Neuronal Ca$^{2+}$ Sensor 1, the Mammalian Homologue of Frequenin, Is Expressed in Chromaffin and PC12 Cells and Regulates Neurosecretion from Dense-core Granules*

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Neuronal Ca$^{2+}$ sensor 1 (NCS-1) is the mammalian homologue of the Ca$^{2+}$-binding protein frequenin previously implicated in regulation of neurotransmission in Drosophila (Pongs, O., Lindemeier, J., Zhu, X. R., Theil, T., Endelkamp, D., Krah-Jentgens, I., Lambrecht, H.-G., Koch, K. W., Schwemer, J., Rivosecchi, R., Mallart, A., Galceran, J., Canal, I., Barbas, J. A., and Ferrus, A. (1993) Neuron 11, 15–28). NCS-1 has been considered to be expressed only in neurons, but we show that NCS-1 expression can be detected in bovine adrenal chromaffin and PC12 cells, two widely studied model neuroendocrine cells. NCS-1 was present in both cytosolic and membrane fractions including purified chromaffin granules, and in immunofluorescence, its distribution overlapped with peripheral punctate staining seen with the synapt-like microvesicle marker synaptophysin in PC12 cells. The possible functional role of NCS-1 in exocytosis of dense-core granules was tested using transient transfection in PC12 cells and assay of co-transfected growth hormone (GH) release. Overexpression of NCS-1 increased evoked GH release in intact cells in response to ATP. No effect of overexpression was seen on GH release because of Ca$^{2+}$ in permeabilized cells suggesting that NCS-1 may have a regulatory but not direct role in neurosecretion.

Regulated exocytosis of neurotransmitters and hormones during neurosecretion is triggered by a rise in cytosolic Ca$^{2+}$ concentration (1). Recently a large number of proteins have been identified that are essential for regulated exocytosis (2, 3). In the multiple stages of the exocytotic pathway (4, 5) it is likely that both secretory vesicle recruitment and membrane fusion are regulated by Ca$^{2+}$, but the identity of the Ca$^{2+}$ receptors involved is still not fully resolved. In neurons, fast neurotransmission appears to require the low affinity Ca$^{2+}$-binding protein synaptotagmin I (6–8). The role of synaptotagmin in slow exocytosis that shows high Ca$^{2+}$ affinity is unclear (1), and other Ca$^{2+}$-binding proteins have been implicated in neurosecretion. Among these is the small EF-hand Ca$^{2+}$-binding protein, frequenin (9). Overexpression of this protein in Drosophila (9, 10) leads to a marked frequency-dependent facilitation of evoked neurotransmission. Injection of recombinant frequenin into Xenopus spinal neurons (11) increases both basal and evoked neurotransmission. The site of action of frequenin is not known nor is it known whether it interacts directly with the exocytotic machinery or if it has an indirect regulatory effect on neurotransmission. In addition, it is not known if frequenin functions in exocytosis in non-neuronal cells.

Frequenin is a member of a family of related Ca$^{2+}$-binding proteins. Within this family are two subclasses of proteins. Type A includes proteins such as visinin (12), recoverin (13), and S-modulin (14), which are expressed only in photoreceptor cells and function in the control of visual transduction pathways. Type B includes proteins expressed in neurons such as neurocalcin (15), hippocalmin (16), frequenin (9), neuronal Ca$^{2+}$ sensor 1 (NCS-1) (17, 18) and VILIP (19). The cellular functions of the type B proteins are unknown. All members of this family possess 2–3 functional Ca$^{2+}$-binding domains and an N-terminal myristoylation site. Mammalian neurocalcin, hippocalmin, and NCS-1 are expressed in various neuronal classes, and it is clear that NCS-1 is the chicken (17) and mammalian (18) frequenin homologue possessing a recognizable and distinct, C-terminal domain also found in Xenopus frequenin (11). NCS-1 is believed to be neuron-specific and has not so far been shown to be expressed outside the nervous system.

Adrenal chromaffin and PC12 cells have proved to be good models for the study of neurosecretion (4, 5). They express many proteins previously believed to be neuron-specific, and exocytosis of dense-core granules in these cells uses the same conserved machinery that functions in synaptic neurotransmission (20). We have, therefore, addressed the question of whether NCS-1 is expressed in these cell types and exploited PC12 cells for functional analysis of the role of NCS-1. We show here that the mammalian frequenin homologue, NCS-1, is expressed in neuroendocrine cells. Overexpression of NCS-1 resulted in an increase in stimulated exocytosis in intact though not permeabilized PC12 cells implicating frequenin/NCS-1 as a general but indirect regulator of neurosecretion.

MATERIALS AND METHODS

Cell Culture—Bovine chromaffin cells were prepared and cell cultures maintained as described previously (21). PC12 cell cultures were maintained as described previously (22).

RT-PCR—Total RNA was extracted from whole brain of Wistar rats and cultured chromaffin bovine cells using an RNasey isolation kit (Qiagen, Surrey, UK), cDNA synthesized with a reverse transcription system, and amplified in PCR reactions using an Omne-E dryblock thermocycler (Hybaid, Middlesex, UK). The primers contained restriction endonuclease sites (underlined) to allow subsequent cloning. The sense and antisense primers used for NCS-1, based on the rat nucleotide sequence (GenBankTM number L27421), were 5'-CATGGATCCATGGGAAAATCCAACAGCAAGT-3' (BamHI) and 5'-CATGGTACCTATACCCAAGCCCCGTCTAGAGG-3' (RpsI), respectively, and for neuro-

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calcin/hippocalcin, based on the bovine nucleotide sequence of neurocalcin (15), were 5′-GCTGGATCCTAGGGAAAGAGAACAGCAAGC-3′ (BamHI) and 5′-ACTGAGGTTCCAGAATGGCCGAGCCTGCTG-3′ (HindIII).

Expression and Purification of Recombinant His<sub>6</sub>-tagged NCS-1—For protein expression, the NCS-1 PCR product from rat brain was ligated into the pQE-30 vector to provide an N-terminal His<sub>6</sub>-tag (Qiagen, Surrey, UK) and transformed into M15 [pREP4] cells. Recombinant protein expression was induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h and His<sub>6</sub>-tagged proteins, after sonication and lysis, purified on nickel-nitrotriacetic acid-agarose by applying a 50–500 mM imidazole gradient. All chromatography was performed at 4 °C using a Pharmacia fast protein liquid chromatography system. Peak fractions containing recombinant NCS-1 were identified by SDS-polyacrylamide gel electrophoresis, and the pooled protein was stored at −80 °C.

Preparation of Affinity-purified NCS-1 Antiserum—His<sub>6</sub>-NCS-1 (300 μg) in complete Freund’s adjuvant (Pierce, Chester, UK) was injected subcutaneously into a rabbit, followed after 4 weeks by a similar injection in incomplete Freund’s adjuvant (Pierce, Chester, UK). Bleeds were taken thereafter at 2–4-week intervals after boosting with His<sub>6</sub>-NCS-1 (300 μg) in incomplete Freund’s adjuvant 7 days beforehand. Affinity purification was performed by incubating 1 mg of His<sub>6</sub>-NCS-1 linked to a 0.5-mL nickel-nitrotriacetic acid-agarose column with unpurified serum for 1 h at room temperature. The column was then washed with 50 mM imidazole, 200 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 10% glycerol (v/v), 20 mM HEPES (pH 7.0) to remove nonspecifically bound proteins. Antibodies were eluted with 3 ml of 5 M LiCl in 10 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.2 and dialyzed against phosphate-buffered saline (PBS) before storage at −80 °C.

Subcellular Fractionation—Bovine adrenal medullae were homogenized in 0.3 M sucrose, 5 mM EDTA, and 5 mM HEPES (pH 7.3), filtered, and centrifuged at 800 g for 20 min at 4 °C. The supernatant from this step was further centrifuged at 100,000 g for 60 min at 4 °C to provide cytosolic (supernatant) and microsomal (pellet) fractions. The pellet from the 100,000 g centrifugation contained mitochondria as well as chromaffin granules, and the granules were further purified by centrifugation at 100,000 g for 60 min over a 1.7 M sucrose cushion. Granule membranes were then prepared by lysis of the granule pellet and washing in 5 mM Hepes, 5 mM EDTA, pH 7.3, to remove proteins. For fractionation of PC12 cells, cells were homogenized in 0.32 M sucrose, 10 mM Hepes, pH 7.4, and nuclei were pelleted at 750 × g for 5 min. The postnuclear supernatant was loaded on a 0.6–1.8 M sucrose gradient and centrifuged at 100,000 × g for 6 h (23).

Immunoblotting with NCS-1 Antiserum—For immunoblotting, samples were separated on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose by transverse electrophoresis, and probed as described previously (24) with anti-NCS-1 antisera (1:400). As a control, preimmune serum from the same rabbit was used at the same dilution.

Immunofluorescence—Cells were grown in culture on glass coverslips for 3 days before fixation in 4% formaldehyde in PBS. The cells were washed twice in PBS and incubated for 30 min in PBTA (0.1% Triton X-100 and 0.3% bovine serum albumin in PBS). Following this, the cells were incubated with affinity-purified anti-NCS-1 antibody (1:10) or anti-secretogranin II antibody (1:75; a gift from Dr. D. Cutler, MRC Laboratory of Molecular Cell Biology, London) in PBTA for 1 h and washed three times in PBTA. The cells were then incubated in anti-rabbit IgG biotinylated (1:100; Amersham, Buckinghamshire, UK) for 1 h, washed three times in PBTA followed by a further incubation in streptavidin-Texas Red (1:50; Amersham, Buckinghamshire, UK) for 30 min. The cells were again washed three times in PBTA, and the coverslip was blotted and allowed to air dry before mounting. For dual labeling of NCS-1 and synaptophysin or NCS-1 and dopamine β-hydroxylase, cells were incubated as above for NCS-1 but with anti-synaptophysin antibody (1:50; Sigma, Dorset, UK) or anti-dopamine β-hydroxylase antibody (1:100; a gift from Dr. D. Apps, Department of Biochemistry, University of Edinburgh) and anti-mouse IgG fluorescein isothiocyanate conjugate (1:75; Amersham, Buckinghamshire, UK) present in addition to the first and second antibodies, respectively.

To determine whether NCS-1 or the related neuronal Ca<sup>2+</sup>-binding proteins neurocalcin and hippocalcin are expressed in chromaffin cells we initially used an RT-PCR approach. Primers were designed based on the rat NCS-1 sequence or alternatively based on the bovine neurocalcin sequence. The neurocalcin primers were also predicted to amplify cDNA for

**RESULTS**

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**FIG. 1. Expression of NCS-1 in adrenal chromaffin and PC12 cells.** A, RT-PCR showing amplification from rat brain or chromaffin (chrom.) cell cDNA using primers for NCS-1 or for neurocalcin. B, immunoblotting with anti-NCS-1 showing that the antiserum recognizes recombinant NCS-1 but not neurocalcin or VILIP. C, immunoblottin with anti-NCS-1 showing labeling of a single band in rat brain (at 1 mg/ml), PC12, and chromaffin cells. D, immunoblotting with anti-NCS-1 showing the lack of any signal in RBL cells. For this comparison the rat brain homogenate was run at 1:5 dilution (0.2 mg/ml). E, immunoblotting with anti-NCS-1 showing the presence of NCS-1 in cytosol (Cyto), microsomal (Micro), and granule membranes (G memb) fractions from adrenal medulla. F and G, dual label immunofluorescence on chromaffin cells in culture using anti-dopamine β-hydroxylase (F) and affinity-purified anti-NCS-1 (G).
hippocalin. A PCR product of the expected size for NCS-1 (around 600 base pairs) was amplified from rat brain and bovine adrenal chromaffin cell cDNA (Fig. 1A) and confirmed as encoding NCS-1 by cloning and sequencing. In contrast, the bovine neurocalcin/hippocalin primers gave a weak PCR signal from rat brain cDNA, as predicted because of species differences, but gave no product from bovine chromaffin cell cDNA (Fig. 1A). These data suggested that NCS-1 but not neurocalcin or hippocalin is expressed by chromaffin cell cultures. The absence of neurocalcin from mouse chromaffin cells despite its presence in adrenal nerve terminals has been reported (26).

The expression of NCS-1 in chromaffin cell cultures and PC12 cells was confirmed using an antiserum raised against recombinant His⁶-tagged NCS-1. This antiserum recognized recombinant NCS-1 but not recombinant VILIP or neurocalcin (Fig. 1B). This antiserum labeled a single co-migrating band in immunoblots of rat brain, chromaffin, and PC12 cell homogenates (Fig. 1C). No polypeptides were detected using a preimmune antiserum. In rat basophilic leukemia (RBL) cells, a well-characterized myeloid secretory cell line, no polypeptides were detected by the anti-NCS-1 antiserum (Fig. 1C). In subcellular fractions from adrenal medulla, NCS-1 was detected in cytosol, and despite the presence of 5 mM EDTA during homogenization and fractionation, was also present in microsomal and to a lesser extent in purified chromaffin granule membrane fractions (Fig. 1E). Expression of NCS-1 in chromaffin cells in the primary cultures was confirmed by dual label immunofluorescence using affinity-purified anti-NCS-1 and antibodies that recognize the chromaffin granule protein dopamine β-hydroxylase (Fig. 1, F and G).

The data from fractionation of adrenal medulla were consistent with NCS-1 expression as a soluble and also a membrane-associated protein. The diffuse localization in chromaffin cells by immunofluorescence would be consistent with such a distribution. In contrast, in PC12 cells in addition to a diffuse cellular staining, anti-NCS-1 also gave a distinct peripheral punctate staining pattern that substantially overlapped with that of the synaptic-like microvesicle vesicle (SLMV) marker synaptophysin (Fig. 2, A and B), suggesting that a portion of the NCS-1 in PC12 cells is present on SLMVs. A marker for dense-core granules in PC12 cells, secretogranin II, gave only a diffuse staining pattern (Fig. 2C). On sucrose gradient fractionation of PC12 cells, a complex distribution was found that included some overlap with secretogranin II (data not shown), and so it is possible, but not conclusive, that NCS-1 may also be associated with dense-core granules in PC12 cells.

To examine a possible function of NCS-1 in control of dense-core granule exocytosis, we tested the effect of its overexpression using the GH transient co-transfection assay in which GH acts as a reporter of exocytosis in only the transfected cells (27). Transfection with sense NCS-1 plasmid had no effect on total GH levels within the cells 3 days after transfection, but the antisense plasmid used as a control in these experiments markedly reduced cellular GH levels (Fig. 3A). When exocytosis was examined in response to stimulation with ATP to activate purinergic receptors, it was clear that the antisense plasmid had little effect on the extent of GH release, expressed as a percentage of total cellular GH content, but that overexpression of NCS-1 because of transfection with sense plasmid increased the extent of stimulated GH release by around 50% (Fig. 3B). This increase was reproducible and seen in four separate transfections, the data from which are pooled in Fig. 3B. Overexpression of NCS-1 had no effect on basal GH release. These results indicate that NCS-1 can act as a positive regulator of the pathway leading to dense-core granule exocytosis in neuroendocrine cells. To examine whether NCS-1 overexpression was because of an effect on an early stage of the pathway activated by ATP or if it could directly increase the extent of exocytosis, GH release was also assayed from digitonin-permeabilized cells that allow analysis of any direct effects on Ca²⁺-regulated exocytosis. Overexpression of NCS-1 did not affect GH release in the absence of Ca²⁺ nor in response to Ca²⁺ over the range of 1–10 μM in permeabilized cells (Fig. 4) suggesting that NCS-1 may not act directly on the exocytotic machinery.

**DISCUSSION**

It has previously been suggested (17, 18) that NCS-1 is a neuron-specific protein, but the data presented here show that NCS-1 is also apparently expressed in adrenal chromaffin and PC12 cells, two widely studied model neuroendocrine cells. Sequence comparison of NCS-1 from chicken, rat (17), and mouse (18) with frequenin and related proteins has led to the conclusion (18) that NCS-1 should be regarded as the mammalian homologue NCS-1 can act as a positive regulator of evoked dense-core granule exocytosis in neuroendocrine cells but that it is unlikely to do so by a direct action on the exocytotic machinery as its overexpression resulted in increased GH release in intact cells, but no effect was seen on Ca²⁺-induced
exocytosis in permeabilized PC12 cells.

NCS-1 expression was demonstrated by RT-PCR amplification from chromaffin cell cDNA, and protein expression was confirmed by immunoblotting and immunofluorescence on both chromaffin and PC12 cells. No evidence for neurocalcin or hippocalcin expression in chromaffin cells was found by RT-PCR, and in fact hippocalcin has been shown to have a very restricted distribution in only hippocampal neurons (16). The antisera raised against recombinant NCS-1 recognized only a single polypeptide that co-migrated in brain, chromaffin, and PC12 cells. It did not recognize neurocalcin, and no polypeptides were detected in RBL cells, demonstrating its specificity. In addition, we have confirmed NCS-1 expression in chromaffin and PC12 cells using an antisera raised against a peptide corresponding to the distinct C-terminal sequence of this protein.2 The localization of NCS-1 was complex, being found in cytosolic fractions and in membrane fractions from adrenal medulla including a crude microsomal fraction and also purified chromaffin granule membranes. Other members of this family including neurocalcin (28) and hippocalcin (29) have been shown to associate with membranes only in the presence of Ca\(^{2+}\), but NCS-1 was associated with membranes from chromaffin and PC12 cells even when prepared in the presence of 5 mM EDTA. Co-localization of NCS-1 in immunofluorescence with the SLMV marker synaptophysin in PC12 cells suggested its presence on SLMVs. NCS-1 also showed an additional diffuse distribution in PC12 cells. Its complex distribution on subcellular fractionation and sucrose gradients precluded unequivocal demonstration of its presence or absence on dense-core granules. The function of NCS-1 associated with SLMVs remains to be established.

The cellular function of NCS-1/frequenin is unknown, but studies in Drosophila and Xenopus strongly suggest that it is a Ca\(^{2+}\) sensor leading to the facilitation of exocytosis. This role would be consistent with its high level of expression in brain. We have examined the possible role of NCS-1 in dense-core granule exocytosis using transient transfection in PC12 cells. This approach was chosen as any expressed protein should be correctly post-translationally modified and NCS-1 has an N-terminal myristoylation site (17, 18). Co-transfection with a plasmid-encoding growth hormone has become widely used in the study of proteins involved in dense-core granule exocytosis in PC12 cells including raphilphin 3A (30), Doc 2 (31), Munc13 (32), Rim (33), and nSec-1 (25). LipofectAMINE results in transfection of up to 10% of the PC12 cells (25), and assay of GH allows analysis of secretion from only those cells that take up plasmid. It has previously been shown that following co-transfection with two plasmids around 90% of the cells expressing GH also express the second protein (30, 31). We have also confirmed such a high efficiency of co-expression in our assays using transfected light chain of botulinum neurotoxin C. This neurotoxin inhibited GH release by 95% suggesting that essentially all cells expressing GH also express this second transfected protein.3 It has been confirmed that GH expression in PC12 cells following transfection is targeted to catecholamine granule exocytosis (27, 30–34). Analogous to the overexpression of frequenin in Drosophila,

\[\text{FIG. 3. Overexpression of NCS-1 in transient transfection increases reporter growth hormone release from PC12 cells. PC12 cells were transfected (4 \mu g/well of each plasmid) with control, sense NCS-1, or antisense NCS-1 plasmids along with plasmid (4 \mu g/well) encoding GH. After 3 days the cells were washed and incubated with no additions or with 300 \mu M ATP for 15 min. Cellular GH and GH present in the medium were assayed. A, total GH content per well (n = 10 for antisense (a/sense) and n = 20 for control (Cont) and sense). B, GH released into the medium was expressed as a percentage of total GH level for each well, and data were normalized with the control value with ATP as 100% to allow data from four separate transfections to be combined (n = 5 for antisense and n = 10 for control and sense).}\]
overexpression of NCS-1 increased stimulated release of reporter GH from intact transfected PC12 cells compared with control transfections. In these experiments, we used an antisense NCS-1 construct as an additional control. It would appear unlikely that an antisense plasmid could result in depression of NCS-1 levels within the short time course of this experiment, and so it was surprising that, in the cells transfected with the antisense plasmid, total GH levels were reduced. This may indicate an additional role of NCS-1, but we cannot rule out that this is because of a loss of PC12 cells transfected with antisense NCS-1 rather than a specific effect on GH synthesis. The low percentage of cells (around 5–10% (25)) that become transfected precludes quantitative analysis of NCS-1 levels within those cells by immunoblotting. The increase in ATP-stimulated GH release from intact PC12 cells was a specific effect as in previous work no effect of plasmids encoding various control proteins or nSec-1 was seen on GH exocytosis (25). In addition, the effect of transfection with NCS-1 was specific to intact cells, and no changes were observed in Ca\(^{2+}\)-induced GH release after permeabilization of the cells.

The data presented here show that NCS-1 is not expressed solely in neurons and for the first time implicate NCS-1/frequenin in the pathway leading to dense-core granule exocytosis in neuroendocrine cells. Studies on neurotransmission in *Drosophila* have suggested that frequenin may act in the synapse via effects on K\(^+\) channels (35) or the plasma membrane Na\(^+\)-Ca\(^{2+}\) exchanger (10). NCS-1/frequenin may, therefore, act as a regulator of neurosecretion by effects at the level of plasma membrane channels and pumps, but it has not been possible to exclude a direct effect on the exocytotic machinery. At physiological Mg\(^{2+}\) concentration, Ca\(^{2+}\) binding to NCS-1 is half-maximal at 0.3 \(\mu\)M free Ca\(^{2+}\) (36), and it, therefore, shows a much higher Ca\(^{2+}\) affinity than that required for the Ca\(^{2+}\)-triggered fusion step in either dense-core granule or synaptic vesicle exocytosis, which requires 10 or 100 \(s^{-1}\) of \(\mu\)M Ca\(^{2+}\), respectively (1). The data in this study suggest that NCS-1 may not function directly in exocytosis, and the most probable role of NCS-1/frequenin is as a general regulator of neurosecretion via effects on Ca\(^{2+}\) signaling pathways or Ca\(^{2+}\) homeostasis.

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