Neuronal calcium sensor 1 and phosphatidylinositol 4-OH kinase β interact in neuronal cells and are translocated to membranes during nucleotide-evoked exocytosis

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Summary

Neuronal calcium sensor 1 (NCS-1) belongs to a family of EF-hand calcium-binding proteins and is mainly expressed in neurons and neuroendocrine cells, where it causes facilitation of neurotransmitter release through unknown mechanisms. The yeast homologue of NCS-1 has been demonstrated to interact with and regulate the activity of yeast phosphatidylinositol 4-OH kinase β (PI4Kβ). However, in neurons and neurosecretory cells NCS-1 has not unequivocally been shown to interact with PI4Kβ. Here we have compared the subcellular distribution of NCS-1 and PI4Kβ and investigated whether they are capable of forming complexes. In neurons, both proteins are widely distributed and are present in perikarya and, to a lesser extent, in nerve terminals. A consistent portion of NCS-1 and PIK4β is cytosolic, whereas a portion of both proteins appears to be associated with the membranes of the endoplasmic reticulum and the Golgi complex. Very small amounts of NCS-1 and PI4Kβ are present in synaptic vesicles. Our results further demonstrate that in neurosecretory cells, endogenous NCS-1 and PIK4β interact to form a complex that can be immunoisolated from membrane as well as from cytosolic fractions. Moreover, both proteins can be recruited to membranes when cells are treated with nucleotide receptor agonists known to increase polyphosphoinositide turnover and concomitantly induce exocytosis of secretory vesicles. Finally, in PC12 cells overexpressing NCS-1, the amount of PI4Kβ associated with the membranes is increased concomitantly with the increased levels of NCS-1 detected in the same membrane fractions. Together, these findings demonstrate that mammalian NCS-1 and PI4Kβ interact under physiological conditions, which suggest a possible role for NCS-1 in the translocation of PI4Kβ to target membranes.

Key words: Calcium-binding proteins, Neuronal calcium sensor 1, Phosphatidylinositol 4-OH kinase, Polyphosphoinositides, Membrane traffic

Introduction

Recent work has demonstrated that a family of myristoylated calcium-binding proteins (the so-called neuronal calcium sensor proteins) may play a role in a variety of processes including phototransduction, neurotransmitter release, the control of cyclic nucleotide metabolism, the regulation of calcium channels and phosphoinositide metabolism (Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001). One member of this large family is neuronal calcium sensor 1 (NCS-1), the mammalian orthologue of frequenin, a protein originally identified in *Drosophila* (Pongs et al., 1993). Studies in *Drosophila* and in *Xenopus* have indicated that frequenin acts as a modulator of synaptic efficacy (Pongs et al., 1993; Rivosecchi et al., 1994; Olafsson et al., 1995). In line with these findings, overexpressed NCS-1 has been shown to enhance regulated secretion from pheochromocytoma (PC12) cells and to be, at least in part, localized to synaptic-like microvesicles (McFerran et al., 1998). The important function of NCS-1 as a regulator of associative learning and memory has recently been demonstrated in vivo in *C. elegans* (Gomez et al., 2001). Moreover, NCS-1 has been described to play a role in the mechanisms underlying long-term neurotrophin regulation of synaptic plasticity (Wang et al., 2001). Together, these observations suggested an important function for NCS-1 in synaptic transmission. However, the mechanisms by which NCS-1 regulates neurotransmitter release and/or synaptic plasticity remain poorly understood.

An interesting insight into the physiological role of NCS-1 has come from recent data (Hendricks et al., 1999) demonstrating that the yeast homologue of NCS-1 can associate with and upregulate the activity of an isoform of phosphatidylinositol 4-OH kinase (PI4K) homologous to the mammalian PI4Kβ [a member of the so-called type III PI4Ks (Balla, 1998; Fruman et al., 1998; Hendricks et al., 1999)].

The members of the PI4K family catalyze the first step in the synthesis of phosphoinositides and polyphosphoinositides that are known to play a crucial role in exocytosis and intracellular traffic (for reviews, see De Camilli et al., 1996; Brodin et al., 2000; Huijbregts et al., 2000; Cremona and De Camilli, 2001). The first evidence that polyphosphoinositides are important in vesicular trafficking reactions independently of their phospholipase C-mediated cleavage came from studies of regulated exocytosis in neuroendocrine cells (Holz et al., 1989; Eberhard et al., 1990; Hay et al., 1995). In these studies, Ca^{2+}-dependent neurotransmitter release has been shown to require an ATP-priming step. Both a PI4K and a phoshoinositide 4*P* 5-kinase [PI(4)*P*5K] are required for the priming reaction, suggesting that phosphoinositides, mainly phosphatidylinositol(4,5)P_2, may play a role in this process. Interestingly, a PI4K activity has been detected on chromaffin granules (Wiedemann et al., 1996) and synaptic vesicles (Wiedemann et al., 1998). Besides exocytosis, phosphoinositides have also been implicated in other aspects of membrane traffic (e.g. synaptic vesicle endocytosis and constitutive secretion), suggesting that their synthesis is highly regulated. The molecular mechanisms underlying the synthesis of different polyphosphoinositide pools, the subcellular localization of these pools and the PI4Ks involved are only partially known. The recent finding in yeast suggests that NCS-1 and PI4Kβ may cooperate in modulating the exocytotic processes. However, it is still unclear whether endogenous NCS-1 and PI4Kβ interact in vivo. The two proteins were found to form a complex after overexpression in epithelial Madin-Darby canine kidney and COS-7 cells, but not in cultured DRG neurons (Weisz et al., 2000; Bartlett et al., 2000; Zhao et al., 2001).

In order to further study the function of NCS-1 in neurosecretory cells and its interaction with PI4Kβ, we analyzed and compared the subcellular distribution of both proteins and tested whether they are capable of forming a complex in neurons and neuroendocrine cells. Moreover, we investigated whether the membrane distribution of NCS-1 and PI4Kβ was modulated in intact neuroendocrine cells under conditions that are known to stimulate polyphosphoinositide turnover and neurotransmitter secretion (Raha et al., 1993; Murrin and Boarder, 1992; Koizumi et al., 1995).

Materials and Methods

Cell cultures and electroporation

Primary neuronal cultures were prepared from the hippocampi of embryonic day 18 rats as described (Verderio et al., 1995). PC12 cells were maintained in DME medium and electroporation was carried out as previously described (Rowe et al., 1999) with minor modifications. Cells from subconfluent cultures were trypsinized, resuspended in DME medium with 8 μg of rat NCS-1 cDNA (in pcDNA3 vector) and 8 μg of pEGFP-N1 vector (Clontech Laboratories, Heidelberg, Germany) or with 8 μg of pEGFP-N1 vector alone. Cells were electroporated with one shock at 250 mV, 960 mF, incubated on ice for 10 minutes and then centrifuged for 10 minutes at 900 ***g*** on a cushion of Ficoll to separate the damaged cells. The live cells at the Ficoll interface were plated onto 35 mm petri dishes and used for biochemical analysis 48 hours after electroporation. Under these experimental conditions transfection efficiency (calculated by counting the number of GFP-expressing cells per petri-dish) was 54.5±14.8%.

Antibodies

The rabbit polyclonal (44162) and monoclonal (3D5) antibodies against NCS-1 were prepared as described (Werle et al., 2000). The polyclonal antibodies against ribophorin, calreticulin and synaptotagmin were kind gifts of G. Kreibich (New York University School of Medicine, New York), J. Meldolesi and A. Malgaroli (Department of Neurosciences, San Raffaele Institute, Milan, Italy), respectively. Polyclonal anti-PI4Kβ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibodies against protein disulphide isomerase (PDI), anti-tubulin, the TGN38 trans-Golgi network protein and synaptobrevin 2 were obtained from Stressgen Biotechnologies (Victoria, BC, Canada), Sigma Aldrich (Milan, Italy), Transduction Laboratories (Lexington, KY) and Synaptic Systems (Gottingen, Germany), respectively. The peroxidase and gold-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA) or Sigma. Rabbit IgGs were purchased from Sigma.

Subcellular fractionation

Differential centrifugation

Rat brain fractionation was carried out essentially as described (Huttner et al., 1983). Cerebral cortices dissected from rat brains were homogenized in homogenization buffer (4 mM Hepes-NaOH, pH 7.3, and 0.32 M sucrose). The total homogenate was centrifuged for 10 minutes at 800 ***g*** and the post-nuclear supernatant (S1) was collected and centrifuged as described to yield a pellet corresponding to the synaptosomal fraction (P2) and a supernatant (S2). The S2 containing the remaining organelles from the total homogenate was centrifuged at 165,000 ***g*** for 2 hours to yield a high-speed supernatant corresponding to the cytosol (S3) and a pellet (P3) enriched in membrane-bound organelles of cell bodies. P2 was subjected to hypo-osmotic shock by means of 10-fold dilution in 7.5 mM Hepes-NaOH buffer, pH 7.2. The P2-lysate was centrifuged for 20 minutes at 25,000 ***g*** to yield a lysate pellet (LP1) containing membrane-bound organelles/vesicles larger than synaptic vesicles and a lysate supernatant (LS1) that was further centrifuged at 165,000 ***g*** for 2 hours. The resulting supernatant (LS2, the cytosolic fraction of the synaptosomal compartment) was removed and the pellet (LP2) containing the small vesicles was resuspended in 40 mM sucrose, loaded on top of a linear sucrose gradient (50-800 mM sucrose) and centrifuged at 65,000 ***g*** for 5 hours. After centrifugation, 20 fractions of 500 μl were collected and those equilibrated in the 200-400 mM sucrose region were pooled and centrifuged 165,000 ***g*** for 5 hours to yield a pellet, SG-V, highly enriched in synaptic vesicles. Equal amounts of proteins from each fraction were separated on SDS-polyacrylamide gels and analyzed by western blotting as described (Rowe et al., 1999).

Velocity gradient centrifugation

P3 was resuspended with a dounce homogenizer in 250 mM sucrose, 1 mM Mg-acetate, 2 mM EDTA and 10 mM Hepes-KOH, pH 7.4 and then loaded on top of a sucrose linear gradient (0.3-1.2 M). After centrifugation at 75,000 ***g*** for 20 minutes, 12 aliquots of 1 ml were collected from the top of the gradient. Proteins from equal volumes of each fraction (300 μl) were precipitated with acetone at –20°C and then separated on SDS-polyacrylamide gels and analyzed by western blotting.

Discontinuous sucrose density gradient centrifugation

The S2 fraction prepared by differential centrifugation was adjusted to 1.2 M sucrose containing 1 mM EDTA loaded into an SW 27 tube and overlayed with 8 ml of 1.1 M sucrose, 10 ml of 0.85 M sucrose and 8 ml of 0.25 M sucrose. The gradients were centrifuged at 100,000 ***g*** for 3 hours and the band at the 0.85-1.1 M sucrose interface (fraction

1), a second band at the 1.1-1.2 M sucrose interface (fraction 2) and the pellet were collected and analyzed by western blotting.

Immunoprecipitation

Brains from adult Sprague-Dawley rats (females) were homogenized in ice-cold immunoprecipitation (IP) buffer (125 mM potassium-acetate, 0.1% (w/v) Triton X-100, 20 mM Tris-HCl pH 7.2, 2 μg/ml pepstatin, 2 μg/ml aprotinin). When required, the IP buffer was supplemented with 1 mM $CaCl_2$ or 5 mM EGTA. The total homogenates were incubated for 1 hour on ice and then clarified by centrifugation (30 minutes at 14,000 ***g***). Supernatant volumes corresponding to 0.5-1 mg of protein were incubated for 1 hour with 50 μl of Protein A sepharose beads (Amersham-Pharmacia). The beads were removed by centrifugation (10 minutes at 3000 ***g***) and the 'precleared' supernatants were added to 50 μl of protein A beads preincubated (2 hours at 4°C) with either the affinity purified anti-NCS-1 IgG (2-4 μg, polyclonal 44162), the anti-PI4Kβ IgG (2-4 μg, Upstate) or rabbit IgG (2-4 μg, Sigma Aldrich) as a control. After 16 hours at 4°C, the beads were collected by centrifugation (5 minutes at 3000 ***g***) and extensively washed with IP buffer and then resuspended in Laemmli sample buffer (Laemmli, 1970). When the immunoprecipitation was performed on rat brain membrane or soluble protein fractions, the tissue was homogenized in 320 mM sucrose, 4 mM Hepes-NaOH pH 7.3 supplemented with protease inhibitors. The postnuclear supernatants (S1) were centrifuged at high speed (1 hour at 200,000 ***g***) in order to obtain total membrane pellets and soluble protein fractions. The S1, membrane (resuspended in sucrose buffer to reconstitute the initial volume) and cytosol fractions were adjusted to 1× IP buffer, incubated for 1 hour on ice and clarified by centrifugation. Immunoprecipitation was carried out as described above by using equal volumes of S1, membrane and cytosol. Immunoprecipitated proteins were then analyzed by western blotting.

Uridine 5′-triphosphate (UTP) stimulation

PC12 cells were grown as described (Rowe et al., 1999). For UTP stimulation, subconfluent cell cultures (in 35 mm petri dishes) were incubated for 3 minutes at 37°C in 1 ml of Hepes-buffered external medium (Krebs-Ringer buffer; KRB) with Ca^{2+} or without Ca^{2+} (+2 mM EGTA) in the absence or presence of 300 μM UTP. Cells were then cooled on ice, scraped in ice-cold homogenization buffer (0.25 M sucrose, 1 mM Mg-acetate, 10 mM Hepes-KOH, pH 7.4, 2 μg/ml pepstatin and 2 μg/ml aprotinin), pelleted, and homogenized in 150 μl of homogenization buffer. The post nuclear supernatants (120 μl) were centrifuged at 200,000 ***g*** for 1 hour. The high-speed supernatants (cytosolic fractions) were collected and solubilized in Laemmli sample buffer (final volume 180 μl). The pellets (membrane fractions) were resuspended in 180 μl Laemmli sample buffer. Equal volumes of cytosolic and membrane fractions were then analyzed by western blotting. The levels of NCS-1, PI4Kβ or synaptophysin were quantified by measuring the density of the bands. Autoradiograms showing the appropriate band intensities were acquired by means of an ARCUS II scanner (Agfa-Gevaert, Mortsel, Germany) and the density of each band was quantitated using the NIH Image program 1.61 (National Technical Information Service, Springfield, VA).

Immunocytochemistry

Immunoelectron microscopy

Adult Sprague-Dawley rats (females, 150 g) were deeply anaesthetised with 2 mg xylazine and 5 mg ketamine and then perfused transcardially with 20 ml of a solution containing 0.9% NaCl, 0.025% heparin and 2.5% polyvinyl pyrrolidone 40,000, followed by 100 ml of freshly prepared 4% paraformaldehyde and 0.2% glutaraldehyde in 0.12 M phosphate buffer pH 7.4. The brains were dissected and cerebral cortices and hippocampi were cut into small pieces of about 1 mm^3 and fixed by immersion in the same fixative for 2 hours at 4°C. After fixation, the tissue pieces were extensively rinsed with phosphate buffer, infiltrated overnight with 2.3 M sucrose in PBS and then frozen in liquid nitrogen. Ultrathin frozen sections were obtained by using a Reichert Jung Ultracut E ultramicrotome equipped with a FC4 cryochamber and collected on Formvar-coated nickel grids. Immunolabeling experiments were performed as described (Bassetti et al., 1995). The specificity of staining was tested by substituting normal rabbit or mouse IgGs for specific antibodies as well as by omitting the primary antibody and incubating the grids with appropriate secondary antibody.

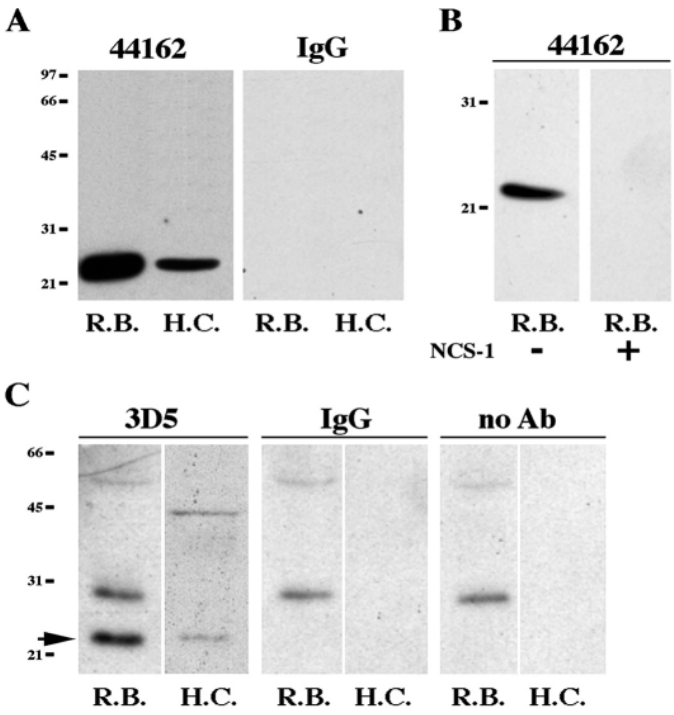

Fig. 1. Characterization of anti-NCS-1 antibodies. Total homogenates (40 μg) from rat brain (R.B.) and human cerebellum (H.C.) were analyzed by western blots using polyclonal (A, 44162; diluted 1:1000) or monoclonal antibodies (C, 3D5; diluted 1:5000) raised against recombinant NCS-1. Parallel samples were immunostained with preimmune rabbit or mouse IgG (IgG in A and C) or with the anti-mouse IgG, omitting the primary monoclonal antibody (C, no Ab). In B, proteins from rat brain total homogenate (R.B.) were labeled with anti-NCS-1 (44162) with (+) or without (–) pre-adsorption with 1 μg of purified recombinant NCS-1. In C, the arrow indicates the position of NCS-1; the bands of 55 and 28 kDa correspond to rat Ig heavy and light chains, respectively, which were present in the brain extracts and were recognized by the secondary antibodies (no Ab).

Statistical analysis

The density of the gold particles in the perikarya is expressed as the number of particles per square micrometer of the different organelle areas measured using the Image 1.61 analysis program. As specified in Table 1 a number of micrographs were acquired for each determination using an Arcus II scanner (Agfa-Gevaert). The data are expressed as mean values±s.e.m. The gold particle densities at the synapses were evaluated in two different experiments using the antibody against NCS-1 at a final dilution of 1:100, and detected using a secondary antibody conjugated to 12 nm colloidal gold particles. Randomly chosen synapse-rich areas were directly evaluated under

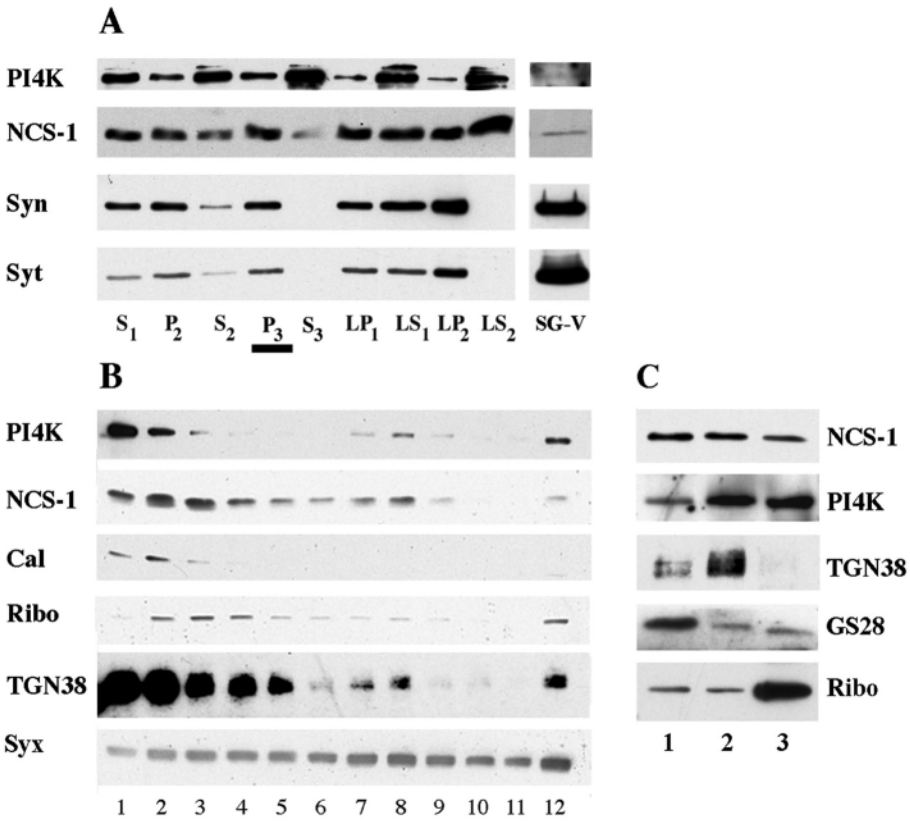

Fig. 2. Distribution of NCS-1 and PI4Kβ in subcellular fractions of rat brain. (A) Differential centrifugation of rat brain total homogenates. Equal amounts of proteins (12 μg) from each fraction (defined as described in Materials and Methods) were analyzed by SDS-PAGE and western blotting. The blots were probed with antibodies directed against PI4Kβ (PI4K, diluted 1:4000), NCS-1, synaptophysin (Syn, diluted 1:2000) or synaptotagmin (Syt, diluted 1:1000). Note the presence of small amounts of NCS-1 and PI4Kβ in a fraction of purified synaptic vesicles (SG-V). Both proteins are present in large amounts in P3 containing membrane-bound organelles of perikarya. (B) Velocity sucrose gradient centrifugation of the P3 (see A). Equal volumes (250 μl) of each fraction of the velocity gradients (fraction 1=top) were analyzed by western blotting using antibodies raised against PI4Kβ (PI4K), NCS-1, calreticulin (Cal, diluted 1:2000), ribophorin (Ribo, diluted 1:250), TGN38 (diluted 1:1000) and syntaxin 1 (Syx, diluted 1:4000). (C) Fractionation of the S2 on discontinous sucrose density gradients. Equal amount of proteins from fractions 1, 2 and pellet (3) were analyzed by immunoblotting using anti-NCS1, PI4Kβ, ribophorin, TGN38 and GOS-28 (GS28, 1:100) antibodies.

the electron microscope at a magnification of 13,500×. Three hundred synapses were analysed in samples immunolabeled with anti-NCS-1 polyclonal antibody and 200 in control sections incubated with rabbit IgG.

Immunofluorescence

After fixation with 3% paraformaldehyde, cells were processed for immunofluorescence as previously described (Rowe et al., 1999). Images were collected on a MRC-1024 laser scanning microscope (Bio-Rad Laboratories, Munich, Germany) and analyzed using the Bio-Rad computer software. For comparison of double-staining patterns, images from the FITC or TRITC channels were acquired independently from the same area of the sample and then superimposed.

Results

Antibody specificity

Since NCS proteins are highly homologous, we characterized the specificity of antibodies raised against rat NCS-1. IgG fractions purified from rabbit antisera and ascite fluids were used to probe protein extracts derived from rat brain or human cerebellum by immunoblotting. A polyclonal antibody raised against recombinant NCS-1 (Werle et al., 2000) immunostained a band of about 22 kDa, similar to the predicted molecular mass of NCS-1, in rat and human tissue extracts (Fig. 1A). As a control for the specificity of the anti-NCS-1 polyclonal antibody, the blots were immunostained with either rabbit IgG or the specific antibodies pre-adsorbed overnight with 1 μg of antigen. As shown in Fig. 1A,B, no immunoreactive bands were detected in either case. This antibody was then used in immunocytochemical and immunoprecipitation studies. The monoclonal antibodies specifically recognized NCS-1 in the rat brain and weakly in the human cerebellar extracts (Fig. 1C). The monoclonal anti-NCS-1 antibody was used in western blot analysis of immunoprecipitated proteins (see below). We also tested the commercially available anti-PI4Kβ antibody, which specifically recognized a band of approximately 110 kDa on western blots (data not shown), similar to the predicted molecular mass of the kinase (Nakagawa et al., 1996b; Meyers and Cantley, 1997; Wong et al., 1997).

NCS-1 and PI4Kβ subcellular distribution in rat neurons

In order to investigate the possible interaction between NCS-1 and PI4Kβ we decided first to investigate the distribution of both NCS-1 and PI4Kβ in rat brain by subcellular fractionation assays using a procedure originally designed for the isolation of synaptic vesicles from rat brain cortices (Huttner et al., 1983). During this procedure, synaptosomes (P2) were separated from the homogenates (S1) by differential centrifugation. The supernatants containing the remaining membrane-bound organelles (S2) were centrifuged at high speed in order to obtain a cytosolic (S3) and a total membrane fraction (P3). The synaptic vesicles (SG-V) were partially purified from the synaptosomal fraction (P2) by hypo-osmotic lysis followed by differential centrifugation and separation on a continuous sucrose density gradient. The fractions collected during the different steps were analyzed by western blotting by using antibodies directed against NCS-1 and PI4Kβ. The effectiveness of the purification procedure was demonstrated

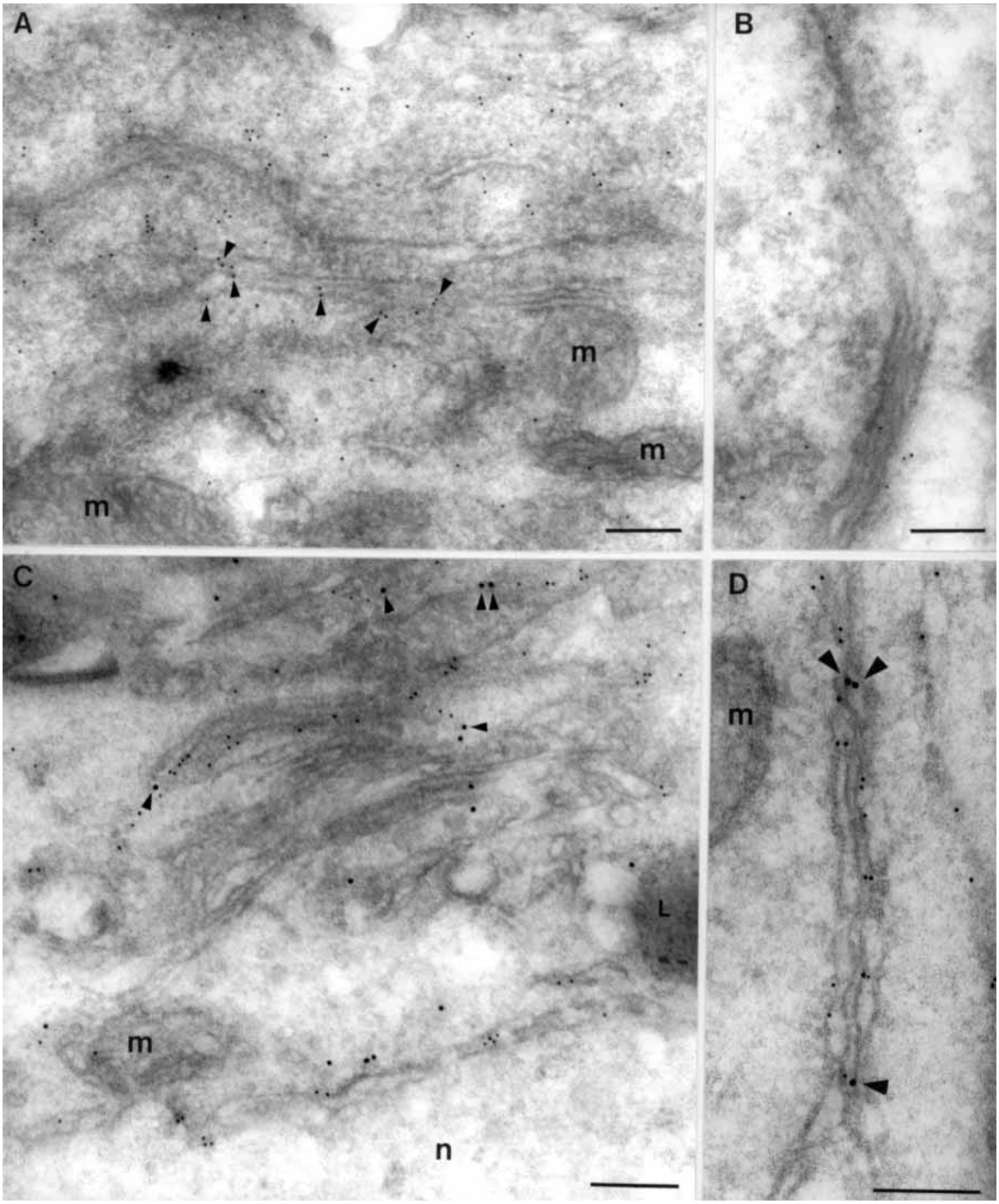

Fig. 3. NCS-1 immunoreactivity is associated with the ER. Ultrathin frozen sections from rat brain cortex (A,C,D) and hippocampus (B) were immunostained for NCS-1 (A,B) or double immunolabeled (C,D) for NCS-1 (18 nm gold particles) and PDI (12 nm gold particles). NCS-1 immunoreactivity is visible in the cytoplasm and, to a lesser extent, on tubulovesicular structures (A-D, arrowheads). In these structures, NCS-1 (arrowheads) colocalizes with the ER marker PDI. No labeling is detectable on mitochondria (m), lysosmes (L) or nuclei (n). Bars, 200 nm.

by the enrichment in the synaptic vesicle fraction (SG-V) of the synaptic vesicle membrane proteins synaptophysin and synaptotagmin (Fig. 2A). NCS-1 was detected in the particulate fractions (P3) containing various membrane bound organelles, other than synaptic vesicles, indicating its widespread distribution. Furthermore, small but consistent amounts of NCS-1 were immunodetected in the synaptic vesicles (SG-V, Fig. 2A) as well as in a highly purified synaptic vesicle preparation (data not shown). Finally, NCS-1 was also found in the fractions containing soluble proteins (S3 and LS2, Fig. 2A), indicating that a consistent portion of the protein is cytosolic. PI4Kβ was also immunodetected in the fractions containing membrane-bound organelles but a larger portion of the kinase was detected in the high-speed supernatants (S3 and

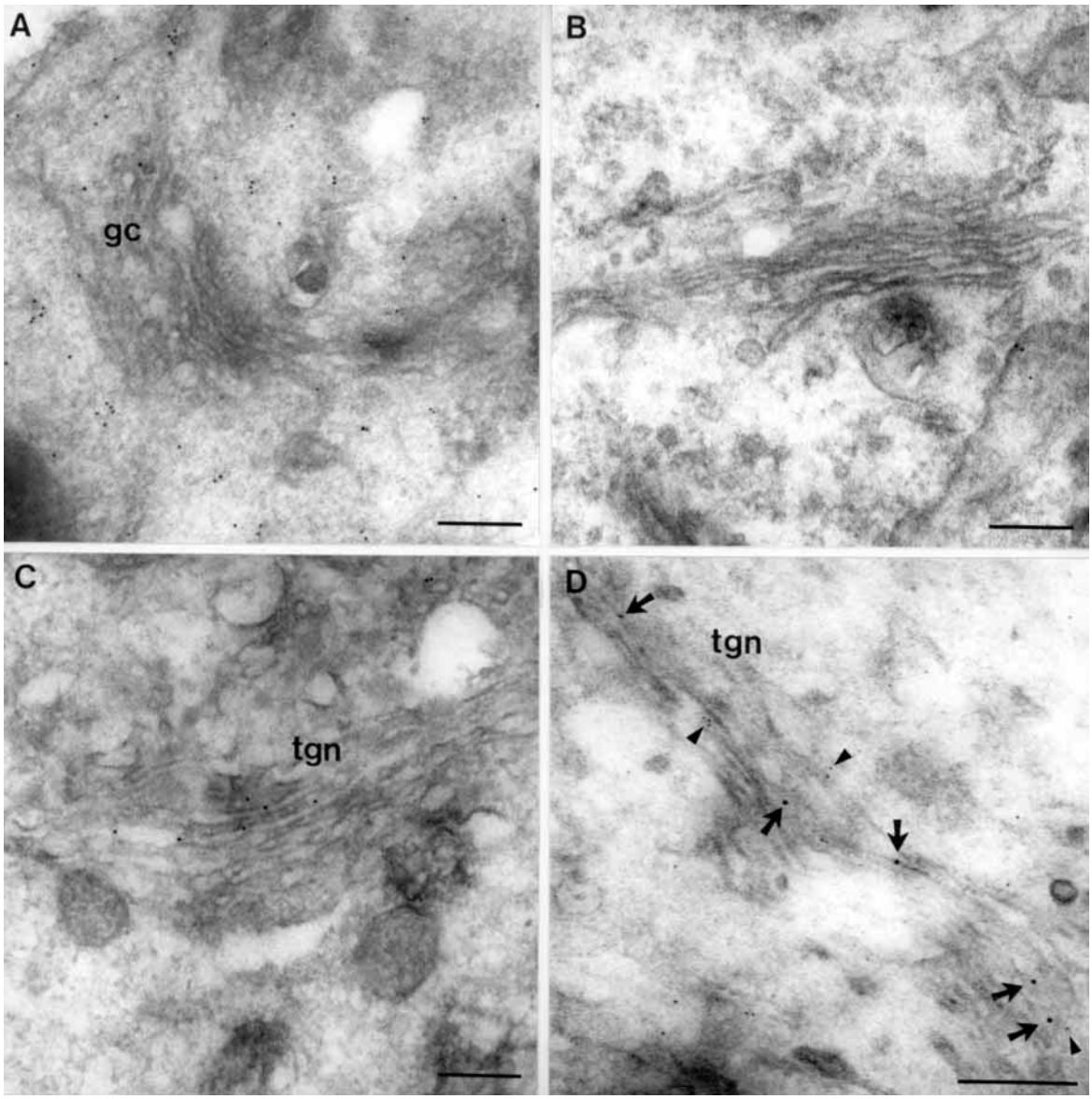

Fig. 4. NCS-1 immunoreactivity in the Golgi area. Ultrathin frozen sections from rat brain cortices were labeled for NCS-1 (A) and TGN38 (C) or double-immunostained (D) for NCS-1 (6 nm gold particles, arrowheads) and TGN38 (12 nm gold particles, arrows). In B, a section immunolabeled with rabbit IgG (control) is shown. NCS-1 immunoreactivity is present in the area of the Golgi complex (gc). The anti-TGN38 antibody specifically immunostains tubular-like elements corresponding to the TGN (tgn, C). Some labeling for NCS-1 (D, arrowheads) is detectable on the tubular-like structures immunolabeled with the anti-TGN38 antibodies (D, arrows). Note the lack of labeling when non-immune rabbit IgGs are used for immunostaining (B). Bars, 200 nm.

LS2). A band corresponding to PI4Kβ was identified in the synaptic vesicle fraction (SG-V) but only after long exposure, indicating that only a small amount of the kinase may be associated with synaptic vesicles.

To study the intracellular distribution of NCS-1 and PI4Kβ associated with membrane-bound organelles other than synaptic vesicles, we analyzed the high speed pellet P3 by using velocity centrifugation on a 0.3-1.2 M continuous sucrose gradient in order to separate the organelles according to their size. The fractions collected were assayed for the presence of various proteins by western blotting. As shown in Fig. 2B, NCS-1 was distributed in two peaks: the first comprising fractions 1-3 (which contain a larger portion of the protein) and the second comprising fractions 7-8. When the distribution of NCS-1 was compared with that of protein markers for the endoplasmic reticulum (ER, calreticulin and ribophorin), TGN (TGN38) and the plasma membrane (syntaxin 1), we found that NCS-1 was distributed similarly to the markers of the ER in the lighter fractions of the gradient (1-3) and to the TGN marker in the denser fractions (7-8). PI4Kβ was also present in the lighter fractions as well as in the denser fractions (fractions 7-8). In contrast, the axonal membrane marker syntaxin 1 was more widely distributed in the gradient than NCS-1, PI4Kβ and the markers of ER and TGN (Fig. 2B).

Under our experimental conditions, however, TGN38 was

Table 1. Quantitation of NCS-1 labeling in neuronal perikarya: gold particles/μm²

	Tubulovesicular elements	Mitochondria	Lysosomes	Nuclei
NCS-1	108±15.2 (0.77 μm²/7)	19±2.3 (1.82 μm²/6)	17±3.8 (1.2 μm²/5)	9±0.6 1.77 μm²/3)
IgG	7±3.8 (0.91 μm²/8)	7±1.3 (3.92 μm²/13)	11±5.5 (0.38 μm²/6)	1 (0.77 μm²/1)

Values given are means±s.e.m. The area (μm²) analyzed and the number of micrographs used for quantitation are shown in brackets. Results are from at least two separate experiments in which rat brain ultra thin sections were immunolabeled using anti-NCS-1 antibody or non-immune IgG and revealed by anti-rabbit IgG conjugated to 12 nm gold particles.

not only detected in the denser fractions, which are expected to contain large organelles (Tooze and Huttner, 1990), but also at the top of the gradient. This altered distribution may be due to fragmentation of the fragile reticular structure of the TGN into smaller vesicles, which may have occurred during the resuspension of P3 before centrifugation. Therefore, the organelles contained in the S2 fraction obtained after differential centrifugation were separated by discontinuous gradient centrifugation. After centrifugation, the two bands at the 0.8-1.1 and 1.1-1.2 M sucrose interface and the pellet were collected and analyzed by immunoblotting with antibodies against NCS-1, PI4Kβ and markers for the ER (ribophorin), Golgi cisternae (GS-28) and TGN (TGN38). The results of western blots demonstrated that adequate fractionation was achieved: Golgi cisternae were mainly localized in fraction 1 (0.85-1.1 M interface), TGN membranes were enriched in fraction 2 (1.1-1.2 M interface) and the ER membranes were almost exclusively found in the pellet (Fig. 2C). NCS-1 and PI4Kβ were clearly present in fractions containing TGN and ER marker proteins (Fig. 2C).

Immunoelectron microscopy studies

To further analyze the subcellular distribution of NCS-1 in neurons, we performed high resolution immunoelectron microscopy studies on cryosections using colloidal gold labeling. Ultrathin frozen sections from rat brain cortices and hippocampi were immunolabeled using the specific NCS-1 polyclonal antibody followed by anti-rabbit IgG antibodies conjugated to gold particles. Immunostaining for NCS-1 was detected in the majority of neurons with gold particles being mainly observed in the perikarya. NCS-1 staining was dispersed in the cytoplasm and partially associated with the membrane of vesicular-like structures and stacks of tubular-like cisternae (108 gold particles/μm²; Fig. 3A,B; Table 1). In order to characterize this compartment, double immunolabeling was performed using antibodies as markers for different organelles. Double immunolabeling with a monoclonal antibody directed against the luminal ER protein PDI demonstrated the localization of NCS-1 near to and on the membrane of ER cisternae (Fig. 3C,D). NCS-1 was also localized in the proximity of the Golgi complex area (Fig. 4A), and some gold particles were detected on a reticular-like structure that, by using double-immunolabeling with anti-TGN38 antibodies, was identified as TGN (Fig. 4D). No significant labeling was observed on the plasma membrane (not shown), mitochondria, lysosomes or nuclei (Table 1; Figs 3, 4). Under control conditions (rabbit IgGs or no primary antibodies) very few gold particles were detected on the ultrathin sections (Table 1; Fig. 4B).

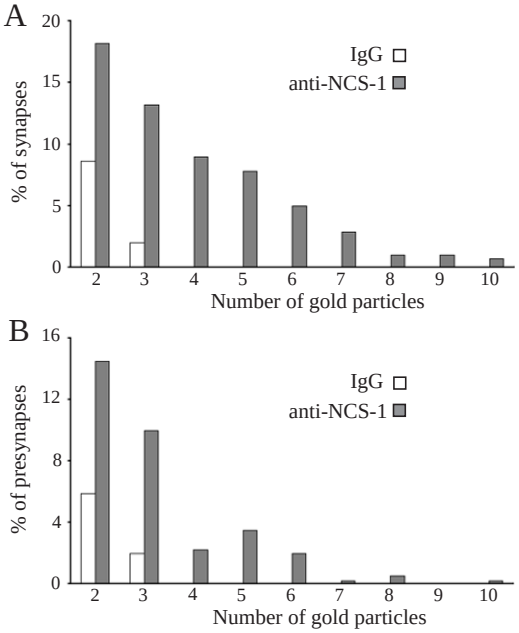

Fig. 5. Quantitation of NCS-1 in total synaptic profiles and presynaptic terminals. Thin sections were immunolabeled with rabbit IgGs or anti-NCS-1 antibodies, followed by anti-rabbit IgG conjugated to 12 nm gold particles. The gold particles were counted over 200 or 300 synaptic profiles, respectively. The values are expressed as the number of gold particles per percentage of synapses (A) and presynaptic profiles (B).

When NCS-1 immunostaining was analyzed in the synaptic regions, not all of the synapses observed were equally immunolabeled. About 40% of the synaptic profiles examined (n=300) were positive for NCS-1, with significant labeling ranging from three to ten 12 nm gold particles/synaptic bouton against the background of less than two gold particles/bouton (Fig. 5A). In the presynaptic compartment (significant labeling ranging from two to ten gold particles/presynaptic region; Fig. 5B), NCS-1 was observed in the cytosolic matrix, near small synaptic vesicles, and also on the synaptic vesicle membranes (Fig. 6A-C).

We next analyzed the intracellular distribution of PI4Kβ by immunoelectron microscopy. Some PI4Kβ immunoreactivity was detected in dendrites and in perikarya but very few, if any, gold particles localized over the Golgi complex (data not shown). This result suggested that either the immunogold labeling was not sensitive enough to detect the kinase localized on the membranes or, more likely, that the anti-PI4Kβ antibodies were working less efficiently in glutaraldehyde-fixed tissue. Therefore, we decided to analyze the intracellular distribution of PI4Kβ by confocal immunofluorescence in cultured hippocampal neurons fixed with 3% paraformaldehyde. Under this condition PI4Kβ immunoreactivity was very intense throughout the perikarya and dendritic trees (Fig. 7) but barely detectable in the axon and axon terminals. No colocalization with the presynaptic marker synaptobrevin 2 was detected (Fig. 7D). In the perikarya, PI4Kβ immunoreactivity had a

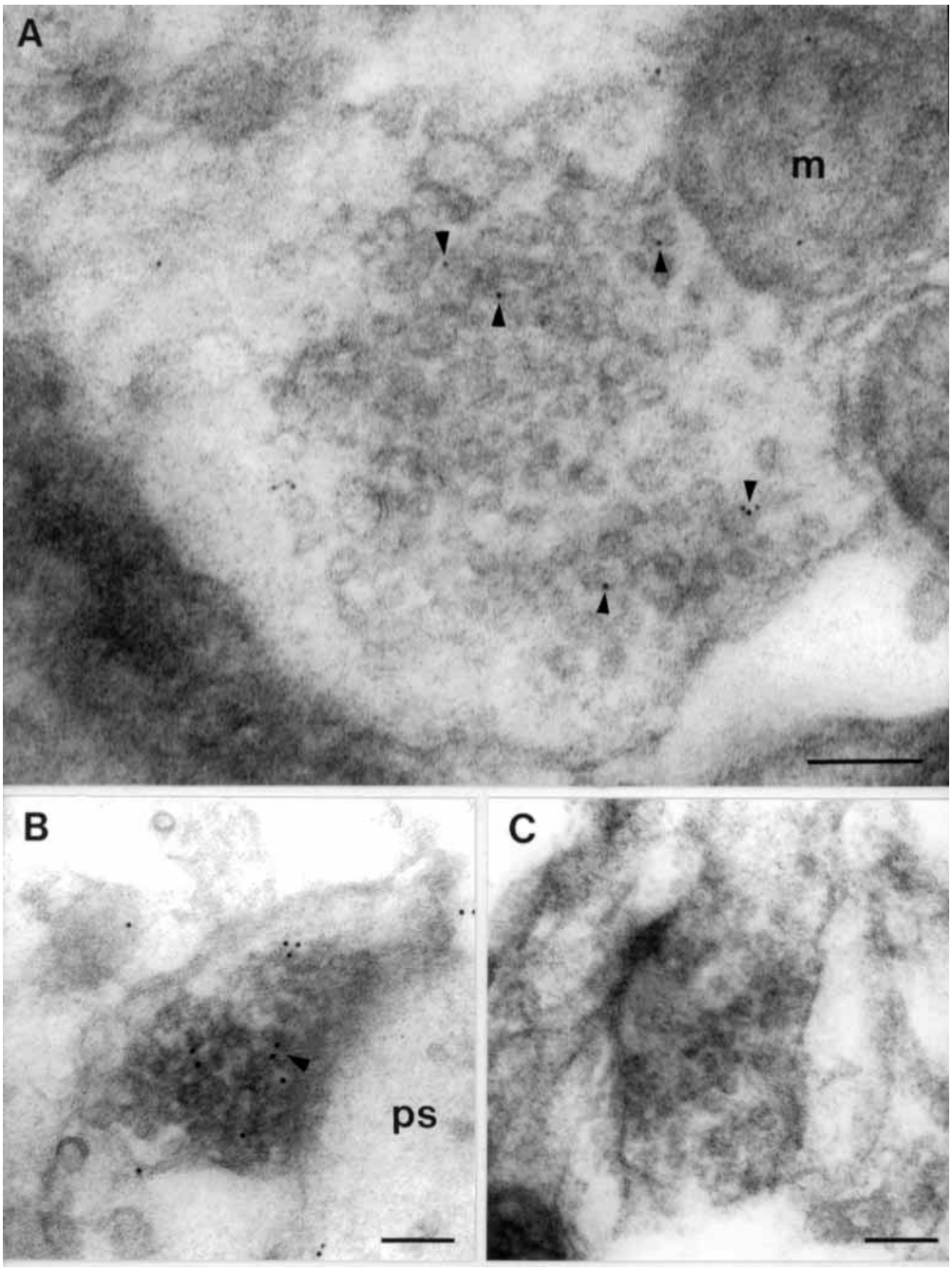

Fig. 6. Localization of NCS-1 in axon terminals. Ultrathin frozen sections were labeled with anti-NCS-1 antibody (A,B) or rabbit IgGs (C). NCS-1 immunolabeling is detectable in the presynaptic region. Note the presence of gold particles on synaptic vesicles (arrowheads); m, mitochondria; ps, postsynaptic region. Bars, 100 nm.

clustered perinuclear distribution that colocalized with the immunostaining of the Golgi-marker TGN38 (Fig. 7B). Moreover, both in perikarya and dendrites the kinase showed a patchy/punctate signal that partially colocalized with the immuno-signal of the ER marker PDI (Fig. 7C). These results are in line with our biochemical data and with previous immunocytochemical studies in rat brain neurons showing the partial distribution of PI4Kβ at the Golgi complex as well as at the membranes of the ER (Balla et al., 2000).

In conclusion, the subcellular fractionation and immunocytochemical data indicate a similar widespread distribution of NCS-1 and PI4Kβ in neurons. Both proteins are partly cytosolic, whereas the membrane-bound portions are localized to the ER and the TGN with only minor amounts present on synaptic vesicles.

NCS-1 interacts with PI4Kβ

Although NCS-1 and PI4Kβ have been shown to form a complex in vitro and, after overexpression in epithelial Madin-Darby canine kidney and Cos-7 cells (Weisz et al., 2000; Zhao et al., 2001), it was still unclear whether endogenous NCS-1 and PI4Kβ interact in neuronal cells under physiological conditions (Bartlett et al., 2000). Therefore we examined whether the two mammalian proteins can be coimmunoprecipitated from neurosecretory cell extracts. We

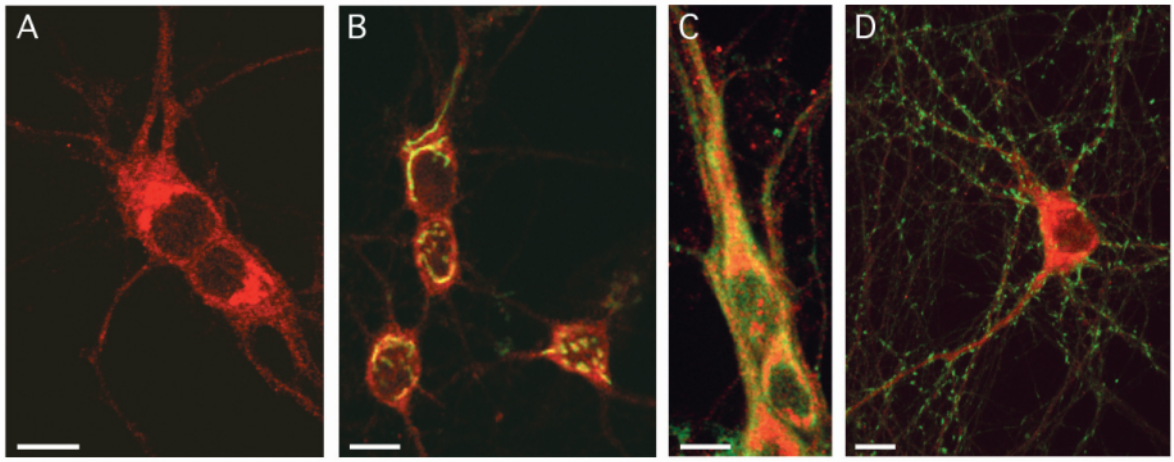

Fig. 7. PI4Kβ in hippocampal neurons. The distribution of PI4Kb in 15-day-old neurons was analyzed by confocal fluorescence microscopy. (A) Neurons immunolabeled with anti-PI4Kβ antibodies. (B-D) Superimposed images (merge) of optical sections collected from neurons doubly immunostained for PI4Kβ (red, B-D) and TGN38 (green, B), PDI (green, C) or synaptobrevin 2 (green, D). Note that immunolabeling for PI4Kβ partially overlaps with that for the Golgi complex marker TGN38 or for the ER marker PDI (B,C). Bars, 10 μm.

incubated rat brain and PC12 cells extracts with anti-NCS-1 antibodies, anti-PI4Kβ antibodies or rabbit IgGs and analyzed the immunoprecipitated proteins on western blots by using antibodies against NCS-1 or PI4Kβ. As shown in Fig. 8, NCS-1 and PI4Kβ were specifically coimmunoprecipitated. When non-immune rabbit IgGs were used for immunoprecipitation purposes, no specific bands were detected.

We next examined whether the interaction between NCS-1 and PI4Kβ occurs preferentially in the cytosol or in membranes. PI4Kβ was co-precipitated with NCS-1 from both rat brain cytosol (high speed supernatants) and membrane fractions (Fig. 9A). To analyze whether the interaction between the two proteins could be modulated, coimmunoprecipitation experiments were carried out in the absence or presence of Ca^{2+} (Fig. 9B). Only a very small increase in the amounts of both NCS-1 and PI4Kβ was observed in immunoprecipitates performed in the presence of 1 mM Ca^{2+} compared with those performed in the presence of EGTA. Thus, NCS-1 and PI4Kβ specifically interact with each other in both cytosol and membrane fractions and, as described in yeast (Hendricks et al., 1999), Ca^{2+} does not increase the binding of NCS-1 to PI4Kβ.

NCS-1 and PI4Kβ membrane recruitment following UTP stimulation

It has been suggested that increases in cytosolic Ca^{2+} may influence the translocation of NCS-1/PI4Kβ to membranes (Meyer and York, 1999). Since our data demonstrating the presence of a NCS1/PI4Kβ complex in the cytosol strengthen that hypothesis, we investigated the possible membrane recruitment of NCS-1 and PI4Kβ using the PC12 neuroendocrine cell line as a model system. The distribution of NCS-1 in cytosol and membrane fractions was analyzed upon stimulation with 300 μM UTP, a G-protein-coupled receptor agonist known to induce a rise in $[Ca^{2+}]i$, an increase in polyphosphoinositide metabolism and concomitant release of dopamine from PC12 cells (Raha et al., 1993; Murrin and Boarder, 1992; Koizumi et al., 1995). Upon stimulation, membrane and cytosol fractions were separated as described in Materials and Methods, and equal volumes of both cytosolic and membrane fractions were analyzed by immunoblotting (Fig. 10B). Quantitative analysis of the blots obtained from non-stimulated cells revealed that 68.4±2.6% (n=7) of total NCS-1 was found in the membrane fractions and 31.6±2.6% in the cytosolic fractions (Fig. 10A), which is similar to previous results (McFerran et al., 1999). On the contrary, upon stimulation with UTP about 95% (94.9±0.6, n=7) of the total NCS-1 was detected in the membrane fractions. Recruitment of NCS-1 to membranes was also observed after ATP treatment (data not shown). This process appeared to be dependent on extracellular Ca^{2+} since upon treatment with UTP or ATP (data not shown), in the absence of extracellular Ca^{2+}, ~66% (65.7±5.4, n=3) of total NCS-1 was detected in the membrane fractions (Fig. 10A). To test for the specificity of the membrane translocation of NCS-1 after UTP treatment, we analyzed the cytosol and membrane distribution of tubulin in the same blots. As shown in Fig. 10B, UTP treatment did not affect the cytosolic localization of the cytoskeletal protein that always appeared only in the cytosolic fractions. Together, these results indicate that stimulation of nucleotide receptors induces the translocation of NCS-1 to membranes.

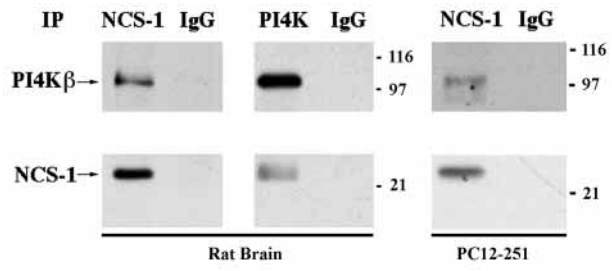

Fig. 8. Association of NCS-1 with PI4Kβ. Triton X-100 rat brain (600 μg) or PC12 cell (1 mg) extracts were immunoprecipitated (IP) with antibodies against NCS-1, PI4Kβ (PI4K) or non-immune rabbit IgG (IgG). The immunoprecipitated proteins were analyzed by western blotting using rabbit anti-PI4Kβ IgG or polyclonal anti-NCS-1 antibodies.

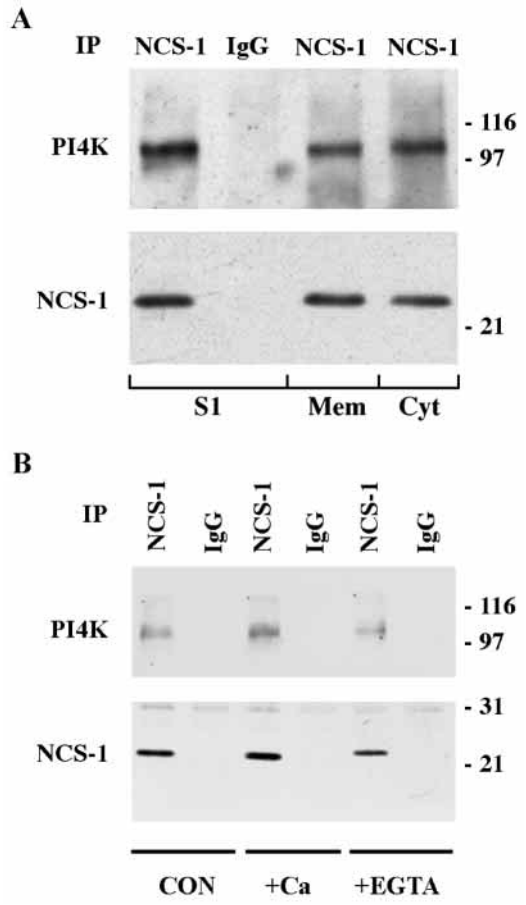

Fig. 9. Association of NCS-1 and PI4Kβ occurs in the cytosol and membrane fractions and is not influenced by Ca^{2+}. In A, equal proportions of Triton X-100 extracts prepared from total rat brain postnuclear supernatant (S1), membrane (Mem) or cytosol (Cyt) were immunoprecipitated (IP) with anti-NCS-1 antibodies, or rabbit IgG (IgG). In B, equal amounts (600 μg proteins) of rat brain extracts (Con) or rat brain extracts supplemented with either 1 mM Ca^{2+} (+Ca) or 5 mM EGTA were immunoprecipitated (IP) with anti-NCS-1 antibodies or rabbit IgG (IgG). The immunoprecipitates were analyzed by western blotting using anti-PI4Kβ or anti-NCS-1 antibodies.

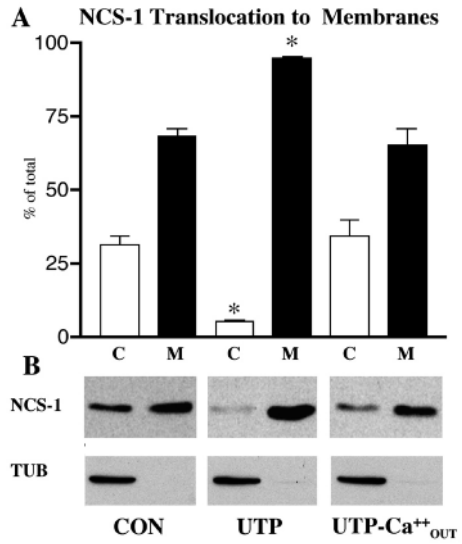

Fig. 10. NCS-1 distribution in UTP-stimulated PC12 cells. The cells grown on petri dishes were incubated for 3 minutes at 37°C in KBR in the absence (CON) or presence of 300 μM UTP (UTP) or in KBR minus Ca^{2+} supplemented with 2 mM EGTA and UTP (UTP-Ca^{++}_{OUT}). The cells were homogenized and the and cytosolic (C) and membrane (M) fractions were obtained. Equal volumes of the fractions were analyzed by western blotting using anti-NCS-1 or anti-tubulin antibodies and the amount of NCS-1 in the cytosol or in the membrane were quantified. (A) Data are expressed as a percentage of total NCS-1 measured in the cytosolic and membrane fractions after no treatment (CON) or treatment with UTP or UTP minus Ca^{2+}. The results represent the mean±s.e.m. (*P<0.001 compared with control). (B) Immunoblots showing the relative membrane/cytosol distribution of NCS-1 and tubulin (TUB) in membranes and cytosol of control and stimulated cells in the presence or absence of Ca^{2+}.

We next determined the effects of UTP-treatment on the membrane distribution of PI4Kβ. As found for NCS-1, UTP treatment affected the membrane translocation of PI4Kβ in a Ca^{2+}-dependent way (Fig. 11A). Since at steady state the largest amount of PI4Kβ was soluble, it was difficult to compare in control samples the levels of kinase in the cytosolic fractions with those in the membrane fractions because of rapid saturation reach by NCS-1 signals in autoradiograms of control cytosolic fractions. Therefore we quantified and compared the level of enzyme present in the membrane fractions of control or UTP-stimulated PC12 cells. Equal aliquots of membrane fractions were analyzed by immunoblotting using antibodies directed against PI4Kβ or synaptophysin (an integral membrane protein of synaptic vesicles completely recovered in the membrane fractions; not shown). The PI4Kβ signals were normalized to those of synaptophysin in the same blots and the data of multiple experiments were averaged. As shown in Fig. 11B, levels of PI4Kβ in the membranes after UTP treatment were about two- to threefold higher than those detected in the untreated cells or cells upon UTP treatment in the absence of extracellular Ca^{2+}.

We then analyzed whether overexpression of NCS-1 may modulate the membrane translocation of PI4Kβ. PC12 cells were transfected with cDNAs coding for NCS-1 and GFP or GFP alone, respectively. Cells were used for biochemical analysis when the transfection efficiency (determined in live cells by counting the percentage of GFP-expressing cells per petri dish) was about 50%. Cytosol and membrane fractions were separated and analyzed by western blotting. In overexpressing cells, the levels of NCS-1 associated with the membrane fractions were largely increased (at least ten times more than in control cells, Fig. 12B). When PIK4β associated with the membrane was quantified, the levels of the kinase detected in the membrane fractions of NCS-1-overexpressing cells were five- to six-times higher than those detected in GFP-transfected cells (Fig. 12A). In NCS-1-overexpressing cells, UTP-treatment was unable to further induce an increase in the levels of PI4Kβ (Fig. 12A) as well as of NCS-1 (data not shown) in the membrane fractions, suggesting that the membrane-binding sites for NCS-1 and/or PI4Kβ were saturated.

Discussion

Although NCS-1 and its orthologue frequenin play an important role in regulating synaptic vesicle and dense-core

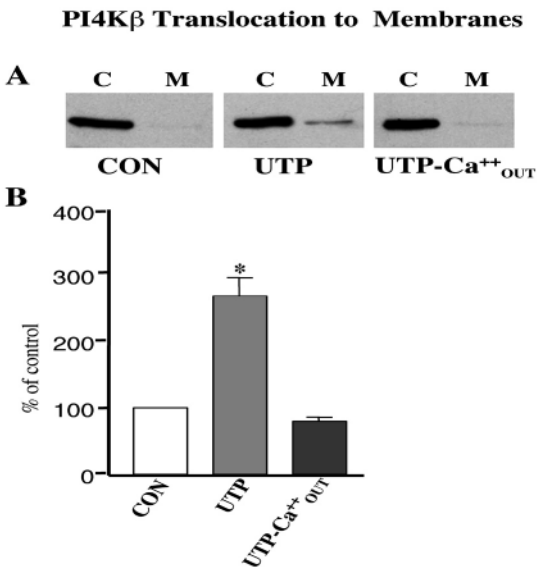

Fig. 11. PI4Kβ distribution in UTP-stimulated PC12 cells. The cells grown on petri dishes were incubated for 3 minutes at 37°C in KBR in the absence (CON) or presence of 300 μM UTP (UTP) or in KBR minus Ca^{2+} supplemented with 2 mM EGTA and UTP (UTP-Ca^{++}_{OUT}). The cells were homogenized and cytosolic (C) and membrane (M) fractions were obtained. Equal volumes of the various fractions were analyzed by western blotting using anti-PI4Kβ. (A) Immunoblots showing the distribution of PI4Kβ in the membranes and cytosol of control and stimulated cells in the presence or absence of Ca^{2+}. (B) The amounts of PI4Kβ in the membrane fractions were analyzed by immunoblotting and the signals for PI4Kβ were normalized to those of synaptophysin present in the same blots. The results are expressed as a percentage of the control and represent the mean±s.e.m. (n=14, *P<0.005 compared with control).

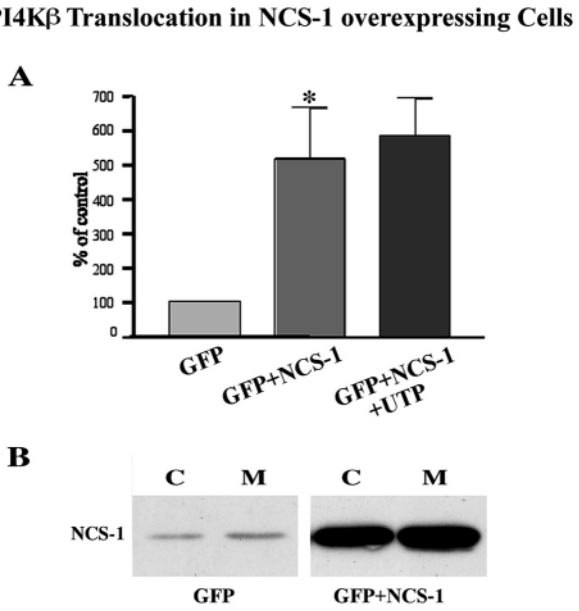

Fig. 12. PI4Kβ distribution in NCS-1-overexpressing PC12 cells. Cells were electroporated with cDNAs coding for NCS-1 and GFP (GFP+NCS-1) or GFP alone (GFP). 48 hours after transfection, the cells were incubated for 3 minutes at 37°C in KBR in the absence or presence of 300 μM UTP (+UTP) and then homogenized. The cytosolic (C) and membrane (M) fractions were prepared and analyzed by western blotting using anti-PI4Kβ or NCS-1 antibodies. In A, the amounts of PI4Kβ in the membrane fractions were quantified as described in Fig. 11 and the results are expressed as a percentage of the control (GFP-transfected cells) and represent the mean±s.e.m. (n=6, *P<0.002 compared with GFP-transfected cells). (B) Immunoblots showing the distribution of NCS-1 in the membranes and cytosol of GFP and NCS-1+GFP-transfected cells.

granule exocytosis, the underlying molecular mechanisms are not clearly understood. NCS-1 was suggested not to directly act on the secretory machinery, but to exert its action on signaling pathways. It has been proposed to modulate the functions of various molecules, including NO synthases and calcineurin (Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001), and recent data demonstrated a possible role for frequenin in directly regulating Pik, the yeast orthologue of mammalian PI4Kβ (Hendricks et al., 1999). In order to obtain insights into the function of NCS-1 in mammalian cells, we investigated the subcellular distribution and interaction of NCS-1 and PI4Kβ in neurons and neuroendocrine cells.

The following main findings are reported: (1) NCS-1 and PI4Kβ show a similar widespread subcellular distribution; (2) endogenous NCS-1 interacts with PI4Kβ to form a complex that is immunoprecipitated from rat brain and PC12 cell extracts using anti-NCS-1 or anti-PI4Kβ antibodies; (3) the NCS-1/PI4Kβ interaction occurs in membranes as well as in the cytosol; and (4) stimulation of regulated secretion facilitates the translocation of NCS-1 and PI4Kβ from cytosolic to membrane fractions, suggesting a role for NCS-1 in the recruitment of the kinase onto target membranes.

NCS-1 and PI4Kβ show a similar widespread subcellular distribution in neurons

Although limited immunohistochemistry and in situ hybridisation data indicate that NCS-1 is expressed in many regions of the brain (Jeromin et al., 1999; Martone et al., 1999; Paterlini et al., 2000; Werle et al., 2000), less is known about its subcellular localization. We used subcellular fractionation and immunoelectron microscopy to investigate the subcellular localization of NCS-1 and both methods revealed that it is partially cytosolic and partially associated with different membrane-bound organelles. NCS-1 was distributed in several (but not all) synaptic boutons, where it was also localized on synaptic vesicles. The presence of NCS-1 in a subpopulation of synapses may be related to the different physiological properties of these synapses. Interestingly, in crustacean neuromuscular junctions, NCS-1 appears more predominantly expressed in the phasic nerve terminals that are capable of releasing more neurotransmitter at low frequencies than their tonic counterparts (Jeromin et al., 1999). Our results show that most NCS-1 was distributed in the perikarya, where it could be detected near to or associated with the membranes of the ER and the TGN. A consistent amount of NCS-1 was also found in dendrites. Although the physiological significance of this complex NCS-1 distribution is unclear, the absence of any specific compartmentalisation suggests that it may have a general function in controlling signaling and/or vesicle trafficking events.

Although PI4K activity has been detected in many cellular compartments including plasma membrane (Cockcroft et al.,

1985), secretory granules (Wiedemann et al., 1996), synaptic vesicles (Wiedemann et al., 1998), Golgi (Jergil and Sundler, 1983; Cockcroft et al., 1985) and glucose transporter 4-containing transport vesicles (Del Vecchio and Pilch, 1991), little is known about the subcellular localization of PI4K proteins in neurons and neuroendocrine cells (Balla et al., 2000). Different types of PI4Ks have been cloned and characterized from yeast and mammalian cells (Balla, 1998; Fruman et al., 1998; Nakagawa et al., 1996a; Nakagawa et al., 1996b; Meyers and Cantley, 1997) and it is clear from these studies that different isoforms of the PI4K family may synthesize different pools of polyphosphoinositides that in turn modulate different cellular functions (De Camilli et al., 1996; Anderson et al., 1999; Balla, 2001; Cremona and DeCamilli, 2001). The localization of distinct kinase isoforms to specific sites in cellular compartments helps to explain the different roles that phosphoinositides play in cells. Therefore we have characterized the distribution of PI4Kβ and compared it with that of NCS-1. Although PI4Kβ is mainly enriched in the cytosolic fraction after rat brain differential centrifugation, our results demonstrate that PI4Kβ is also present on membranes of the ER and the late Golgi complex. This latter result is consistent with previous data obtained in mammalian cell-free systems and yeast cells, respectively (Godi et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000), which showed that PI4Kβ plays an essential role in Golgi complex organization and protein secretion. Our data indicate that PI4Kβ is also associated with synaptic vesicles, albeit in very small amounts and, to our knowledge, this is the first report showing the physical presence of an isoform of the PI4K family on synaptic vesicles. However, the small amount of PI4Kβ associated with the synaptic vesicles suggests that this enzyme might not be the only PI4K isoform responsible for the PI4K activity detected on synaptic vesicles (Wiedemann et al, 1998). In addition, or alternatively, other kinases may be involved since several PI4K activities have been characterized and recently a PI4K type II has been discovered on secretory granule membranes (Barylko et al., 2001; Wenk et al., 2001).

Interaction of NCS-1 with PI4Kβ and its possible function

The observed presence of both proteins on membrane-bound organelles, together with the observed interaction of NCS-1 with PI4Kβ strongly suggests that NCS-1 could function in regulating PI4Kβ activity and/or localization. Recent data have described that myristoylated NCS-1, but not its unmyristoylated form, is capable of stimulating the kinase activity of recombinant PI4Kβ in vivo (Balla, 2001). Similarly, a significant increase in PI4K activity was observed when both NCS-1 and PI4Kβ were exogenously expressed in COS-7 cells (Zhao et al., 2001). Thus the molecular interaction with NCS-1 appears to be important for activation of the kinase. Less data are available on the possible role of NCS-1 in the targeting of PI4Kβ. Our results showing that endogenous NCS-1 and PI4Kβ can form a complex in the cytosol and that both proteins can be translocated to membranes upon stimulation of exocytosis strongly suggest that NCS-1 modulates the translocation of PI4Kβ to membranes. Two mechanisms by which NCS-1 may function in PI4Kβ membrane localization or activation have recently been proposed (Meyer and York, 1999). In the first model, NCS-1 is prelocalized on the membrane and a Ca^{2+} induced-conformational change triggers its interaction with and activation of a membrane-pre-bound pool of PI4Kβ; in the second, Ca^{2+} binding triggers the exposure of the myristoyl group and the concomitant translocation of the NCS-1/PI4Kβ complex to the membrane. Our present data showing that the NCS-1/PI4Kβ complex is also present in the cytosol and that both proteins are translocated to membranes upon a secretory stimulus support the second mechanism.

At steady state, PI4Kβ is mainly found in the cytosol and may thus require protein-carrier(s) to be translocated to possible sites of action. Recent studies have demonstrated that the small GTP-binding protein ARF-1 is involved in the translocation of PI4Kβ to Golgi membranes (Godi et al., 1999), where the PI4K activity is important for cisternae organization and constitutive protein trafficking from the Golgi to the cell surface. However, at present no ARF proteins have been shown to be concentrated at the synapse (Cremona and De Camilli, 2001). We suggest that NCS-1, which is mainly expressed in neuronal and neuroendocrine cells, may modulate the localization and activity of PI4Kβ and thereby the level of a phosphoinositide pool required for intracellular trafficking events that specifically occur in neurosecretory cells. Neurons and neuroendocrine cells express two types of secretory vesicles specialized in storage and release of neurotransmitters: the dense core vesicles (or secretory granules) produced from the TGN (for reviews, see Eaton et al., 2000; Tooze et al., 2001), and the small synaptic vesicles (or synaptic-like microvesicles) that are derived from a recycling compartment near, or at, the plasma membrane (Hannah et al., 1999; Slepnev and De Camilli, 2000). Upon regulated exocytosis and consumption of either secretory vesicles, the NCS-1/PI4Kβ complex may be translocated and stimulate the synthesis of phosphoinositide pools involved in the generation of neurosecretory vesicles. In line with this model, we found that after NCS-1 overexpression in PC12 cells, the amount of membrane-associated PI4Kβ increases concomitantly with the increase of NCS-1 in the membrane fractions. Moreover, NCS-1 overexpression enhances ATP-stimulated release from PC12 cells (McFerran et al., 1998) and the levels of phosphatidylinositol 4-phosphate and phosphatidylinositol (4,5)-bisphosphate in PC12 cells (Koizumi et al., 2002). Although further work is required to identify the membrane targets of NCS-1/PI4Kβ and to fully understand their role in membrane traffic, our data demonstrate that NCS-1/PI4Kβ are translocated to membranes and suggest that the two proteins may regulate the levels of phosphoinositides involved in regulated secretion processes; for example, by modulating the formation of neurosecretory vesicles. This action could lead to downstream effects on neurosecretion, and in turn could also be modulated by Ca^{2+} and thus by the secretory activity of the cells.

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