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# The stathmin phosphoprotein family: intracellular localization and effects on the microtubule network

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## SUMMARY

Stathmin is a small regulatory phosphoprotein integrating diverse intracellular signaling pathways. It is also the generic element of a protein family including the neural proteins SCG10, SCLIP, RB3 and its two splice variants RB3' and RB3''. Stathmin itself was shown to interact *in vitro* with tubulin in a phosphorylation-dependent manner, sequestering free tubulin and hence promoting microtubule depolymerization. We investigated the intracellular distribution and tubulin depolymerizing activity *in vivo* of all known members of the stathmin family. Whereas stathmin is not associated with interphase microtubules in HeLa cells, a fraction of it is concentrated at the mitotic spindle. We generated antisera specific for stathmin phosphoforms, which allowed us to visualize the regulation of phosphorylation-dephosphorylation during the successive stages of mitosis, and the partial localization of stathmin phosphorylated on serine 16 at the mitotic spindle. Results from overexpression experiments of wild-type and novel phosphorylation site mutants of stathmin further suggest that it induces depolymerization of interphase and mitotic microtubules in its

unphosphorylated state but is inactivated by phosphorylation in mitosis. Phosphorylation of mutants 16A25A and 38A63A on sites 38 and 63 or 16 and 25, respectively, was sufficient for the formation of a functional spindle, whereas mutant 16A25A38A63E retained a microtubule depolymerizing activity. Transient expression of each of the neural phosphoproteins of the stathmin family showed that they are at least partially associated to the Golgi apparatus and not to other major membrane compartments, probably through their different NH<sub>2</sub>-terminal domains, as described for SCG10. Most importantly, like stathmin and SCG10, overexpressed SCLIP, RB3 and RB3'' were able to depolymerize interphase microtubules. Altogether, our results demonstrate *in vivo* the functional conservation of the stathmin domain within each protein of the stathmin family, with a microtubule destabilizing activity most likely essential for their specific biological function(s).

Key words: Stathmin family, SCG10, SCLIP, RB3, Protein phosphorylation, Microtubule, Cell cycle

## INTRODUCTION

Stathmin (Sobel, 1991) is a ubiquitous cytoplasmic phosphoprotein of 19 kDa that was initially identified as a protein phosphorylated in response to extracellular signals (Sobel and Tashjian, 1983; Pasmantier et al., 1986; Cooper et al., 1991) and overexpressed in acute leukemias (Hanash et al., 1988). Extensive studies have shown that this protein, also called Op18 (Hailat et al., 1990) and p19 (Pasmantier et al., 1986), has a complex pattern of phosphorylation in response to various extracellular signals, in particular growth and differentiation factors (for a review, see Sobel, 1991). Moreover, its phosphorylation varies during the cell cycle, especially in mitosis where stathmin is phosphorylated on one to four sites (Beretta et al., 1992; Strahler et al., 1992; Luo et al., 1994; Brattsand et al., 1994). It was thus previously suggested that stathmin could act as a relay integrating the activation of diverse intracellular signaling pathways, and

participating to the control of cell proliferation, differentiation and activities (Sobel et al., 1989; Sobel, 1991).

The primary structure of stathmin suggests that it is organised into two domains: an NH<sub>2</sub>-terminal 'regulatory' domain containing the four phosphorylation sites identified *in vivo*, and a COOH-terminal 'interaction' domain containing a predicted  $\alpha$ -helical structure, potentially forming coiled-coil interactions with other proteins (Doye et al., 1989; Maucuer et al., 1990; Beretta et al., 1993; Curmi et al., 1994). The search for stathmin partners allowed the identification of a potential RNA binding kinase KIS, a protein of the Hsp70 family, and two proteins predicted to form coiled-coil interactions: CC1 and the likely tumor susceptibility protein CC2/tsg101 (Maucuer et al., 1995, 1997; Li and Cohen, 1996).

Among the four phosphorylation sites of stathmin identified *in vivo*, serine 16 is phosphorylated by CaM kinases II and IV/Gr (Marklund et al., 1994a; le Gouvello et al., 1998). Serine 25 is a substrate of MAPK in response to stress, growth and

differentiation factors (Leighton et al., 1993; Marklund et al., 1993; Beretta et al., 1995) and serine 38 is a target for p34<sup>cdc2</sup> (Beretta et al., 1993; Luo et al., 1994; Brattsand et al., 1994). Finally, serine 63 is the major site for PKA (Beretta et al., 1993).

Extensive phosphorylation of stathmin occurs during mitosis (Beretta et al., 1992; Strahler et al., 1992; Luo et al., 1994; Brattsand et al., 1994) and seems essential for the progression of the cell cycle, as revealed by the effects of overexpression of various stathmin phosphorylation site mutants (Marklund et al., 1994; Larsson et al., 1995; Lawler et al., 1998). The importance of stathmin in the cell cycle is also supported by the induction of a G<sub>2</sub>/M arrest in response to anti-sense inhibition of stathmin expression (Luo et al., 1994; Marklund et al., 1994b).

Recent studies have revealed that stathmin can induce the depolymerization of microtubules *in vitro* and *in vivo*, in a way that is dependent on its phosphorylation state (Belmont and Mitchison, 1996; Marklund et al., 1996; Melander Gradin et al., 1997, 1998; Jourdain et al., 1997; Larsson et al., 1997; Di Paolo et al., 1997a). To account for these effects, it was subsequently shown that stathmin (S) interacts *in vitro* with two free heterodimers of tubulin (T) to form a tubulin sequestering complex (T<sub>2</sub>S) in the sub-micromolar range (Curmi et al., 1997; Jourdain et al., 1997), and that a fully pseudo-phosphorylated 4E-mutant displayed a lower apparent affinity for tubulin and a reduced microtubule depolymerizing activity. These results suggested a model whereby stathmin, in its unphosphorylated state, interacts with and sequesters free tubulin, inducing a depolymerization of microtubules. Its phosphorylation on different combinations of sites during the cell cycle or in response to cell stimulation reduces its ability to interact with tubulin and hence its microtubule depolymerizing activity.

Stathmin is also the generic element of a protein family including SCG10, SCLIP, RB3 and its two splice variants RB3' and RB3'' (Anderson and Axel, 1985, 1988; Schubart et al., 1989; Maucuer et al., 1993; Ozon et al., 1997, 1998). In contrast to stathmin, which is ubiquitous, the stathmin-related proteins are specifically expressed in the nervous system (Stein et al., 1988; Schubart et al., 1989; Mori et al., 1990; Ozon et al., 1997, 1998). All of them share a COOH-terminal stathmin-like domain with a 'regulatory' region containing one or several of the stathmin phosphorylation sites and an 'interaction' subdomain with a putative coiled-coil forming an  $\alpha$ -helical structure (Fig. 1). They also possess an additional specific NH<sub>2</sub>-terminal domain of varying size (Fig. 1). The NH<sub>2</sub>-terminal domain of SCG10 anchors the protein to intracellular membranes, including the Golgi apparatus, through palmitoylation of two cysteines (Di Paolo et al., 1997c). SCG10 was recently shown to induce the depolymerization of microtubules through its stathmin-like domain (Riederer et al., 1997; Antonsson et al., 1998). Anti-sense inhibition of stathmin expression and overexpression of SCG10 showed that they are both involved in the neuronal-like differentiation of PC12 cells (Di Paolo et al., 1996; Riederer et al., 1997).

In the present study, we investigated the intracellular distribution of endogenous stathmin and of its serine 16 phosphoform during the cell cycle, reporting its partial concentration to the mitotic spindle. We also demonstrate the

Golgi localization of ectopically expressed SCLIP, RB3, RB3' and RB3''. We analyzed the phosphorylation dependence of the microtubule depolymerizing activity of stathmin *in vivo*, and demonstrate the conservation of this activity in each member of the stathmin family. Altogether, our results demonstrate the *in vivo* functional conservation of the stathmin domain within the stathmin-related protein family, with a microtubule destabilizing activity that is most likely essential for the specific biological function(s) of each protein, under the control of its specific molecular properties, expression and phosphorylation.

## MATERIALS AND METHODS

### Antibodies

Antibodies to proteins of the stathmin family used were polyclonal anti-recombinant RB3, anti-COOH-terminal peptide of SCG10 (Ozon et al., 1997), anti-COOH-terminal (antisera C) and internal (antisera I) peptides of stathmin (Koppel et al., 1990). Rabbit polyclonal anti-phosphorylated serine 16, 25 and 38 (anti-16P, -25P, -38P, respectively) were generated against the following synthetic peptides conjugated to KLH (Neosystem, France): [Y-L-E-K-R-A-S (PO<sub>3</sub>H<sub>2</sub>)-G-Q-A-F-E], [Y-L-E-L-I-L-S (PO<sub>3</sub>H<sub>2</sub>)-P-R-S-K-E] and [Y-P-E-L-P-L-S (PO<sub>3</sub>H<sub>2</sub>)-P-P-K-K-K], respectively. Human anti-cis-Golgi network RM (Ohta et al., 1990), monoclonal anti-medial Golgi CTR 433 (Howell et al., 1997) and rat monoclonal anti-tyrosinated tubulin (Kilmartin et al., 1982) were kind gifts of Dr M. Bornens (Institut Curie, France). Monoclonal anti-myc 9E10 and anti- $\alpha$ -tubulin N356 were from Tebu (France) and Amersham (UK), respectively.

### Immunofluorescence analysis

Paraformaldehyde fixation was chosen as it generally preserves the native structures of proteins. We sometimes preferred methanol fixation as paraformaldehyde does not preserve microtubules very well. For methanol fixation, HeLa cells were treated with methanol at -20°C for 6 minutes. Treatment with 5  $\mu$ M taxol or nocodazole was done overnight before methanol fixation. Triton extraction before methanol fixation was performed with 0.5% Triton X-100 in PHEM buffer: 45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 1 mM MgCl<sub>2</sub>, pH 6.9. For paraformaldehyde fixation, cells were treated with PBS containing 2% paraformaldehyde for 20 minutes at room temperature, subsequently permeabilized with 0.2% Triton X-100 and blocked with 100 mM glycine. Coverslips were blocked with PBS containing 3% BSA and incubated overnight with primary antibodies. After three washes with 0.1% Tween 20, coverslips were incubated for 1 hour with the appropriate rhodamine or fluorescein-conjugated anti-rabbit (1:300), mouse (1:300), rat (1:200) or human (1:300) secondary antibodies (Jackson Immunoresearch). Stathmin and stathmin family members were revealed with rhodamine-coupled secondary antibodies. DNA was stained by incubation with DAPI for 5 minutes. After three washes, coverslips were mounted with AF1 antifade mountant solution (Citifluor). Preparations were observed with a Provis Olympus fluorescence photomicroscope equipped with a Princeton Instruments camera.

### DNA constructs

DNA manipulations were carried out using standard techniques (Sambrook et al., 1989). Stathmin mutant cDNAs were constructed as previously described (Lawler et al., 1998). All the stathmin forms and the RB3, RB3', RB3'', SCLIP and SCG10 cDNAs were amplified by PCR. The 5' and 3' primers were chosen in order to introduce a 5' *KpnI* and a 3' *BamHI* restriction site to subclone into the eucaryotic expression vector pcDNA3myc. The 5' primers used were: 5'-CCC-

CGG-TAC-CAT-GAC-CCT-CGC-AGC-CTA-TAA-3' for RB3, RB3' and RB3''; 5'-GGG-GGG-TAC-CAT-GGC-CAG-CAC-CGT-ATC-T-3' for SCLIP; 5'-GGG-GGG-TAC-CAT-GGC-TAA-AAC-AGC-AAT-G-3' for SCG10. The 3' primers were: 5'-CCC-CGG-ATC-CCC-TGG-AGG-CCT-CTT-CCT-TGA-3' for RB3 and RB3''; 5'-CCC-CGG-ATC-CAC-GTG-CAG-CAG-GCG-GCT-CCT-T-3' for RB3'; 5'-CCC-CGG-ATC-CGC-CAG-ACA-TTT-CCT-CCC-GCT-G-3' for SCLIP; 5'-CCC-CGG-ATC-CGC-CAG-ACA-GTT-GAA-CCT-GCA-3' for SCG10. All the constructs were verified by sequencing (Sanger et al., 1977).

**Cell culture and DNA transfection**

Human HeLa cells were grown as monolayers in DMEM containing 10% (v/v) fetal calf serum (Life Technologies) at 37°C in 5% CO<sub>2</sub>. Transfections were performed using Lipofectamine (Life Technologies) according to the manufacturer's recommendations. Synchronization was achieved using a double thymidine block (Celis, 1994) and subsequent cell cycle progression was monitored by a FACStarPLUS cytofluorometer (Becton-Dickinson).

**Cell extracts**

Transfected cells were collected in homogenization buffer (10 mM Tris, pH 7.4, 10 mg/ml leupeptin, 25 mg/ml aprotinin, 10 mg/ml pepstatin, 1 mM EDTA) containing 20 mM NaF and sonicated for 45 seconds. The extracts were centrifuged at 10,000 g for 5 minutes at 4°C and supernatants were re-centrifuged at 400,000 g for 6 minutes at 4°C (S2) in a Beckman TL-100 ultracentrifuge. Proteins were quantified by the BCA method (Pierce), using BSA as a standard.

**Brain extracts**

Neonatal mouse brains were homogenized in homogenization buffer supplemented with 1% NP40, agitated for 1 hour at 4°C, and centrifuged at 400,000 g for 6 minutes at 4°C to yield the Σ2 supernatant. To generate more phosphorylated forms of stathmin, the Σ2 extract was incubated with 2 mM ATP, 5 mM MgCl<sub>2</sub> for 30 minutes at room temperature and for 4 hours at 4°C, yielding Σ'2.

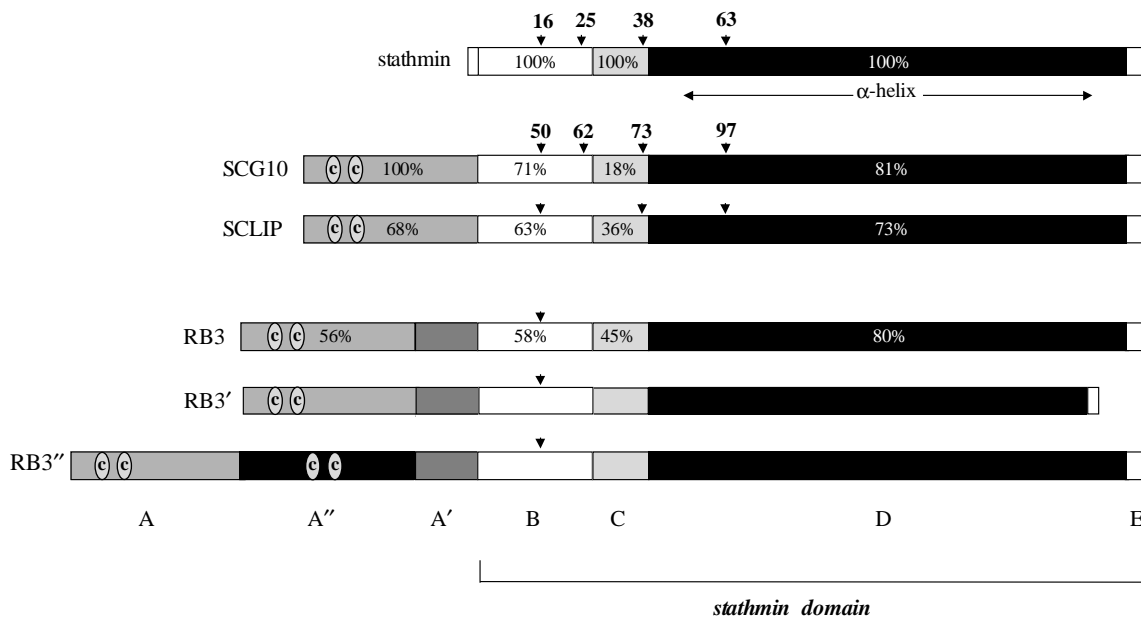
**Polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis**

One- (12.5% acrylamide) and two-dimensional gel electrophoresis (pH 5-7, 12.5% acrylamide) and immunoblots were performed as described (Laemmli, 1970; Ozon et al., 1997), except that protein blots were fixed with 0.25% glutaraldehyde (room temperature, 20 minutes) and blocked with 5% dry milk. Primary antibodies were detected by appropriate secondary antibodies coupled to peroxidase (1:5,000) (Dako) and revealed by the chemiluminescent ECL kit protocol (Amersham).

**RESULTS**

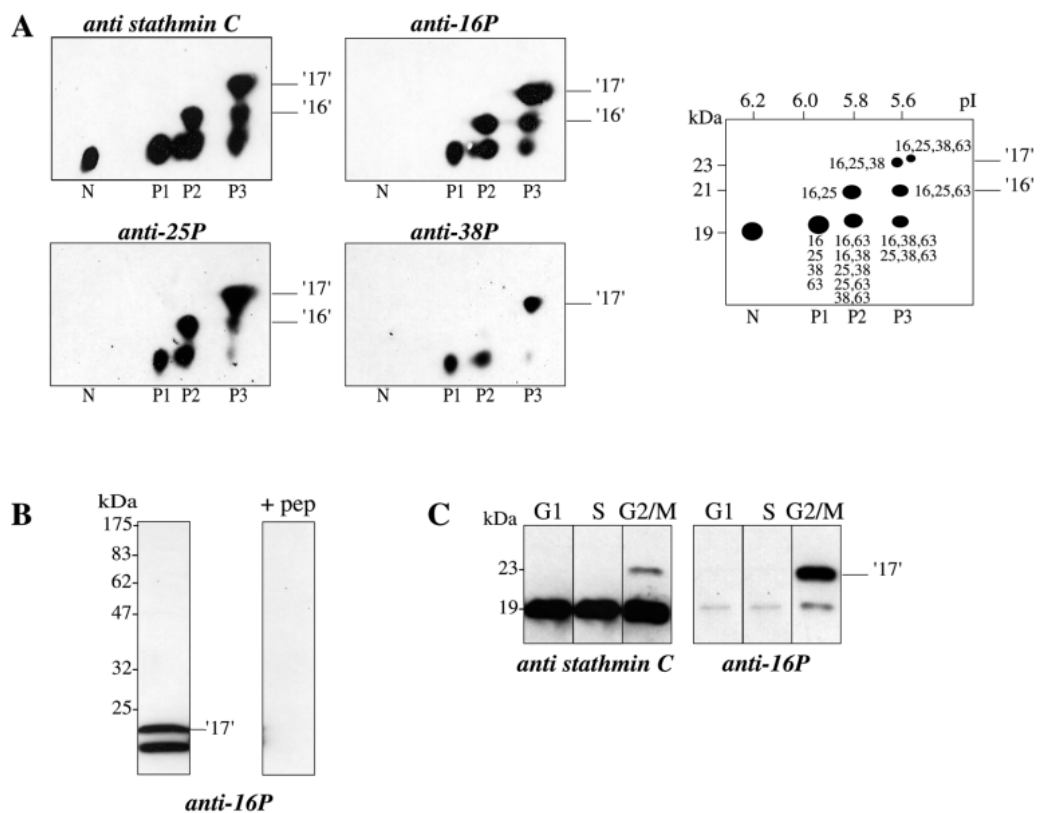
**Characterization of antisera directed against the phosphorylated sites of stathmin**

To further investigate the function and mechanism of action of stathmin during the cell cycle, we examined its intracellular localization in HeLa cells by immunofluorescence microscopy using polyclonal antibodies directed against a COOH-terminal peptide of stathmin (antiserum C) (Koppel et al., 1990). We also generated polyclonal antisera directed against each of the phosphorylated sites 16, 25 and 38 (anti-16P, -25P or -38P, respectively; see Materials and methods) to study the subcellular localization of the corresponding phosphorylated forms of stathmin. Unfortunately, the peptide corresponding to phosphorylated site 63 appeared not to be immunogenic. Two-dimensional gel analysis of a mouse brain extract containing the non-phosphorylated and all phosphorylated forms of stathmin showed that the antibodies directed against phosphorylated serines 16, 25 or 38 recognized only the phosphorylated forms and not the non-phosphorylated form (N) of stathmin (Fig. 2A). The signal with each antiserum was



**Fig. 1.** Domain organization and sequence similarities of the stathmin family phosphoproteins. The stathmin domain and the additional NH<sub>2</sub>-terminal domains of each member of the stathmin family are shown schematically with their subdomains (see text). The degrees of amino acid identity of each subdomain with the corresponding stathmin sequence and with the A domain of SCG10 are indicated. Conserved consensus phosphorylation sequences are indicated by arrows. c, cysteine amino acids shown for SCG10 to be palmitoylated.

**Fig. 2.** Antisera directed to stathmin-phosphorylated sites. (A) Two-dimensional immunoblots of a mouse brain extract mixture containing non-phosphorylated (N) and all phosphorylated forms of stathmin ( $\Sigma 2 + \Sigma' 2$ , 50  $\mu$ g protein each, see Materials and methods) hybridized with anti-stathmin antiserum C (1:20,000) or antisera directed against phosphorylated serines 16 (1:300,000), 25 (1:1,000) or 38 (1:1,000) (anti-16P, -25P, -38P), respectively. Two-dimensional migration of the various stathmin phosphoforms and the corresponding phosphorylated sites are shown in the right panel diagram (Beretta et al., 1993). As expected, anti-16P and -25P recognized spots P1, P2, P3, '16' and '17' whereas anti-38P did not recognize spots '16'. The non-phosphorylated form N was not recognized by anti-16P, -25P and -38P. (B) One-dimensional immunoblot of asynchronous HeLa cell extracts (50  $\mu$ g protein): anti-16P recognized only stathmin (its 19 kDa and '17' forms), the signal being inhibited with the corresponding antigenic peptide (+ pep). (C) One-dimensional immunoblots of extracts (50  $\mu$ g protein) from HeLa cells synchronized in G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle (see Materials and methods), with anti-stathmin antiserum C or anti-16P. The phosphorylation of stathmin on site 16 (right panel, form '17') was mostly detected in G<sub>2</sub>/M.



inhibited by the corresponding antigenic peptide, as shown in a HeLa cell extract for anti-16P (Fig. 2B). The reduced electrophoretic mobility forms '16' and '17' of stathmin are phosphorylated on sites 16, 25 ( $\pm$  site 63) and 16, 25, 38 ( $\pm$  site 63), respectively (Beretta et al., 1993). Accordingly, forms '16' are recognized in the brain extract by anti-16P and anti-25P and forms '17' by anti-16P, anti-25P and anti-38P (Fig. 2A). Altogether, these results show that the anti-16P, -25P and -38P specifically recognized stathmin phosphorylated on sites 16, 25 and 38, respectively. As these antisera were aimed in the present study at localizing phosphoforms of stathmin in HeLa cells throughout the cell cycle, we further examined their specificity by western blot analysis on HeLa cell extracts. Whereas a small number of other proteins could be recognized occasionally with anti-25P and anti-38P (not shown), probably due to the presence in these proteins of similar phosphorylation sites, only stathmin was recognized by the anti-16P antiserum (Fig. 2B), which was thus further used for immunolocalization studies of phosphorylated stathmin.

#### Immunolocalization of endogenous stathmin

In interphase HeLa cells, anti-stathmin antiserum C yielded a punctate staining in the cytoplasm, which was more concentrated around the nucleus (Fig. 3, top). This labeling was independent of the fixation procedure and was inhibited when the antiserum was preincubated with the antigenic peptide (Fig. 3). The punctate staining was not organized into a clear network and no colocalization with microtubules, or with other

cytoskeletal filaments (not shown), was detected by double immunostaining. The reorganization of microtubules into bundles after taxol treatment or the depolymerization of microtubules by nocodazole had no significant effect on the stathmin distribution, further showing that stathmin is not associated with the polymerized fraction of tubulin (Fig. 3). Moreover, the stathmin labeling was strongly decreased after a 1-minute Triton X-100 extraction before cell fixation, indicating that stathmin is not associated with detergent-insoluble cytoplasmic structures, such as the cytoskeletal filaments (Fig. 3). A punctate staining persisted throughout cells in mitosis. Moreover, we observed a diffuse staining of the spindle (as checked in parallel by costaining with an anti- $\alpha$ -tubulin, not shown) in some mitotic cells, especially in metaphase where the spindle is more apparent (Fig. 4A).

We next examined the subcellular localization of stathmin phosphorylated on site 16. In interphase cells, a punctate staining was very weakly detected with anti-16P, whereas mitotic cells were strongly labeled (Fig. 4B). The staining of mitotic cells increased from prophase to metaphase and strongly decreased at cytokinesis (Fig. 4C). These observations are in agreement with western blot analysis of synchronized cell extracts, which showed that the phosphorylation of site 16 was mostly detected in G<sub>2</sub>/M phases of the cell cycle (Fig. 2C). They further demonstrate that a very rapid dephosphorylation of stathmin on site 16 occurs at cytokinesis, as soon as the cells have finished their transition through the mitotic phase.

In all mitotic cells examined, from prometaphase to

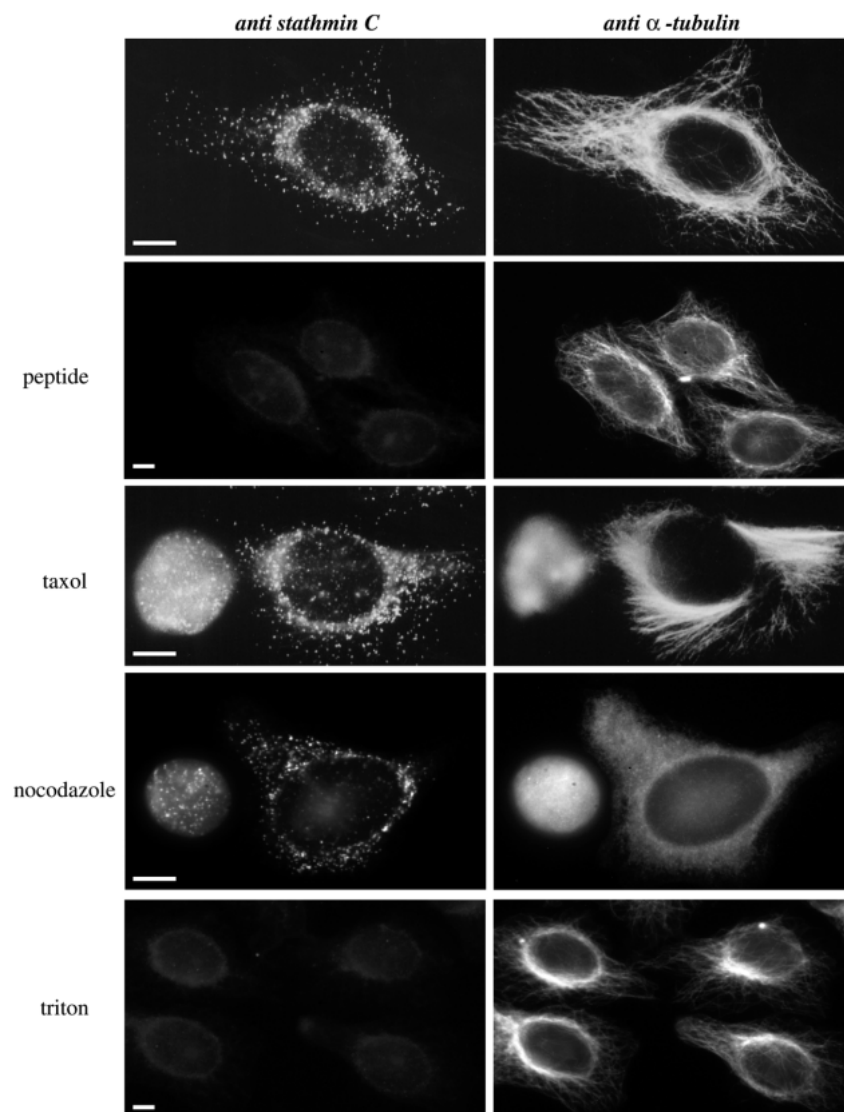
telophase, we observed again a diffuse staining of the spindle (checked by a double staining with an anti- $\alpha$ -tubulin, not shown), more clearly than with antiserum C (Fig. 4B,C, arrows). We investigated whether the spindle staining followed a reorganization of mitotic microtubules induced by incubating the cells at 30°C for 5 to 30 minutes before fixation. This treatment lead to the formation of two asters which were both labeled with anti-16P (not shown). Together, these results suggest that, during mitosis, a fraction of stathmin, including stathmin phosphorylated on site 16, is concentrated and associated with the mitotic spindle.

### Effects of stathmin phosphorylation site mutants on the microtubule network

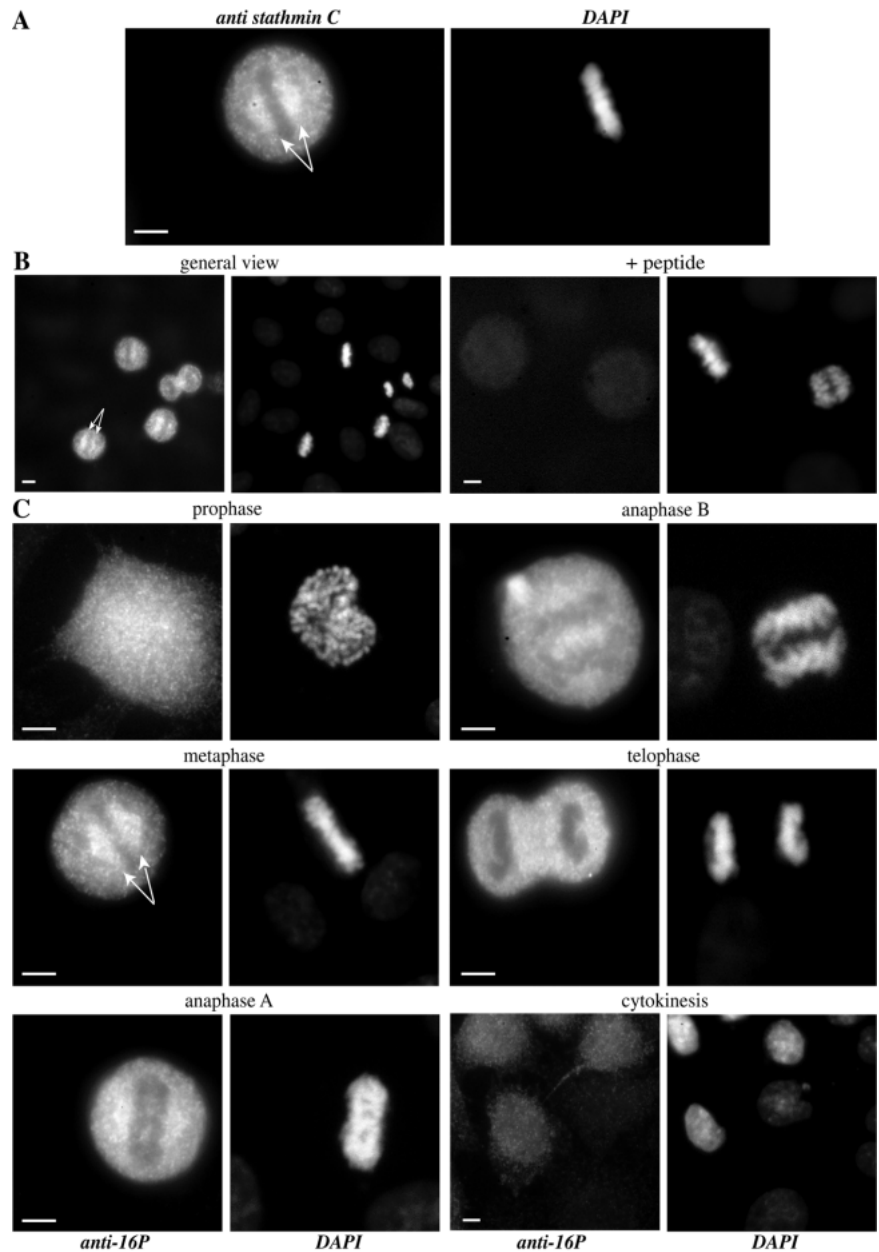
We examined the effect on the microtubule network of the overexpression of wild-type (WT) stathmin and mutants of its four phosphorylation sites. Cells transfected (from 5-10% of the cell population) with COOH-terminal myc-tagged forms of stathmin were detected by using either monoclonal anti-myc antibodies (not shown) or antiserum I (Koppel et al., 1990) (used at a high dilution to detect only the overexpressed

exogenous form). 24 hours after transfection, all the transfected cells examined were in interphase. The intracellular localization of each form of stathmin was diffuse, with no observable accumulation at any defined structure. Moreover, the nucleus was strongly stained, probably as a result of overexpression, with the exception of the nucleoli (Fig. 5A). Expression of WT-stathmin and of the 4A-mutant, whose four phosphorylation sites were replaced by alanine, induced a depolymerization of interphase microtubules in the fraction of cells expressing high levels of exogenous stathmin (Fig. 5A, arrows). This effect on the microtubule network was clearly dependent on the level of stathmin expression (not shown). The remaining microtubules were frequently not straight but 'sinuous' (Fig. 5A) and could represent more stable subpopulations of microtubules (Geuens et al., 1986). In contrast, the pseudo-phosphorylated 4E-stathmin, whose four phosphorylation sites were replaced by glutamic acid, had no visible effect on the interphase microtubule network (Fig. 5A).

During mitosis, 48 hours after transfection, 4E- and WT-stathmin had no effect on the spindle formation and cells in anaphase or telophase were observed, having normally passed



**Fig. 3.** Immunolocalization of stathmin in interphase HeLa cells. HeLa cells were pretreated or not with taxol or nocodazole (5  $\mu$ M) or with a Triton extraction buffer, before fixation with methanol (see Materials and methods). Cells were double-stained with anti-stathmin antiserum C (1:5,000) (preincubated or not with the corresponding antigenic peptide) and with anti- $\alpha$ -tubulin (1:300). Stathmin displays a punctate, Triton-sensitive labeling, which is not associated with interphase microtubules. Bars, 10  $\mu$ m.



**Fig. 4.** Immunolocalization of stathmin in mitotic HeLa cells. HeLa cells were fixed with paraformaldehyde and stained with anti-stathmin antiserum C (1:5,000) (A) or with anti-16P (1:50,000) directed to stathmin phosphorylation site 16 (B and C). The two antisera detected a fraction of stathmin located at the mitotic spindle (arrows). Only mitotic cells were stained with anti-16P (B, left panel) and this staining was inhibited by the corresponding antigenic peptide (B, right panel). The staining of anti-16P is shown with more detail at the successive steps of mitosis (C). DNA was visualized by co-staining with DAPI. Bars, 10  $\mu$ m.

the metaphase checkpoint (not shown). In contrast, we observed with the 4A-mutant a progressive increase of round cells (<2, 20 and 50% of 4A-stathmin expressing cells at 24, 30 and 48 hours after transfection). DNA staining revealed that, in these cells, chromatin was condensed and the chromosomes were detectable. However, the chromosomes were not aligned on a metaphase plate or separated in two groups but were often observed as aggregates (Fig. 5B, DAPI). These cells exhibited either short spindles with a low density of microtubules, or more severe phenotypes ranging from two small microtubule asters to no microtubules at all (Fig. 5B, arrows).

We examined whether the lack of effect of exogenous WT-stathmin in mitosis correlates with its increased phosphorylation. Cells were cotransfected with WT-stathmin and 4A-mutant. Western blot analysis showed that the phosphorylation of exogenous WT-stathmin increased with the

accumulation of transfected cells in mitosis induced by expression of the 4A-stathmin (not shown). Altogether, these results suggest that, in interphase and mitotic cells, the effect of stathmin on microtubules can be controlled by phosphorylation and that its normal phosphorylation during mitosis is essential for the correct formation of the mitotic spindle.

We next tried to determine the potential regulatory role of the different phosphorylation sites during mitosis by overexpressing stathmin mutants containing complementary combinations of one, two or three sites changed to alanine. We generated single 16A- or 63A-, double 16A25A- or 38A63A-, and a triple alanine 16A25A38A63E-mutant with a glutamic acid at position 63. We checked by western blot that the expression level of all stathmin mutants was very similar (not shown). In interphase, 24 hours after transfection, we

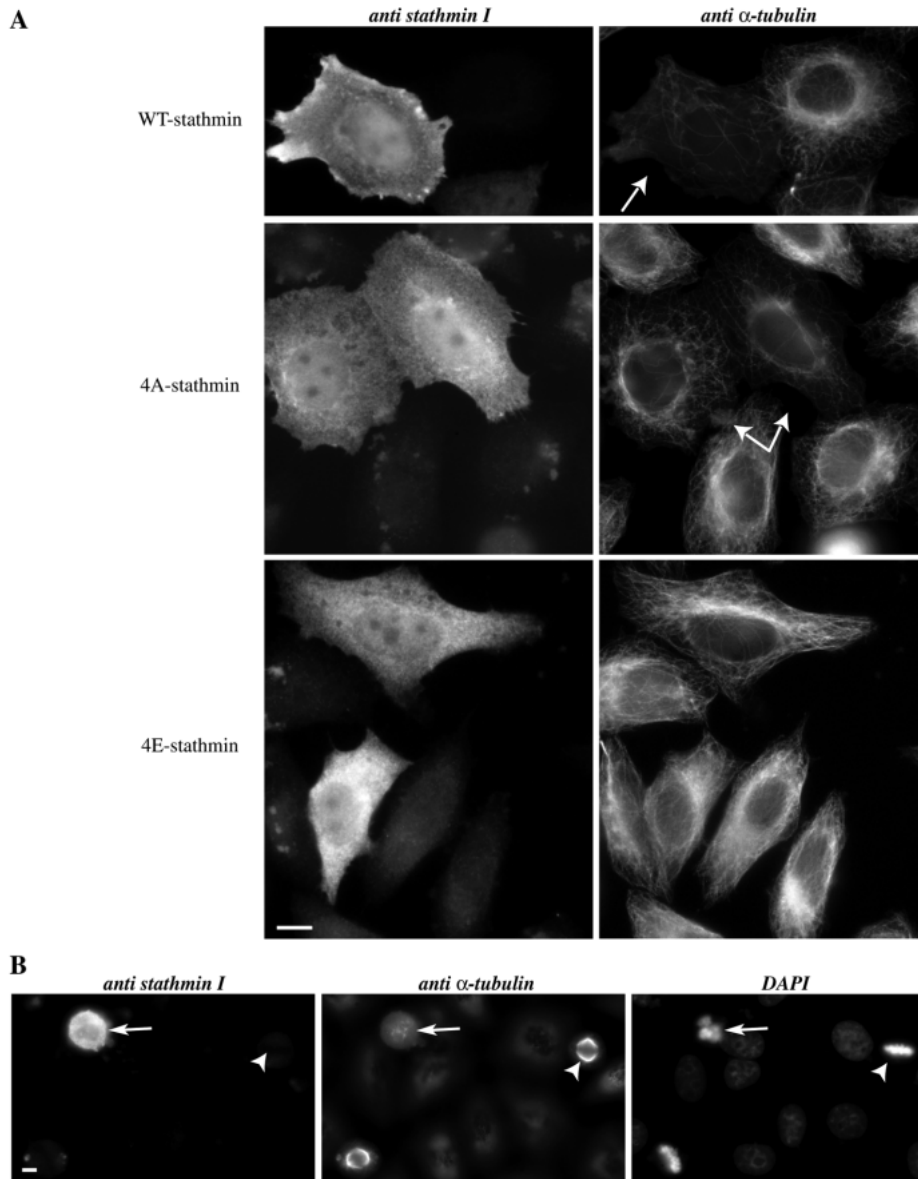
determined among the transfected cells the proportion of cells displaying a depolymerized microtubule network (less dense than in neighboring non transfected cells) (Fig. 6A). The expression of the five partial alanine mutants yielded similar results compared to WT-stathmin, whereas the full 4A-mutant yielded a slightly higher proportion of cells with a depolymerized microtubule network. In contrast, 4E-stathmin had no effect on microtubules, as the few cells detected with an abnormal microtubule network could be cells dying as a result of the transfection procedure.

These results are in agreement with the fact that stathmin is normally weakly phosphorylated in interphase (Beretta et al., 1992, 1993; Strahler et al., 1992; Luo et al., 1994; Brattsand et al., 1994). However we also observed, as it has been previously reported (Marklund et al., 1996), that approximately 50% of the exogenous wild-type stathmin was phosphorylated on one or two sites in transfected cells (5-10% of the total cell population), in contrast with the low phosphorylation state of

endogenous stathmin in the non-transfected cell population (Fig. 7A). This increased phosphorylation might reflect a modification in the activation of kinases and/or phosphatases induced by microtubule depolymerization.

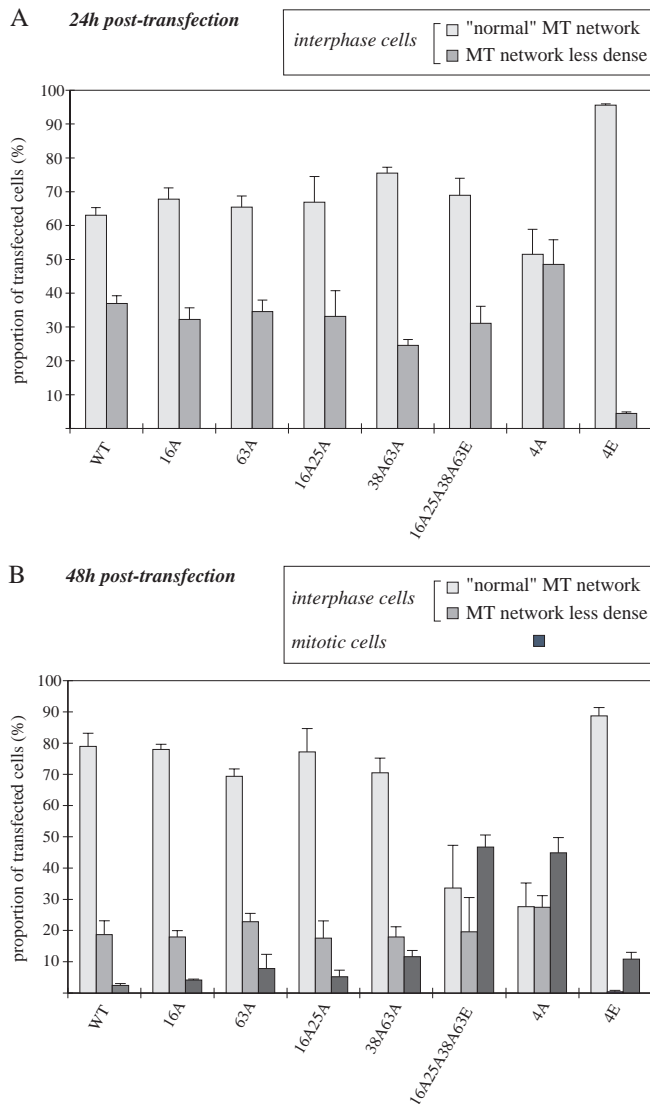
48 hours after transfection (Fig. 6B), the proportion of interphase cells with a depolymerized microtubule network was similar in cells expressing either WT- or one of the single or double alanine stathmin mutants. The proportion of mitotic cells was, on average, between 5% and 10% of the transfected cell population and all the spindles observed seemed normal. These results are very similar to that obtained with non-transfected cells (not shown) and with cells expressing the 4E-stathmin mutant. In contrast, we observed a strong accumulation in mitosis of cells expressing the 4A- or the 16A25A38A63E-mutant, the latter also exhibiting the same spindle phenotypes as described in the 4A-stathmin transfected cells (not shown).

We investigated if the lack of effect of the double alanine



**Fig. 5.** Effects of wild-type and phosphorylation site mutant stathmin overexpression on the interphasic and mitotic microtubule networks. HeLa cells transfected to overexpress wild-type stathmin (WT), or the 4A- and 4E-mutants, were fixed with paraformaldehyde 24 hours (A) or 48 hours (B) later, and stained with anti-stathmin antiserum I (1:1,500) and anti- $\alpha$ -tubulin (1:300). (A) In contrast to the pseudo-phosphorylated 4E-stathmin, WT- and 4A-stathmin induced a depolymerization of interphase microtubules (arrows). (B) In mitosis, only non-phosphorylatable 4A-stathmin prevented formation of the mitotic spindle (arrow), as compared to a non-transfected normal mitotic cell (arrowhead). Bars, 10  $\mu$ m.





**Fig. 6.** Comparison of wild type and phosphorylation-site-mutant stathmin effects on the microtubule network. HeLa cells transfected to overexpress wild-type stathmin and phosphorylation site mutants were stained for stathmin and  $\alpha$ -tubulin as in Fig. 5. The transfected interphase cells with, respectively, a 'normal' or a less dense microtubule (MT) network (in comparison with neighboring non-transfected cells) were quantified 24 hours (A) and 48 hours (B) after transfection. At 48 hours, the transfected mitotic cells were also quantified (no transfected mitotic cell being detected at 24 hours) (B). Results are shown as percentiles of transfected cells examined; they are the mean values from three counts of at least 150 cells in different fields, and are representative of at least three independent experiments. In interphase, all stathmin forms had a comparable effect on microtubules with the exception of 4E-stathmin. In mitosis, only 16A25A38A63E- and 4A-stathmin mutants induced a depolymerization of microtubules and an accumulation of mitotic cells.

mutants was due to their phosphorylation on their unmodified phosphorylation sites. For the 38A63A-mutant, phosphorylation on serines 16 and 25 was easily observed, as we detected the '16' reduced electrophoretic mobility forms of transfected stathmin, characteristic of this double

phosphorylation (Beretta et al., 1993) (Fig. 7B, right panel). Accordingly, the phosphorylation on site 16 was also detected with the anti-16P antiserum. For 16A25A-stathmin, the phosphorylation on serine 38 was detected with anti-38P antiserum (Fig. 7B, left panel). Moreover, 2D-gel analysis allowed the detection of a single and a double phosphorylation of this mutant (forms P1 and P2, respectively), indicating that serines 38 and 63 were phosphorylated. Together, these results suggest that the mutations generated in 16A25A or 38A63A-stathmin had no qualitative inhibitory effect on the phosphorylation of the other sites. In conclusion, phosphorylation of the 16A25A- or 38A63A-stathmin mutants on sites 38 and 63 or 16 and 25, respectively, seems to be sufficient to reduce their depolymerizing effect on microtubules and to allow the formation of a functional spindle. In contrast 16A25A38A63E-stathmin, which is pseudo-phosphorylated on site 63, retained the ability to induce the depolymerization of mitotic microtubules.

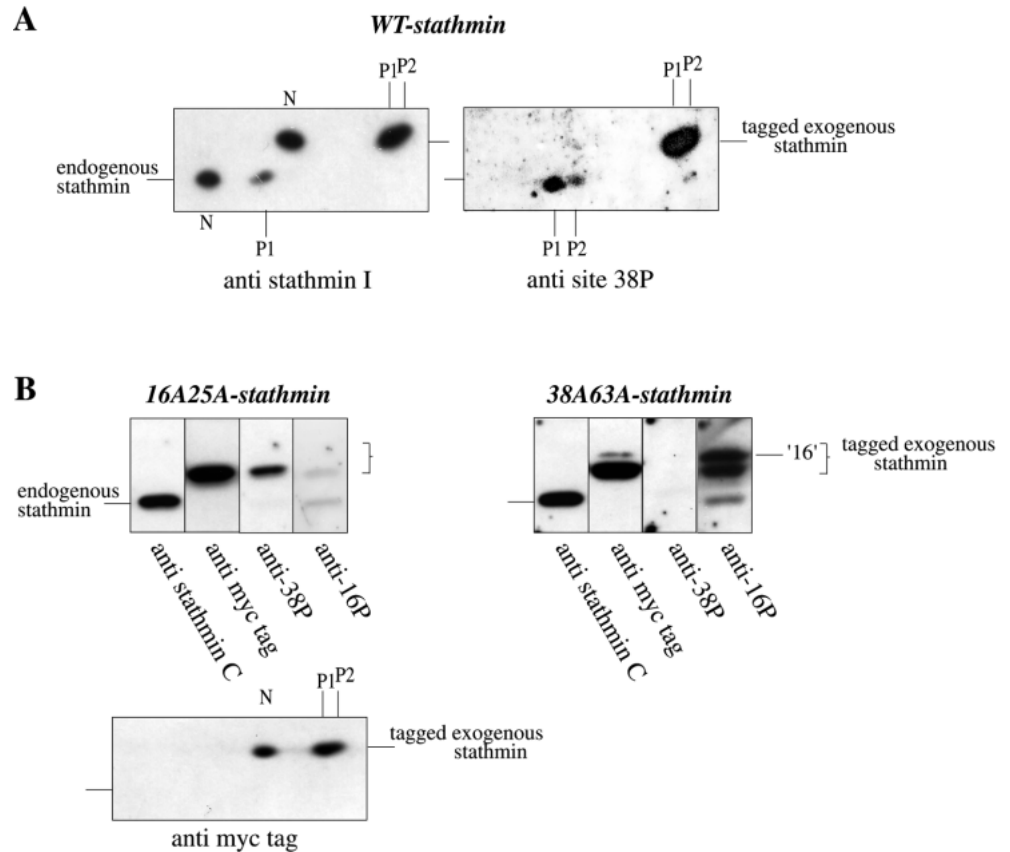
### Intracellular localization of stathmin related proteins

To examine whether other proteins of the stathmin family also possess a tubulin depolymerizing activity, we expressed each protein ectopically in HeLa cells.

First, the intracellular localization of the myc epitope-tagged proteins was followed with anti-myc antibodies or with two antisera directed against a COOH-terminal peptide of SCG10 or against the recombinant RB3 protein (this last antiserum recognized RB3, RB3' and RB3'') (Ozon et al., 1997). In cells expressing relatively low levels of transfected protein, we detected for each protein a diffuse staining of the cytoplasm and a strong staining, in the perinuclear area, of vesicles and tubular-like structures (Fig. 8). This staining suggested a possible association of SCLIP, RB3, RB3' and RB3'' with the Golgi compartments, as previously reported for SCG10 (Di Paolo et al., 1997c). Double immunostaining with antibodies specific for the medial Golgi compartment or for the cis-Golgi network confirmed that each stathmin-related protein was, at least partially, localized to the Golgi apparatus (Fig. 8). The colocalization was never perfect, as some vesicle structures stained with the antibodies recognizing the transfected proteins were not recognized by the anti-Golgi antibodies (Fig. 8, arrows). The Golgi localization of each protein seems specific, as they did not appear to be associated with other major cell membrane compartments, such as the plasma membrane and the endoplasmic reticulum. In agreement with their localization to the Golgi apparatus, the transfected proteins SCG10, SCLIP, RB3, RB3' and RB3'' were partially recovered in the high speed centrifugation pellets containing membrane-associated proteins (not shown), like endogenous rat brain SCG10, RB3, RB3' and RB3'' (Ozon et al., 1997). In mitosis, during the reorganization of the microtubule network, the Golgi apparatus is fractionated into vesicular structures and at the end of cytokinesis these vesicles fuse to reform a Golgi apparatus. Interestingly, we also observed in some cells that the staining of the transfected proteins followed the reorganization steps of the Golgi apparatus during mitosis (not shown).

Together, these results suggest that in addition to SCG10, SCLIP and members of the RB3 subfamily are membrane-associated proteins, in part localized to the Golgi apparatus. This membrane anchorage takes place probably through palmitoylation of the two well-conserved cysteines within the

**Fig. 7.** Phosphorylation state of overexpressed wild type and phosphorylation-site-mutant stathmin forms. The phosphorylation state of endogenous and overexpressed myc-tagged stathmin was analyzed on one- (30  $\mu$ g protein) and two-dimensional (200  $\mu$ g protein) immunoblots with anti-stathmin I (1:10,000) and C (1:20,000) (recognizing only the endogenous and not the myc-tagged form of stathmin), anti-myc tag (1:2,500), anti-16P (1:300,000) and anti-38P (1:1,000). S2 extracts from HeLa cells were analyzed 20 hours after transfection for wild type (WT) stathmin (A), and after 48 hours for the 16A25A- and 38A63A-mutants (B). Exogenous, overexpressed WT-stathmin in transfected cells was more phosphorylated on one or two sites (spots P1 and P2, respectively) than endogenous stathmin in the overall cell population (A). The phosphorylation of 16A25A-stathmin on serines 38 and 63 was revealed with anti-38P and by the two phosphorylated spots P1 and P2 visualized on the two-dimensional blot. The phosphorylation of 38A63A-stathmin on serines 16 and 25 was revealed by the signal with anti-16P and by the detection of the low mobility '16' form characteristic for this double phosphorylation (see Fig. 1).



NH<sub>2</sub>-terminal domain (Fig. 1), as described for SCG10 (Di Paolo et al., 1997c).

### Effects of the stathmin-related proteins on the microtubule network

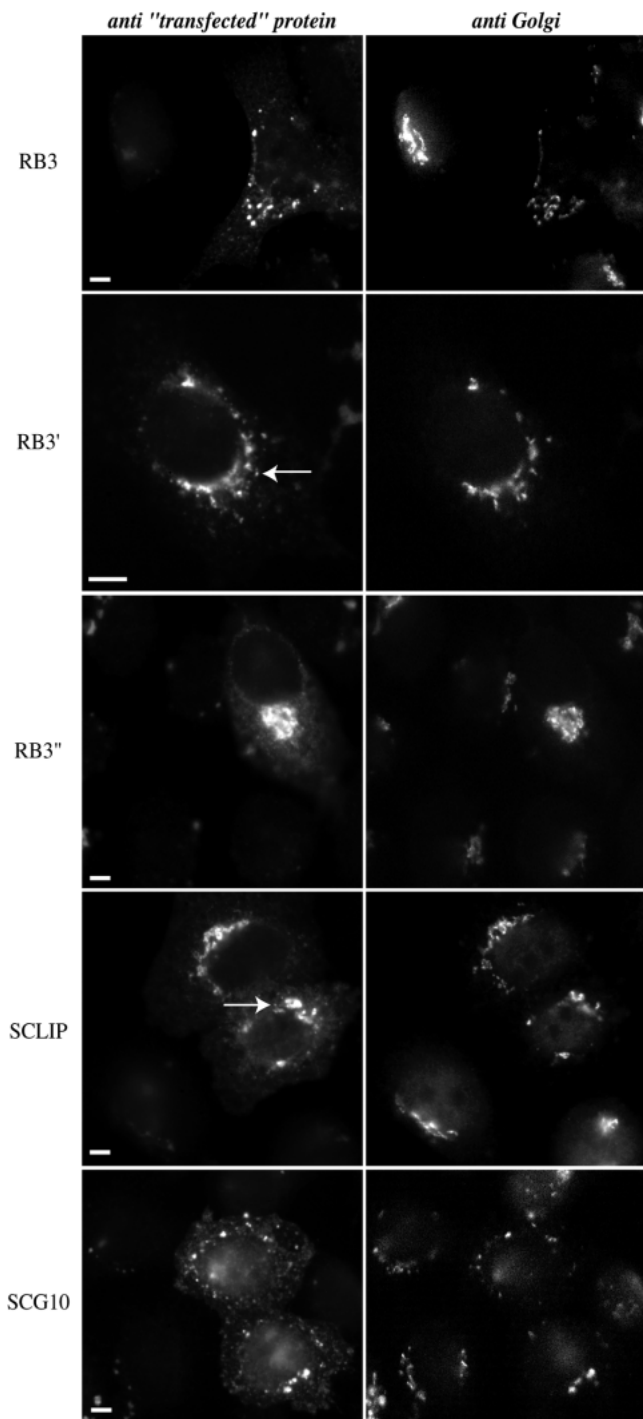
24 hours after transfection, the fraction of transfected cells expressing the highest levels of SCG10, SCLIP, RB3 or RB3'' displayed a punctate staining throughout the cytoplasm with some large dispersed spots, rather than only a perinuclear staining (Fig. 9). These large spots colocalized with vesicles of a disorganized Golgi apparatus (not shown). In the same cells, the majority of interphase microtubules were depolymerized. In contrast, we were unable to detect any RB3' expressing cells with a depolymerized microtubule network (Fig. 9). Finally, no precise colocalization of any of the stathmin-related proteins with microtubules was detected. Western blot analysis showed that the level of expression of each stathmin-related protein was very different (not shown) and it was difficult to directly and quantitatively compare the effect of the different proteins on microtubules. The level of RB3' expression was significantly lower, possibly because of a reduced stability of the molecule, and could thus be at the origin of the absence of visible effect on the microtubule network. Accordingly, preliminary experiments in vitro suggest that the stathmin-like domain of RB3' indeed shares a microtubule depolymerizing and a tubulin binding activity (not shown). 48 hours after transfection, cells transfected with SCG10, SCLIP, RB3, RB3'

or RB3'' showed no strong accumulation in mitosis, as observed with some stathmin mutants, and the few mitotic cells detected had an apparently normal spindle with chromosomes aligned on a metaphase plate.

These results show that the stathmin-related proteins SCG10, SCLIP, RB3 and RB3'' share a microtubule depolymerizing activity through their COOH-terminal stathmin-like domain, when overexpressed in interphase cells. Moreover, for these proteins, we were unable to detect a microtubule depolymerizing activity in mitosis. This result suggests that the microtubule depolymerizing activity of these proteins could be, as for stathmin, regulated by phosphorylation.

### DISCUSSION

Among its numerous likely roles related to the integration and relay of intracellular signaling pathways in diverse physiological situations, stathmin (Sobel, 1991) is involved in the control of the cell division process in a phosphorylation-dependent manner. Its action on the cell cycle probably occurs through the sequestration of free tubulin (Curmi et al., 1997; Jourdain et al., 1997) and the resulting microtubule depolymerizing activity (Belmont and Mitchison, 1996; Marklund et al., 1996; Melander Gradin et al., 1997, 1998; Curmi et al., 1997; Jourdain et al., 1997; Larsson et al., 1997;



**Fig. 8.** Immunolocalization of neural proteins of the stathmin family expressed in interphase HeLa cells. HeLa cells were transfected to express the various neural members of the stathmin phosphoprotein family, and fixed with paraformaldehyde 24 hours later. Colocalization of RB3, RB3' and RB3'' with the Golgi apparatus was investigated with anti-recombinant RB3 (1:3,000) and anti-medial Golgi CTR 433 (1:1). Cells expressing SCG10 were co-stained with an antiserum (1:2,000) directed to a COOH-terminal peptide of SCG10 and with CTR 433, and SCLIP expressing cells with anti-myc tag (1:10,000) and human anti-cis-Golgi antiserum RM (1:1,000). Each stathmin-related protein was mostly localized at the Golgi level. Arrows indicate some vesicular structures not stained with the anti-Golgi antibodies. Bars, 10  $\mu$ m.

Di Paolo et al., 1997a). Stathmin is also the generic element of a conserved protein family including the neural proteins SCG10, SCLIP, RB3 and its two splice variants RB3' and RB3'' (Anderson and Axel, 1985; Stein et al., 1988; Schubart et al., 1989; Maucuer et al., 1993; Ozon et al., 1997, 1998). Here, we examined the intracellular localization and microtubule destabilizing activity of stathmin *in vivo*, and their control by phosphorylation. Furthermore, we examined the subcellular localization and microtubule depolymerizing activity of all the other, including the recently identified, neural proteins of the stathmin family.

In interphase HeLa cells, the observed lack of association of stathmin with the microtubule network is in good agreement with its ability to interact with free tubulin rather than with polymerized microtubules *in vitro* (Belmont and Mitchison, 1996; Melander Gradin et al., 1997, 1998; Curmi et al., 1997; Jourdain et al., 1997; Larsson et al., 1997; Di Paolo et al., 1997a). In mitosis, whereas stathmin is detected throughout the cell, a fraction of the protein, including stathmin phosphorylated on serine 16, appears to be concentrated and associated with the mitotic spindle. The targeting mechanism of stathmin to the spindle is unknown but it could result from an association with proteins already localized to the mitotic spindle, such as protein kinase(s), which could phosphorylate stathmin. Such an example of targeting to the spindle was described for p34<sup>cdc2</sup> through the interaction between cyclin B and MAP4 (Ookata et al., 1995). Interestingly, it was shown that the addition of mitotic chromatin to *Xenopus* egg extracts induces an increase of stathmin phosphorylation, probably resulting from the local activation of a kinase (Andersen et al., 1997). Moreover, stathmin was shown by the two-hybrid system to interact with CC2/tsg101 (Maucuer et al., 1995), and it was reported recently that the latter is localized at the mitotic spindle (Xie et al., 1998).

A more precise investigation of the phosphorylation of site 16 *in situ* during the successive mitotic stages was made possible by a specific phosphorylation site-directed antiserum. Whereas hardly any signal is detectable in interphase, phosphorylation of site 16 increases from prophase to metaphase and strongly decreases at cytokinesis. These results suggest that a protein kinase which phosphorylates serine 16 is activated during prophase and probably inactivated at cytokinesis. Serine 16 is an *in vitro* substrate of CaM kinase II and is also phosphorylated *in vivo* by CaM kinase IV/Gr (Marklund et al., 1994; Melander Gradin et al., 1997; le Gouvello et al., 1998). CaM kinase II is localized to the mitotic apparatus in metaphase and anaphase (Ohta et al., 1990), and could thus be a candidate for the phosphorylation of stathmin on site 16 during mitosis. Furthermore, as stathmin is rapidly dephosphorylated at cytokinesis, one has to assume that a corresponding phosphatase is also active at that stage. Interestingly, it was shown recently that stathmin could be dephosphorylated, at least partially, by phosphatase 2A in a mitotic *Xenopus* egg extract (Tournebize et al., 1997).

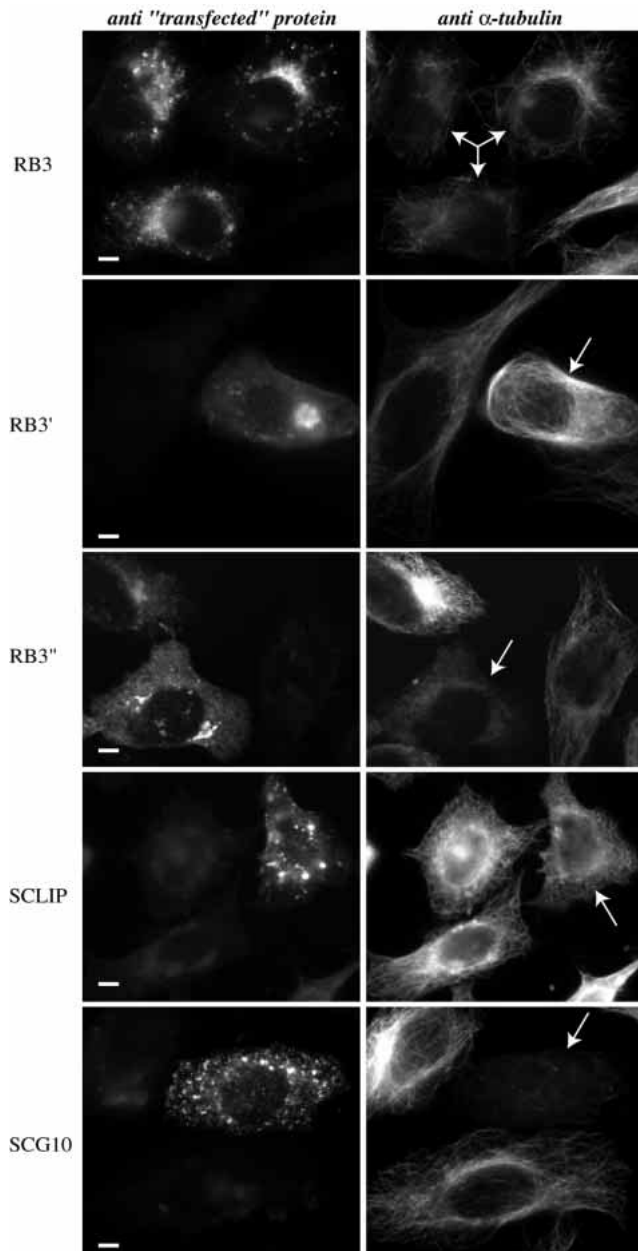
We investigated the phosphorylation dependence of the microtubule depolymerizing activity of stathmin *in vivo* by overexpressing stathmin mutants of the four phosphorylation sites. In interphase, overexpression of the unphosphorylated wild-type stathmin or of the non-phosphorylatable 4A-mutant induced a depolymerization of microtubules whereas the pseudo-phosphorylated 4E-mutant had no effect. During

mitosis, the 4E-mutant and wild-type stathmin that became phosphorylated exhibited no depolymerizing activity towards microtubules, in contrast with the 4A-mutant. Our present results confirm, in agreement with other studies (Marklund et al., 1996; Horwitz et al., 1997; Melander Gradin et al., 1997, 1998; Larsson et al., 1997; Di Paolo et al., 1997a), that in interphase and in mitosis the activity of stathmin on microtubules is

regulated by phosphorylation and that its physiological phosphorylation during mitosis is essential to allow the formation of the spindle (for a review, see Lawler, 1998). It is known that in interphase the turnover of microtubules is relatively low, but it is still higher than for microtubules polymerized from pure tubulin *in vitro* (Walker et al., 1988; Cassimeris et al., 1988; Shelden and Wadsworth, 1993). These observations suggested that some intracellular proteins can modulate microtubule dynamics in interphase, in particular by increasing the catastrophe frequency. The interaction of stathmin with tubulin and the subsequent sequestration of free tubulin could indeed be responsible for an indirectly increased catastrophe frequency (Curmi et al., 1997; Jourdain et al., 1997). In agreement with this hypothesis, it was recently reported that, in interphase cells, the microinjection of antibodies directed against stathmin induces a decrease in the catastrophe frequency and an increase in total microtubule polymer (Howell et al., 1997). In interphase, there is probably an equilibrium between the depolymerizing activity of proteins, such as stathmin, and the stabilizing activity of microtubule associated proteins (MAPs), such as the ubiquitous protein MAP4. During mitosis, stathmin and MAP4 are phosphorylated and probably inactivated (Beretta et al., 1992; Strahler et al., 1992; Luo et al., 1994; Brattsand et al., 1994; Ookata et al., 1995).

We also examined the effects of various, including novel, combinations of stathmin phosphorylation site mutants. Overexpression of the 16A25A38A63E-mutant induced a depolymerization of the mitotic spindle and a mitotic block, whereas the 16A25A and 38A63A-mutants had no detectable effect on mitotic microtubules and on cell cycle progression. Since the 16A25A- and 38A63A-mutants are phosphorylated on their remaining phosphorylatable sites, these results indicate that phosphorylation of sites 16 and 25 or 38 and 63 (but not 63 alone) is sufficient to reduce the action of stathmin on mitotic microtubules and to allow the formation of a functional spindle. In HeLa cells, endogenous stathmin is phosphorylated to completion on serine 38 in mitosis (Beretta et al., 1992; Strahler et al., 1992; Luo et al., 1994; Brattsand et al., 1994) and our results suggest that its double phosphorylation on sites 38 and 63 is sufficient to reduce its microtubule depolymerizing activity. Accordingly, it was reported recently that the triple phosphorylation of sites 16, 25 and 38 or 25, 38 and 63, or the double phosphorylation of serines 16 and 63, reduces the depolymerizing activity of stathmin, whereas single phosphorylation of site 16 or 63 does not (Larsson et al., 1997). In conclusion, sites 16 and 63 seem to play a key role in the regulation of the microtubule depolymerizing activity of stathmin when they are both phosphorylated or each phosphorylated in association with phosphorylated site 25 or 38, respectively.

It was also reported that the injection into cells of a 63D-mutant of stathmin, with serine 63 replaced by an aspartic acid, had no effect on interphase microtubules (Horwitz et al., 1997), in contrast to our results with the 16A25A38A63E-mutant. We observed that, in interphase (24 hours after transfection), more than 50% of the transfected wild-type stathmin was phosphorylated on one or two sites, in particular on serine 38. It is thus possible that a significant fraction of the injected 63D mutant was phosphorylated on site 38, resulting in an inactivated form of stathmin which would account for the lack of effect on the microtubule network.



**Fig. 9.** Effect of neural proteins of the stathmin family on the interphase microtubule network. HeLa cells were transfected to express the various neural members of the stathmin phosphoprotein family, and fixed with methanol 24 hours later. RB3, RB3', RB3'', SCLIP and SCG10 were detected as described in Fig. 8. Microtubules were stained with anti- $\alpha$ -tubulin, except for SCLIP expressing cells where a rat anti-tyrosinated tubulin (1:4,000) was used. Arrows indicate transfected cells, showing that RB3, RB3', SCLIP and SCG10 induced a depolymerization of interphase microtubules. Bars, 10  $\mu$ m.

In order to further investigate the functional properties of the stathmin domain, we examined the conservation of the microtubule destabilizing activity of the neural members of the stathmin family by ectopic expression in HeLa cells. Like SCG10, SCLIP and RB3/RB3'/RB3'' are localized diffusely throughout the cytoplasm but showed a marked accumulation at the Golgi apparatus. Although it was reported that SCG10 copurifies with microtubules (Riederer et al., 1997), no colocalization of any of the stathmin-related proteins with microtubules was observed. Their Golgi localization seems to be biologically relevant considering that (1) it was previously reported for SCG10 and attributed to the palmitoylation of two cysteines in its NH<sub>2</sub>-terminal domain (Di Paolo et al., 1997c), (2) these two cysteines are conserved in SCLIP and RB3/RB3'/RB3'' (Ozon et al., 1997, 1998) and (3) RB3/RB3'/RB3'' were recovered in rat brain high speed centrifugation fractions containing membrane-associated proteins (Ozon et al., 1997). The Golgi association of the neural members of the stathmin family is remarkable in its specificity versus other cellular membranes such as the plasma membrane or the endoplasmic reticulum. The NH<sub>2</sub>-terminal domains of the various proteins of the stathmin family could be used to unravel the mechanisms responsible for this novel sorting of non-transmembrane proteins towards the cytosolic side of Golgi membranes.

Overexpression of SCLIP, RB3 and RB3'', like that of SCG10, clearly induced the depolymerization of the interphase microtubule network. In transfected cells, the effect on the microtubule network was dependent on the level of expression of each protein of the stathmin family. The relatively low level of expression of RB3' is thus likely to explain its lack of effect, and this interpretation is in agreement with preliminary *in vitro* experiments suggesting that RB3' also interacts with free tubulin and shares a microtubule depolymerizing activity.

Altogether our results with the stathmin family proteins allow us to propose a shared mechanism for the *in vivo* microtubule depolymerizing activity of all proteins (P) of the stathmin family, in which P interacts with a fraction of the intracellular pool of free tubulin (T) to form a T<sub>2</sub>P tubulin sequestering complex, as observed for stathmin *in vitro* (Curmi et al., 1997; Jourdain et al., 1997).

For each family member, this interaction could be regulated by phosphorylation, as several of the consensus phosphorylation sites of stathmin are conserved in the stathmin-related proteins. It was shown very recently that SCG10 is phosphorylated *in vitro* on serines 50, 62, 73 and 97, corresponding to stathmin sites at serines 16, 25, 38 and 63, respectively (Antonsson et al., 1998) and, as for stathmin, the phosphorylation of SCG10 regulates its microtubule destabilizing activity. SCLIP contains consensus sites corresponding to stathmin serines 16, 38, 63 and possibly 25, whereas RB3 and its two splice variants contain the consensus site corresponding to site 16 (Fig. 1) (Ozon et al., 1997, 1998). Nevertheless, well-conserved tyrosines present in the NH<sub>2</sub>-terminal extension domain of these proteins might also be phosphorylated. Accordingly, SCG10 is an *in vitro* substrate for the tyrosine protein kinase Src (Antonsson et al., 1997). Two-dimensional gel analysis of brain extracts showed that several spots with increasing isoelectric points were detected for SCG10, RB3, RB3' and RB3'', which could correspond to their different phosphorylated forms (Ozon et al., 1997). Cells

expressing the various neural members of the stathmin family displayed no mitotic block and a normal appearance of the spindles. Further experiments are needed, however, to determine if these proteins are actually phosphorylated in mitosis.

Although we show here that all stathmin family members share a common microtubule depolymerizing activity, they each probably fulfil specific roles. Stathmin is the only ubiquitous member of the family and could participate in the regulation of microtubule dynamics in relation with diverse biological regulations and in many cell types. In contrast, the five other members of the family are specific to the nervous system. Their level of expression during brain development is differentially regulated (Ozon et al., 1998) and the pattern of expression of each corresponding mRNA is different in the whole rat embryo or in the adult rat brain, with only RB3 and stathmin being detected in glial cells (Peschanski et al., 1993; Himi et al., 1994; Ozon et al., 1997, 1998). In cultured cortical neurons, SCG10 is localized to the Golgi apparatus and to the growth cones of axons and dendrites (Stein et al., 1988; Di Paolo et al., 1997b), and it was suggested that SCG10 could be transported to the growth cones via its association with Golgi vesicles (Stein et al., 1988; Di Paolo et al., 1997c). In neurons, the structure and the dynamics of microtubules are different in the cell body, axon and dendrite shafts, and growth cones (Lim et al., 1989; Okabe and Hirokawa, 1990). Since the other stathmin-related neural proteins are associated with Golgi vesicles, they may be transported and accumulated in different cell compartments, participating locally in the regulation of microtubule dynamics. Nevertheless, these proteins could also fulfil partially similar roles, as the stathmin null mutation in mice induced no clearly disturbed phenotype (Schubart et al., 1996).

In conclusion, we report here the intracellular distribution of stathmin, in particular stathmin phosphorylated on serine 16, and the specific localization of all the other known proteins of the stathmin family. We further document the phosphorylation dependence of the microtubule depolymerizing activity of stathmin and report a similar activity for the other members of the family. Altogether, our results demonstrate the *in vivo* functional conservation of the stathmin domain within the entire stathmin protein family, with a microtubule destabilizing activity that is most likely essential for the specific biological function(s) of each protein, and related to the control of cell proliferation and differentiation, or for differentiated cellular activities. The role of each protein is probably controlled by its specific molecular properties, as well as its expression, subcellular localization and phosphorylation profile.

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