Stathmin and its Phosphoprotein Family: General Properties, Biochemical and Functional Interaction with Tubulin

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ABSTRACT. Stathmin, also referred to as Op18, is a ubiquitous cytosolic phosphoprotein, proposed to be a small regulatory protein and a relay integrating diverse intracellular signaling pathways involved in the control of cell proliferation, differentiation and activities. It interacts with several putative downstream target and/or partner proteins. One major action of stathmin is to interfere with microtubule dynamics, by inhibiting the formation of microtubules and/or favoring their depolymerization. Stathmin (S) interacts directly with soluble tubulin (T), which results in the formation of a T2S complex which sequesters free tubulin and therefore impedes microtubule formation. However, it has been also proposed that stathmin's action on microtubules might result from the direct promotion of catastrophes, which is still controversial. Phosphorylation of stathmin regulates its biological actions: it reduces its affinity for tubulin and hence its action on microtubule dynamics, which allows for example progression of cells through mitosis.

Stathmin is also the generic element of a protein family including the neural proteins SCG10, SCLIP and RB3/RB3'/RB3". Interestingly, the stathmin-like domains of these proteins also possess a tubulin binding activity *in vitro*. *In vivo*, the transient expression of neural phosphoproteins of the stathmin family leads to their localization at Golgi membranes and, as previously described for stathmin and SCG10, to the depolymerization of interphasic microtubules. Altogether, the same mechanism for microtubule destabilization, that implies tubulin sequestration, is a common feature likely involved in the specific biological roles of each member of the stathmin family.

Key words: Microtubule dynamics/Protein phosphorylation/SCG10/SCLIP/RB3

Stathmin is a small regulatory protein for which we proposed a general role as a relay integrating diverse intracellular signaling pathways controlling cell proliferation, differentiation and functions (81, 82). It was shown more recently that it contributes to the control of microtubule dynamics (MT) by inhibiting the polymerization of microtubules and/or favoring their depolymerization. The scope of the present review is to survey the current knowledge, as well as our contribution and our ideas concerning stathmin and the related phosphoproteins of its molecular family in regard to their tubulin and microtubule interfering properties.

Stathmin, an ubiquitous cytosolic regulatory phosphoprotein

Stathmin (from $\sigma\tau\alpha\theta\mu\sigma$: relay (82)), also referred to as Op18 (32) was initially identified by independent investigators working in the field of signal transduction. They were searching for intracellular proteins whose phosphorylation was either related to the multi-hormonal regulation of prolactin secretion and release in the anterior pituitary tumor derived GH cell lines (83) or to the stimulus-secretion coupling in insulinoma cells (76). Following these pioneer studies, stathmin was recognized as a regulatory phosphoprotein in a wide variety of cells such as neurons (17, 18, 66, 74), muscle cells (6, 88) and lymphocytes (19, 31, 33, 46). Phosphorylation of stathmin was observed in parallel to the triggering of intracellular molecular cascades by diverse extracellular signaling molecules such as hormones (10, 11, 83), growth factors (25, 48) and neurotransmitters

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(18). More recently, it was reported that an extensive phosphorylation of stathmin occurs during mitosis (9, 15, 52, 85), which seems essential for the progression of the cell cycle (42, 45, 57).

Taken together, the results of these studies allowed to propose that stathmin is a general regulatory protein involved in diverse ways in the control of cell growth and differentiation, or of specific functional activities of differentiated cells (Fig. 1). This model was strengthened by the analysis of the primary sequence of stathmin which revealed that it possesses an organization compatible with its being a relay protein integrating diverse intracellular signaling cascades. Stathmin is a 148 amino-acid cytosoluble protein, highly conserved in vertebrates (39, 61) organized in two domains which slightly overlap between amino-acids 50–70: an NH₂-terminal “regulatory” domain containing several phosphorylation sites, and a COOH-terminal “interaction”

domain that presents a predicted α -helical structure potentially forming coiled-coil interactions with other proteins (12, 20, 27, 60) (Fig. 1).

In line with the putative integration properties of stathmin, four phosphorylation sites have been identified within its NH₂-terminal regulatory domain, accounting for all its combinatorial phosphorylation states observed *in vivo*, together with kinases responsible for the phosphorylation of the same sites *in vivo* and/or *in vitro*: serine 16 phosphorylated by CaM kinases II and IV/Gr (47, 55, 64); serine 25, a substrate for MAPKs in response to stress, growth and differentiation factors (13, 48, 54); serine 38, a target for p34^{cdc2} (12, 15, 52) or p33^{cdk2} (15); and serine 63 which is the major site for PKA (12).

Finally, the search for stathmin targets and/or partners led to the identification of candidate proteins that might interact with the COOH-terminal interaction

Fig. 1. Stathmin, a small regulatory phosphoprotein that integrates and relays diverse intracellular signaling pathways. Stathmin is a 148 amino acid (the initial methionine is cleaved) ubiquitous cytosoluble protein, integrating diverse intracellular signaling pathways through combinatorial phosphorylation of the four sites of its “regulatory” domain, and relaying them towards the appropriate and coordinated biological responses through its interaction with target/partner proteins.

domain of stathmin: KIS, a novel kinase with an unusual RNA recognition motif; proteins of the Hsp70 chaperone family; and two proteins predicted to form coiled-coil interactions, namely CC1 and the likely tumor susceptibility protein CC2/tsg101 (50, 51, 53, 59, 62). More recently, stathmin was found to interact with tubulin, which might account for its actions on the control of microtubule dynamics (1, 8, 21, 44). All these proteins are potential downstream target or partner proteins which may relay the action of stathmin towards the appropriate and coordinated control of cellular activities.

Stathmin and the control of cell proliferation. Implications in cancer

A growing set of arguments indicates that stathmin may participate to the control of cell proliferation. They are based on data from development, tissue regeneration, cell biology and cancer studies. During development, the expression pattern of stathmin is variable. Whereas stathmin expression is high in undifferentiated multipotent cells of the early embryo (26) and in most tissues at the neonatal period (27, 39), it strongly decreases in the adult, remaining abundant mostly in the nervous system and in the testis (39, 74). An important regulation of stathmin expression was also noted during induction of differentiation in a variety of cell types, with a high expression level in undifferentiated cells followed by a dramatic decrease during induction of differentiation (79). Another series of information was collected from the model of liver regeneration (40, 70). It was shown that stathmin expression, which is barely detectable in adult liver, is rapidly induced to a level comparable to that observed in neonatal liver after partial hepatectomy. Stathmin expression peaks

after about 60 hours, clearly after the major and characteristic mitotic wave at 24 hours, and then decreases rapidly to its basal level within 7 days. Stathmin expression appears thus related to the slowdown of cell proliferation. These results converged with the observation that in cell culture experiments, high cell density induces a transient burst of stathmin expression, probably triggered by cell-cell contacts (6), after which stathmin expression decreases with differentiation. All these observations led us to propose a model to account for stathmin expression during the process of tissue proliferation/differentiation (Fig. 2): stathmin expression may probably be up-regulated as a means to limit cell overgrowth at the stage preceding cell commitment and/or differentiation, before decreasing to low levels in most tissues except brain and testis.

At the cellular level, an extensive phosphorylation of stathmin occurs during mitosis (9, 15, 52, 85), which seems essential for the progression through the cell cycle, as revealed by the strong cell cycle block following overexpression of various stathmin phosphorylation site mutants (42, 45, 57). Altogether, it appears that stathmin is subject to a set of complex biological regulations related to the control of cell proliferation: regulation of its expression level during development, during the process of differentiation and tissue regeneration, and regulation of its phosphorylation in response to growth factors and most extensively for the correct progression through the cell cycle.

In regard to possible dysfunctions that may both help to extend our knowledge of stathmin and give new targets for medical treatments, stathmin overexpression has been reported in different types of cancers such as acute leukemia (16, 30, 33, 52, 65), lymphomas (16, 30, 69), various carcinomas (30), adenocarcinomas (14, 28) (Curmi *et al.*, 1999, in press), and neuroblastomas

Fig. 2. Regulation of stathmin expression in relation with cell proliferation and differentiation. On the basis of a large number of experimental and physiological or pathological observations, it appears that the expression of stathmin can be basal in low density proliferating cells, and that it increases in highly proliferating cells before and while their proliferation slows down, to reach low (B: as in muscle cells) or higher (A: as in neurons) levels in mature, differentiated cells. Stathmin might thus play a role in limiting cell "overproliferation", and in the control of physiological cell activities.

(32). Stathmin overexpression was found clearly linked to the highest mitotic index groups in two studies (28) (Curmi *et al.*, 1999, in press), although in others this link was not underlined, as in leukemia cells (16), or in non Hodgkin's lymphoma and Hodgkin's disease (although Hodgkin and Reed-Sternberg cells frequently express stathmin with strong staining intensity (69)).

The fact that stathmin overexpression seems to be encountered in tumors with high proliferation rate might seem in contradiction with our above mentioned model which suggests that stathmin tends to slow down cell proliferation. This apparent contradiction might however only be apparent if one considers that, in cancer, stathmin expression is stimulated as part of a regulatory program aimed at limiting cell overproliferation but which is inefficient, for reasons still to be understood but likely in relation with the transformed nature of the cell. Another explanation could be that overexpression of an altered stathmin is capable of causing the malignant phenotype; this interpretation is however unlikely since mutations in the structural gene of stathmin have never been detected in a significant proportion of cancer. Finally, one might also consider that cells could react to changes concerning a stathmin protein partner in a feedback pathway (14) (Curmi *et al.*, 1999, in press).

Stathmin, a phosphorylation controlled, tubulin binding and sequestering protein

An important progress has been made in understanding some functions and mechanisms of action of stathmin with the observation that it may be an important regulator of microtubule dynamics (8). On the basis of these original results, it was proposed that stathmin interacts with tubulin and acts as a catastrophe promoting agent, although no molecular mechanism was suggested to account for this property. A number of studies have since addressed the characterization of the mechanisms and regulation of stathmin's actions on microtubules. We performed a set of experiments to clearly demonstrate and characterize the interaction between stathmin and soluble tubulin, which led us to conclude that stathmin (S) can contribute to the regulation of microtubule dynamics by sequestering free tubulin (T) through the formation of a T2S complex (21, 38).

Stathmin and tubulin form a T2S complex

When bovine brain tubulin is mixed with recombinant human stathmin in solution, they form a complex with a Stokes radius of about 60 Å as determined by size exclusion chromatography (21). In parallel, the Stokes radii of tubulin and stathmin alone were found equal to about 41.5 and 37 Å, respectively. This latter value indicates that stathmin, a 17 kDa protein, is of

Fig. 3. The stathmin-tubulin interaction. Stathmin (S) and tubulin (T) are at the center of an array which includes at least three equilibria. In its non phosphorylated state, stathmin may drive tubulin towards sequestration in a T2S complex (1); stathmin phosphorylation may then result in the release of sequestered tubulin (3) which will then contribute to and enhance microtubule polymerization (2). It has been suggested that chromatin might regulate this array by inducing stathmin phosphorylation through either stimulation of a protein kinase (PK) or inhibition of a protein phosphatase (PP); Microtubule polymerization itself could stimulate stathmin phosphorylation and provide thus a positive feedback for microtubule growth.

elongated shape, as suggested previously (77). In these experiments, the stoichiometry of the stathmin-tubulin complex was established both by quantitative western blotting and chromatographic analysis, one stathmin molecule (S) binding 2 tubulin $\alpha\beta$ -heterodimers (T) to form a T2S hetero-trimer of 217 kDa (calculated value) (21). This result was confirmed recently with the use of stathmin affinity-beads (36). The 60 Å stathmin-tubulin complex having a sedimentation coefficient of about 7.7 S as determined by analytical ultracentrifugation (21, 38), its molecular mass can be evaluated to 197 kDa, a value consistent with the 217 kDa calculated molecular mass of the T2S complex.

Stathmin interacts with tubulin in the submicromolar range, and in a phosphorylation-dependent manner

The direct interaction of stathmin with soluble tubulin has been confirmed by several groups with a panel of experimental approaches. We demonstrated a direct and specific interaction between stathmin and tubulin by plasmon resonance technology (21). This technology allows to monitor binding and dissociation kinetics of the interaction, and hence to derive quantitative parameters such as an apparent dissociation constant, K_D . It should be noted that this constant does not describe an equilibrium in free solution, and that it is calculated on the basis of a classical bimolecular equilibrium not taking into account the formation of a trimolecular T2S complex. However, the quantitative plasmon resonance derived kinetic and "equilibrium" parameters are very useful for comparison purposes. For example, as stathmin is regulated by phosphorylation, we compared the interaction with tubulin of wild type stathmin and of a fully "pseudo-phosphorylated" 4E mutated stathmin (where all four Ser phosphorylation sites have been replaced by Glu). Whereas the stathmin-tubulin K_D reaches an optimal value of about 0.5 μ M at pH 6.5, it is of about 1.6 μ M for its 4E mutated form, corresponding to a 3 times lower apparent affinity. This analysis suggested thus that the affinity of the stathmin-tubulin interaction might be modulated by stathmin phosphorylation *in vitro* and hence *in vivo*. Furthermore, it should be noted that when studying tubulin binding to stathmin with other techniques such as size exclusion chromatography, dissociation is observed below the micro or sub-micromolar range suggesting that the solution K_D is likely within the same magnitude as the apparent dissociation constant derived from the plasmon resonance experiments. Furthermore, this micromolar or submicromolar range for the stathmin-tubulin interaction is in good agreement with the intracellular concentration of tubulin which is precisely in the micromolar range (68). The stathmin-tubulin interaction could thus take place and be reversible in the cell, and therefore be adjustable and

of physiological relevance.

The direct stathmin-tubulin interaction was confirmed more recently by cross-linking (22, 41, 63, 64) as well as affinity bead retention (36, 43) experiments. On the basis of cross-linking observations, it was proposed that stathmin interacts mainly with α -tubulin: two cross-linked forms are generated, migrating as a 71–75 kDa and an 83 kDa band on SDS-PAGE, recognized by both anti-stathmin and anti- α -tubulin antibodies and fitting the sum of stathmin and α -tubulin molecular masses. Stathmin phosphorylation results also in a decreased formation of the stathmin- α -tubulin complex: phosphorylation of the four kinase target sites abolishes complex formation whereas separate Ser-25 and 38 phosphorylation have only a modest inhibitory action. On the opposite, dual phosphorylation on Ser-16 and Ser-63 induces a strong inhibition of complex formation, an effect which is partial with single Ser-16 or Ser-63 phosphorylation.

Stathmin, a phosphorylation regulated microtubule destabilizing protein *in vitro* and *in vivo*

The original *in vitro* functional assay revealing the involvement of stathmin in the control of microtubule dynamics (8) was followed by numerous *in vitro* and *in vivo* experiments aimed at understanding the basis of stathmin's activity towards microtubules as well as appreciating the *in vivo* pertinence of its actual implication in the regulation of microtubule dynamics.

In vitro

Stathmin inhibition of microtubule growth can be accounted for by tubulin sequestration in the stathmin-tubulin complex

In vitro, the direct, phosphorylation-controlled interaction between stathmin and soluble tubulin (see above) as well as the destabilizing potential of stathmin towards microtubules is now firmly established. After the initial qualitative demonstration on the basis of a polymerization inhibition assay (8), semi-quantitative studies showed that stathmin prevents assembly and promotes disassembly of microtubules in a concentration-dependent manner, and that this effect is variously inhibited by stathmin phosphorylation (22, 34, 63). Quantitative polymerization analyses (38) revealed that the dose-dependent inhibition of tubulin polymerization by stathmin, at any tubulin concentration above the critical concentration, corresponded to the depolymerization of two moles of tubulin per mole of stathmin added, in good agreement with the formation of a T2S sequestering complex. Video microscopy analysis of *in vitro* microtubule dynamics showed that, in our conditions, stathmin reduced the microtubule growth rate with no effect on the catastrophe frequen-

cy (21). The microtubule growth dependence on tubulin concentration observed in the presence of various stathmin concentrations were parallel, and reflected a sequestration of tubulin, with a stathmin to tubulin heterodimer molar ratio fitting, again, the 2:1 stoichiometry of the T2S complex. Furthermore, stathmin does not alter the steady state GTPase activity of microtubules or the critical concentration, which is consistent with a pure tubulin sequestering activity (38).

Effects of stathmin on microtubule catastrophes

The latter studies differ from those reported by Belmont and Mitchison (8), who found in a similar assay that the catastrophe rate was 5–10 fold higher in the presence of purified *Xenopus* stathmin in a pH 7.5 buffer. The reasons for this divergence may be several. It may be partially explained by differences in the source of the protein or, more likely, in the experimental conditions used, including buffers, which possibly condition the dynamic instability of microtubules. The sequestration of soluble tubulin itself might actually result in an increase in the catastrophe frequency, due to a decrease of tubulin available for polymerization and hence a decreased stability at microtubule ends.

It has been recently proposed that stathmin can promote microtubule catastrophes independently of tubulin sequestration (36, 43). According to these studies, varying the pH could tune the balance between a major sequestration effect at pH 6.8 and mostly catastrophe promotion at pH 7.5, the NH₂-terminal region of stathmin bearing the catastrophe promotion activity, whereas tubulin sequestration would be provided by the rest of the molecule. However, the reality of this duality has to be confirmed by complementary experiments. In addition, although optimal at pH 6.5, the stathmin-tubulin interaction is still substantial at pH values (6.8–7.2) within the physiological intracellular range, whereas its proposed sequestration independent catastrophe promoting activity is observed significantly only at a higher, less physiological pH.

Although the precise mechanisms of stathmin's action on microtubules remain to be further studied and controversial, the results from *in vitro* experiments contribute to understand the roles of stathmin *in vivo*.

In vivo

In living cells, the effects of stathmin on the microtubule network and their regulation by phosphorylation have been addressed either by micro-injections or transfections of wild type or various mutated forms of stathmin, as well as of kinases known to phosphorylate stathmin.

In interphase, micro-injection of antibodies directed against stathmin decreases the catastrophe frequency and increases the total microtubule polymer (35).

On the opposite, microtubule depolymerization is observed when wild-type or a non phosphorylatable mutated 4A-stathmin (serines of the four phosphorylation sites replaced by alanine) is overexpressed or micro-injected in the cell (29, 34, 56). Interestingly, this effect is not seen with the fully pseudo-phosphorylated 4E form of stathmin (29), in agreement with its lower affinity with tubulin, which further suggests a role of stathmin phosphorylation in the control of its physiological regulatory activities. In interphase, the microtubule depolymerization potential of stathmin may be considered as a novel element able to balance the stabilizing activity of other proteins such as microtubule associated proteins (67).

In mitosis, when wild-type or 4E-stathmin is overexpressed, mitotic spindles assemble normally and cells pass the metaphase checkpoint. Importantly, the lack of effect of stathmin in mitosis correlates with its increased phosphorylation status (29). In contrast, 4A-stathmin transfection leads to the progressive appearance of round cells containing aggregated chromosomes and a range of microtubule alterations including short spindles or total lack of organized microtubules, as the cells are blocked in G2/M (22, 29, 34, 41, 56, 63, 64). This effect was described independently and before the knowledge of the stathmin-tubulin interaction. A strong G2/M block is also induced with other phosphorylation site-deficient mutants of stathmin: the cdk target site-deficient mutant S25A/S38A, or S16A/S63A-stathmin (42, 45, 55). It thus appears that if stathmin is present in the cell in some non phosphorylatable forms, the cell cycle is blocked with a parallel alteration of spindle formation. It follows that when present in a cell, stathmin has to be phosphorylated for the correct progression throughout the cell cycle.

These *in vivo* effects are nowadays interpreted in the light of *in vitro* studies on stathmin influence on microtubules. It becomes thus very attractive to search for a parallelism between the *in vitro* conditions where stathmin phosphorylation lowers the stathmin-tubulin affinity and the *in vivo* phosphorylation(s) of stathmin that are able to switch off its interphase depolymerization potential or its spindle alteration and cell cycle blocking power. *In vivo* analyses performed with single or multiple stathmin site deficient mutants, deletion constructs or kinase transfections provide patchy answers to this question. Stathmin activity towards microtubules seems switched off by the phosphorylation of diverse combinations of sites (29, 34, 41, 63, 64): dual phosphorylation on Ser-16/Ser-38, Ser-16/Ser-63, Ser-16/Ser-25 or Ser-38/Ser-63; triple phosphorylation on Ser-16/Ser-25/Ser-38 or Ser-25/Ser-38/Ser-63; and finally phosphorylation on all four phosphorylation sites. In contrast, the phosphorylation of stathmin on either Ser-16 or Ser-63 alone, or the double phospho-

A. The Stathmin family

B. Neuronal differentiation / microtubule regulation

Fig. 4. The Stathmin phosphoprotein family. Stathmin is the ubiquitous generic element of a conserved phosphoprotein family whose other members are restricted to the nervous system. (A) Their structural domain organization is shown with their common stathmin domain and its conserved features (arrowheads: phosphorylation sites/percentiles: similarity with the corresponding 100% labeled domain) and their specific NH2-terminal domains (A–A'') responsible for their membrane association partly through palmitoylation of cysteines (c) in the A subdomain. (B) On the basis of their biological expression profiles, the various members of the stathmin family seem to be involved in the successive steps of neuronal differentiation, all of which might involve their tubulin/microtubule regulation properties.

rylation on Ser-25/Ser-38 appear not to be sufficient to inactivate its microtubule destabilizing activity *in vivo* (29, 41, 63, 64). The situation *in vivo* is thus not clear-cut, and comparison with *in vitro* results not easy. This is probably due to the fact that all these phosphoforms present a continuum of affinities for tubulin. If true, the net *in vivo* effect of an individual phosphoform will therefore be function of its intracellular concentration and degree of phosphorylation. Only careful quantitative measurements *in vitro* (or *in vivo* if in comparable situation) will answer this question.

Interestingly, novel regulatory pathways and/or mechanisms have been proposed recently for the control of stathmin phosphorylation and hence its microtubule interfering potential. It has been suggested that stathmin phosphorylation might be influenced very locally by mitotic chromatin (2), which may also regulate stathmin phosphorylation through the control of a phosphatase activity (direct inhibition of PP2A or activation of a PP2A dependent kinase) (87). On the other hand, microtubule polymerization itself results in an increased stathmin phosphorylation, which might provide a positive regulatory loop in favor of microtubule formation (Kuntziger *et al.*, 1999, submitted). Finally it was also suggested that phosphorylation of stathmin might increase its degradation, thus emphasizing its inactivation (63, 64).

Stathmin, a core shared by a protein family with individual specialized roles

Stathmin is actually the generic element of a family of well conserved phosphoproteins, which are the products of four distinct genes. They include SCG10, SCLIP, and RB3/RB3'/RB3''', all of which possess a stathmin-like domain and additional specific NH₂-terminal extensions (61, 71, 73, 78, 84). Whereas stathmin is expressed ubiquitously, although more intensely in the nervous system, the other members of the stathmin family are exclusively restricted to the nervous system. They are differentially regulated during neuronal differentiation and brain development (71), and differentially expressed and regulated in the adult brain (7, 71-73). The various proteins of the stathmin family seem thus to play distinct, possibly complementary roles in relation with the control of the proliferation, differentiation, and specific activities of cells of the nervous system.

Molecular organization of stathmin family proteins

Sequence comparisons show that the stathmin family consists of three protein sub-groups (Fig. 4) (58, 61, 71, 73, 78, 84). The first group is made of stathmin itself which is the generic element of the family for it corresponds to the minimal consensus sequence shared

by all members. The second group includes SCG10, a neuron-specific developmentally regulated protein (3) and of SCLIP (71). The third group is made of RB3 and its two splice variants RB3' and RB3''' (61, 71, 73): RB3' possesses an additional exon (4') between exons 4 and 5 which bears an early stop codon and results in a shorter protein; RB3''' includes an additional exon (2') between exons 2 and 3 of RB3.

As displayed in Figure 4, each member of the family possesses an entire stathmin-like domain with its "regulatory" and "interaction" sub-domains, the degree of similarity of which varies along the sequence. Sequence comparisons with stathmin shows that for all members, the highest identity score concerns domain D, which is made essentially of the α -helical region. Furthermore, the probability of this region to form coiled-coil interaction structures is conserved among all members of the stathmin family. This suggests that each member of the family could act as a relay interacting with either common or distinct target or partner proteins.

Several of the phosphorylation sites essential for the integration and regulatory properties of stathmin are also conserved in other members of the family. Ser-16 is conserved perfectly throughout the entire family, which may again indicate the possibility of a specific regulation shared by all its members. Differences affect the other phosphorylation sites: while the RB3 sub-group proteins have no other common phosphorylation site with stathmin, SCG10 and SCLIP have the equivalent of stathmin Ser-38 (a proline directed site) and Ser-63 (a PKA site), SCG10 having furthermore the equivalent of Ser-25 (a proline directed site). This conservation of phosphorylation site sequences seems relevant since SCG10, RB3 and RB3' display several likely phosphoforms *in vivo* and it has been shown that SCG10 is phosphorylated *in vitro* with a kinase/phosphorylation site relationship close to that known for stathmin (4).

Finally, all the stathmin-related proteins possess additional specific NH₂-terminal domains of varying size, implicated in intracellular membrane addressing to Golgi membranes (29, 73). This membrane addressing mechanism most probably involves palmitoylation of two conserved cysteine residues as it has been demonstrated for SCG10 (24).

The effects of stathmin-related proteins on microtubules *in vitro* and *in vivo*

Although the effects of the stathmin-related proteins on microtubules have not yet been studied as extensively as for stathmin, recent reports show that they actually possess a microtubule destabilizing potential that may fulfill specific roles.

In vitro, a truncated form of SCG10 comprising essentially its stathmin-like domain (SCG10_{Δ1-34}, lack-

ing 34 N-term aa) induces the depolymerization or prevents polymerization of microtubules in a dose-dependent manner with a kinetics that resembles that of stathmin (75). Size exclusion chromatography shows that the SCG10₃₁₋₃₈-tubulin complex has a Stokes radius close to that of the stathmin-tubulin complex suggesting that it has the same 1:2 stoichiometry (Charbaut *et al.*, in preparation). The stathmin-like domains of the other members of the stathmin family also interact with tubulin qualitatively like stathmin, as evidenced by surface plasmon resonance or size exclusion chromatography, with some quantitative differences concerning binding kinetics or Stokes radii (Charbaut *et al.*, in preparation). It is thus expected that the stathmin-like domains of all proteins of the stathmin family are able to destabilize microtubules *in vitro*. Their specific features are likely related to their respective roles and actions in their physiological environment.

In vivo, each stathmin-related protein has been explored at least partially, and appears to display a microtubule depolymerization capacity. Ectopic expression of SCG10, SCLIP and RB3/RB3'/RB3'' in HeLa cells (29) results in a diffuse cytoplasmic localization and a heavy labeling of the Golgi complex, with no co-localization of any of these proteins with microtubules. In these cells, overexpression of SCG10, SCLIP, RB3 and RB3'' induces a depolymerization of the interphasic microtubule network whose extent varies with the expression level of each protein. No mitotic block accompanies these transfections and the spindles appear unaltered. The absence of effect of these ectopic expressions on the cell cycle may be due to the localization of the proteins mainly at the Golgi, or/and to their inactivation by phosphorylation during mitosis. In COS-7 cells, overexpression of SCG10 also leads to the disruption of the microtubule network in a phosphorylation-dependent manner (4).

In PC12 cells, it was known for a long time that SCG10 is strongly increased upon NGF mediated differentiation towards sympathetic neurons. When these cells are stably transfected with SCG10, neurite outgrowth is enhanced and SCG10 becomes localized in growth cones in addition to the Golgi apparatus. It was suggested that SCG10 could be transported to the growth cones via its association with Golgi vesicles (24, 84), and that this localization is microtubule dependent as drug disruption of microtubules causes dispersion of the SCG10 labeling. Localization of SCG10 in growth cones is of great interest since growth cone needs highly dynamic microtubules for advancing (49, 86). SCG10 could thus behave as a local regulator of neurite extension. This is also suggested by the observation that in cortical neuron cultures, after differentiation, SCG10 labels the growth cones of axons and den-

drites, where microtubules are more dynamic (23).

Altogether *in vitro* and *in vivo* observations reveal that each member of the stathmin family actually possesses a microtubule depolymerization activity. Preliminary data obtained in our laboratory allows us to propose a common mechanism to account for this activity. This mechanism implies that all proteins (P) of the stathmin family interact with a fraction of the intracellular pool of free tubulin (T) to form a T2P tubulin sequestering complex, as observed for stathmin *in vitro*. For each family member, this interaction could be regulated by phosphorylation of P, the site corresponding to stathmin Ser-16 being most probably involved as a common target to switch off the depolymerization potential. It should also be noted that another type of regulation may concern the NH₂-terminal extensions of the stathmin-related proteins, for they share well conserved tyrosines that might also be phosphorylated. Accordingly, SCG10 is an *in vitro* substrate for the tyrosine protein kinase Src (5). It could be speculated that phosphorylation of the N-terminal extension may regulate membrane anchoring of these proteins and thus provide an additional way to regulate more precisely their microtubule activity. Moreover, since these proteins are probably associated with Golgi derived vesicles, they may be transported and accumulated in different cell compartments, locally participating in the regulation of microtubule dynamics. In this manner each protein of the stathmin family may probably fulfill common as well as specific roles in relation to its intracellular localization and its pattern of expression in the nervous system.

Conclusion

From signal transduction to microtubule regulation, the last 15 years have provided a rich set of information on stathmin and its family. This small phosphoprotein family shares a stathmin motif possibly interacting with diverse target and/or partner proteins. Its actual interaction with tubulin provides these proteins with the ability to participate in the control of microtubule polymerization/depolymerization, and hence dynamics, in a phosphorylation-dependent fashion. The wealth of data accumulated makes now the stathmin family of high interest for a number of cell regulations involved in the physiological control of the proliferation, differentiation and specific activities of cells in the organism. Stathmin, as it is expressed in most tissues and cell types, could participate in the general regulation of microtubule dynamics in many biological situations. The five other members of the family are specific to cells of the nervous system, neurons and, for some of them, glial cells. Their participation to the regulation of microtubule dynamics in the different cell compart-

ments, cell body, growth cones, axon and dendrites, is most probably involved in the development, plasticity, stability and integrated functioning of the nervous system.

In cancer, whatever the reasons for stathmin overexpression, targeting specifically the activity of stathmin to alter its action on tubulin and microtubules may help developing therapeutics aimed at cell cycle inhibition. The working hypothesis is that an acute change of the stathmin-tubulin equilibrium might lead to a blockade of the cell cycle. Research in that direction is encouraged by the substantial cell cycle inhibition observed after transfections with stathmin antisense sequences (37, 52, 57) or with non-phosphorylatable stathmin (29, 41, 45, 57).

Numerous questions concerning stathmin and its roles in cell regulation still remain unanswered. They include the challenging finding that inactivation of the stathmin gene does not seem to perturb significantly normal mouse development and life (80). Most of the research in the stathmin field now focuses on its role in relation with the control of microtubule dynamics, as the precise mechanism and regulation of the action of stathmin on microtubule dynamics is still not fully understood and controversial. Although it is undoubted that stathmin plays a significant role in relation with tubulin and microtubules, we believe that the function and roles of stathmin extends beyond this field, as a general regulatory protein potentially acting through interaction with other partner proteins. These other interactions might themselves influence stathmin's action on microtubule dynamics. The full understanding of the biological roles of stathmin and of the other members of its phosphoprotein family includes thus the full characterization their diverse functional interactions and regulations within the complex network of intracellular signaling pathways.

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