Two separate motifs cooperate to target stathmin-related proteins to the Golgi complex

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Summary

The appropriate targeting of membrane-associated proteins involves a diversity of motifs including post-translational modifications and specific protein sequences. Phosphoproteins of the stathmin family are important regulators of microtubule dynamics, in particular in the developing and mature nervous system. Whereas stathmin is cytosolic, SCG10, SCLIP and the splice variants RB3/RB3′/RB3″ are associated with Golgi and vesicular membranes, through their palmitoylated N-terminal A domains. In order to identify essential motifs involved in this specific targeting, we examined the subcellular distribution of various subdomains derived from domain A of SCG10 fused with GFP. We show that the Golgi localization of SCG10 results from the cooperation of two motifs: a membrane-anchoring palmitoylation motif and a newly identified Golgi-specifying sequence. The latter displayed no targeting activity by itself, but retained a Golgi-specifying activity when associated with another membrane-anchoring palmitoylation motif derived from the protein GAP-43. We further identified critical residues for the specific Golgi targeting of domain A. Altogether, our results give new insight into the regulation of the subcellular localization of stathmin family proteins, an important feature of their physiological functions in differentiating and mature neural cells. More generally we provide new information on essential mechanisms of functional protein subcellular targeting.

Key words: Golgi targeting sequence, Palmitoylation, Stathmin family, Neurons

Introduction

The establishment of functionally differentiated compartments within the cell is dependent on the accurate sorting of proteins. This provides each compartment with a specific protein composition enabling it to fulfil its specialized cellular role. Reciprocally, the spatial localization of a protein is a major determinant of its function, by locally specifying its molecular partners. Such protein targeting is especially important in neuronal cells, which possess a striking diversity of functional spaces including dendrites, axons, growth cones or synapses. Studies on integral membrane proteins have unravelled two main protein targeting principles, based on retention and recycling. Both mechanisms are thought to involve specific structural motifs or signals located within the protein, like the retrieval KDEL motif in luminal ER proteins (Pelham, 1995) or the plasma membrane tyrosine-based recycling signal in the cytoplasmic tail of proteins (e.g. TGN38) (Ponnambalam et al., 1994). With regard to peripheral membrane-bound proteins, post-translational modifications with fatty acids function as membrane-binding signals. In the case of Src-related proteins, a single lipid anchor (such as a myristoyl) is not sufficient to provide stable membrane binding (Peitzsch and McLaughlin, 1993; Resh, 1999), which is only achieved with a second signal that can be either another lipid modification (such as palmitoylation) or a polybasic stretch (Sigal et al., 1994; Resh, 1996; Wedegaertner, 1998). Altogether, the diversity in subcellular localization of peripheral proteins seems to be governed by a diversity of lipid modifications and associated targeting signals, many of which have not been identified yet.

A good example of proteins whose function is modulated by subcellular localization is provided by the stathmin family, which includes stathmin (Sobel et al., 1989; Hailat et al., 1990), SCG10 (Anderson and Axel, 1985), SCLIP and the splice variants RB3/RB3′/RB3″ (for a review, see Cassimeris, 2002; Ozon et al., 1997; Ozon et al., 1998). These proteins are involved in the regulation of microtubule dynamics, as suggested by their shared ability to sequester free tubulin (Belmont and Mitchison, 1996; Jourdain et al., 1997; Riederer et al., 1997; Gavet et al., 1998; Howell et al., 1999; Charbaut et al., 2001). However, they display specific functional properties arising from their distinct tubulin interaction properties (Charbaut et al., 2001; Brannstrom et al., 2003), their different regulation by phosphorylation (Sobel, 1991; Antonsson et al., 1998; Neidhart et al., 2001), and most of all, from their distinct subcellular localization. Whereas stathmin is ubiquitous and cytosolic (Sobel et al., 1989), the other proteins of the stathmin family are expressed essentially in the nervous system and are peripherally bound to intracellular membranes (Stein et al., 1988; Di Paolo et al., 1997a; Di Paolo et al., 1997b; Ozon et al., 1998). They are mainly localized to the Golgi complex and on vesicles found in the neuron cell body, along the neuronal processes and enriched at the centre

of growth cones (Stein et al., 1988; Di Paolo et al., 1997a; Gavet et al., 1998; Lutjens et al., 2000; Gavet et al., 2002). SCG10 was also shown to associate with lipid rafts (Maekawa et al., 2001). This specific subcellular distribution has led to proposals that stathmin-related proteins are involved in the local regulation of cellular processes, including microtubule dynamics and possibly others in relation with their other identified partners (Nixon et al., 2002; Liu et al., 2002; Greka et al., 2003).

The membrane localization of stathmin-related proteins is achieved by their N-terminal extension composed of one or several specific domains (Fig. 1). Domains A′ and A″, with unknown functions, are specific to the splice variants RB3, RB3′ and RB3″. By contrast, domain A, which is the most N-terminal, is conserved in all stathmin-related proteins (60-70% identity) (Stein et al., 1988; Ozon et al., 1997; Ozon et al., 1998) and has been identified as a key element to target SCG10 to the Golgi complex (Di Paolo et al., 1997b). Indeed, it was shown that not only the deletion of domain A in SCG10 redistributed the protein to the cytosol (Di Paolo et al., 1997b), but also that the addition of this same domain to heterologous proteins was able to target the fusion protein to the Golgi complex in COS-7 cells and in neurons (Di Paolo et al., 1997b; Lutjens et al., 2000). Two palmitoylation sites have been identified in domain A of SCG10, but their mutation did not result in the complete solubilization of the protein (Di Paolo et al., 1997b), suggesting that other signals must be involved in the subcellular localization of stathmin-related proteins to the Golgi complex.

In this study, we investigated the molecular mechanisms involved in the Golgi targeting of stathmin family proteins. By characterizing the subcellular distribution mediated by various subdomains derived from domain A of SCG10 fused to GFP, we show that it is achieved by the combination of two adjacent motifs, one being responsible for membrane association and the other specifying targeting to the Golgi complex. In addition, we demonstrate that this second motif is able to cooperate with another palmitoylated membrane-anchoring sequence for Golgi targeting and we identified critical residues for its functional integrity.

Materials and Methods

Plasmid constructs

Standard recombinant DNA techniques were carried out as described (Sambrook et al., 1989). The plasmids pEGFP-N1 and pEGFP-N3 (ClonTech, Palo Alto, CA) were modified for the expression of GFP fusion proteins containing either full-length or truncated SCG10, or the N-terminal domains A of SCG10, SCLIP or RB3, or subdomains of the domain A of SCG10 (see Fig. 1). The amino acid sequence of the inserts for constructs derived from domain A is given in Table 1. Sequences of the oligonucleotides used are available upon request.

For the various A-GFP fusion proteins, a DNA fragment corresponding to domain A of SCG10, SCLIP or RB3 was amplified by PCR, using a plasmid encoding rat SCG10, SCLIP or RB3 respectively (Gavet et al., 1998) as a template. To generate SCG10-GFP and Δn-SCG10-GFP, the nucleotide regions 1-625 or 134-625 respectively (nucleotide 1 being the start of the SCG10 coding sequence) were amplified separately by PCR using the SCG10-myc plasmid as a template (Gavet et al., 1998). Oligonucleotides (synthesized by Genset, Paris, France) were designed in order to insert a *Kpn*I restriction site at the 5′ end and a *Bam*HI restriction site at the 3′ end of the DNA fragment, and to generate a coding sequence in

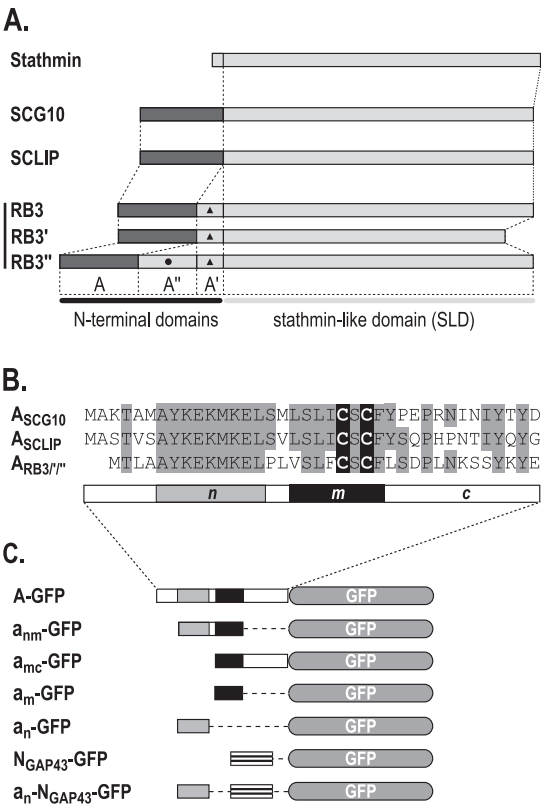

Fig. 1. Domains and constructs investigated in this study. (A) Stathmin family proteins: except for stathmin, each protein possesses, in addition to its stathmin-like domain, an N-terminal extension including the conserved domain A (domain A, dark grey; domains A′ and A″, symbols). (B) Sequence alignment of domains A from SCG10, SCLIP and RB3/RB3′/RB3″: conserved residues are highlighted in grey, and the two palmitoylated cysteines in black. The delineation of the three regions n (grey), m (black) and c (white) was driven by the sequence conservation pattern. (C) A first series of GFP fusion proteins was constructed with the domain A from SCG10 or with various combinations of its derived subdomains (n+m, m+c, m, n); an additional pair of constructs encoding the palmitoylated N-terminal domain of GAP-43 (hatched) fused or not to region n (lower two constructs) was also engineered to further investigate the mechanism of action of this region.

Table 1. Amino-acid sequence of the domains fused to the N-terminus of GFP

Domain	Amino-acid sequence
A	MAKTAM AYKEKMKEL S MLSLICSCF YPEPRNINIYTYD
a_{nm}	MAKTAM AYKEKMKEL S MLSLICSCF
a_{mc}	MLSLICSCF YPEPRNINIYTYD
a_m	MLSLICSCF
a_n	M AYKEKMKEL
N_{GAP43}	M LCCMRRTKQV
a_n-N_{GAP43}	M AYKEKMKEL SV LCCMRRTKQV
Y/A	MAKTAM A**A**KEKMKEL S MLSLICSCF YPEPRNINIYTYD
Y/F	MAKTAM A**F**KEKMKEL S MLSLICSCF YPEPRNINIYTYD
KKK/AAA	MAKTAM AY**A**E**A**M**A**EL S MLSLICSCF YPEPRNINIYTYD
KKK/RRR	MAKTAM AY**R**E**R**M**R**EL S MLSLICSCF YPEPRNINIYTYD
EE/AA	MAKTAM AYK**A**KMK**A**L S MLSLICSCF YPEPRNINIYTYD
EE/DD	MAKTAM AYK**D**KMK**D**L S MLSLICSCF YPEPRNINIYTYD

Each domain is separated from GFP by a GDPPVAT linker, except in the case of a_m-GFP where the linker sequence is GSIAT.

frame with GFP after ligation in the plasmid pEGFP-N1 or pEGFP-N3. The PCR reaction was carried out with the Expand High Fidelity System (Roche Diagnostics, Mannheim, Germany) for 28 amplification cycles. The PCR product was then digested by the endonucleases *Kpn*I and *Bam*HI (Invitrogen Life Technologies, Carlsbad, CA), as well as the plasmid pEGFP-N1 (or the plasmid pEGFP-N3 in the case of SCG10-GFP and Δn-SCG10-GFP), which was then dephosphorylated using calf intestinal phosphatase (CIP, New England Biolabs, Beverly, MA). The ligation reaction was carried out with T4 DNA ligase (Invitrogen Life Technologies) overnight at 16°C.

For the fusion proteins derived from SCG10 domain A (a_m-GFP, a_n-GFP, a_{nm}-GFP, a_{mc}-GFP, N_{GAP43}-GFP and a_n-N_{GAP43}-GFP), complementary 5′-phosphorylated oligonucleotides (synthesized by Genset) were designed to form a double strand DNA fragment containing the whole sequence of the insert, with *Kpn*I and *Bam*HI cohesive ends at the 5′ and 3′ ends respectively. The annealing of complementary oligonucleotides was performed as follows: an equimolar mix of 10 μM sense and antisense oligonucleotides in 17 mM sodium citrate-HCl pH 7.0, 150 mM NaCl was heated at 95°C for 5 minutes, then progressively cooled to 4°C for 1 hour and kept on ice. The ligation reactions were carried out as above, with the plasmid pEGFP-N1 or pEGFP-N3 (for a_m-GFP) prepared as above.

For all constructs, the ligase was then inactivated, and the ligation reactions were digested with *Sma*I (Invitrogen Life Technologies) in order to linearize plasmids with no insert. The resulting products were used to transform XL1-Blue competent *Escherichia coli* cells. Kanamycin-resistant clones were cultivated in liquid Luria-Bertani medium containing 25 μg/ml kanamycin and plasmid DNA was prepared using the Nucleobond kit (Machery-Nagel, Düren, Germany). The fusion protein open reading frames were checked by DNA sequencing (Genome Express, Meylan, France).

Mutations of A-GFP within region n (AYKEKMKEL) were generated by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) with pairs of complementary mutagenic primers. All constructs were confirmed by DNA sequencing. When the sequence was correct, large preparations of plasmid DNA were carried out using the QiaTip-550 kit (Qiagen, Hilden, Germany) or the Nucleobond AX2000 kit (Machery-Nagel), and their concentrations were assessed by spectrometry at 260 nm. The plasmid pEGFP-F (ClonTech, Palo Alto, CA), which encodes a protein EGFP fused to the farnesylation signal contained in the 20 C-terminal residues of c-HA-Ras, was used as a control for plasma membrane targeting.

Primary hippocampal neuron culture

Embryos (E18) from a pregnant female rat (OFA, Iffa Credo, France) were dissected in HBSS-HEPES media (1× HBSS, 20 mM HEPES) to isolate the hippocampi. Hippocampi were then digested at 37°C by incubation in 3 ml trypsin (Invitrogen Life Technologies) with agitation every 5 minutes. After 15 minutes, the reaction was stopped by addition of 5 ml HBSS. The hippocampi were sedimented and the supernatant was aspirated. This step was repeated once to remove all the trypsin. 3 ml HBSS were added with 300 μl DNase (Roche Diagnostics) and incubated for 5 minutes at 37°C to disaggregate the hippocampi. Cells were then dissociated mechanically by 30 gentle passages through the tip of a eroded pipette. The mix was left to settle down for 5 minutes and the supernatant transferred to a second tube. Then 3 ml HBSS were added to the remaining pellet, which was dissociated by 20 more passages. After a 5-minute sedimentation, the supernatant was removed and pooled with the first one. Cell number was determined with a Malassez cell. The appropriate volume of Neurobasal™ medium (Invitrogen Life Technologies) supplemented with 4% B27 (Invitrogen Life Technologies) and 0.5 mM L-glutamine was added in order to reach a final concentration of 2×10^5 cells/ml. Cells were then plated on 14-mm glass coverslips coated with 25 μg/ml poly-L-lysine (Sigma) in 1.5 cm^2 wells (0.5 ml of cell suspension per well). The cells were placed in an incubator (37°C, 5% CO_2) until transfection 5 days later (D5). Half of the medium was changed the next day.

Cell culture

HeLa cells and MDCK cells were grown in DMEM-Glutamax with 10% decomplemented fetal calf serum and 1% penicillin/streptomycin (Invitrogen Life Technologies), at 37°C in a humidified atmosphere containing 5% CO_2. Twice a week, cells were dissociated in 0.25% trypsin, 1 mM EDTA (Invitrogen Life Technologies), then diluted at 1:10 before replating.

Transfection

Primary hippocampal neurons were transfected with the A-GFP plasmid as follows: for each 14-mm glass coverslip, 25 μl Opti-MEM® I was combined with 0.5 μl Lipofectamine™ 2000 (Invitrogen Life Technologies) or with 0.5 μg plasmid DNA (encoding A-GFP) and allowed to sit at room temperature for 5 minutes. These solutions were then combined and the DNA-lipid complex was allowed to form for 20 minutes at room temperature. While the complexes were forming, the cultures were removed from the incubator and the medium from each well aspirated and replaced with 450 μl fresh medium. These plates were then returned to the incubator until addition of the complexes. After 20 minutes of complexing, 50 μl per coverslip of the Opti-MEM® I/DNA/Lipofectamine™ 2000 mixture were added to the culture, which was kept for another 24 hours.

For transfection, HeLa cells were seeded at 12,500 cells/cm^2 in 35 mm-diameter dishes containing three 12 mm-diameter glass coverslips. They were transfected 16 hours later using the liposoluble reagent Fugene 6 (Roche Diagnostics), according to the manufacturer's instructions: 3 μl reagent and 1 μg plasmid DNA were used to transfect one dish. MDCK cells were electroporated using a gene pulser (Bio-Rad, Hercules, CA) at 260 V and 1500 μF for 5×10^6 cells and 10 μg plasmid DNA supplemented with 60 μg salmon sperm DNA as a carrier, then plated onto glass coverslips.

Immunocytochemistry and epifluorescence microscopy observation

HeLa and MDCK cells were fixed 24 hours after transfection with 2% paraformaldehyde, 30 mM sucrose in PBS for 15 minutes at room temperature. Primary hippocampal neurons were fixed 24 hours after transfection with 4% paraformaldehyde in PBS for 20 minutes. After fixation, coverslips were washed three times in PBS before being processed for immunochemistry or directly mounted in a Mowiol solution supplemented with DABCO antifading agent.

Immunocytochemistry was performed as follows: cells on coverslips were incubated with 50 mM NH_4Cl in PBS for 5 minutes, thereafter permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and then blocked for 30 minutes with 3% BSA in PBS. The coverslips were then incubated for 1 hour with the appropriate antibodies diluted in blocking buffer: either the monoclonal antibody CTR433, a marker of the median Golgi (Jasmin et al., 1989) (generous gift of M. Bornens, Institut Curie, France), diluted 1:2, or a polyclonal rabbit antiserum raised against SCG10 (Ozon et al., 1998) diluted 1:500 and a monoclonal antibody against GFP (Roche Diagnostics) diluted 1:200. After five washes with 0.1% Tween-20 in PBS, the coverslips were incubated for 1 hour with either a TRITC-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:300 in blocking buffer, or with an Alexa 488-conjugated anti-mouse IgG antibody and an Alexa 546-conjugated anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) both diluted 1:400 in blocking buffer. The coverslips were finally washed five times with 0.1% Tween-20 in PBS and mounted as described above.

Observation of coverslips was performed either with a Provis fluorescence photomicroscope (Olympus, Tokyo, Japan) equipped with a digital camera (Princeton Scientific Instruments, Monmouth Junction, NJ), or with a SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). For quantification of subcellular localization of a_n-N_{GAP43}-GFP, N_{GAP43}-GFP and A-GFP, series of random pictures were acquired with the Provis microscope at a given exposure time chosen to overexpose highly transfected cells. Non-overexposed cells were then classified according to three categories and the number of cells in each category was counted manually using the corresponding function in the Metamorph software (Universal Imaging Corporation, Downingtown, PA). A total of 100-300 cells was counted for each condition.

Results

Domains A of stathmin-related proteins target GFP to the Golgi complex in neurons and various cell lines

All stathmin-related proteins are localized to the Golgi complex and to punctate vesicle-like structures. Their subcellular distribution is thought to be mediated by their homologous N-terminal domain A. In order to identify sequence motifs essential for this specific targeting, we analysed the subcellular distribution of domain A as well as various derived subdomains in fusion with GFP (Fig. 1, Table 1).

As stathmin-related proteins are mainly expressed in neurons, we validated our strategy by overexpressing the full-length domain A of SCG10 fused to GFP (A-GFP, Table 1, Fig. 1) in primary embryonic hippocampal neurons. We compared the subcellular distribution of A-GFP to the endogenous SCG10 and observed (Fig. 2A) that A-GFP immunoreactivity was concentrated mainly at a perinuclear, Golgi-like compartment, where it overlapped with that observed for SCG10. As expected for SCG10 (Stein et al., 1988; Di Paolo et al., 1997a), the neurites and their growth cone tips were also stained for both proteins (Fig. 2A). This shows that A-GFP faithfully reproduces the subcellular distribution of endogenous SCG10, which validates its use as a molecular tool to study the mechanisms involved in the Golgi targeting of stathmin-related proteins.

The fusion protein corresponding to domain A of SCG10 displayed a similar distribution when it was expressed in non-neuronal cell lines, such as the human HeLa cells (Fig. 2B, Fig. 3) or the dog epithelium-derived MDCK cells (Fig. 4A). It was detected at a perinuclear compartment and as small punctate structures distributed throughout the cytoplasm. Co-staining with the CTR433 antibody, a median Golgi marker (Jasmin et al., 1989), identified the perinuclear compartment as the Golgi complex (Fig. 3), as expected from previous studies (Di Paolo et al., 1997b; Gavet et al., 1998). The small punctate structures also stained with A-GFP were not positive with the CTR433 antibody, and may correspond to trafficking vesicles. Similar constructs, corresponding to domain A of SCLIP or RB3 in fusion with GFP, showed the same subcellular distribution in HeLa cells as the one derived from SCG10 (Fig. 2B). This is in agreement with the subcellular distribution previously described for endogenous SCLIP and RB3 in neurons (Gavet et al., 1998).

These data show that all three domains A from stathmin-related proteins possess the capacity to target GFP to a subcellular compartment, which includes the Golgi complex and possibly a population of vesicles. Considering the high sequence identity of these domains (Fig. 1B), we chose domain A of SCG10 as representative to further investigate the nature of the targeting motifs present in the domains A of stathmin-

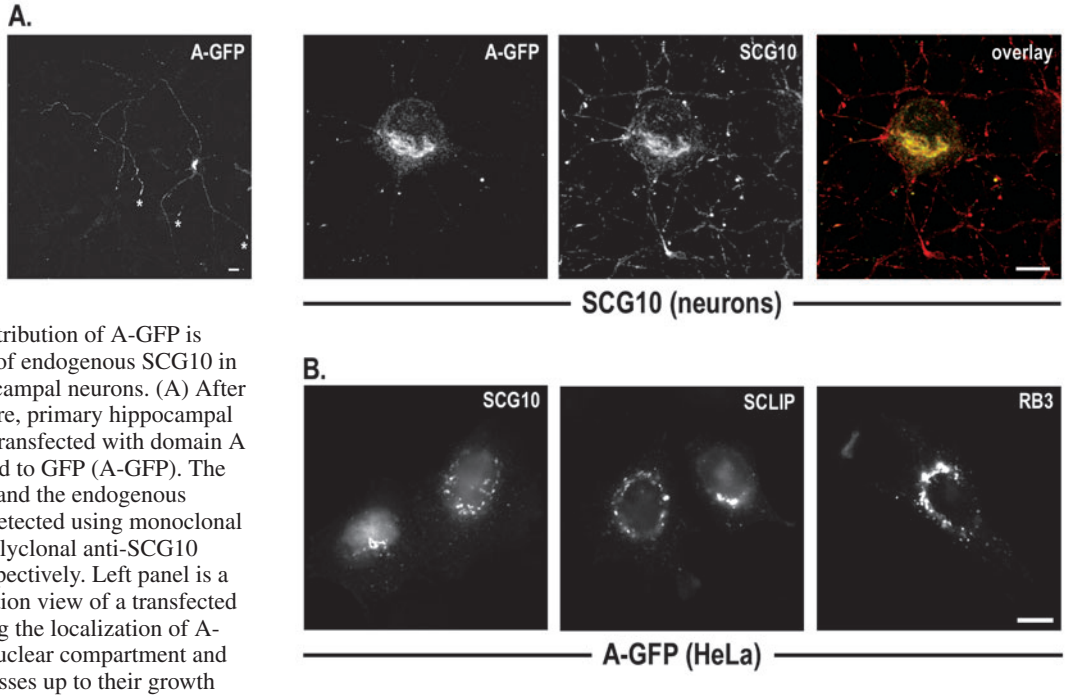

Fig. 2. The distribution of A-GFP is similar to that of endogenous SCG10 in primary hippocampal neurons. (A) After 5 days in culture, primary hippocampal neurons were transfected with domain A of SCG10 fused to GFP (A-GFP). The fusion protein and the endogenous SCG10 were detected using monoclonal anti-GFP or polyclonal anti-SCG10 antibodies, respectively. Left panel is a low-magnification view of a transfected neuron showing the localization of A-GFP at a perinuclear compartment and along all processes up to their growth cone tips (asterisks). The three images to the right show a high magnification detail of a neuron double-labelled for A-GFP and endogenous SCG10, with the corresponding overlay image showing the colocalization of A-GFP and SCG10 in the perinuclear, Golgi-like region. All images are projections of confocal stacks. (B) Domains A of SCG10, SCLIP and RB3 were fused to GFP and transfected into HeLa cells. In all cases, GFP fluorescence showed a perinuclear as well as vesicular distribution, similar to the subcellular localization described for the corresponding stathmin-related proteins (Gavet et al., 1998). Bar, 10 μm.

related proteins. Furthermore, the similar distribution of A-GFP in HeLa cells compared to primary neurons validate the use of these cell lines as appropriate experimental models for this investigation.

Domain A of SCG10 contains two distinct motifs involved in efficient Golgi targeting

In order to specify which regions in domain A are responsible for the specific subcellular localization of stathmin family proteins, we divided it into various subdomains on the basis of sequence comparison between SCG10, SCLIP and RB3/RB3′/RB3″ (Fig. 1B). We delineated three regions: the central region m, which is highly conserved between the three proteins and contains two cysteines known to be palmitoylated in SCG10 (Di Paolo et al., 1997b); the N-terminal region n, which is perfectly conserved between the three stathmin-related proteins, and also highly conserved among vertebrate species; and the C-terminal region c, which is poorly conserved.

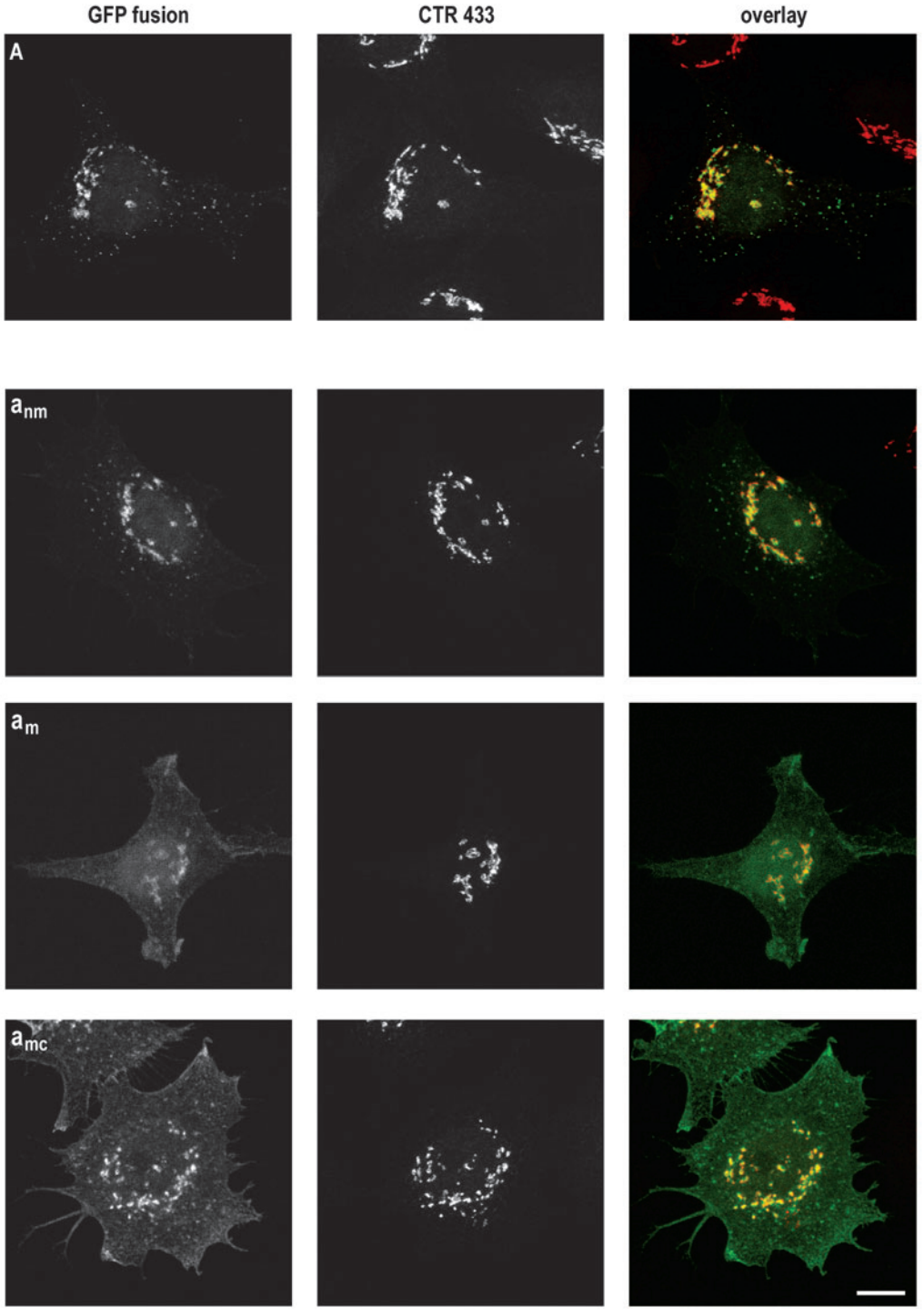

Fig. 3. Subcellular distribution of GFP fusion proteins containing domain A of SCG10 or derived subdomains in HeLa cells. A-GFP, a_{nm}-GFP, a_{m}-GFP, a_{mc}-GFP were expressed in HeLa cells. After 24 hours, the cells were fixed and labelled with the CTR433 monoclonal Golgi marker, and examined by fluorescence confocal microscopy. GFP fluorescence and CTR433 immunofluorescence images for each fusion protein, with the corresponding overlay image in the right-hand column (GFP, green; CTR433, red). A-GFP (A) is localized to the Golgi complex, as shown by its colocalization with the CTR433 labelling, and on punctate structures, which may correspond to vesicles. a_{m}-GFP (a_{m}) and a_{mc}-GFP (a_{mc}) are present at the Golgi complex but also at the plasma membrane, whereas a_{nm}-GFP (a_{nm}) is restricted to the Golgi and punctate structures, like the full-length domain A construct. All images are projections from confocal stacks. Bar, 10 µm.

We designed constructs encoding one or two of these regions within domain A of SCG10 in fusion with GFP (Fig. 1C): a_m-GFP contains only region m, whereas a_{nm}-GFP and a_{mc}-GFP contain regions n and m, or m and c, respectively. We assessed the subcellular distribution of these three constructs after transfection in both HeLa and MDCK cells (Fig. 3, Fig. 4A). The subcellular localization of a_m-GFP was partly different from that of A-GFP: it was detected at the Golgi complex, as outlined by its colocalization with the CTR433 labelling (Fig. 3), but was also present at the plasma membrane (Fig. 3, Fig. 4A) in a way similar to the localization of the C-terminal N-Ras-GFP fusion protein used as a membrane targeting reference (not shown). The a_{mc}-GFP fusion protein displayed a Golgi and plasma membrane distribution very similar to that of a_m-GFP. By contrast, a_{nm}-GFP was localized to the Golgi complex and to punctate structures, and was virtually absent from the plasma membrane in a way similar to A-GFP.

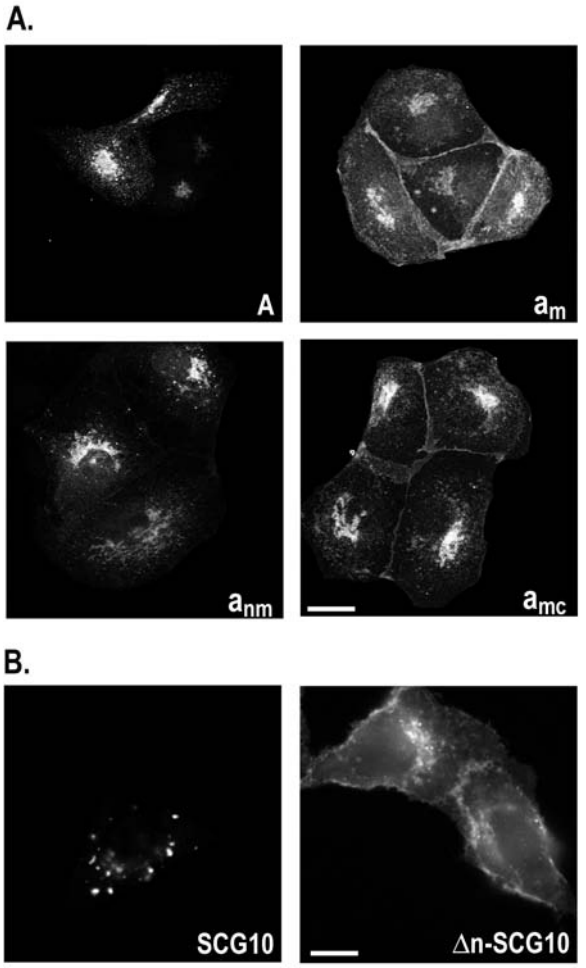

Fig. 4. Subcellular distribution of GFP fusion proteins containing either the N-terminal domain A or derived subdomains, or full-length or Δn-truncated SCG10 cells. MDCK cells were transfected with (A) A-GFP, a_{nm}-GFP, a_m-GFP, a_{mc}-GFP and (B) SCG10-GFP or Δn-SCG10-GFP constructs, fixed after 24 hours and GFP fluorescence was examined by fluorescence confocal microscopy (A) (images are projections from confocal stacks) or (B) conventional fluorescence microscopy. The subcellular distribution of the proteins is very similar to that observed in HeLa cells: A-GFP (A), a_{nm}-GFP (a_{nm}) and the full-length SCG10-GFP are mainly restricted to the Golgi complex and punctate structures (in the case of SCG10-GFP, the Golgi is disseminated owing to the microtubule-destabilizing activity of the transfected protein), whereas a_m-GFP (a_m), a_{mc}-GFP (a_{mc}) and Δn-SCG10-GFP are also present at the plasma membrane. Bar, 10 μm.

In order to verify the importance of region n in the Golgi targeting of full-length stathmin-related proteins, we compared the subcellular distribution in MDCK cells of the full-length SCG10 (SCG10-GFP) and of a truncated form in which region n has been deleted (Δn-SCG10-GFP), both in fusion with GFP. SCG10-GFP was mainly localized to the Golgi complex (Fig. 4B), which appeared dispersed as a result of the microtubule destabilizing activity of the transfected full-length protein (Gavet et al., 1998). By contrast, Δn-SCG10-GFP displayed a broader membrane distribution since it was present both at the plasma membrane and at the Golgi complex.

Altogether, our data suggest that the two regions n and m are involved, with distinct activities, in the shuttling of stathmin-related proteins to the Golgi complex. Region m is sufficient to target GFP to cell membranes, but to a broader extent than the full-length domain A, whereas region n, which seems to have no membrane targeting potential by itself (see below, Fig. 5), restricts the broad membrane localization to the Golgi complex and vesicle-like punctate structures (Figs 3, 4: compare a_{nm}-GFP with a_m-GFP and SCG10-GFP with Δn-SCG10-GFP). By contrast, region c does not appear to contribute to the Golgi targeting, as its presence did not significantly modify the subcellular distribution of the corresponding fusion proteins (Fig. 3; Fig. 4A: compare a_{mc}-GFP with a_m-GFP). It has to be noted that as region n is fully (100%) conserved among all stathmin-related proteins (Fig. 1B), its Golgi specifying property can be extended from SCG10 to SCLIP and RB3.

Region n of domain A is able to restrict another palmitoylated membrane targeting sequence to the Golgi complex

Region n within domain A appeared essential to achieve the specific targeting of stathmin family proteins to the Golgi complex and to punctate structures, and to prevent their localization at the plasma membrane. To gain insight into the mechanism of action of this newly identified motif, we examined the influence of region n alone. The observed distribution of the a_n-GFP fusion protein (Fig. 5A) was similar to the classical distribution of GFP alone (including some nuclear localization), suggesting that region n has no targeting activity per se. Therefore, its contribution to targeting seemed dependent on its association with a membrane-anchoring domain, such as the region m within domain A. We tested this hypothesis by appending region n to another membrane-anchoring signal, the palmitoylated N-terminal domain of GAP-43. The corresponding fusion protein a_n-N_{GAP43}-GFP was expressed in HeLa cells and compared with a protein containing only the N-terminal domain of GAP-43 (N_{GAP43}-GFP) (Fig. 1, Table 1). As expected (McCabe and Berthiaume, 1999), the subcellular distribution of N_{GAP43}-GFP included both the Golgi complex and the plasma membrane (Fig. 5A), with relative distributions depending on individual cells. The a_n-N_{GAP43}-GFP fusion protein was much more concentrated at the Golgi complex, with only some cells displaying fluorescence at the plasma membrane (Fig. 5A). In order to

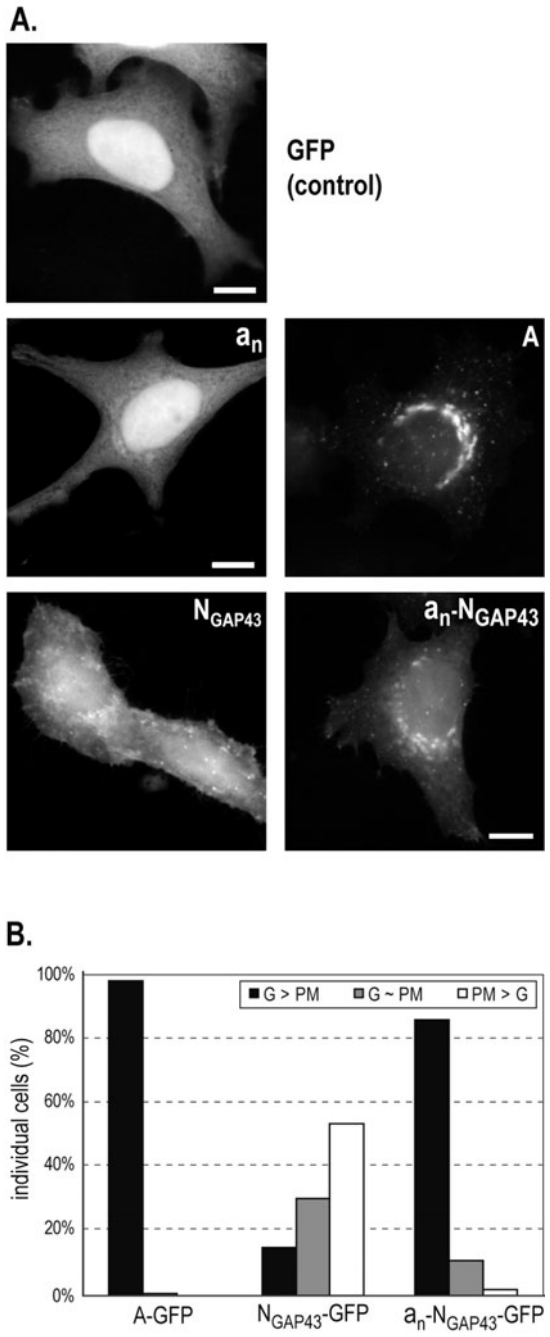

Fig. 5. Region n of domain A displays a Golgi-specifying activity when appended to the palmitoylation signal of GAP-43. GFP fusion proteins containing either region n, domain A, the palmitoylated N-terminal domain of GAP-43, or a combination of region n and the N-terminal domain of GAP-43 were transfected in HeLa cells. (A) Conventional fluorescence microscopy image for GFP alone as a control and for each construct. (B) Weakly expressing cells were classified according to the relative distribution of the GFP fusion proteins between the Golgi complex and the plasma membrane. The presence of region n appended to the N-terminal domain of GAP-43 resulted in a higher proportion of fusion protein at the Golgi complex, compared to the protein with the N-terminal domain of GAP-43 alone. G>PM, mainly localized to the Golgi complex; G~PM, equally distributed between the Golgi complex and the plasma membrane; PM>G, mainly localized to the plasma membrane. Bar, 10 μm.

quantify the Golgi-specifying effect of region n, we counted individual cells according to three categories describing the repartition of the GFP fusion proteins between the Golgi and the plasma membrane (mainly at the Golgi complex, equally distributed, or mainly at the plasma membrane) (Fig. 5B). In the case of the A-GFP construct used as a control, almost all cells displayed a signal restricted mainly to the Golgi complex and to the previously described punctate structures. As mentioned above, N_{GAP43}-GFP was distributed more heterogeneously: it was observed mainly at the plasma membrane in 55% of the considered cells, equally distributed in 30% of them, and mainly at the Golgi complex in only 15% (Fig. 5B). Interestingly, a_n-N_{GAP43}-GFP was mainly restricted to the Golgi complex in 85% of the considered cells, which demonstrated that region n retains a Golgi-specifying activity when appended to another palmitoylated membrane-anchoring domain.

Mutations of charged residues within region n lead to unspecific membrane localization

As already mentioned, region n (AYKEKMKEL) is perfectly conserved among stathmin-related proteins and also very well conserved among vertebrate species. To further identify which residues delineate the Golgi-specifying motif present in region n, we carried out site-directed mutagenesis on this region and analysed the subcellular distribution of the resulting A-GFP variants in MDCK cells.

Replacement of the single tyrosine residue present in region n (which could be a potential phosphorylation and hence regulatory site) by either alanine or phenylalanine resulted in no disturbance of the Golgi localization of A-GFP: these variants still colocalized with CTR433 immunolabelling, were present in punctate structures and were not observed at the plasma membrane (not shown). As the AYKEKMKEL contains a large proportion of charged residues, we then assessed whether these were involved in Golgi targeting: we mutated the three lysine residues (Lys8, Lys10 and Lys12 in Fig. 1) or the two glutamic acid residues (Glu9 and Glu13) to alanines within the A-GFP fusion protein (KKK/AAA or EE/AA, respectively). As shown in Fig. 6, these mutations had a similar effect to removing the whole region n (a_{mc}-GFP and Δn-SCG10-GFP, see Figs 3, 4), the mutant proteins being localized both to the Golgi and to the plasma membrane. Then, to determine whether the respective charge of lysine and glutamic acid residues was responsible by itself for their role in the Golgi-specifying activity of region n, we replaced them by residues with the same charge: all lysines by arginines (KKK/RRR), or all glutamic acid by aspartic acid residues (EE/DD). Most interestingly, these charge-conservative mutations also altered the Golgi targeting of A-GFP, as the corresponding mutants were both localized to the plasma membrane in addition to the Golgi complex (Fig. 6).

Altogether, our data suggest that the charged residues present in region n are essential for its Golgi-specifying activity, but in a way more specific than through their mere charge. These results highlight the importance of the specific nature of region n in governing the functional localization of stathmin family proteins to the Golgi complex and punctate structures.

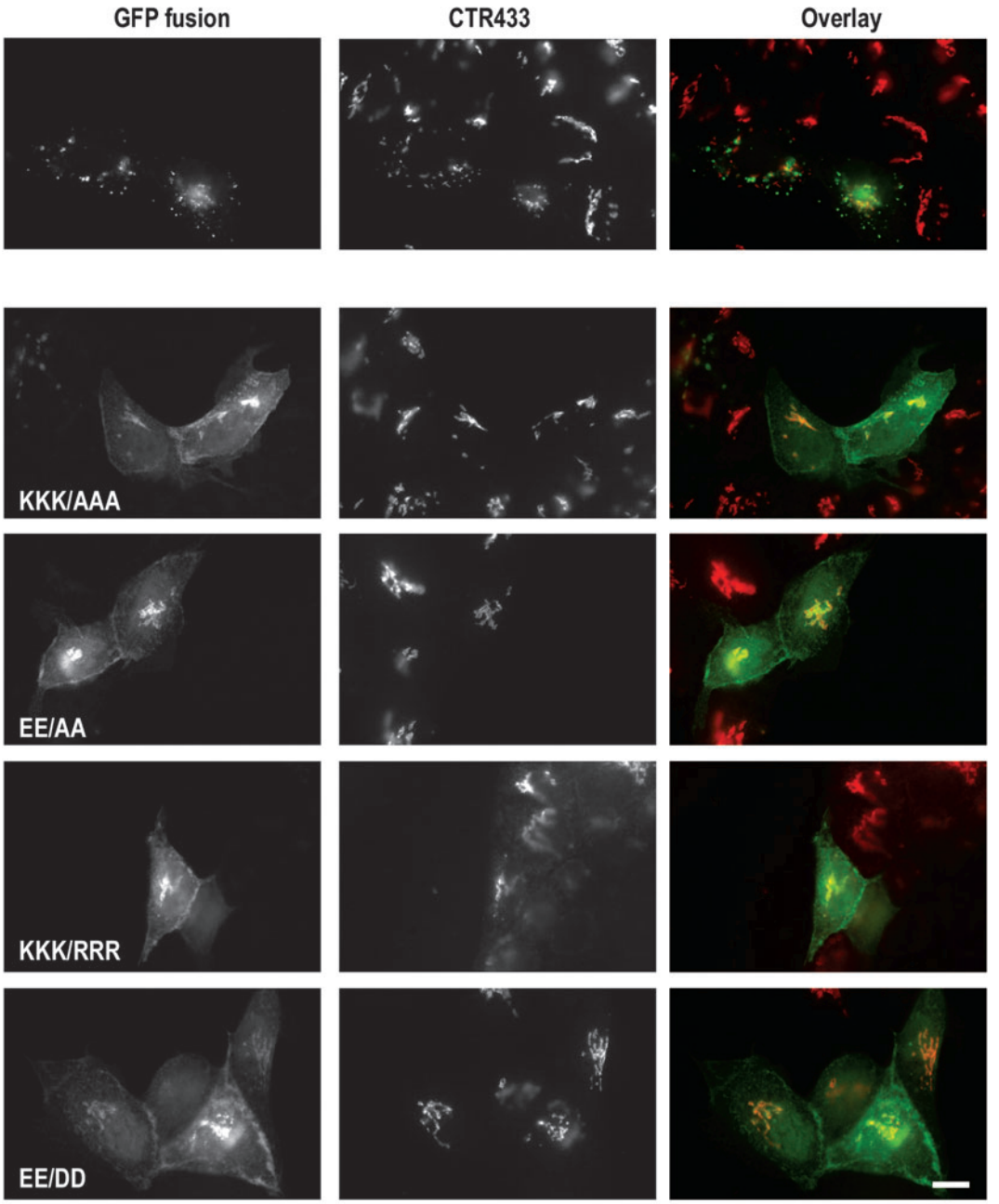

Fig. 6. Subcellular localization of A-GFP mutated at specific residues within region n. The indicated mutants (see text for details) were expressed in MDCK cells. Twenty-four hours after transfection, cells were fixed and stained with the CTR433 monoclonal Golgi marker. The subcellular localization of the mutants was examined by conventional fluorescence microscopy. For each fusion protein, GFP fluorescence and CTR433 immunofluorescence images are shown, with the corresponding overlay image (GFP, green; CTR433, red) in the right-hand column. When compared to the wild-type A-GFP (top panels), mutation of charged residues within region n (AYKEKMKEL) to either alanine (KKK/AAA; EE/AA) or to conservatively charged amino acids (KKK/RRR; EE/DD) disturbed the subcellular localization of the resulting fusion proteins. In contrast to the wild type, which is only localized to the Golgi and punctate structures, these mutants were distributed both at the Golgi complex and at the plasma membrane, as clearly observed at cell junctions. Bar, 10 μm.

Discussion

The sorting of peripheral proteins to distinct subcellular compartments is thought to be driven by various combinations of lipid modifications and additional motifs (Resh, 1999; McCabe and Berthiaume, 2001; El Husseini and Bredt, 2002). In this study, we investigated the molecular mechanisms involved in the Golgi targeting of stathmin family proteins. By characterizing the subcellular localization mediated by various subdomains derived from domain A of SCG10 in fusion with GFP, we delineated and characterized two targeting motifs acting in a cooperative fashion, with membrane-anchoring and Golgi-specifying activities respectively. Altogether, our results open new perspectives concerning the mechanisms by which stathmin family proteins are targeted to their functional compartments and, more generally, concerning specific determinants for Golgi targeting of peripheral membrane proteins.

Stathmin-related proteins are peripheral membrane-bound proteins localized to the Golgi complex and on vesicular structures present in the cell body, along neurites, and concentrated within the growth cones of neurons (Stein et al., 1988; Di Paolo et al., 1997a; Lutjens et al., 2000; Gavet et al., 2002). Domain A, common to all stathmin family proteins other than stathmin, was shown here to be sufficient to target GFP to the Golgi complex and vesicles in primary hippocampal neurons, but also in HeLa and MDCK cells, in agreement with previous studies (Di Paolo et al., 1997b; Lutjens et al., 2000). Thus, although stathmin family proteins bearing domain A are essentially expressed in the nervous system, their subcellular targeting appears to involve a

ubiquitous mechanism shared by most cell types. The dissection of domain A of SCG10 into three regions further showed that both the N-terminal n and the middle m regions contain targeting determinants, whereas no significant contribution of region c was observed. However, we cannot exclude a role of this last region, which in spite of a significant sequence divergence among stathmin family proteins, is highly conserved among vertebrate species for each protein, and may thus be responsible for conserved properties specific to each member of the stathmin family.

The middle, nine residue region m is sufficient to target GFP to the Golgi complex, vesicles and plasma membrane. Membrane binding through this region is probably achieved by its two palmitoylated cysteines (Di Paolo et al., 1997b), which are surrounded by several hydrophobic residues that may be important for palmitoylation, as shown for other proteins (El Husseini et al., 2000; El Husseini and Bredt, 2002). It is noteworthy that the subcellular localization induced by region m is very similar to that observed with other palmitoylation motifs. Indeed, several such motifs fused to GFP have been localized to the Golgi complex and to the plasma membrane (McCabe and Berthiaume, 1999), and we also observed that the palmitoylation motif of GAP-43 targets GFP to the Golgi complex and to the plasma membrane, as previously described (McCabe and Berthiaume, 1999). Region m can thus be considered as an autonomous palmitoylation motif, sufficient to achieve the membrane binding of a protein to the Golgi complex and the plasma membrane.

The information present in region m is not sufficient to account for the specific Golgi and vesicles targeting mediated by the full-length domain A. This result is consistent with the general observation that the subcellular distribution of full-length palmitoylated proteins is often more restricted than that mediated by their palmitoylation motifs alone. For example, the palmitoylation motif of GAP-43 broadly targets GFP to the Golgi complex and to the plasma membrane, whereas the full-length protein is mainly restricted to the axon membrane (Burry et al., 1992). In the case of the proteins GAD-65 and PSD-95, it was shown that other signals in addition to the palmitoylation motif are necessary to achieve the fine targeting of these proteins to presynaptic clusters and postsynaptic densities, respectively (Craven and Bredt, 2000; El-Husseini et al., 2001; Kanaani et al., 2002; Kanaani et al., 2004). For SCG10, we identified region n as a second signal, which participates in the specific sorting of domain A to the Golgi complex, in addition to the palmitoylation motif within region m. Indeed, constructs containing region n (A-GFP, a_{nm}-GFP and SCG10-GFP) were restricted to the Golgi complex and vesicles, whereas the others did not display this limited distribution (a_m-GFP, a_{mc}-GFP or Δn-SCG10-GFP). On the other hand, region n by itself was unable to display any specific targeting activity, suggesting that this region is necessary but not sufficient to shuttle stathmin-related proteins to the Golgi complex. Rather, our results strongly suggest that the functionality of region n is dependent on the presence in its vicinity of a membrane-binding signal, such as the region m of stathmin-related proteins or the palmitoylation motif of GAP-43. It has to be noted that the linker regions in the corresponding constructs are different, hence making it unlikely that the linker sequence or its length play a role in the observed targeting.

Region n (AYKEKMKEL) is highly conserved among stathmin-related proteins and among vertebrate species but is not found in other proteins. It is a targeting motif so far specific to the stathmin family. It displays several remarkable features, amongst which is the presence of a high proportion of positively or negatively charged residues. Interestingly, polybasic sequences have been shown before to function as non-specific membrane association motifs interacting with acidic membrane phospholipids, for example in the cases of Src and Ras family proteins (Sigal et al., 1994; Roy et al., 2000). In the case of region n, mutation of the charged residues, either to alanine or to similarly charged homologous residues, disturbed the localization of the A-GFP fusion protein, with an effect similar to removing the whole region n. These results suggest that the nature and not the charge of the residues present in region n are involved in the Golgi-specifying activity of this region, which appears thus to act differently from polybasic stretches commonly found in other peripheral proteins. Rather, our data hint at the existence of a specific partner interacting with region n, which would restrict the localization of domain A, and therefore of SCG10, to the Golgi complex. Identification of such a partner, which may or may not be a protein, would be of high relevance for elucidating the targeting mechanisms of stathmin family proteins, but also more generally of other proteins localized to the Golgi complex and vesicles.

Similar to integral membrane proteins, the targeting of stathmin family proteins could involve retention or recycling mechanisms. In the first case, the postulated partner of region n would be localized to the Golgi complex, and would prevent region n-containing proteins to exit the compartment and reach the plasma membrane. In the case of a recycling mechanism, the partner would be present at the plasma membrane and act as a repulsion signal to trigger the rapid return of region n-containing proteins to the Golgi complex. Both hypotheses are consistent with our data. In all cases, the molecules involved in the Golgi-specifying activity of the region n are probably ubiquitous, as the targeting mechanisms of domain A are conserved in most cell types.

The details of the route taken by stathmin-related proteins may have consequences for their function. Indeed, their enrichment on organelles at the centre of the growth cone, as well as their expression peak during the neuronal differentiation period, has led very early to proposals of a role for these proteins in growth cone regulation (Di Paolo et al., 1997a; Gavet et al., 1998; Gavet et al., 2002). Their interaction with the cytoskeleton component tubulin (Andersen et al., 1997; Charbaut et al., 2001; Gavet et al., 2002; Brannstrom et al., 2003), with the protein G regulators RGSZ1 and RGS6 (Nixon et al., 2002; Liu et al., 2002) or the ion channel TRPC5 (Greka et al., 2003) further suggests that they may participate in the modulation of signaling pathways and cytoskeleton dynamics, which are of critical importance for guided growth cone advance and neuritic growth. In this respect, the subcellular localization of stathmin-related proteins and its regulation can strongly affect the way they interact with their partners and have functional consequences on their activities.

In conclusion, our results provide new insight into the molecular mechanisms involved in the subcellular sorting of intracellular peripheral membrane-bound proteins. Indeed, only a few peripheral proteins have been described to be

localized exclusively to the Golgi complex. Here we show that the cooperation of two distinct motifs within the same molecule can control its targeting to the Golgi complex, with one motif allowing membrane anchoring and a second motif specifying the localization, probably through protein-protein interaction. We identified a novel Golgi-specifying motif, which restricts subcellular localization to the Golgi complex when targeted to intracellular membranes by another signal, such as a palmitoylation motif. From a functional point of view, our study reinforces the current view on stathmin-related proteins as spatial regulators able to regulate microtubule dynamics locally. Elucidation of the fine mechanisms by which these proteins are spatially controlled will help us to understand how and where they regulate the activities of their partners, especially in neurons and growth cones.

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