Neuronal Ca\(^{2+}\) Sensor 1

CHARACTERIZATION OF THE MYRISTOYLATED PROTEIN, ITS CELLULAR EFFECTS IN PERMEABILIZED ADRENAL CHROMAFFIN CELLS, Ca\(^{2+}\)-INDEPENDENT MEMBRANE ASSOCIATION, AND INTERACTION WITH BINDING PROTEINS, SUGGESTING A ROLE IN RAPID Ca\(^{2+}\) SIGNAL TRANSDUCTION

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Overexpression of frequenin and its orthologue neuronal Ca\(^{2+}\) sensor 1 (NCS-1) has been shown to increase evoked exocytosis in neurons and neuroendocrine cells. The site of action of NCS-1 and its biochemical targets that affect exocytosis are unknown. To allow further investigation of NCS-1 function, we have demonstrated that NCS-1 is a substrate for N-myristoyltransferase and generated recombinant myristoylated NCS-1. The bacterially expressed NCS-1 shows Ca\(^{2+}\)-induced conformational changes. The possibility that NCS-1 directly interacts with the exocytotic machinery to enhance exocytosis was tested using digitonin-permeabilized chromaffin cells. Exogenous NCS-1 was retained in permeabilized cells but had no effect on Ca\(^{2+}\) release of catecholamine. In addition, exogenous NCS-1 did not regulate cyclic nucleotide levels in this system. These data suggest that the effects of NCS-1 seen in intact cells are likely to be due to an effect on the early steps of stimulus-secretion coupling or on Ca\(^{2+}\) homeostasis. Myristoylated NCS-1 bound to membranes in the absence of Ca\(^{2+}\) and endogenous NCS-1 was tightly membrane-associated. Using biotinylated NCS-1, a series of specific binding proteins were detected in cytosol, chromaffin granule membrane, and microsome fractions of adrenal medulla. These included proteins distinct from those detected by biotinylated calmodulin, demonstrating the presence of multiple specific Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent binding proteins as putative targets for NCS-1 action. A model for NCS-1 function, from these data, indicates a constitutive membrane association independent of Ca\(^{2+}\). This differs from the Ca\(^{2+}\) myristoyl switch model for the closely related recoverin and suggests a possible action in rapid Ca\(^{2+}\) signal transduction in response to local Ca\(^{2+}\) rises.

Among the many EF-hand-containing Ca\(^{2+}\)-binding proteins is a family known as the recoverin/neurocalcin or neuronal calcium sensor family (1). These small Ca\(^{2+}\)-binding proteins include members expressed only in photoreceptor cells such as recoverin (2), the best characterized of this family (3). Recoverin has a well defined function in the control of rhodopsin phosphorylation due to direct inhibition of rhodopsin kinase (3, 4). Other members such as VILIP (6) neurocalcin (7), hippocalcin (8), frequenin (9), and neuronal Ca\(^{2+}\) sensor 1 (NCS-1) (10) are highly expressed in neurons and were originally all believed to be neuron-specific implying a key role in Ca\(^{2+}\)-regulated events in the nervous system. The cellular function of many of the neuronal proteins of this family remains a mystery, but, in contrast, some insight has been gained into the role of frequenin/NCS-1 in the regulation of presynaptic function.

Frequenin is a Drosophila protein that has been demonstrated in molecular genetic and other studies to regulate synaptic neurotransmission. Overexpression of frequenin in Drosophila, in the V7 mutant (9), was found to facilitate evoked neurotransmission at the neuromuscular junction, and a direct stimulation of both spontaneous and evoked neurotransmission was found following injection of Xenopus frequenin into Xenopus spinal neurons (11). NCS-1, first identified in chickens (10) and later in rodents (12, 13) and Caenorhabditis elegans (13), appears to be the frequenin orthologue expressed in those species. Recently, it has been shown that NCS-1 is not expressed solely in neurons, since it was detected in glial cells in the central nervous system (12). In addition, it is present in adrenal chromaffin cells and PC12 neuroendocrine cells, where its overexpression also results in an increase in Ca\(^{2+}\)-regulated exocytosis but from dense-core granules (14), analogous to the effect reported for frequenin in Drosophila for synaptic vesicle exocytosis (9). It is clear, therefore, that frequenin/NCS-1 has an important regulatory role in the steps leading to Ca\(^{2+}\)-dependent exocytosis of synaptic vesicles and dense core granules in neurons and neuroendocrine cells. The step in the exocytotic sequence at which frequenin/NCS-1 acts is, however, not known.

The biochemical processes on which NCS-1 might act in vivo are also unclear. From in vitro experiments, frequenin has been shown to activate membrane-bound guanylate cyclase, but only at low Ca\(^{2+}\) concentration, in rod outer segments (9) and NCS-1 to inhibit rhodopsin kinase (13). These in vitro effects on photoreceptor proteins may not be relevant to the function of frequenin/NCS-1 in neurons and neuroendocrine cells. In addition, NCS-1 has been shown to activate various calmodulin targets including cyclic nucleotide phosphodiesterase, calcin,ineurin, and nitric-oxide synthase (15). NCS-1 has also been implicated as a direct or indirect (via cGMP) activator of Ca\(^{2+}\)-dependent K\(^+\) channels (9, 15, 16) and also Na\(^{+}\)/Ca\(^{2+}\) exchange (17). Despite these interactions, the molecular targets for frequenin/NCS-1 action in vivo are not known for certain, and thus the mechanism by which evoked exocytosis is increased remains to be elucidated.

From analysis of its biochemical properties (18, 19) and structure (3), recoverin has been described as a calcium-myristoyl switch, and this was initially assumed to typify the behavior of all members of this family of proteins. Ca\(^{2+}\) binding to two of the four EF-hand-like domains of recoverin leads to the exposure of an N-terminal myristoyl group (3) believed to
allow membrane attachment (19). The movement of the myristoyl group also exposes a hydrophobic pocket that may then interact with target proteins (3). All other members of this family possess consensus myristoylation motifs (20) and, in the case of recoverin (19), neurocalcin (21, 22), and hippocalcin (23), only the myristoylated and not the nonmyristoylated protein shows Ca\(^{2+}\)-dependent binding to membranes consistent with the calcium-myristoyl switch model. In contrast, another family member, the guanyl cyclase-activating protein 2, shows the distinct property of only binding to membranes and activating its target protein at low Ca\(^{2+}\) concentration and dissociates from membranes at micromolar Ca\(^{2+}\) (24). Freqenin and NCS-1 possess a putative N-myristoylation motif (10–12), but their myristoylation and Ca\(^{2+}\) dependence of membrane binding have not been examined for either native or recombinant protein.

The aims of this study were to examine whether NCS-1 is a substrate for myristoyltransferase by bacterial co-expression with the yeast enzyme (25), to characterize myristoylated NCS-1, and to use the recombinant protein to investigate cellular functions of NCS-1. The results show that NCS-1 does indeed act as a substrate for N-myristoyltransferase, and the recombinant protein shows Ca\(^{2+}\)-dependent conformational changes but Ca\(^{2+}\)-independent interaction with membranes, and thus its behavior differs from recoverin. In addition, we subcloning of the product into the pET-5a expression vector (Promega, Madison, WI), and stored at 80 °C until required.

NCS-1: Membrane and Protein Interactions

MATERIALS AND METHODS

Plasmid Production—Total RNA was extracted from whole brain of Wistar rats using an RNAeasy isolation kit (Qiagen, Surrey, UK) and cDNA synthesized with a reverse transcription system. cDNA encoding NCS-1 was amplified in polymerase chain reactions using F1 antisense primer (Stratagene, Cambridge, UK), according to the supplier’s protocol, and an OmniE dryblock thermocycler (Hybaid, Middlesex, UK). The sense and antisense primers used were 5′-CATCATAATGAGATATC-CAACAGCAAGTTGA-3′ and 5′-CATGATCTCTTACATACCGTGTCG-3′, respectively. These were based on the nucleotide sequence of rat NCS-1 (Ref. 15; GenBank\textsuperscript{13} accession no. L27424) and incorporated Rose-HI restrictions (underlined) to facilitate subcloning of the product into the pET-5a expression vector (Promega, Southampton, UK). The nucleotide sequence was established by automated sequencing.

Expression and Purification of Recombinant NCS-1—BL21 (DE3) cells (Promega) were transformed with pBB131 (25), a plasmid encoding N-myristoyltransferase (NMT; myristoyltransferase-CoA; protein N-myristoyltransferase (EC 2.3.1.97)) and subsequently with the above pET-5a/NCS-1 construct. Expression of both recombinant NCS-1 and NCS-1 was amplified in polymerase chain reactions using B-lactamase fragment of Zozulya pepstatin A) and stored at 80 °C until required.

TGAGAGG-3′

Expression of recombinant NCS-1 was confirmed by means of fluorography following induction of expression in the presence of 25 μCi/ml \(^{3}H\)myristate (Amersham Pharmacia Biotech, Buckinghamshire, UK). For production of labeled purified protein, a 500-ml culture was grown in the presence of 1.5 μCi/ml \(^{3}H\)myristate. Cells were harvested by centrifugation and resuspended in buffer A (50 mM HEPES (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, and 1 mM MgCl\(_2\)) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM pepstatin A) and stored at –80 °C until required.

Murine NCS-1 was purified using methods adapted from those of Zozulya et al. (26) for recoverin and neurocalcin. Upon thawing, cells were added to 1 volume of buffer A supplemented with 0.2 mM EGTA, incubated with 1 mg/ml lysosome for 30 min, ultrasonicated, and incubated with 2 μg/ml DNase for 15 min, and finally cell debris was removed by centrifugation at 100,000 × g for 1 h. The cleared lysate was supplemented with 1 mM CaCl\(_2\) and applied to a phenyl-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) column. The column was washed, and NCS-1 was eluted with buffer A containing 5 mM EGTA. For further purification, pooled eluted fractions containing NCS-1 were diluted with 3 volumes of water and applied to a Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated with buffer B (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 50 mM EGTA) and NCS-1 was eluted with a 0–60% buffer B gradient in a single peak. All chromatography was performed at room temperature (22–25 °C) using an Amersham Pharmacia Biotech FPLC system. Peak fractions containing recombinant proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the pooled fractions were stored at –80 °C until required. Analysis by HPLC used reverse phase chromatography with a 50% acetonitrile gradient. Samples for electrophoresis were routinely dissolved in sample buffer (125 mM HEPES (pH 6.8), 1.25% SDS (w/v), 2 mM EDTA, 10% sucrose (w/v), 1% β-mercaptoethanol, 10% glycerol (v/v), and 0.001% bromophenol blue), boiled, and separated on a 10 or 15% SDS-polyacrylamide gel. For experiments investigating calcium binding to NCS-1, EDTA was omitted and replaced with either 5 mM EGTA or 5 mM CaCl\(_2\). For detection of \(^{[3]}H\)myristate incorporation, gels were impregnated with Amplify (Amersham Pharmacia Biotech), dried, and exposed on x-ray film overnight at –70 °C.

Tryptophan Fluorescence Emission Spectra—Purified recombinant NCS-1 (1 μM) in 20 mM HEPES (pH 7.4), 139 mM NaCl, 2 mM ATP, 5 mM nitrotriatic acid, and 5 mM EGTA was excited at room temperature at 280 nm, and the emission spectra from 290 to 410 nm were detected using a Perkin-Elmer LS-5 luminescence spectrometer. The free Ca\(^{2+}\) concentration was increased by incremental additions of CaCl\(_2\), and the subsequent spectra were similarly recorded.

Preparation of Membranes from Rat Brain—Whole brains of Wistar rats were homogenized in HEPES (pH 7.2), 140 mM sucrose, 70 mM potassium acetate, 1 mM dithiothreitol, and 230 μM phenylmethylsulfonyl fluoride and centrifuged at 1600 × g for 10 min at 4 °C. The supernatant was removed and centrifuged at 100,000 × g for 60 min at 4 °C and the supernatant pellet was resuspended in 125 mM Na\(_2\)CO\(_3\) (pH 11.5). After incubation at 4 °C for 30 min, membranes were obtained by centrifugation at 100,000 × g for 60 min at 4 °C; resuspended in 20 mM HEPES (pH 7.4), 100 mM NaCl, and 2 mM EDTA; and stored at 4 °C until required.

Determination of NCS-1 Association with Membranes—Membranes were washed twice by centrifugation at 13000 × g for 10 min at room temperature, with 20 mM HEPES (pH 7.4), 139 mM NaCl, 2 mM ATP, 5 mM nitrotriatic acid, and 5 mM EGTA and finally resuspended in the same buffer with added MgCl\(_2\) and CaCl\(_2\) to give a calculated free Mg\(^{2+}\) concentration of 2 mM and free Ca\(^{2+}\) concentration of 0 or 10 μM. After incubation for 30 min with 250 mM nigericin, 10% glycerol, and 0.001% bromophenol blue). The pH was adjusted to 8.0 with 1 M Na\(_2\)CO\(_3\), and the sample was placed on ice for 30 min. After centrifugation at 100,000 × g for 30 min to remove cell debris, and then both supernatants and cells were precipitated with an equal volume of methanol at –20 °C to concentrate protein samples before solubilization in 300 μl of SDS dissociation buffer and boiling. Samples were separated on a 15% SDS-polyacrylamide gel and analyzed by immunoblotting as above.

Assay of Catecholamine Secretion from Permeabilized Chromaffin Cells—Bovine chromaffin cell cultures were prepared and maintained as described previously (27). Cells were permeabilized in 300 μl of permeabilization buffer (20 mM PIPES (pH 6.5), 139 mM potassium glutamate, 5 mM EGTA, 2 mM ATP, and 2 mM MgCl\(_2\)) containing 20 μM digitonin (Novabiochem, Nottingham, UK). After the indicated times, the supernatant was centrifuged at 13,000 × g for 3 min to remove cell debris, and then both supernatants and cells were precipitated with an equal volume of methanol at –20 °C to concentrate protein samples before solubilization in 300 μl of SDS dissociation buffer and boiling. Samples were separated on a 15% SDS-polyacrylamide gel and analyzed by immunoblotting as above.

Assay of cAMP and cGMP Production in Permeabilized Chromaffin Cells—Cells were permeabilized and treated as above. For assay of cAMP, the incubation medium was removed after step 3, mixed with an equal volume of 10 mM Tris-HCl (pH 7.5) and 8 mM EDTA, boiled for 2
min, sonicated for 15 s, and centrifuged at 13,000 × g for 3 min. Levels of cAMP in the subsequent supernatants were determined using the appropriate enzyme immunoassay kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. For the assay of cGMP, 1/10 volume of lysis buffer (5% dodecyltrimethylammonium bromide in 0.05 M sodium acetate, pH 5.8) was added to the wells at the end of the incubation in step 3, and samples were taken for assay of cGMP after the acetylation of samples using an enzyme immunoassay kit (Amersham Pharmacia Biotech).

**Characterization of Binding of Biotinylated Proteins to Adrenal Medulla Subcellular Fractions—Cytool.** Chromaffin granule membrane, and microsome fractions were prepared from bovine adrenal medulla by standard techniques (29). For biotinylation of recombinant NCS-1, ADP-ribosylation factor 1 (ARF1), or purified bovine brain calcineurin (Sigma), the proteins (200 μg/ml) were mixed with 6-(biotinamidocaproxylamino)-N-hydroxysuccinimide ester (Sigma) from a stock solution in Me2SO to give a 100-fold molar excess of biotinylating reagent. Incubation was performed at room temperature for 2 h and stopped by the addition of a final concentration of 100 mM glycine. To remove excess biotin, biotinylated proteins were dialyzed against 20 mM HEPES, 139 mM NaCl, 2 mM ATP, 5 mM EGTA, 5 mM nitritetriacetic acid, pH 7.4. The concentration of the biotinylated proteins was determined by taking A280 and converting to mg/ml based on the predicted molar extinction coefficient. Biotinylated probes were stored at −20 °C until use. Proteins separated on SDS gels were blotted onto nitrocellulose membranes for analysis. Membranes were incubated in blocking solution (the above buffer plus 5% milk, 5% bovine serum albumin, 5% fetal calf serum, and 0.5% Tween 20) at room temperature for 2 h with three changes and then incubated overnight at 4 °C with biotinylated probes at 3.6–8 μg/ml in blocking solution (with the addition of MgCl2 and CaCl2 to obtain the appropriate free Ca2+ concentration and 2 mM free Mg2+). Membranes were washed four times in buffer plus the appropriate free Ca2+ concentration and 0.5% Tween 20 and incubated with Streptavidin-horseradish peroxidase at 1:400 in blocking solution for 30 min. The membranes were washed four times as above, and bound biotinylated fragments were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). In other experiments, binding of biotinylated proteins to bovine brain calcineurin (Sigma) was assayed using a similar protocol.

**RESULTS**

**Expression of Myristoylated NCS-1—NCS-1 has a potential N-terminal myristoylation site (10−12), related to the consensus motif recognized by N-myristoyltransferase (20). To determine whether NCS-1 can be a substrate for N-myristoyltransferase, NCS-1 was co-expressed in bacteria with the yeast enzyme, and [3H]myristic acid was added to the growth medium to allow detection of myristic acid incorporation. Bacterial cultures expressing the enzyme alone revealed no labeled polypeptides following an overnight exposure of the fluorography. In contrast, when both plasmids for N-myristoyltransferase and for NCS-1 were present, an intensively labeled polypeptide of the expected size for NCS-1 was detected by fluorography after induction of protein expression with isopropyl-1-thio-β-D-galactoside (IPTG) from bacteria containing plasmid encoding NMT alone or both plasmids for NMT and NCS-1 as indicated. B. elution of expressed NCS-1 from phenyl-Sepharose by 5 mM EGTA and analysis of fractions by SDS-PAGE and Coomassie Blue staining. C, characterization of NCS-1, purified on phenyl-Sepharose, by reverse phase HPLC. The inset shows the presence of NCS-1 in the loaded material (L) and in the first (1) and second (2) peaks from the HPLC.

determine the relative amounts of myristoylated and nonmyristoylated protein in the pool of purified protein. Analysis of purified NCS-1 by reverse-phase HPLC revealed two discrete peaks both of which contained NCS-1 when analyzed by SDS-PAGE (Fig. 1C). The first corresponded to the position of [3H]myristate-labeled NCS-1 in other experiments, so the myristoylated pool of NCS-1 from Fig. 1C amounted to 58% of the total NCS-1.

**Ca2+ Binding Properties of Recombinant NCS-1—**To establish the functional nature of the bacterially expressed myristoylated NCS-1, its ability to bind Ca2+, leading to Ca2+-dependent conformational change, was assessed. First, the effect of Ca2+ on migration of NCS-1 on SDS-PAGE was examined. As shown for other related Ca2+-binding proteins such as Dro sophila frequenin (9), NCS-1 underwent a conformational change in the presence of Ca2+ that resulted in faster migra-
tion on SDS-PAGE (Fig. 2A). In addition, conformational change could be demonstrated due to increasing Ca\(^{2+}\) concentration from the monitoring of tryptophan fluorescence emission. NCS-1 possesses two tryptophans at positions 30 and 103 (10–12), and previous work using nonmyristoylated bacterially expressed NCS-1 showed an increase in peak emission due to tryptophan fluorescence over a range of Ca\(^{2+}\) concentrations. The concentration of free Ca\(^{2+}\) was increased by the sequential addition of CaCl\(_2\), and the emission spectrum was measured after each addition. The data shown are the increase in peak emission as a percentage of the control trace and are shown as mean ± S.E. (n = 3).

Ca\(^{2+}\)-independent Binding of NCS-1 to Brain Membranes—Closely related Ca\(^{2+}\)-binding proteins such as recoverin (19), hippocalcin (23), and neurocalcin (21, 22) bind to membranes when myristoylated but not in the nonmyristoylated state. This binding is strictly Ca\(^{2+}\)-dependent, and the endogenous proteins can be stripped from membranes by Ca\(^{2+}\) chelators. We therefore determined whether the recombinant NCS-1 protein would show Ca\(^{2+}\)-dependent interaction with cellular membranes in the presence of physiological (millimolar) Mg\(^{2+}\) concentration. It should be noted that we previously observed that endogenous NCS-1 can be tightly associated with adrenal medulla membranes in the presence of Ca\(^{2+}\) chelator (14), and even after washing with chelator and subsequently with carbonate buffer at pH 11.5, endogenous membrane-associated NCS-1 was still detectable (Fig. 3A). To specifically determine binding of myristoylated NCS-1, the recombinant protein was prepared as a \(^{3}H\)myristate-labeled protein (Fig. 3B). Incubation of carbonate-washed rat brain membranes with recombinant \(^{3}H\)NCS-1 resulted in further binding of myristoylated NCS-1 in a Ca\(^{2+}\)-independent manner to the membranes (Fig. 3C). No differences in the extent of binding were seen over the Ca\(^{2+}\) range of 0–100 \(\mu\)M. These data show that NCS-1 has a pattern of membrane interaction distinct from closely related family members and that does not fit the recoverin Ca\(^{2+}\)/myristoyl switch model.

NCS-1 Does Not Directly Stimulate Ca\(^{2+}\)-dependent Exocytosis in Permeabilized Chromaffin Cells—In order to examine whether NCS-1 exerts its stimulatory effects on Ca\(^{2+}\)-regulated exocytosis directly via interaction with the exocytotic machinery, we used digitonin-permeabilized adrenal chromaffin cells. This is a well characterized model system for the analysis of dense core granule exocytosis, which allows exchange of soluble proteins within the cells and demonstration of stimulatory effects of various added soluble proteins (31, 32). The possibility that bacteria expressed NCS-1 might stimulate exocytosis after the addition to permeabilized cells was examined. Initially we determined the cellular content of endogenous NCS-1 following permeabilization. Within 15 min of
digitonin permeabilization, about 50% of NCS-1 was lost from the cells, but the remainder was retained in the cells following a longer permeabilization time even in the absence of Ca\(^{2+}\) (Fig. 4A). When permeabilized cells were incubated with 100 \(\mu\)g/ml (5 \(\mu\)M) bacterially expressed myristoylated NCS-1, increased levels of NCS-1 were detected, after prolonged washing of the cells in either 0 or 10 \(\mu\)M Ca\(^{2+}\), due to retention of the added protein with an approximate 3-fold increase compared with control cells at the end of the incubations (Fig. 4B). For analysis of exocytosis, the cells were permeabilized, preincubated for 15 min with or without NCS-1, and stimulated with 0 or 10 \(\mu\)M free Ca\(^{2+}\). In some experiments, NCS-1 was also included in the stimulation step. The concentration of NCS-1 was based on maximally effective concentrations of the non-myristoylated protein in published in vitro assays (16). Under no conditions did exogenously added NCS-1 stimulate exocytotic release of catecholamine. No effect was seen with at least some of its target proteins. In order to examine this possibility and to investigate the nature of NCS-1-binding proteins, we established an assay based on the binding of biotinylated NCS-1 to proteins separated by SDS-PAGE. In initial experiments, multiple putative binding partners were detected using biotinylated NCS-1 overlays, and, therefore, to establish the specificity of this binding it was compared with that with a distinct biotinylated protein of similar molecular mass, the myristoylated GTP-binding protein ARF1, also expressed as a recombinant protein (33). In these experiments,
the ARF1 was not activated by the addition of GTP and, therefore, was used simply as a control for nonspecific binding of a biotinylated probe. From a comparison of overlays, a series of bands, particularly of less than about 30 kDa, were detected by both probes, but the two Ca\textsuperscript{2+}-dependent binding proteins also specifically detected distinct proteins (Fig. 6B).

A well characterized calmodulin-binding protein is the 61-kDa subunit of calcineurin (34). NCS-1 has been shown to activate calcineurin in vitro (16), so we compared the Ca\textsuperscript{2+} dependence of binding of NCS-1 and calmodulin to purified calcineurin in the overlay assay. No binding was detected with the ARF1 control. With calmodulin, binding to calcineurin was completely Ca\textsuperscript{2+}-dependent (Fig. 7). In contrast, while binding of NCS-1 to calcineurin was increased at 10 \( \mu \text{M Ca}^{2+} \), it was also detectable at 0 \( \text{Ca}^{2+} \). Calcineurin may account for one of the polypeptides identified in adrenal fractions at about 60 kDa that did not show marked Ca\textsuperscript{2+}-dependence of NCS-1 binding (Fig. 6).

**FIG. 6. Identification of NCS-1-binding proteins in adrenal subcellular fractions using biotinylated NCS-1 overlays.** Cytosol (Cyto), chromaffin granule membrane (G. memb), and microsome (Micro) fractions from adrenal medulla were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose. A, left panel, binding proteins detected with biotinylated NCS-1 in the absence of Ca\textsuperscript{2+} with the positions of molecular mass standards shown on the left. Center panel, binding proteins detected with biotinylated NCS-1 in the presence of 10 \( \mu \text{M Ca}^{2+} \). Ca\textsuperscript{2+}-dependent binding proteins are indicated on the right of each track. Right panel, proteins detected with biotinylated ARF1 in the presence of 10 \( \mu \text{M Ca}^{2+} \), revealing a group of nonspecifically detected bands. B, comparison of proteins detected in the presence of 10 \( \mu \text{M Ca}^{2+} \) by biotinylated calmodulin (CaM) and biotinylated NCS-1 in the microsome fraction. Proteins specific for one or the other probe are indicated with diamonds (Ca\textsuperscript{2+}-dependent binding) or closed circles (Ca\textsuperscript{2+}-independent binding).

**FIG. 7. Binding of biotinylated NCS-1 and calmodulin to calcineurin.** Using biotinylated protein overlays, the ability of NCS-1, calmodulin, or ARF1 to bind to purified bovine brain calcineurin (0.1 \( \mu \text{g/track} \)) in the absence or presence of 10 \( \mu \text{M Ca}^{2+} \) was compared. No binding was detected with ARF1 or with calmodulin in the absence of Ca\textsuperscript{2+}. Binding to the 61-kDa subunit is shown.

Similar results were found in three separate experiments. We compared binding proteins detected in the absence or presence of 10 \( \mu \text{M Ca}^{2+} \) by biotinylated NCS-1 and biotinylated calmodulin. As illustrated for binding to microsomes in 10 \( \mu \text{M Ca}^{2+} \), some bands were detected by both probes, but the two Ca\textsuperscript{2+}-binding proteins detected with biotinylated NCS-1 and calmodulin were not specifically detected distinct proteins (Fig. 6B).

**DISCUSSION**

The functional importance of frequenin and its orthologue NCS-1 in the regulation of calcium-dependent exocytosis of synaptic vesicles and dense-core granules in vivo has been established (9, 11, 14), but it is not clear whether overexpression of frequenin/NCS-1 modifies some aspect of membrane excitability, Ca\textsuperscript{2+} homeostasis, or some other aspect of signal transduction or if it has a direct effect on the exocytotic machinery.

For further examination of NCS-1 action and identification of its specific biochemical targets, we have expressed and characterized recombinant myristoylated NCS-1. Following co-expression with yeast N-myristoyltransferase, about 50% of the NCS-1 became myristoylated. This is a lower proportion than for other family members (19, 21–23) but still considerably higher than for other expressed myristoylated proteins such as the GTP-binding protein ARF (33), for example.

The bacterially expressed NCS-1 protein appeared to be active as a Ca\textsuperscript{2+}-binding protein based on changed migration on SDS-PAGE in the presence of Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-induced changes in tryptophan fluorescence. Previous work on NCS-1 has demonstrated a change in tryptophan fluorescence, due to a Ca\textsuperscript{2+}-dependent conformational change, that consisted of an increase in peak emission (30). In contrast, with myristoylated NCS-1, we saw a more complex pattern of changes consistent with two conformational changes of differing Ca\textsuperscript{2+} sensitivity. Similar differences in the effect of Ca\textsuperscript{2+} on tryptophan fluorescence for myristoylated versus nonmyristoylated species have been reported for recovery (18). The initial increase in peak emission occurred over the range of Ca\textsuperscript{2+} concentration from 0.1 to 1.0 \( \mu \text{M} \), and this is consistent with previous work on direct assay of Ca\textsuperscript{2+} binding to NCS-1. These data have shown binding over this Ca\textsuperscript{2+} range due to two sites with high cooperativity. NCS-1 contains three potential functional EF-hand domains with an additional N-terminal domain lacking key residues required for Ca\textsuperscript{2+} coordination (9). The changes in tryptophan fluorescence
we observed at higher Ca\(^{2+}\) concentrations may reflect Ca\(^{2+}\) binding to a third low affinity site not detected by direct Ca\(^{2+}\) binding. Further interpretation of the Ca\(^{2+}\)-induced changes in tryptophan fluorescence will require information on structural change during Ca\(^{2+}\)-binding. Nevertheless, these data confirm that the recombinant myristoylated NCS-1 is a functional Ca\(^{2+}\)-binding protein.

An effect of overexpression of a protein on neurotransmission in vivo as seen for frequenin (9) could be due to effects on many different aspects of presynaptic function. We have previously demonstrated that overexpression of NCS-1 in PC12 cells resulted in an increase in the extent of dense core granule exocytosis evoked by ATP acting on a purinergic receptor (14), indicating that the effect of frequenin/NCS-1 was not limited to synaptic vesicle exocytosis. These data also show that NCS-1 is not normally present in limiting amounts in intact PC12 cells, since increased levels of expression can exert a stimulatory effect. In contrast, when the overexpressing PC12 cells were permeabilized prior to stimulation by calcium, no effect of NCS-1 overexpression on exocytosis was detected. These findings may be consistent with NCS-1 acting on some early step in stimulus-secretion coupling. Alternatively, the possibility could not be ruled out that permeabilization resulted in loss of NCS-1 overexpression on dense core granule exocytosis. These findings demonstrate that NCS-1 makes a tight Ca\(^{2+}\)-dependent membrane interaction. Recent work has shown that the binding of the related protein S-modulin to membranes, which is Ca\(^{2+}\)-dependent, requires the presence of a series of charged residues at the C terminus (40). These charged residues are absent in NCS-1, suggesting a distinct mechanism for membrane interaction. These findings demonstrate, therefore, that NCS-1 makes Ca\(^{2+}\)-independent interactions with target membrane and that Ca\(^{2+}\) does not regulate its membrane association. Presumably, conformational change following Ca\(^{2+}\)-binding in membrane-bound NCS-1 will lead to activation of target membrane proteins. This situation is distinct from the Ca\(^{2+}\)/myristoyl switch model based on the properties of recoverin.

Little information was available on the nature and number of possible target proteins for NCS-1 or other members of this family of Ca\(^{2+}\)-binding proteins or on whether their binding proteins are distinct from or overlap with those for calmodulin. The use of biotinylated NCS-1 has now allowed the detection of several binding proteins in subcellular fractions of adrenal medulla, some of which showed Ca\(^{2+}\)-independent and others Ca\(^{2+}\)-dependent interactions. These were shown to be specific based on lack of detection by biotinylated ARF1. In addition, a distinct pattern of Ca\(^{2+}\)-dependent binding partners was detected using biotinylated calmodulin. The multitude of binding partners detected is consistent with the various biochemical effects of NCS-1/frequenin that have been reported. It has been suggested that NCS-1 overlaps with calmodulin in its target protein interactions (16), but our data on biotinylated NCS-1 binding suggest that NCS-1 is unlikely to act simply as a calmodulin replacement but also has specific target proteins distinct from those for calmodulin. In the case of one common target protein, calcineurin, binding of calmodulin was strictly Ca\(^{2+}\)-dependent, but binding of NCS-1 was not entirely Ca\(^{2+}\)-dependent. These data suggest, therefore, a distinct mode of action for NCS-1 in which it is constitutively associated with membranes and certain target proteins at resting Ca\(^{2+}\) concentration, allowing it to act as a rapid transducer of Ca\(^{2+}\) signals in response to localized changes in Ca\(^{2+}\) concentration.

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Note Added in Proof—It has recently been shown that the yeast homologue of frequenin/NCS-1 activates phosphatidylinositol-4-OH kinase and binds to the enzyme in a Ca\(^{2+}\)-independent manner (41).

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NCS-1: Membrane and Protein Interactions

An effect of overexpression of a protein on neurotransmission in vivo as seen for frequenin (9) could be due to effects on many different aspects of presynaptic function. We have previously demonstrated that overexpression of NCS-1 in PC12 cells resulted in an increase in the extent of dense core granule exocytosis evoked by ATP acting on a purinergic receptor (14), indicating that the effect of frequenin/NCS-1 was not limited to synaptic vesicle exocytosis. These data also show that NCS-1 is not normally present in limiting amounts in intact PC12 cells, since increased levels of expression can exert a stimulatory effect. In contrast, when the overexpressing PC12 cells were permeabilized prior to stimulation by calcium, no effect of NCS-1 overexpression on exocytosis was detected. These findings may be consistent with NCS-1 acting on some early step in stimulus-secretion coupling in intact cells that would be bypassed in permeabilized cells. Alternatively, the possibility could not be ruled out that permeabilization resulted in loss of soluble NCS-1, explaining the difference in results between intact and permeabilized cells. We set out, therefore, to test these possibilities by directly manipulating cellular NCS-1 levels after permeabilization.

Digitonin-permeabilized adrenal chromaffin cells have been shown to be a useful model to examine the effects of exogenous soluble proteins on Ca\(^{2+}\)-dependent exocytosis, allowing demonstration of the stimulatory effects of annexin II (35), 14-3-3 proteins (32), α-SNAP (36), calmodulin (37), and cAMP-dependent protein kinase (38). Following digitonin permeabilization, about 50% of endogenous NCS-1 leaked from the cells. Preincubation with exogenous NCS-1 resulted in a 3-fold increase in cell-associated NCS-1, demonstrating its ability to enter the cells and remain cell-associated through all of the steps of the exocytosis assay. This association was Ca\(^{2+}\)-independent, consistent with the ability of NCS-1 to bind to membranes in a Ca\(^{2+}\)-independent manner. Using protocols similar to those used successfully with other proteins, we did not see any stimulatory effect of exogenous NCS-1 on exocytosis at low or high Ca\(^{2+}\) concentrations. These results are unlikely to be due to the levels of NCS-1 remaining following permeabilization already being sufficient, since intact cell studies with overexpression clearly show that NCS-1 levels are not saturating in neurons or PC12 cells (9, 11, 14). These data in combination with previous work on cells permeabilized after transfection (14) argue that NCS-1 does not directly regulate the machinery for Ca\(^{2+}\)-triggered exocytosis but may act on signal transduction pathways that control exocytosis or an early step in stimulus-secretion coupling.

Frequenin has been shown to stimulate membrane-bound photoreceptor guanylate cyclase at low but not high Ca\(^{2+}\) concentrations in rod outer segment membranes (9). It is not known if it can stimulate the forms of guanylate cyclase expressed in neuronal or other cell types. We did not detect any changes in cGMP levels following introduction of NCS-1 into permeabilized chromaffin cells. Similarly, while NCS-1 (16) and the related protein VILIP (38) have been suggested to be potential regulators of cAMP, we did not see any effects on cAMP levels. We had examined these cyclic nucleotides, since both have been shown to modulate dense core granule exocytosis in chromaffin cells (39). It seems unlikely, therefore, that cyclic nucleotide generation is part of the pathway by which NCS-1 overexpression enhances dense core granule exocytosis.

Related members of this family of Ca\(^{2+}\)-binding proteins have been reported to only bind to membranes in the presence of Ca\(^{2+}\) (19, 21, 22, 23, 24), consistent with the Ca\(^{2+}\)/myristoyl switch model for recoverin (3). In contrast, one member of the neuronal calcium sensor family, guanyl cyclase-activating protein 2, has been shown to have a reversed Ca\(^{2+}\) dependence in that it binds membranes at low Ca\(^{2+}\) and dissociates as Ca\(^{2+}\) is elevated (24). NCS-1 behaves in a manner distinct from these proteins in that the myristoylated protein shows Ca\(^{2+}\)-independent membrane binding. It is significant that endogenous NCS-1 remains membrane-associated in adrenal medullary fractions (14) and with rat brain membranes, even following extensive washing in the presence of Ca\(^{2+}\) chelators and even with carbonate buffer to extract extrinsic membrane proteins demonstrating a tight Ca\(^{2+}\)-independent membrane interaction. Recent work has shown that the binding of the related protein S-modulin to membranes, which is Ca\(^{2+}\)-dependent, requires the presence of a series of charged residues at the C terminus (40). These charged residues are absent in NCS-1, suggesting a distinct mechanism for membrane interaction. These findings demonstrate, therefore, that NCS-1 makes Ca\(^{2+}\)-independent interactions with target membrane and that Ca\(^{2+}\) does not regulate its membrane association. Presumably, conformational change following Ca\(^{2+}\)-binding in membrane-bound NCS-1 will lead to activation of target membrane proteins. This situation is distinct from the Ca\(^{2+}\)/myristoyl switch model based on the properties of recoverin.

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