of the biotinylating reagent [6-(biotinamidocaproylamido) *N*-hydroxysuccinimide ester; Sigma] for 2 h at approx. 24 °C. The reaction was stopped by adding glycine to a final concentration of 100 mM. The biotinylated probes were dialysed against 20 mM Hepes, 139 mM NaCl, 2 mM ATP, 5 mM EGTA and 5 mM nitriloacetic acid, pH 7.4, and stored at −20 °C until further use. Nitrocellulose membranes were incubated with blocking solution [5 % (w/v) milk, 5 % (w/v) BSA, 5 % (v/v) fetal calf serum and 0.5 % (v/v) Tween 20 in the above buffer] for 2 h with three changes. For incubation with biotinylated probes, blocking solution containing $MgCl_2$ and $CaCl_2$ to obtain the required free Ca^{2+} concentration was used (in these experiments, 10 μM). Membranes were incubated with 4 μg/ml biotinylated myr-neurocalcin δ or 9.6 μg/ml myr-ARF overnight at 4 °C (the protein concentrations used were established by dot-blotting with the probes to give signals of corresponding intensity). In all the subsequent washes and incubations, the appropriate free Ca^{2+} concentration was maintained. Membranes were rinsed in the above buffer containing 0.5 % Tween 20, then washed in the above buffer three times for 15 min. Membranes were incubated with streptavidin-horseradish peroxidase (1:400 dilution) in blocking solution for 30 min, and washed as above. Biotinylated probes were detected using enhanced chemiluminescence.

Plasmid preparation

The following primers were used to amplify neurocalcin δ from the pDL1312 vector: forward primer, 5′-CCAGGATCCATGGGCAAGCAGAACAGCAA-3′; and reverse primer, 5′-CCGAAGCTTTCAGAACTGGCTAGCACT-3′. The underlined sequences indicate restriction sites (for *Bam*HI and *Hin*dIII respectively) incorporated to facilitate cloning. The amplified product was ligated into pcDNA3.1(−) vector. The following primers were used to introduce the Gly2 → Ala (G2A) mutation in the neurocalcin δ pcDNA3.1(−) plasmid: forward primer, 5′-GGACTAGTGGATCCATGGCAAAACAGAACAGCAAGCTGCG-3′; and reverse primer, 5′-CGCAGCTTGCTGTTCTGTTTTGCCATGGATCCACTAGTCC-3′. Plasmid DNA was analysed by automated sequencing.

Immunoblotting

Samples were boiled for 5 min in SDS-dissociation buffer, separated by SDS/PAGE and transferred on to nitrocellulose membranes. The membranes were incubated in blocking solution [3 % (w/v) milk in PBS] for 45 min, and then with the appropriate antibody in 3 % (w/v) milk and 0.5 % (v/v) Tween 20 in PBS for 1 h [anti-(neurocalcin δ) at 1:400 dilution, anti-(clathrin heavy chain) (ICN Biochemicals) at 1:500 dilution, anti-β-actin (Sigma) at 1:7000 dilution, anti-α-tubulin (Sigma) at 1:4000 dilution, anti-GAPDH (Insight Biotechnology) at 1:300 dilution and anti-NCS-1 [19] at 1:1000 dilution]. The membranes were washed once in 0.5 % (v/v) Tween 20 in PBS, and then three times in PBS. The membranes were incubated with the appropriate peroxidase-conjugated secondary antibody at 1:400 dilution and washed as before. Bound antibodies were detected using enhanced chemiluminescence.

HeLa cell culture and transfection

HeLa cells were cultured in 75 cm^2 flasks in 20 ml of Dulbecco's modified Eagle's medium with pyruvate and high glucose (Gibco) containing 1 % (v/v) non-essential amino acids and 5 % (v/v) foetal calf serum. Cells were passaged when approaching confluency, and plated into 24-well plates at 4×10^4 cells/well. Cells were transfected 16–24 h after plating using 1 μg of each plasmid DNA and GeneJuice (Novagen), at a ratio of 1:3 (μg:μl), according to the manufacturer's protocol. For immunoblotting, cells were lysed and boiled in SDS-dissociation buffer 3 days after transfection.

Immunofluorescence and confocal laser scanning microscopy

Three days after transfection, HeLa cells grown on coverslips were washed twice in a Krebs buffer [19], and incubated in 1 μM ionomycin and 3 mM Ca^{2+}, or Ca^{2+} only as a control, in Krebs buffer for 10 min. The buffer was removed, the cells were fixed with 3.7 % (v/v) formaldehyde in PBS for 30 min, and kept at 4 °C until use. Cells were washed once in PBS, then incubated with 0.1 % (v/v) Triton X-100 and 0.3 % (w/v) BSA in PBS (PBT) for 45 min. Cells were incubated with affinity-purified anti-(neurocalcin δ) at 1:100 dilution and anti-(clathrin light chain) (Santa Cruz Biotechnology) at 1:1500 dilution in PBT for 2 h. After three washes in PBT, cells were incubated with biotinylated anti-mouse secondary antibody (1:100 dilution) and fluorescein-conjugated anti-rabbit secondary antibody at 1:75 dilution (both from Sigma) in PBT for 1 h. Cells were washed three times in PBT, then incubated with 1:50 streptavidin-Texas Red in PBT for 30 min. The coverslips were washed three times in PBT, air-dried, mounted in glycerol/PBS (9:1, v/v) containing 0.25 % 1,4-diazadicyclo[2.2.2]octane and 0.002 % *p*-phenyldiamine, and cells were imaged on a Zeiss Universal microscope using standard Zeiss filter sets. For confocal laser scanning microscopy, fixed transfected HeLa cells were examined with a Leica TCS-SP-MP microscope (Leica Microsystems, Heidelberg, Germany) using a 63× water immersion objective with a 1.2 numerical aperture. For Texas Red, the cells were excited at 545 nm, and light was collected at 650–750 nm; for fluorescein, the cells were excited at 488 nm, and the light was collected at 450–550 nm.

Immunoprecipitation

Anti-(neurocalcin δ) or pre-immune sera were incubated with Protein G–Sepharose Fast Flow beads (Amersham Biosciences) in PBS at 4 °C for 45 min. After washing, the antibodies were cross-linked to the beads by incubation in 20 mM dimethylpimeli-

midate/0.1 M sodium borate (pH 9.2) for 30 min. The suspension was centrifuged and sodium borate washes and the dimethylpimelimidate incubation were repeated. The beads were washed twice in 50 mM glycine (pH 2.5) and then three times in 50 mM Tris/HCl (pH 8.0) and stored at 4 °C with 0.05 % sodium azide. Rat brain cytosol was pre-cleared by incubating with Protein G–Sepharose beads (50 μl/1 ml of cytosol) at 4 °C for 1 h. Antibody beads (40 μl) and 50 μl of pre-cleared cytosol were incubated at 4 °C overnight in 0.5 ml of immunoprecipitation buffer [20 mM Tris/acetate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 5 % (w/v) glycerol, 270 mM sucrose, 0.5 % (v/v) Tween 20, 1 mM benzamidine, 0.1 % (v/v) 2-mercaptoethanol and 10 μg/ml leupeptin, pH 7.0] for 0 Ca^{2+}, or with added $CaCl_2$ to give 10 μM free Ca^{2+}. The suspension was applied to CytoSignal spin filters (Affiniti), washed in immunoprecipitation buffer (maintaining the appropriate Ca^{2+} concentrations) and proteins were eluted in SDS-dissociation buffer.

RESULTS

Search for binding partners of neurocalcin δ in rat brain using neurocalcin δ–Sepharose column chromatography

Purified recombinant myr-neurocalcin δ was produced and bound to CNBr-activated Sepharose 4B beads as described in the Materials and methods section. Supernatants recovered from beads before and after incubation with myr-neurocalcin δ were separated on an SDS/15 % (w/v) polyacrylamide gel and silver-stained; no protein was found in supernatants from beads that had been incubated with myr-neurocalcin δ overnight, indicating that the protein had fully bound to the beads. Rat brain cytosol or membrane extracts were incubated with the myr-neurocalcin δ beads in the presence of Ca^{2+} and bound proteins were eluted with EGTA, and further with high salt. In initial experiments, high background levels of binding of a solubilized membrane extract to control columns precluded identification of specific myr-neurocalcin δ-binding proteins from these membrane extracts when using the myr-neurocalcin δ columns. In contrast, conditions were established, notably the inclusion of 0.5 % Tween 20 in binding and wash buffers, in which almost no non-specific binding was detected to control columns that had been incubated with the brain cytosol fraction. Under these conditions, a number of cytosolic proteins were found to bind specifically to myr-neurocalcin δ, but not to control beads, in a Ca^{2+}-dependent manner, and could be eluted with EGTA (lanes 3 and 4 in Figure 1). In these experiments the silver staining was considerably overdeveloped to enhance the detection of any proteins that might have bound to control beads; as a consequence the pattern of eluted proteins from the myr-neurocalcin δ beads does not accurately represent the relative amounts of the various proteins present within the eluate (see in contrast the Coomassie Brilliant Blue-stained gel in Figure 3). Following elution with EGTA no additional proteins were eluted with high salt buffer (lanes 7–11 in Figure 1), and nor could any additional proteins be recovered following boiling of beads in SDS-dissociation buffer (results not shown), indicating that all protein interactions on the myr-neurocalcin δ column were Ca^{2+}-dependent. The analysis of proteins bound to myr-neurocalcin δ columns was repeated several times with different preparations of beads and rat brain cytosol extracts and on each occasion similar results were obtained.

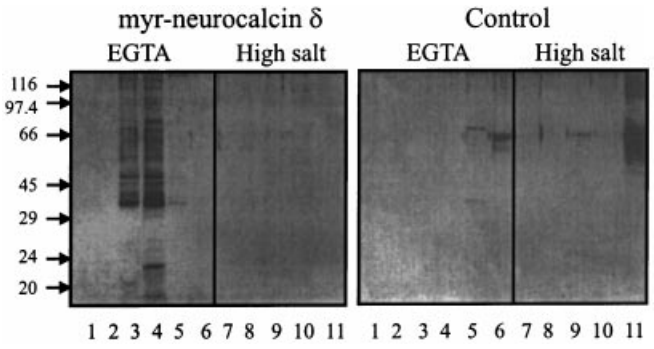

Figure 1 Linkage of myr-neurocalcin δ to CNBr-activated Sepharose 4B beads and Ca^{2+}-dependent binding of rat brain cytosol proteins

Columns consisting of myr-neurocalcin δ bound to Sepharose beads, or control beads (with no protein bound) were incubated with rat brain cytosol in the presence of Ca^{2+}. After washing the columns, six 1 ml fractions were eluted with EGTA, and a further six 1 ml fractions were eluted with high salt. Eluted samples were boiled in SDS-dissociation buffer, separated on an SDS/10% (w/v) polyacrylamide gel, and the gel was silver-stained. Lanes 1–6, EGTA-eluted fractions, lanes 7–11, high salt-eluted fractions.

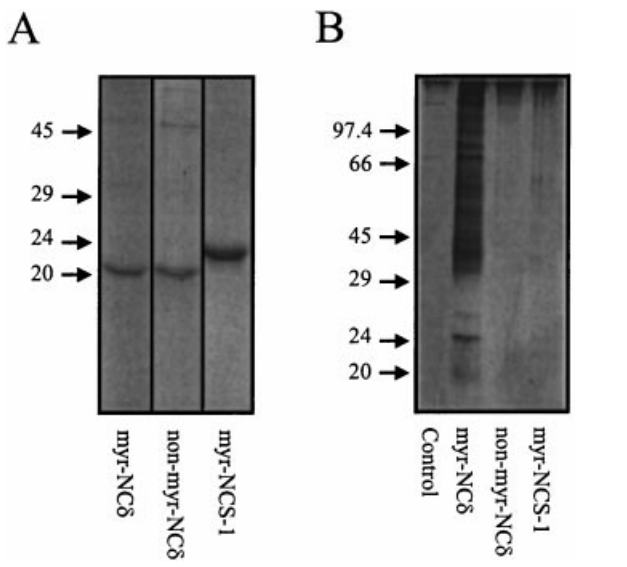

Figure 2 Specificity of the binding and elution of rat brain cytosol proteins from myr-neurocalcin δ in comparison with non-myr-neurocalcin δ and myr-NCS-1

(**A**) SDS/polyacrylamide gel analysis of purified recombinant proteins used to prepare protein–Sepharose columns. (**B**) Recombinant myr-neurocalcin δ, non-myr-neurocalcin δ (NCδ) or myr-NCS-1 were bound to CNBr-activated Sepharose beads and incubated with rat brain cytosol in the presence of Ca^{2+}, and six 1 ml fractions were eluted with EGTA. Corresponding fractions from all columns were pooled, separated by electrophoresis on an SDS/10% (w/v) polyacrylamide gel, and the gel was then silver-stained. Lane 1, EGTA-eluate from control column (beads only); lane 2, EGTA eluate from the myr-neurocalcin δ column; lane 3, EGTA eluate from the non-myr-neurocalcin δ column; lane 4, EGTA eluate from the myr-NCS-1 column.

In order to test further the specificity of protein binding to myr-neurocalcin δ, Sepharose beads were also prepared containing immobilized non-myr-neurocalcin δ or a myristoylated preparation of the closely related protein NCS-1 (Figure 2A). In contrast with the results with myr-neurocalcin δ, little Ca^{2+}-dependent binding of rat brain cytosolic proteins was obtained with the non-myristoylated form of the protein (Figure 2B). The Ca^{2+}-dependent binding of brain cytosolic proteins to myr-neurocalcin δ was additionally shown to be specific as no binding was detected to the myr-NCS-1–Sepharose. This lack of

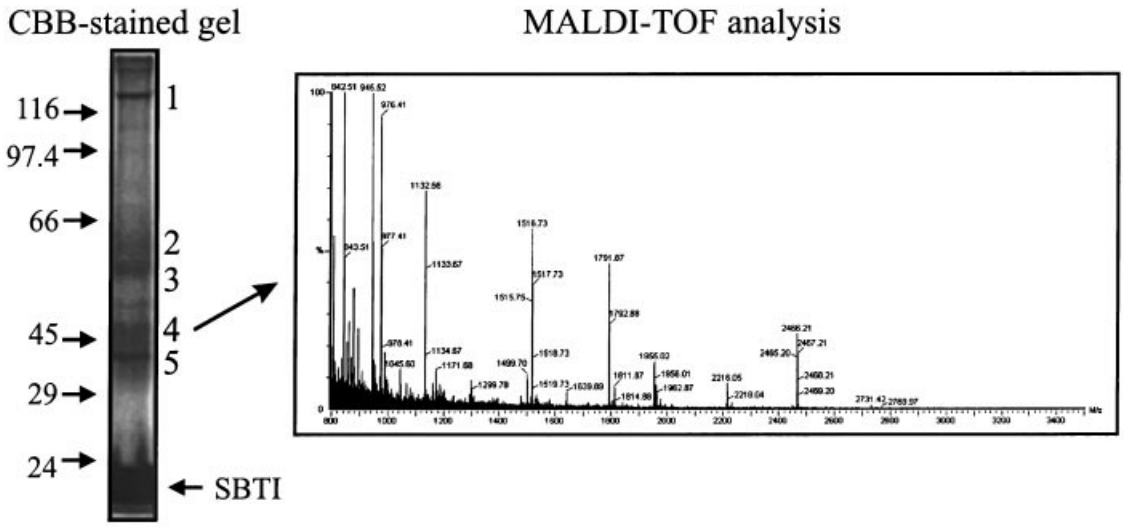

Figure 3 Identification of polypeptides in concentrated EGTA-eluted fractions from large-scale myr-neurocalcin δ column

Myr-neurocalcin δ was bound to CNBr-activated Sepharose beads and incubated with rat brain cytosol proteins in the presence of Ca^{2+}. Bound proteins were eluted with EGTA. Protein-containing fractions were pooled and concentrated by methanol precipitation (see the Materials and methods section). The concentrated samples were boiled in SDS-dissociation buffer, separated on an SDS/10% (w/v) polyacrylamide gel and the gel was stained with Coomassie Brilliant Blue (CBB). The bands excised for MALDI–TOF MS analysis are numbered. Soya-bean trypsin inhibitor (SBTI) was used as a carrier protein during the concentration of the samples. An example of the MALDI–TOF data for band 4 (β-actin) is shown on the right.

Table 1 Summary of binding partners for myr-neurocalcin δ from rat brain cytosol identified by MALDI–TOF MS

Five bands were excised from a gel and analysed by MALDI–TOF MS. Each identified protein is listed with its accession number. The number of peptides given is the number of peptides identified by the MS analysis that matched the corresponding protein. Coverage is expressed as the percentage of the protein sequence covered by the matched peptides. The observed M_r was determined by SDS/PAGE.

Protein	Accession number	Number of peptides	Coverage (%)	M_r predicted	M_r observed
Clathrin heavy chain	P1142	16	12.42	191 599	174 000
α1-Tubulin	P02551	12	39.25	50 136	58 000
β2-Tubulin	P04691	8	21.12	49 963	50 000
β-Actin	P02570	7	28.53	41 737	51 000
GAPDH	P04797	6	29.52	35 705	36 000

binding to NCS-1 was seen with two separate batches of myr-NCS-1 where efficient myristoylation was confirmed (at least 50% of protein) using MS analysis and complete linkage of the protein to Sepharose was demonstrated. In addition, no additional proteins could be eluted from the myr-NCS-1 column by a high salt buffer.

Identification by MALDI–TOF MS of the major cytosolic proteins eluted with EGTA from the myr-neurocalcin δ column

To obtain sufficient amounts of protein for MALDI–TOF analysis, the preparation was scaled up and the protein-rich fractions eluted with EGTA from the large-scale myr-neurocalcin δ column were concentrated approx. 27-fold (see the Materials and methods section). The concentrated pooled fractions were separated on a 10% (w/v) polyacrylamide gel and stained with Coomassie Brilliant Blue (Figure 3). In contrast with the over-developed silver-stained gels that demonstrated the presence of many minor polypeptides, Coomassie Brilliant Blue-stained gels showed a smaller number of predominant polypeptide bands. It was assumed that these would be more likely to represent significant binding partners. The major bands were therefore excised and subjected to MALDI–TOF MS after trypsin digestion. Figure 3 shows an example of the mass spectrum for band 4 (β-actin). The proteins were identified as clathrin heavy chain, α- and β-tubulin, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). In other experiments, analysis of the more minor polypeptides resolved on one-dimensional or two-dimensional gels [and more easily detected in over-developed silver-stained gels (Figures 1 and 2)] did not result, in any case, in unambiguous identification.

Biotinylated myr-neurocalcin δ overlay assays to confirm interactions between neurocalcin δ and the proteins identified by MALDI–TOF MS

To provide independent evidence for the interactions detected above and to establish whether neurocalcin δ binds directly to the major proteins eluted from the column a distinct assay was used. Recombinant myr-neurocalcin δ was biotinylated and used to probe the identified proteins after transfer to a nitrocellulose membrane. The small GTPase ARF was biotinylated and used as a control. ARF is only active in its GTP-bound form, and, as no GTP is present during the overlay assays, any binding detected

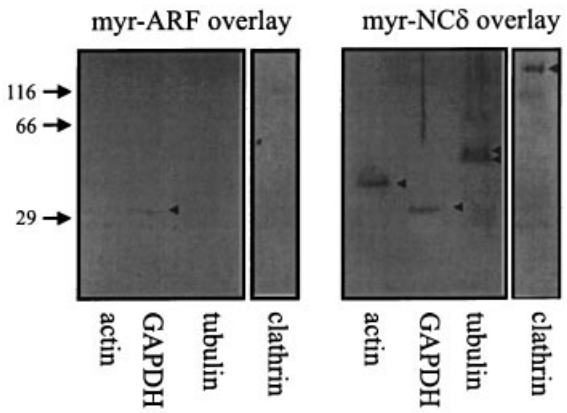

Figure 4 Investigation of the binding of myr-neurocalcin δ using a biotinylated overlay assay

Actin, GAPDH, tubulin and clathrin heavy chain were separated on an SDS/10% (w/v) polyacrylamide gel (0.4 μg/lane) and transferred on to nitrocellulose membranes. Biotinylated myr-ARF as the control, and biotinylated myr-neurocalcin δ (NCδ) were used to probe the nitrocellulose membranes in the presence of 10 μM free Ca^{2+}. Arrowheads indicate detected polypeptides.

with myr-ARF can be considered non-specific. The overlay assays were carried out in buffer containing 10 μM free Ca^{2+}. Clathrin heavy chain, α- and β- tubulin, and actin were found to bind myr-neurocalcin δ directly and specifically (Figure 4). However, GAPDH was detected by myr-ARF to the same extent as by myr-neurocalcin δ, and therefore the interaction of GAPDH with neurocalcin δ in this assay may be non-specific. In similar overlay assays, binding of biotinylated myr-neurocalcin δ was detected to only a subset of cytosolic proteins but not to calcineurin (which does bind to biotinylated NCS-1) or to Hsc70 (results not shown) even at high concentrations of loaded proteins demonstrating the specificity of the overlay assay.

Co-immunoprecipitation of clathrin, tubulin and actin with neurocalcin δ

The above experiments demonstrated direct binding of clathrin, tubulin and actin to bacterially expressed myr-neurocalcin δ. In order to determine whether endogenous neurocalcin δ in brain cytosol could interact with the identified proteins, and to provide further evidence for these interactions, an immunoprecipitation approach was established. Anti-(neurocalcin δ) serum was prepared in rabbits using recombinant neurocalcin δ as immunogen. This antiserum specifically recognized a single band corresponding to neurocalcin δ in Western blots of brain extracts (Figure 5B) and did not detect closely related members of the NCS protein family (Figure 5A). Anti-(neurocalcin δ) antibodies, immobilized on Protein G–Sepharose, were found to efficiently immunoprecipitate native neurocalcin δ from cytosol with close to 100% recovery. This immunoprecipitation was specific as no neurocalcin δ was detectably recovered using immobilized pre-immune serum from the same rabbit and anti-(neurocalcin δ) serum did not immunoprecipitate NCS-1 from cytosol (Figure 5C). The immunoprecipitates were then probed with antisera for clathrin heavy chain, α-tubulin, actin and GAPDH. A proportion of all four proteins were co-immunoprecipitated with neurocalcin δ. This was a specific association as none of these proteins was detected in control immunoprecipitates. To assess the Ca^{2+}-dependency of the interactions, immunoprecipitations were carried out in 1 mM EGTA/1 mM EDTA or in the presence of 10 μM free Ca^{2+}. The co-immunoprecipitation of clathrin, actin and GAPDH was increased in the presence of 10 μM Ca^{2+}, but the amount of α-tubulin recovered was not Ca^{2+}-dependent (Figure 5C).

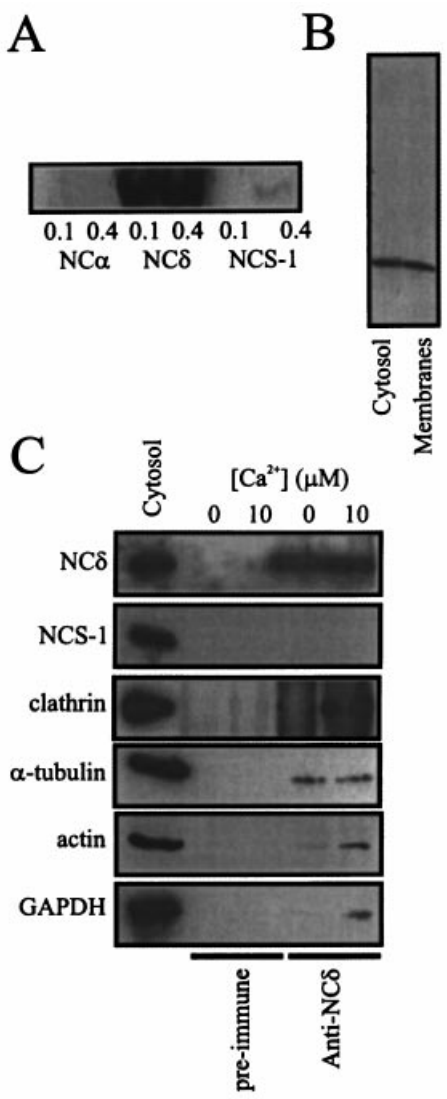

Figure 5 Characterization of anti-(neurocalcin δ) serum and co-immunoprecipitation of clathrin, tubulin and actin with neurocalcin δ

(**A**) Samples of recombinant neurocalcin α (NCα), neurocalcin δ (NCδ) and NCS-1 at two concentrations (0.1 and 0.4 μg/lane) were probed with anti-(neurocalcin δ) serum. (**B**) Rat brain cytosol and membrane fractions were probed with anti-(neurocalcin δ). (**C**) Anti-(neurocalcin δ) serum or pre-immune serum was immobilized on Protein G–Sepharose. The beads were incubated with rat brain cytosol in nominally 0 Ca^{2+} or with 10 μM free Ca^{2+}. Following extensive washing in buffers with the appropriate Ca^{2+} concentrations, bound proteins were solubilized prior to SDS/PAGE. Bound proteins were detected by Western blotting with the antisera indicated on the left.

Ca^{2+}-and myristoylation-dependent localization of neurocalcin δ in HeLa cells

In order to assess the localization of neurocalcin δ and the importance of Ca^{2+} concentration and myristoylation, we expressed neurocalcin δ in HeLa cells. These cells do not normally

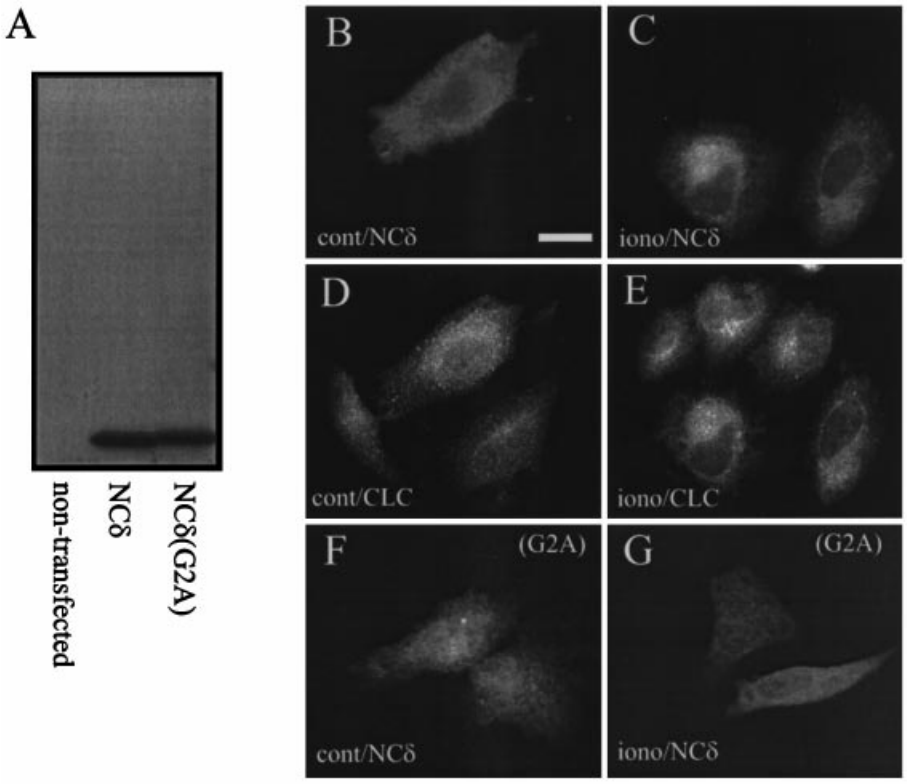

Figure 6 Expression of neurocalcin δ by transfection of HeLa cells and immunofluorescence localization

HeLa cells were transfected with a plasmid encoding wild-type neurocalcin δ (NCδ) or with a plasmid harbouring the G2A mutation to prevent myristoylation. (**A**) Western blot using anti-(neurocalcin δ) serum on control and transfected HeLa cells. (**B** and **C**) Anti-(neurocalcin δ) staining of HeLa cells transfected with wild-type neurocalcin δ. (**D** and **E**) Anti-[clathrin light chain (CLC)] staining of transfected HeLa cells. (**F** and **G**) Anti-(neurocalcin δ) staining of HeLa cells transfected with the neurocalcin δ (G2A). The cells were either treated with control buffer (cont) or with 1 μM ionomycin (iono) for 15 min before fixation. The scale bar represents 20 μm. Note that non-transfected cells are visible which are stained by anti-(clathrin light chain), but not by anti-(neurocalcin δ).

express neurocalcin δ but as they do express clathrin, tubulin and actin they are a valid cell type to explore potential co-localization of neurocalcin δ with these proteins. Neurocalcin δ was effectively expressed in HeLa cells following transfection (Figure 6A). The specificity of the anti-(neurocalcin δ) serum is shown by the fact that it did not recognize any polypeptides in non-transfected cells. In immunofluorescence experiments, the affinity-purified antibodies did not stain non-transfected cells. Under resting conditions, transfected cells gave a diffuse immunofluorescence pattern with affinity-purified anti-(neurocalcin δ) (Figure 6B) consistent with a cytoplasmic localization. This was expected based on previous biochemical experiments that showed neurocalcin δ to be soluble at resting Ca^{2+} concentrations [28]. Elevation of cytosolic Ca^{2+} by treatment with ionomycin resulted in a translocation of neurocalcin δ with a resulting predominantly perinuclear staining pattern (Figure 6C). This localization pattern differed from that for tubulin or actin (results not shown). In contrast, there was apparent extensive overlap (Figures 6D and 6E) with the localization of clathrin (based on the use of a monoclonal antibody raised against the clathrin light chain). Clathrin is found at the plasma membrane and the *trans*-Golgi network (TGN) and the co-localization in the perinuclear region (TGN) was most apparent in these images. The translocation of neurocalcin δ was dependent upon its ability to be myristoylated, as a mutation (G2A) to prevent myristoylation resulted in the expressed protein having a diffuse cytoplasmic localization even after ionomycin treatment (Figures 6F and 6G). Note that non-transfected cells were not stained by anti-(neurocalcin δ) (Figures 6B and 6C). It was clear from a comparison with the staining of non-transfected cells visualized with anti-(clathrin light chain) (Figures 6C and 6D) that expression of neurocalcin δ did not modify the localization of clathrin in the transfected HeLa cells.

To further investigate the potential co-localization of neurocalcin δ and clathrin, ionomycin-treated cells were examined by confocal laser scanning microscopy. Confocal sections are shown at the level of the plasma membrane (Figures 7A–7F) and through the equator of the cell (Figures 7G–7I). At the plasma membrane, neurocalcin δ localization partially overlapped that of clathrin (Figures 7D–7F). At the TGN, close co-localization of neurocalcin δ and clathrin was evident (Figures 7G–7I).

DISCUSSION

The NCS proteins are a conserved family of Ca^{2+}-binding proteins expressed only or predominantly in retinal photoreceptors and in neurons [6]. They are likely to play important roles in the transduction of Ca^{2+} signals leading to changes in neuronal function. The functions of some of these proteins, such as recoverin in photoreceptor cells, have been well characterized. In contrast, others, including neurocalcin δ, have no known cellular

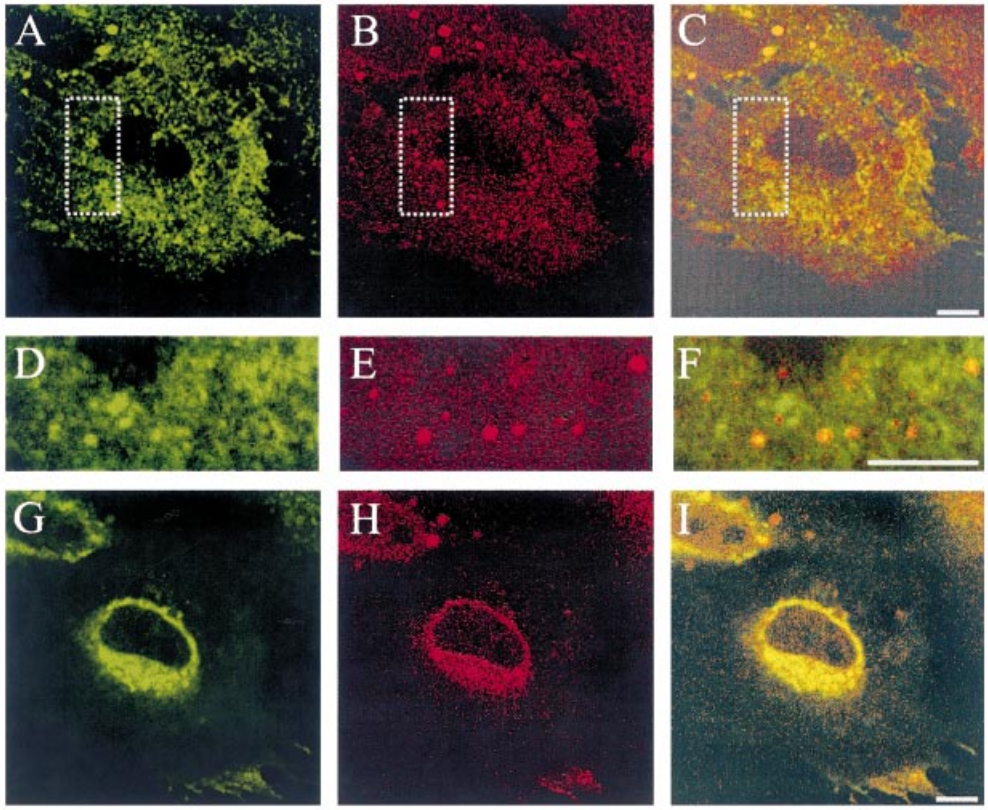

Figure 7 Confocal laser scanning microscopy showing co-distribution of neurocalcin δ and clathrin in transfected HeLa cells

HeLa cells were transfected with a plasmid encoding wild-type neurocalcin δ and treated with Ca^{2+}/ionomycin for 10 min before fixation. Following dual staining with anti-(neurocalcin δ) and anti-(clathrin light chain) the cells were imaged by confocal microscopy. (**A**, **D** and **G**) Anti-(neurocalcin δ) staining. (**B**, **E** and **H**) Anti-(clathrin light chain) staining. (**C**, **F** and **I**) Overlaid images from both antisera. The scale bars represent 10 μm.

function. Of the NCS proteins expressed in brain, NCS-1 has been shown to have a general distribution [5], but, intriguingly, hippocalcin is expressed most highly in hippocampal pyramidal cells [34] and neurocalcin δ in cerebellar Purkinje cells [5,24–26] This suggests that these latter proteins may serve cell-specific functions. Knowledge of the proteins with which they interact will be essential as a first step in determining how they contribute to regulation of neuronal activity in the cell types in which they are expressed. For this reason, we have set out to determine the identity of the binding partners in brain for neurocalcin δ.

Using a myr-neurocalcin δ column, binding partners for this protein were isolated from rat brain cytosol. Proteins bound in the presence of Ca^{2+} were eluted with EGTA from the column. The major proteins visualized on Coomassie Brilliant Blue-stained gels were chosen for further study as these would be more likely to be specific and direct binding partners, although we could not exclude that the more minor eluted proteins are also physiological targets for neurocalcin δ. The major proteins were unequivocally identified by tryptic-digestion followed by MALDI–TOF MS as clathrin, α- and β-tubulin, β-actin and GAPDH. The following reasons indicate that neurocalcin δ binds these proteins in a specific manner. First, no binding was detected to the beads in the control column. Secondly, the binding was Ca^{2+}-dependent. Thirdly, the binding was dependent on the presence of the myristoyl group on neurocalcin δ. Fourthly, no Ca^{2+}-dependent binding was detected to the closely related NCS protein myr-NCS-1 that has approx. 60 % sequence identity with neurocalcin δ. The direct nature of the neurocalcin δ interactions with these proteins was confirmed using a biotinylated overlay assay. This demonstrated that clathrin, tubulin and actin bind neurocalcin δ directly. In contrast, binding to GAPDH in this assay was no higher than with the control myr-ARF probe and, therefore, the binding of neurocalcin δ to GAPDH is likely to be indirect. GAPDH is an enzyme of the glycolytic pathway but it appears to have multiple functions. Indeed, GAPDH has been found to interact with actin [35] and this could underlie an indirect interaction of GAPDH on the myr-neurocalcin δ column. It may be surprising that Ca^{2+}-dependent binding interactions were not detected with the myr-NCS-1 column. It is possible that the conditions required for binding of brain cytosolic proteins to NCS-1 are very different from those for neurocalcin δ. It has been seen, for example, that NCS-1 undergoes a conformational change over the range of free Ca^{2+} concentration 0.1–1.0 μM and that this is reversed at higher Ca^{2+} concentrations [32] such as that used for binding in the present study. NCS-1 may, therefore, bind other proteins optimally at lower Ca^{2+} levels.

The finding that the protein interactions on the neurocalcin δ column were dependent on the myristoylation state of the protein is interesting as it indicates that the N-terminal myristoyl group is not only required for interaction with membrane lipids, as previously assumed [14], but may also be directly important for protein–protein interactions involving neurocalcin δ. The importance of myristoylation for protein–protein interactions by other NCS proteins is unclear but the data on neurocalcin δ

suggest that the Ca^{2+}-myristoyl switch may not only allow translocation of the Ca^{2+}-bound protein to membranes where it can then interact with membrane proteins but may also allow interactions with cytosolic proteins. The myristoyl group alone is not sufficient, however, as the interactions found in the present study were specific for neurocalcin δ.

In addition to the binding of recombinant neurocalcin δ to clathrin, tubulin, actin and GAPDH *in vitro*, we also demonstrated an interaction of endogenous neurocalcin δ in rat brain cytosol with these proteins. We prepared an anti-(neurocalcin δ) serum that proved to be specific, recognizing only a single polypeptide in brain; it did not recognize the closely related proteins neurocalcin α (70 % identity) or NCS-1. This antiserum efficiently immunoprecipitated neurocalcin δ, but not NCS-1. In addition, clathrin heavy chain, tubulin, actin and GAPDH were co-immunoprecipitated. None of these proteins was detected in control immunoprecipitates using pre-immune serum. The binding of clathrin, actin and GAPDH in this assay was Ca^{2+}-dependent, being increased at 10 μM Ca^{2+}, but the interaction of α-tubulin was not. The difference for tubulin between this assay and the column-binding/elution assay may be related to the much higher Ca^{2+} concentration used during binding in the latter approach. Only a portion of each of clathrin, tubulin, actin and GAPDH were recovered in the immunoprecipitates as expected given the multiple interactions made by these proteins. Nevertheless, the preservation of the interaction through the immunoprecipitation protocol suggests that these interactions are of high affinity.

Transfection of HeLa cells with a plasmid encoding neurocalcin δ allowed comparison of neurocalcin δ localization with that of clathrin, tubulin and actin. As expected from previous biochemical studies ([28], and L. Ivings and R.D. Burgoyne, unpublished work), neurocalcin δ gave an apparent cytoplasmic localization in resting cells and translocated to membranes following Ca^{2+} elevation by ionomycin treatment. In treated cells, neurocalcin δ showed co-localization with clathrin. Partial co-localization was evident at the plasma membrane and extensive overlap was seen at the TGN. The translocation of neurocalcin δ was not only Ca^{2+}-dependent but required its myristoylation as the myristoylation-defective G2A mutant did not translocate. These data suggest that at elevated Ca^{2+}, in a cellular context, neurocalcin δ is localized to the same sites as clathrin and could potentially interact with this protein. While the neurocalcin δ localization was not similar to the pattern of microtubules or actin stress fibres seen in the transfected HeLa cells (results not shown) we cannot rule out the possibility that low levels of neurocalcin δ interact with tubulin or actin within these cells.

The major protein interaction for neurocalcin δ that has been characterized previously was with the S100β protein [36]. This interaction is most likely not physiologically relevant as neurocalcin δ is expressed exclusively in neurons and S100β is only expressed in glial cells. This previous study did, however, also detect an interaction with tubulin, consistent with the data reported in the present study. An interaction with actin [37] has also been described for another NCS protein, VILIP-1 (neurocalcin α) which has approx. 70 % identity with neurocalcin δ and recently interaction of neurocalcin δ with actin has been demonstrated using an independent approach [38]. A less closely related EF-hand Ca^{2+}-binding protein known as p22 has been found to interact with microtubules [39]. This differed from the interaction of neurocalcin δ with tubulin as p22 binding was independent of Ca^{2+} concentration. No interactions of any NCS proteins with clathrin heavy chain have previously been reported. The interactions of neurocalcin δ with clathrin, tubulin and actin appear to be direct, based on the biotinylated neurocalcin δ overlay assay. These interactions may not be unrelated, however, as it has been established that clathrin-mediated endocytosis is actin-dependent [40] and can be regulated by proteins that show dual binding to actin and to clathrin [41]. Within cerebellar Purkinje cells, neurocalcin δ is present at high levels in the cell body and throughout the dendrites [24–26]. The interaction of neurocalcin δ with clathrin and actin could contribute to the regulation of endocytic events or alternatively to Ca^{2+}-dependent changes in cytoskeletal organization in these cellular compartments. In addition, these interactions could also be a mechanism for targeting Ca^{2+}-bound neurocalcin δ to membrane organelles (clathrin-coated vesicles or membranes) where it could interact indirectly with other target membrane proteins.

The expression of neurocalcin δ, a high-affinity Ca^{2+} sensor, at substantially higher levels in cerebellar Purkinje cells than any other neuronal cell type raises the question as to whether this protein plays any role in one of the several forms of Ca^{2+}-dependent plasticity demonstrated by Purkinje cells [42]. The identification of interacting targets for neurocalcin δ that we have described will be an important underpinning for future studies on the role of neurocalcin δ in the regulation of Purkinje cell physiology. The interaction of neurocalcin δ with clathrin in three distinct assays and its co-localization following Ca^{2+} elevation in HeLa cells points most strongly to a possible role of neurocalcin δ in regulating coated vesicle traffic.

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