Stathmin overexpression in 293 cells affects signal transduction and cell growth

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Abstract Stathmin is a ubiquitous cytoplasmic protein whose phosphorylation state changes markedly in response to extracellular signals, and during the cell cycle. To clarify the function of stathmin, its four phosphorylation sites were mutated to either alanines (4A-stathmin) or glutamates (4E-stathmin). In transfected cells, 4A-stathmin caused a strong G2/M block and also inhibited the responsiveness of a co-transfected fos promoter/luciferase reporter plasmid to serum stimulation, whereas wild type and 4E-stathmin had relatively minor effects. These results support the idea that stathmin plays a role in multiple cellular processes and indicate that the regulation of the phosphorylation state of stathmin is likely to determine its action.

Key words: Stathmin; Signal transduction; Cell cycle; Protein phosphorylation

1. Introduction

An intricate network of proteins controls the multitude of processes required for proper cellular function and behavior. The activity of these proteins is often regulated by post-translational modifications, particularly by phosphorylation. The ubiquitous 19 kDa cytoplasmic phosphoprotein stathmin [1], also designated p19 [2], Op18 [3], and prosolin [4], has been proposed to play an important role in regulating cellular processes [5]. The rapid phosphorylation of stathmin in response to a wide range of extracellular effectors [6] and the diversity of phosphoforms observed has prompted the hypothesis that it may act as a relay integrating various intracellular signaling pathways [5]. Stathmin expression is highly regulated during development, and is most abundant in the adult brain [7–9]. Interestingly, its expression is increased in a number of neoplastic cells [3,10,11].

Stathmin consists of a C-terminal, mainly α-helical domain which may participate in coiled-coil interactions [12] and of an N-terminal domain with four serine phosphorylation sites, at positions 16, 25, 38 and 63 [6]. The sites at serines 25 and 38 have consensus sequences for proline directed kinases, and it has been shown that serine 25 is a target for MAP kinase [6,13–15], whereas serine 38 is a target for phosphorylation by cdks [6], although there is some overlap in the specificity at these sites. Serines 16 and 63 can be phosphorylated by protein kinase A [6,16], and serine 16 has been shown to be a target for both the Ca^{2+}/calmodulin dependent kinase-Gr [17], and CaM kinase II (S. le Gouvello, V. Manceau and A. Sobel, unpublished).

Stathmin phosphorylation has also been shown to vary during the cell cycle with a major increase at all four sites occurring during mitosis [11,18,19]. Anti-sense inhibition of stathmin expression inhibits cell growth and causes a block in the G2/M phase of the cell cycle [11,20]. Overexpression of mutant forms of stathmin with any of the four serines changed to alanines causes a G2/M block in K562 cells [19,20]. The cause of this phenotype is likely to be related to the observation that stathmin is important in the regulation of microtubule dynamics [21–25].

In this study, we have overexpressed both wild type and mutant forms of stathmin in the human embryonic kidney 293 cell line and examined the effects on cell growth and signal transduction. We have extended previous studies by comparing the activities of a mutant in which each of the four phosphorylation sites was mutated to an alanine, with a mutant containing a glutamic acid at each site designed to mimic hyper-phosphorylated stathmin. Overexpression of the alanine mutant has a strong effect on both cell division and the response to serum-induced signal transduction, whereas the effect of the glutamic acid mutant is much weaker, thus indicating the importance of the multiple phosphorylation of stathmin in diverse cellular processes.

2. Materials and methods

2.1. Plasmid construction

DNA manipulations were carried out using standard techniques [26]. Stathmin mutants in which four serine phosphorylation sites were mutated to either alanine (4A-stathmin) or glutamic acid residues (4E-stathmin) were constructed by the 'megaprimer' PCR technique [27] with oligonucleotide primers from Genset (France), and the human stathmin cDNA [28] as a substrate.

To express a C-terminal myc epitope-tagged form of stathmin in mammalian cells, pcDNA3stath/myc was constructed with primers encoding the myc tag [29] cloned into *Bam*HI/*Hin*dIII cut pcDNA3 (Invitrogen), and the stathmin coding sequence was introduced with PCR generated 5′-*Kpn*I and 3′-*Bam*HI restriction sites. The 4A- and 4E-stathmin/myc expression vectors were constructed similarly. Expression vectors for the truncated forms were generated with primers amplifying either the N-terminal 65 amino acids (ΔC-stathmin) or the C-terminal 85 amino acids (ΔN-stathmin), with the same restriction sites used for the full length forms.

For stable episomal expression, the stathmin/myc unit constructed above was subcloned into the episomal mammalian expression vector pCEP4 (Invitrogen) as a *Hin*dIII/*Xho*I fragment. pCEPβgal was constructed as a control to express β-galactosidase in this system. The β-galactosidase cDNA was excised from pUHG 16-3 (a gift from H. Bujard) using *Xba*I sites, and subcloned into pCEP4.

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2.2. Cell culture, transfections and fos promoter assays

Cells of the human embryonic kidney 293 cell line transfected with the EBNA-1 gene (293-EBNA) (Invitrogen) were cultured in DMEM containing 10% fetal calf serum and 200 μg/ml G418 (Gibco BRL) at 37°C in 5% CO_2. 293 cells were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's recommendations, with 2 μg of a pCEP4 derived stathmin expression vector. 24 h post transfection, medium containing 400 μg/ml hygromycin (Sigma) was added. The medium was changed every 2 days for a week and then cells were passaged by vigorous pipetting to a 10 cm plate. After 2 more days selection the cells were washed with DMEM and viable cells were counted by trypan blue exclusion prior to further experiments.

For the fos promoter assays, transfections were performed using 1 μg stathmin expression vector and 0.5 μg each of a fos luciferase reporter vector [30] (a gift from Dr. A.D. Sharrocks, Newcastle-Upon-Tyne, UK) and pRSVβ-gal (Pharmacia). 6 h after the start of transfection the medium was removed and the cells washed twice in DMEM. Fresh medium containing 0.5% fetal calf serum was then added. After a further 36 h incubation, stimulation was carried out by the addition of DMEM containing 20% fetal calf serum. 8 h later the cells were harvested into 200 μl of Reporter Lysis Buffer (Promega), and the levels of luciferase (Promega assay system) and β-galactosidase [26] were measured. The final activity reading was obtained by dividing each luciferase reading by the corresponding β-galactosidase reading.

2.3. SDS-PAGE and Western blotting

SDS-PAGE on 13% polyacrylamide gels and immunoblotting were performed as described [31]. The primary antibody was added at a suitable dilution in blocking buffer with 1% dry milk, and the blot was probed with an appropriate HRP conjugated secondary antibody (Dako) before development using ECL (Amersham).

2.4. Microscopic analysis and flow cytometry

Immunofluorescence microscopy was performed using an Olympus Provis fluorescence microscope. Cells grown on glass coverslips were fixed for 6 min with methanol at −20°C. After washes with PBS and blocking in PBS containing 3% BSA, cells were incubated overnight with the primary antibody (either anti-myc monoclonal 9E10 (Oncogene Science) or anti α-tubulin monoclonal N356 (Amersham). After washes with PBS containing 0.1% Tween 20, rhodamine conjugated anti-mouse secondary antibodies (BioSys) were used to visualize the antibody staining pattern, and Hoechst 33258 stain (Molecular Probes) at 5 μg/ml for cell nuclei.

Cells selected in hygromycin were analyzed for DNA content after overnight fixation in 70% ethanol and incubation in propidium iodide. Flow cytometry was carried out using a Becton Dickinson FACScan counting at least 10 000 cells per sample. The data were collected using the CELLQuest program (Becton and Dickinson). Cell cycle data was analyzed using the program ModFit LT version 1.0 (Verity Software, US).

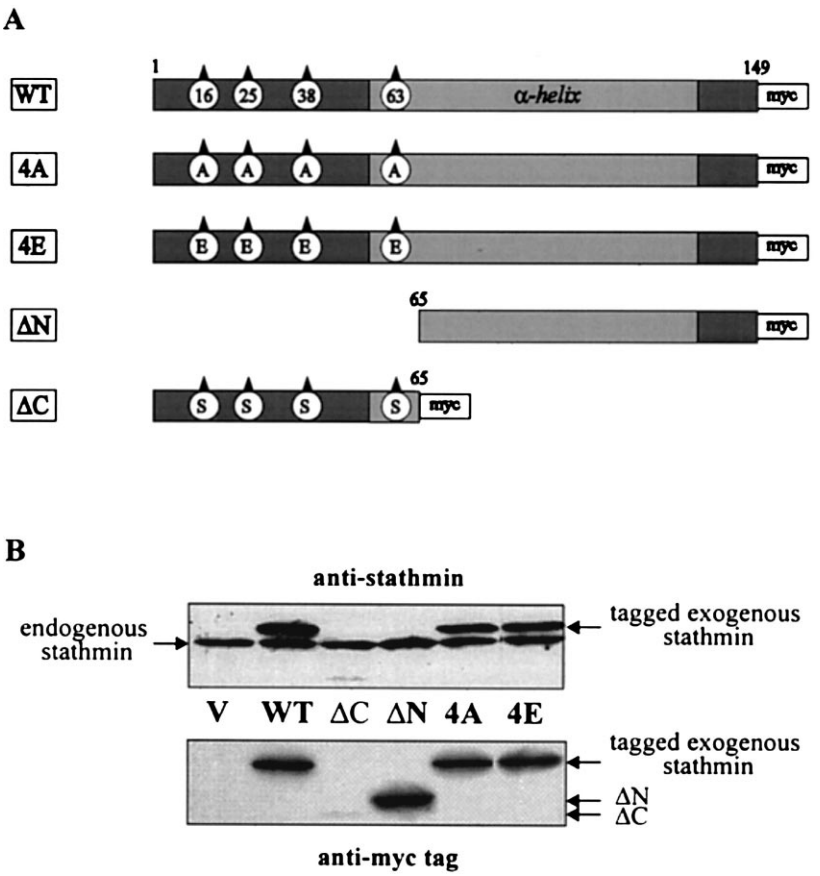
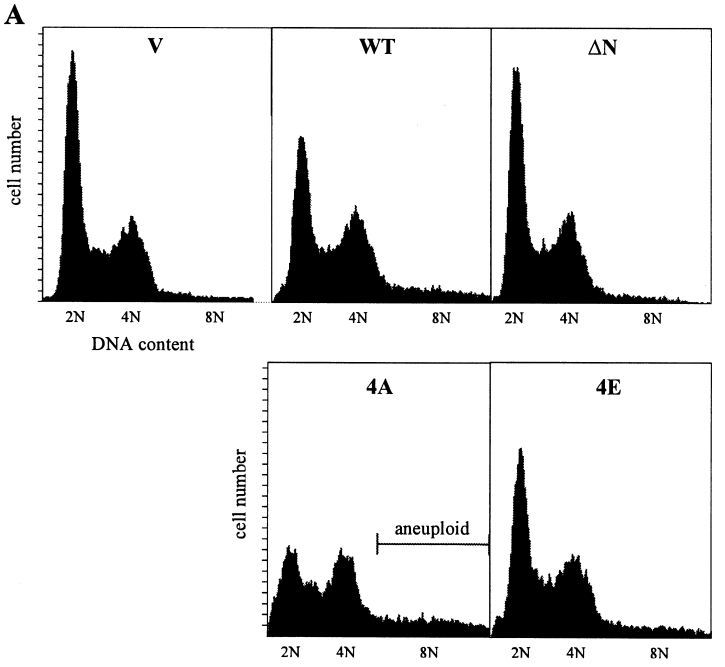


Fig. 1. Construction of mutant stathmin forms and expression in 293 cells. A: Schematic representation of the forms of stathmin expressed in 293 cells with a C-terminal myc epitope tag. The changes introduced into the wild type stathmin (WT) to make each mutant are: replacement of all four phosphorylation sites with alanine (4A) or with glutamic acid (4E), and truncation of either the N-terminal (ΔN) or C-terminal (ΔC) moieties of the molecule. B: 293 cells transfected transiently with expression plasmids for stathmin or its mutant forms were grown for 10 days in hygromycin containing selection medium to enrich the population in transfected cells. Equal amounts of protein were separated by SDS-PAGE and a Western blot was then probed with a polyclonal antiserum directed against the N-terminus of stathmin [9] or an anti-myc monoclonal antibody. The ΔC-stathmin band appears very faint, probably due to degradation of the corresponding protein. Limited degradation is probably also the cause of the presence of some low molecular weight bands revealed by the anti-myc antibody in the 4A and 4E mutant expressing cells. ΔN-stathmin is revealed only by the anti-myc antibody, as it lacks the N-terminal region recognized by the anti-stathmin antiserum.



B

Transfection	*Proportion of cells (%) in G0/G1*	*S*	*G2/M*
V	*39.0 +/- 2.0*	*42.5 +/- 2.3*	*18.2 +/- 0.4*
WT	*38.4 +/- 1.6*	*34.5 +/- 6.4*	*27.1 +/- 7.4*
ΔN	*39.5 +/- 0.1*	*42.3 +/- 2.6*	*18.2 +/- 0.2*
4A	*28.8 +/- 9.5*	*21.5 +/- 11.5*	*46.5 +/- 5.5*
4E	*34.8 +/- 2.2*	*40.2 +/- 1.1*	*25.1 +/- 1.3*

Fig. 2. Flow cytometry cell cycle analysis of selected 293 cell populations expressing stathmin or its mutant forms. After 10 days selection in hygromycin containing medium of 293 cells transfected with expression vectors for stathmin or its mutant forms (see Fig. 1), cells were seeded at 4×10^5 cells per 35 mm well. 48 h later they were harvested and the cell cycle profile was analyzed by flow cytometry after DNA staining with propidium iodide. The graphic data (A) represent the results of a series of typical experiments. The table (B) summarizes the mean values obtained from three experiments with each stathmin form, after calculation of the proportion of cells in the G0/G1, S and G2/M phases of the cell cycle (see Section 2).

3. Results

3.1. The effects of different stathmin forms on cell growth in 293 cells

We investigated the importance of the regulation of stathmin phosphorylation on cell growth by overexpression of various mutants. Constructs were made in which the phosphorylation sites were mutated to either alanine (4A-stathmin) or glutamic acid residues (4E-stathmin). Two truncated forms of stathmin were also generated: ΔC-stathmin consisting of the N-terminal domain (amino acids 1–65) encompassing the four phosphorylation sites, and ΔN-stathmin consisting of the C-terminus (amino acids 65–149) encompassing most of the α-helical domain and lacking the phosphorylation sites (Fig. 1A).

We had difficulty obtaining stable clones overexpressing the different stathmin forms to a high level (S. Lawler, O. Gavet and A. Sobel, unpublished). However, following transfection of 293 cells with an episomal expression vector and 10 days selection in hygromycin, it was possible to obtain populations of strongly expressing cells. A β-galactosidase expression vector (pCEPβgal) transfected in parallel showed that virtually all the cells in the culture were expressing recombinant protein after the selection period. Western blotting demonstrated that each form was expressed at a similar high level, with the exception of ΔC-stathmin which was expressed poorly, probably due to degradation (Fig. 1B).

After the 10 day selection period, flow cytometric analysis of DNA content showed a strong accumulation of 4A-stathmin expressing cells in G2/M, reaching 50% of the total population (Fig. 2). Moreover, a population of cells with a DNA content of 8N was also seen and probably represents cells undergoing endoreduplication (see below). Study of the growth of these populations showed that the 4A-stathmin expressing cells grew extremely slowly in correlation with the block in the cell cycle (Fig. 3). The expression of wild type or 4E-stathmin caused a moderate increase in the percentage of cells in G2/M compared with cells transfected with vector

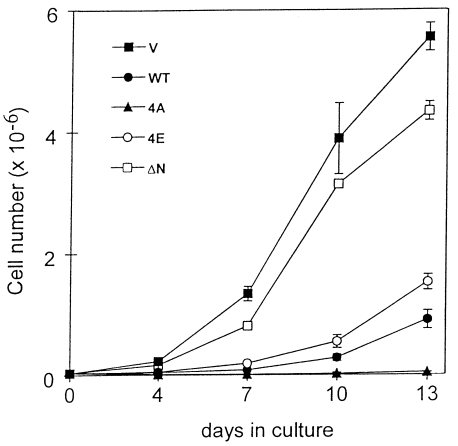


Fig. 3. Growth of selected 293 cells expressing stathmin or its mutant forms. After 10 days selection in hygromycin containing medium of 293 cells transfected with expression vectors for stathmin or its mutant forms (see Fig. 1), 3×10^4 cells per 35 mm well were seeded and allowed to grow for 14 days in hygromycin. Growth curves comparing the rates of growth of the various hygromycin resistant populations are presented. Cell numbers were counted each 3 days in triplicate.

alone and these cells also grew more slowly compared with the control.

Phase-contrast microscopy showed that in addition to the decreased number of cells obtained after transfection, a number of the cells in the culture were larger than the rest of the population. The proportion of large cells was related to the growth rate of the cells, with up to 25% of the cells expressing 4A-stathmin exhibiting this increase in size. Furthermore, many of the normal sized cells which were present in this culture had a spherical appearance and were easily detached from the culture dish, possibly indicating a block in mitosis. In cultures expressing wild type or 4E-stathmin a smaller number of enlarged cells were observed, but overall the cells in the culture appeared normal.

Immunofluorescence microscopy was then carried out to investigate further the phenotype of 4A-stathmin expressing cells (Fig. 4). Hoechst staining showed that the nuclei of the large cells were greatly enlarged and sometimes multilobal. BrdU staining showed that DNA synthesis was taking place in many of these cells (data not shown), indicating that they might result from endoreduplication in agreement with the presence of cells with a DNA content higher than 4N.

3.2. The effect of different stathmin forms on the responsiveness of a fos promoter/luciferase reporter

In addition to its role in the cell cycle, stathmin may be important in signal transduction processes as seen by its rapid phosphorylation in response to diverse extracellular stimuli [5]. Therefore we tested our mutants in an assay for efficacy of signaling. The fos promoter is known to be affected by many stimuli, and was used to examine the effects of stathmin on serum stimulation by measuring the activity of a co-transfected fos promoter/luciferase reporter plasmid, pfosfosTKluc [30].

pcDNA3stath/myc or its derivatives were transiently co-transfected into 293 cells with pfosfosTKluc and pRSVβ-gal. In normally growing cells, the expression of wild type stathmin caused a small but reproducible increase in co-transfected

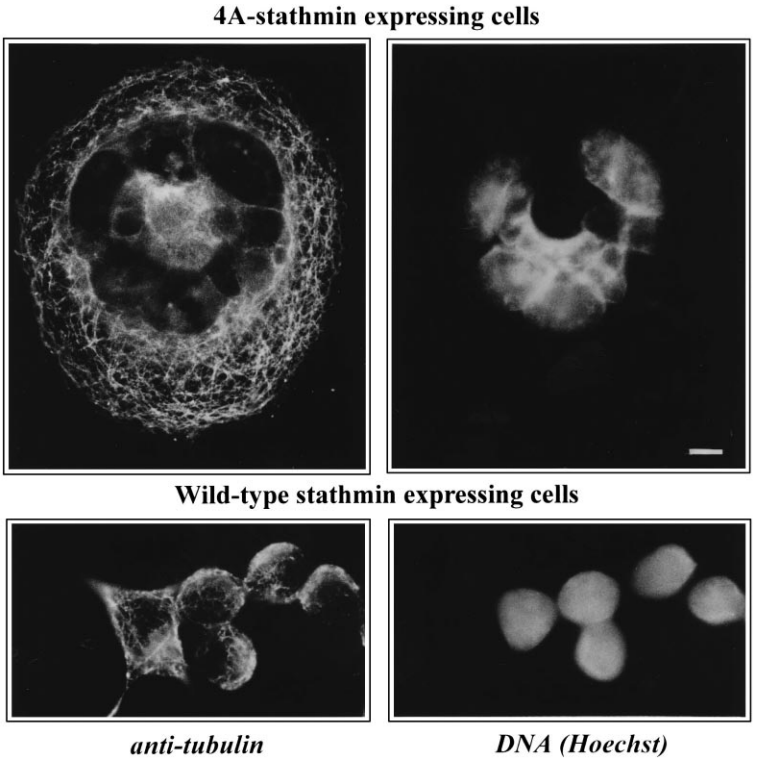


Fig. 4. Morphology of 4A-stathmin expressing cells. Cells transfected with the WT or 4A-stathmin expression vector (see Fig. 1) were examined by immunofluorescence microscopy with anti-tubulin and by Hoechst DNA staining. A significant proportion of the 4A-stathmin expressing cells displayed a striking morphology with large cells containing multilobal nuclei, as compared with the WT-stathmin expressing cells which displayed a normal phenotype. Bar = 15 μm.

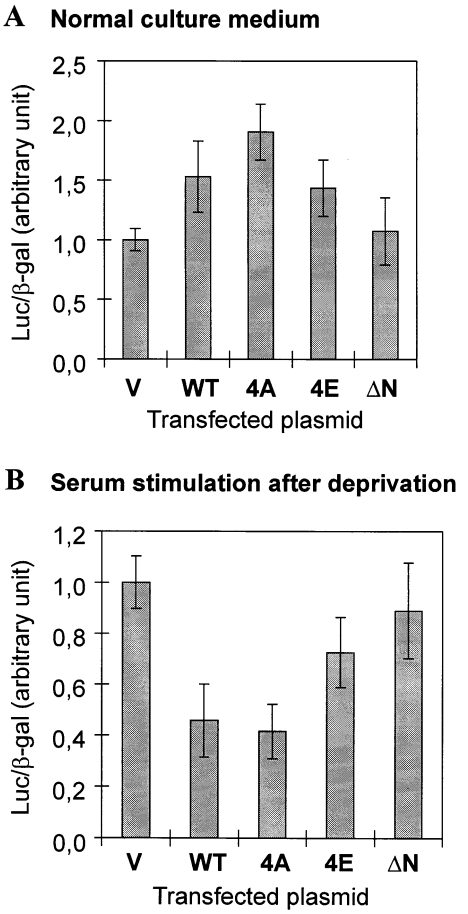


Fig. 5. Effects of overexpression of stathmin and its mutant forms on cotransfected fos-promoter activity. Expression plasmids for stathmin or its mutant forms (see Fig. 1) were co-transfected with pTKfosfosluc and pRSVβgal. After transfection, the cells were maintained in normal culture medium for 24 h (A) or in low (0.5%) serum containing medium for 36 h followed by 20% serum stimulation for 8 h (B). Cells were harvested and luciferase reflecting fos promoter activity and β-galactosidase activities were measured. Results are expressed as the ratio of luciferase/β-galactosidase for each sample, thus corresponding to a level in arbitrary units, 1 unit being the ratio observed with cells transfected with vector alone. Each transfection was done in triplicate, and each graph is the mean of three separate experiments.

fos promoter activity, as revealed by luciferase levels, which reached a level approximately 1.5-fold more than in cells transfected with vector alone (Fig. 5A). 4A-stathmin had a stronger effect, the increase over vector being up to 2-fold whereas 4E-stathmin had a similar or slightly weaker effect than the wild type. ΔN-stathmin had no significant effect on the level of fos activity.

In cells grown in low serum for 36 h after transfection, fos promoter activity was determined in response to stimulation with serum. This revealed an inhibition of fos promoter stimulation in cells transfected with wild type or 4A-stathmin, the level reaching 40% of that observed using the vector alone or ΔN-stathmin (Fig. 5B). 4E-stathmin had a much weaker effect on fos promoter activity than the other full length forms. Western blot analysis showed that the observed effects were not due to differences in expression levels, which were equivalent (data not shown).

These results show that stathmin has complex effects on the fos promoter, causing a small stimulation in normally growing cells but preventing subsequent stimulation in response to growth inducing signals. Although these effects are not very large, they were extremely reproducible, and compare well with effects seen in similar kinds of studies using luciferase reporter systems.

4. Discussion

Our studies show that stathmin plays a role in both the cell cycle and signal transduction processes. A number of mutants were expressed each of which had a distinct effect. Overexpression of wild type stathmin in 293 cells significantly affected intracellular signaling as revealed by a fos promoter directed reporter gene, whereas it only moderately affected cell growth. In contrast, the non-phosphorylatable 4A-stathmin strongly inhibited both serum responsiveness and growth, blocking the cells in G2/M. The 4E mutant, designed to mimic the fully phosphorylated form of stathmin seen in mitosis, only moderately perturbed both cell signaling and the cell cycle, and the truncated form corresponding to the C-terminal 'interaction' domain [12] of stathmin had no significant effect. Altogether, our results underline the involvement of stathmin and the importance of the regulation of its phosphorylation in different cellular processes.

The G2/M block induced by overexpression of 4A-stathmin in 293 cells is in agreement with similar observations in K562 cells [20]. We have extended these studies by using the 4E mutant whose effects appear indistinguishable from the wild type. This result suggests that the phosphorylated form of stathmin may be permissive for the G2/M transition, and that the unphosphorylated form of stathmin may contribute to a kind of 'checkpoint' effect, overrun only after extensive phosphorylation of stathmin by several activated kinases.

Stathmin is known to be highly expressed in some non-proliferating cells, for example in the nervous system [7,16,32], and has other roles related to the relay and integration of intracellular signaling pathways controlling diverse physiological processes not necessarily related to cell proliferation [5,33]. We therefore tested the mutants in a signal transduction assay, by measuring their effect on the serum responsiveness of a co-transfected fos promoter. The effects on the fos promoter appear distinct from those on the cell cycle as seen with the 4E mutant which behaves similarly to the wild type in the cell cycle assays, but has much less effect in the signaling assay. Stathmin appears to have a complex effect on this process as the 4A- and wild type stathmin block signaling, but cause a stimulation of the fos promoter in normally growing cells possibly due to a stress-like effect of stathmin overexpression.

The recently proposed role of stathmin as a potential regulator of microtubule dynamics [21–25] can explain the effects we observed in this study, as it has been shown also that treating cells with agents that disrupt microtubule function can activate the SRE of the fos promoter [34], and that impaired spindle function in *Xenopus* can activate MAP kinase which is known to activate fos expression [35]. Microtubule rearrangements also play key roles during the cycle [36].

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