Probing the Native Structure of Stathmin and Its Interaction Domains with Tubulin

COMBINED USE OF LIMITED PROTEOLYSIS, SIZE EXCLUSION CHROMATOGRAPHY, AND MASS SPECTROMETRY*

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Stathmin is a cytosoluble phosphoprotein proposed to be a regulatory relay integrating diverse intracellular signaling pathways. Its interaction with tubulin modulates microtubule dynamics by destabilization of assembled microtubules or inhibition of their polymerization from free tubulin. The aim of this study was to probe the native structure of stathmin and to delineate its minimal region able to interact with tubulin. Limited proteolysis of stathmin revealed four structured domains within the native protein, corresponding to amino acid sequences 22–81 (I), 95–113 (II), 113–128 (III), and 128–149 (IV), which allows us to propose stathmin folding hypotheses. Furthermore, stathmin proteolytic fragments were mixed to interact with tubulin, and those that retained affinity for tubulin were isolated by size exclusion chromatography and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The results indicate that, to interact with tubulin, a stathmin fragment must span a minimal core region from residues 42 to 126, which interestingly corresponds to the predicted α-helical “interaction region” of stathmin. In addition, an interacting stathmin fragment must include a short N- or C-terminal extension. The functional significance of these interaction constraints is further validated by tubulin polymerization inhibition assays with fragments designed on the basis of the tubulin binding results. The present results will help to optimize further stathmin structural studies and to develop molecular tools to target its interaction with tubulin.

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1 The abbreviations used are: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high pressure liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
taking advantage of the high accuracy and sensitivity of this method (28, 29). The functional value of the interaction results was validated by tubulin polymerization inhibition assays performed with three recombinant stathmin fragments constructed on the basis of the tubulin binding results. Altogether, our results reported here give clues for understanding the structural organization of stathmin as well as the mechanism of interaction of stathmin with tubulin. They may lead to the design of molecular tools tailored to mimic or block the microtubule depolymerization activity of stathmin.

EXPERIMENTAL PROCEDURES

Stathmin Limited Proteolysis—In order to obtain a good resolution of the stathmin structured domains, Lys-C and Glu-C endoproteases (sequencing grade proteases, Roche Molecular Biochemicals) were chosen because both have a high number of cleavage sites that are evenly distributed along the stathmin sequence except in its N terminus (23 and 30 sites, respectively (see Fig. 4B)). The concentration of the proteases is expressed for each proteolysis experiment throughout this paper as the enzyme:stathmin ratio (w/w). Lys-C and Glu-C were added to 9 μg of stathmin (recombinant human stathmin (30)) in a 10-μl final volume of AB or NH₄HCO₃, buffer, respectively (AB buffer: 80 mM K-Pipes, pH 6.5, 1 mM EGTA, 5 mM MgCl₂, 25 mM ammonium bicarbonate, pH 7.8). Different enzyme to stathmin ratios were used, 1:32,000, 1:16,000 (Lys-C and Glu-C), and 1:8000 (Lys-C), and incubated at 37 °C (Lys-C) or at room temperature (Glu-C) for 15 min. Digestion was stopped by adding 90 μl of 0.1% trifluoroacetic acid, 5% acetonitrile buffer. Evolution of the stathmin proteolysis was assayed by reverse phase HPLC and MALDI-TOF mass spectrometry.

Production and Sorting Out of Stathmin Fragments for Their Capacity to Interact with Tubulin—Stathmin fragments were produced by Lys-C or Glu-C proteolysis. The ability of the fragments to interact with tubulin was assessed by comparing their size exclusion chromatography elution volumes observed with or without prior incubation with tubulin. Fragments were then classified in two subtypes. The first comprises fragments that had a clear diminution of their elution volume when the presence of tubulin as compared with that observed in control conditions. They were considered to interact with tubulin. The second corresponds to fragments that eluted in the same position in the presence or absence of tubulin and that were considered as unable to interact with tubulin. Finally, a consensus region for stathmin interaction with tubulin was derived from the comparison of the sequences of these two subtypes.

For each protease, two parallel stathmin digestion mixtures were generated. Proteolysis mixtures contained 125 μg of stathmin and either Lys-C (1:12,000) or Glu-C (1:4000) enzyme:stathmin w/w ratio, in AB (Lys-C) or NH₄HCO₃ (Glu-C) buffer. They were incubated at 37 °C (Lys-C) or room temperature (Glu-C) for different times (0, 40, 80, 120 min) (Lys-C) or to 25 min (Glu-C). Stathmin was digested with Lys-C (1:12,000) or Glu-C (1:4000) enzyme:stathmin w/w ratio, in AB (Lys-C) or NH₄HCO₃ (Glu-C) buffer. They were incubated at 37 °C (Lys-C) or room temperature (Glu-C) for 15 min. Digestion was stopped by adding 90 μl of 0.1% trifluoroacetic acid, 5% acetonitrile buffer. Evolution of the stathmin proteolysis was assayed by reverse phase HPLC and MALDI-TOF mass spectrometry.

Reverse phase HPLC analyses were performed using a Zorbax SB-C18 column (4.6 × 150 mm, 5 microns) with the following scheme: buffer A, 150 mM KH₂PO₄, pH 3.6, and buffer B, 95% acetonitrile, 5% KH₂PO₄, pH 3.6. Stathmin fragments were identified after determination of the molecular masses of the polypeptides fragments, using the stathmin sequence and the known cleavage specificity of the endoproteases. Stathmin amino acid residues were numbered from 2 to 149, residue 1 representing the N-terminal cleaved methionine encoded by stathmin mRNA. The sample solution was mixed with a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxyphenylacetic acid, Aldrich) in 30% acetonitrile, 0.1% trifluoroacetic acid. The spectra of positive ions were recorded in linear mode on a MALDI-TOF mass spectrometer (Voyager Elite, Perseptive Biosystem, Inc. Framingham, MA) equipped with a delayed extraction device. For laser desorption, a nitrogen laser beam (λ = 337 nm) was focused on the target. Delayed extraction time was set at 150 ns. About 200 shots were averaged for each acquired spectrum. External calibration was performed with pepsinogen using the monoprotonated ion of the dimer, and of the monomer, and the biprotonated ion of the monomer with average mass to charge (m/z) ratios corresponding to 33,904, 16,952.5, and 8476.75 respectively. The difference between the calculated average mass and the experimental mass determination (0.05% to 0.1%) is consistent with the accuracy of MALDI-TOF mass spectrometry in linear mode.

Reverse Phase Chromatography—Reverse phase HPLC analyses were performed using a Zorbax SB-C18 column (4.6 × 150 mm, 5 microns) with the following scheme: buffer A, 15 min; linear solvent gradient from 0 to 100% buffer B in 75 min, 100% buffer B 5 min (buffer A, 0.1% trifluoroacetic acid, 5% CH₃CN; buffer B, 0.1% trifluoroacetic acid, 80% CH₃CN). Detection was at 214 nm.

Preparation of Recombinant Stathmin Fragments—DNA manipulations were carried out using standard recombinant techniques (32). CDNA encoding stathmin fragments were constructed as the “megaslicer” polymerase chain reaction technique (33) with oligonucleotides primers from Genset (France) and the human stathmin cDNA as a

2 P. A. Curmi, unpublished data.
Three different stathmin fragments were generated, whose limits were chosen for being as close as possible to either “the core” (fragment 44–125) (see under “Results” for a definition of the “stathmin core”), the core extended on its N-side to the Lys-C site Lys-13 (fragment 12–125), and the core with a C-extension to Glu-138 (fragment 44–138), an amino acid residue that represents the point of sequence divergence in the stathmin family proteins (17). The polymerase chain reaction fragments were cloned in the *Escherichia coli* expression vector pET-8c, expressed in the *E. coli* strain BL21, and purified as described (30). Fragment concentrations were determined by amino acid analysis.

**FIG. 2.** MALDI-TOF MS analysis of stathmin limited proteolysis with Lys-C. A, the indicated enzyme to stathmin ratio mixtures (w/w) were incubated at 37 °C for 15 min except for the control (upper panel) where digestion (1:8000, w/w) was stopped immediately after addition of the enzyme Lys-C. Stathmin, 17,172 Da calculated average mass for its protonated ion, appeared as a singly protonated ion, labeled stathmin or (1+), and as bi- and tri-protonated ions labeled (2+) and (3+), respectively. Each of the other peaks corresponds to a stathmin fragment, and, for the sake of clarity, only the singly charged fragments are labeled. Stathmin peptides produced by Lys-C digestion were identified with their average molecular masses using the stathmin sequence and the known cleavage specificity of the endoproteinase. Some ions may correspond to either a small protonated stathmin peptide or a larger biprotonated peptide (2+). This was the case for peptide 43–104 (marked with an asterisk). Its mass cannot be distinguished from the biprotonated ion of fragment 2–128. Since we did not observe any other peptide that begins or ends at position 43/42 or 105/104, respectively, and its intensity is correlated to that of fragment 2–128, we did not consider sites 42 and 104 as actually cleaved. The 2nd panel shows the peptides generated under very limited conditions (1:32,000). These peptides identified as corresponding to amino acid sequences 2–85, 2–95, 2–128, and 14–149 indicate that cleavage sites 13, 81, 89, and 113 are accessible in the native stathmin conformation. B, for each enzyme dilution, cleaved sites are represented superimposed with the stathmin α-helix score predicted with the Chou and Fasman algorithm, showing the uneven distribution of cleaved sites at high enzyme dilution. Arrowheads in parentheses point to positions that are not considered as cleaved.

**FIG. 3.** MALDI-TOF MS analysis of stathmin limited proteolysis with Glu-C. A, different enzyme to stathmin ratio mixtures (1:32,000 and 1:16,000, w/w) were incubated at room temperature for 15 min, except in the control (upper panel), where digestion (1:8000, w/w) was stopped immediately after addition of the enzyme Glu-C. Ions corresponding to mono-, bi-, or triprotonated forms of intact stathmin or stathmin fragments were identified and labeled as indicated in Fig. 2. Some ions may correspond to either a small stathmin peptide or a larger peptide doubly charged (2+). This was also the case for peptide 78–145 which correspond to the mass of the biprotonated fragment 11–49. However, sites 77 and 145 were not considered since we did not observe any other peptide that begins or ends at positions 78/77 or 146/145, respectively. The 2nd panel shows the peptides generated under very limited conditions. These peptides identified as corresponding to amino acid sequences 2–81, 2–89, 2–113, 22–149, and 14–149 indicate that cleavage sites 13, 21, 81, 89, and 113 are accessible in the native stathmin conformation. B, for each enzyme dilution, cleaved sites are represented superimposed with the stathmin α-helix score predicted with the Chou and Fasman algorithm, showing the uneven distribution of cleaved sites at high enzyme dilution. Arrowheads in parentheses point to positions that are not considered as cleaved.
### Stathmin Domains and Interaction Regions with Tubulin

**Tubulin Polymerization Assay**—Tubulin was purified from bovine brain by two cycles of polymerization and depolymerization followed by phosphocellulose chromatography (34). Tubulin concentration was determined by amino acid analysis, and the protein was stored at ~80°C in 50 mM MES-KOH, pH 6.8, 0.5 mM EGTA, 0.25 mM MgCl₂, 0.1 mM GTP until use. Tubulin polymerization was monitored turbidimetrically at 350 nm in an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech) thermostated at 37°C (1 cm light path). Experiments were carried out in 50 mM MES-KOH, pH 6.8, 30% glycerol, 0.5 mM EGTA, 6 mM MgCl₂, and 0.5 mM GTP (buffer M). Critical concentration plots representing the amount of polymerized tubulin observed at steady state versus the total concentration of tubulin, measured in the absence or presence of stathmin and stathmin fragments, were constructed as described (26).

### RESULTS

**Stathmin Presents Four Structured Domains in Solution**—Limited proteolysis is a classical strategy to isolate discretely folded domains of proteins taking advantage that the exposed regions between folded domains are the most sensitive protease cleavage sites. Therefore, we performed limited proteolysis of stathmin, using Lys-C and Glu-C, two proteases exhibiting a high number of cleavage sites randomly distributed along the stathmin sequence. Conditions of limited proteolysis with these two enzymes were determined by analyzing the progress of stathmin proteolysis by reverse phase HPLC and MALDI-TOF MS. Stathmin digestion is actually a progressive process as shown in Figs. 2 and 3 are shaded. The four stathmin structured domains that can be delimited by comparison of the theoretical and actual cleavage maps are indicated on the sequence of stathmin.

**Hydrodynamic Properties of Stathmin Proteolytic Fragments**

**Stathmin Limited Proteolysis**

![Fig. 4. Correlation between proteolysis accessibility and α-helix prediction. A, superimposed view of stathmin limited cleavage map and α-helix score. Arrows and arrowheads give the positions of cleaved sites observed after 15 min digestion with Lys-C or Glu-C (1:32,000), respectively. The cleaved site distribution is remarkably concentrated. B, linear representation of the stathmin primary sequence: theoretical Lys-C and Glu-C cleavage sites are underlined and those actually cleaved under the limited conditions shown in Figs. 2 and 3 are shaded. The four stathmin structured domains can be identified by comparison of the theoretical and actual cleavage maps are indicated on the sequence of stathmin.](image)

Lys-C and Glu-C cleave stathmin only at discrete sites unevenly distributed along the sequence. Two clusters of cleavage sites lie in the regions between amino acid residues 10–21 and 81–95. In addition, isolated cleavages occurred with Lys-C at residue Lys-128 and with Glu-C at residue Glu-113. The mass profiles for each enzyme looked very similar at 1:32,000 and at 1:16,000 with, however, an increased intensity of the peaks at 1:16,000. New cleavage sites appeared either immediately next to the initial sites (Lys-80 and -126 for Lys-C) or located at the beginning of a predicted α-helix (Lys-42, Lys-C) and in a region where the α-helix score is lower (Lys-104 and Lys-62 and Glu-65). Interestingly, the latter region surrounds Ser-63 which is an important stathmin phosphorylation site. At 1:8000, Lys-C proteolytic cleavage was more extensive and probably reflected the beginning of stathmin denaturation. The superimposed Lys-C and Glu-C concentration-dependent data with α-helix secondary structure prediction (bottom of Figs. 2B and 3B) reveals the uneven localization of initial protease attack. Fig. 4 gives a synthetic view of initial cleavage site distribution together with a sequence map of stathmin showing the delimitation of the deduced stathmin structured domains. Four structured domains can be identified on the basis of our limited proteolysis conditions. Domain I extends from amino acid residue 22 to 81. Secondary structure predictions show that it is probably built from its N-terminal end of about 20 amino acid residues folded in random coil followed by an α-helix stretch of about 39 amino acids. This domain contains three of the four stathmin phosphorylation sites observed in vitro and in vivo (Ser-25, -38, and -63). Domains II and III cover roughly the two halves of a predicted α-helix from amino acid residues 95–113 and 113–128, respectively. Finally domain IV covers the C-terminal stretch of stathmin beyond amino acid residues 128, a region predicted as random coil. The N-terminal end of stathmin (residues 2–21) was not considered as a stable structured domain since it contains two pairs of potential Lys and Glu sites, and each was cut at 1:32,000.
corresponding Stokes radius (149) region of stathmin. For each fraction, the elution volume and the rectified either to the amino-(anti-(15–27)) or C-terminal (anti-(134–

Stathmin proteolytic fragments were chromatographed on a size exclusion column to assess their hydrodynamic parameters. A representative Western blot analyses of fractions obtained after extensive stathmin Lys-C proteolysis (1:4000, 80 min), with antibodies directed either to the amino- (anti-(15–27)) or C-terminal (anti-(134–149)) region of stathmin. For each fraction, the elution volume and the corresponding Stokes radius ($R_s$) is indicated. Proteolysis generated both a wide variety and detectable amounts of stathmin fragments. B, the Stokes radius of stathmin was measured precisely in a control run (¶), whereas Stokes radii of stathmin fragments (¶) were estimated as described under "Experimental Procedures" using MALDI-TOF MS (Horizontal bars reflect the uncertainty in this determination). Stokes radii decreased regularly with stathmin fragment length from 39 Å for stathmin (¶) to about 28 Å for the smallest fragment detected in fraction 16.

enzyme concentrations (1:4000) that lead to advanced but not complete proteolysis. After digestion, stathmin fragments were first chromatographed on a size exclusion column to assess their hydrodynamic parameters. In a control run, we found that the peak of intact recombinant stathmin elutes with a 39-Å Stokes radius that corresponds to an approximately 70-kDa globular protein, as described previously (16). This stathmin feature is currently attributed to the fact that stathmin is an asymmetrical shaped monomer (39) rather than to its existence as a dimer or multimer. Western blot analyses (Fig. 5A) revealed that stathmin fragments generated during proteolysis are eluted in an ordered fashion with a continuum decrease of Stokes radii (for Stokes radius evaluation of the various fragments, see "Experimental Procedures") and no abrupt step. Identification of the fragments was achieved by MALDI-TOF MS. By using this technique, we noticed that the continuous decrease of Stokes radii was indeed proportional to fragment size reduction (Fig. 5B). These results suggested that the stathmin proteolytic fragments most probably retain their native structure. In addition, this indicated that stathmin fragments migrated free of any residual small peptide and that they do not interact either with each other or with intact stathmin. All these results further support the conclusion that stathmin migrates as a monomer peak. Stokes radii distribution ranged from 39 Å for stathmin to about 28 Å for the smallest detectable fragments that were about 60–70 amino acids in length (fragments 14–80, 14–85, 81–143, and 86–149). Again, it is worthy of note that a Stokes radius of 28 Å corresponds to a globular protein of about 34 kDa, when the corresponding stathmin fragments have an actual molecular mass of 7–10 kDa. This clearly showed that the stathmin asymmetry trait is retained even by the smallest fragments analyzed.

**Delimitation of Stathmin Interacting Region with Tubulin—** When the stathmin proteolysis products were mixed to interact with tubulin prior to chromatography, we observed a multimodal shift of the tubulin peak that ranged from 12.5 ml (tubulin control elution volume) to 10 ± 0.1 ml (Fig. 6). The relative importance and the intensity of the shifted peaks were only dependent on the completion of stathmin digestion. The further stathmin was digested the less the tubulin peak was displaced. Fig. 6A shows a representative elution profile obtained after addition of an 80-min Lys-C (1:12,000) stathmin digest to tubulin. Western blot analyses of fractions from such runs (Fig. 6B) showed that stathmin or some of its fragments were present from the leading edge of the shifted peak to the end of the peak (elution was monitored at 280 nm, a wavelength where only tubulin absorbs due to the absence of aromatic residues in stathmin). Stathmin fragments displaced in this assay (reduction of their elution volume) were identified by MALDI-TOF MS analysis of the corresponding fractions (Fig. 6C). These polypeptides presented a reduction of their elution volume and correspond to fractions 2–8.

Stathmin fragments were classified according to their shift in the presence of tubulin, and a shifted fragment was considered to interact with tubulin. Fig. 7 summarizes the results obtained with Lys-C and Glu-C digestions of stathmin using this assay for different proteolysis times. This classification shows that the stathmin potential for interaction with tubulin may be lost in two ways as follows: first by an important loss of its N-terminal region as seen with the 50–149, 63–149, or 81–149 fragments and second by the cleavage of its C-terminal region as revealed by the 2–113, 2–109, or 2–85 fragments. On the other hand, it appears that fragments still interacting with tubulin may have a limited N-terminal shortening as seen with the 31–149 and the 42–149 fragments or a partial deletion of the C-terminal region as observed with the 2–126 and 2–135 fragments. These observations allow us to propose a minimal consensus stathmin fragment necessary for tubulin interaction. It is composed of a core region from amino acids 42 to 126 which must have an N- or a C-terminal extension. The minimal limits of these extensions are not definitively positioned, but they should be between amino acids 14 and 42 in the N-terminal region and cannot be predicted for the C-terminal region.

The **Stathmin Core with an Extension on Its N- or C-side Is Necessary for Inhibition of Tubulin Polymerization**—Stathmin interaction with tubulin has been shown to inhibit tubulin polymerization into microtubules. To assess the functional value of the delimited stathmin regions for interaction with tubulin, we examined the effects on tubulin polymerization of three recombinant stathmin fragments representative of either the core region (fragment 44–125), the core region extended on its N-side (fragment 12–125), or on its C-side (fragment 44–138), and we compared their efficiency to that of wild type stathmin (Fig. 8). In the presence of 2.5 μM stathmin, spontaneous polymerization of tubulin into microtubules was inhib-
ited at all tubulin concentrations leading to a corresponding
shift of the critical concentration plot parallel to the control
plot. The apparent critical concentration shifted from about 4
to 9 \(\mu M\), consistently with the formation of a T2S complex as
previously reported (26). Under the same conditions, 2.5
\(\mu M\) fragments 12–125 and 44–138 had similar effects on the steady
state microtubule levels, whereas 2.5
\(\mu M\) fragment 44–125 did
not inhibit microtubule assembly at any tubulin concentration
tested (Fig. 8). The effects of the recombinant stathmin frag-
ments tested is thus in good agreement with the stathmin
interaction experiments, as the extended cores are efficient to
inhibit tubulin polymerization, whereas the core itself is not.

**DISCUSSION**

In order to progress in the understanding of the molecular
domain organization of stathmin as well as the stathmin-tubu-
lin interaction and its regulation, we investigated the struc-
tural properties of stathmin in solution by means of limited
proteolysis, and we proposed an approach to solve the stathmin
interaction regions with tubulin.

**Stathmin Displays Several Structured Domains in Solution**—Proteolytic cleavage is governed by solvent accessibility
and protein flexibility. Proteolytic protection is conferred to
regions of the protein that are either buried within a rigid
structure or interacting with other parts of the molecule. In
contrast, proteolytic sensitivity is localized in regions that are
solvent-accessible, unstructured, or flexible (40–44). Some
structural information may thus be deduced from determina-
tion of protection against enzyme proteolysis. By using this
method, we have found that stathmin displays four structured
domains, and we have shown that the cleavage accessibility
map fits reasonably with secondary structure prediction given
by several methods. Domain I (residues 22–81) is schemati-
cally divided in two types of predicted secondary structures as
follows: random coil from residues 22 to 45 (domain Ia) and
\(\alpha\)-helix from residues 45 to 81 (domain Ib). Its resistance to
limited proteolysis suggests that its two halves may be folded
over each other to confer this self-protection. Another possibil-
y could be that part Ia is compact with no accessible cleavage
site between it and part Ib. Interestingly, domain I harbors
three of the four stathmin phosphorylation sites, whose phos-
phorylation status might lead to major changes in stathmin
folding. Domains II (residues 95–113) and III (residues 113–
126) fit reasonably within the limits of the second predicted
\(\alpha\)-helical region of stathmin. Domain IV (from residues 128 to
149) has a high content of Lys-C and Glu-C potential cleavage
sites. Its resistance toward proteolysis was ascertained by the
observation that it behaved as a proteolysis end product iso-
lated by reverse phase HPLC. Furthermore, the limits of this
domain are identical to those of the region corresponding to
exon V of stathmin (45), which reinforces its individuality.
minimal lengths of which are not exactly determined. "Stathmin core" together with an N- and/or C-terminal extension, the tubulin interaction is predicted for stathmin fragments that possess a bidimensionally at 350 nm. The steady state buffer M at 37 °C for 40 min. Microtubule assembly was assayed tutat the indicated concentrations was polymerized into microtubules in tubulin, as it is common to all fragments of the interaction group. Comparing the stathmin fragment length in the two groups, we deduced the presence of tubulin were classified in the "interaction" group. Those that had a clear diminution of their elution volume in the presence or absence of tubulin are displayed in the "no interaction" group. Those that had a clear diminution of their elution volume in the presence of tubulin were classified in the "interaction" group. B, by comparing the stathmin fragment length in the two groups, we deduced the existence of a stathmin core that is necessary for interaction with tubulin, as it is common to all fragments of the interaction group. C, tubulin interaction is predicted for stathmin fragments that possess a "stathmin core" together with an N- and/or C-terminal extension, the minimal lengths of which are not exactly determined.

**Fig. 7.** Stathmin minimal consensus region for interaction with tubulin. A, using a size exclusion chromatography interaction assay, stathmin fragments, generated either with Lys-C or Glu-C, were classified in two categories relative to their capacity to interact with tubulin. Fragments that presented a similar elution volume in the presence or absence of tubulin are displayed in the "no interaction" group. Those that had a clear diminution of their elution volume in the presence of tubulin were classified in the "interaction" group. B, by comparing the stathmin fragment length in the two groups, we deduced the existence of a stathmin core that is necessary for interaction with tubulin, as it is common to all fragments of the interaction group. C, tubulin interaction is predicted for stathmin fragments that possess a "stathmin core" together with an N- and/or C-terminal extension, the minimal lengths of which are not exactly determined.

**Fig. 8.** The stathmin core extended on its N- or C-side is necessary for inhibition of the tubulin polymerization. Pure tubulin at the indicated concentrations was polymerized into microtubules in buffer M at 37 °C for 40 min. Microtubule assembly was assayed turbidimetrically at 350 nm. The steady state A250 turbidity observed at each tubulin concentration is plotted in the absence of stathmin or fragments (■) or in the presence of either 2.5 μM stathmin (■) or 2.5 μM recombinant stathmin fragments: 44–125 (▲), 44–138 (○), or 12–125 (△). The apparent critical concentration (cc) shifted from about 4 to 9 μM, in the presence of stathmin, or of fragments 12–125 or 44–138. This is consistent with the formation of a 2-tubulin 1-fragment complex. On the opposite, critical concentration was not modified with 2.5 μM fragment 44–125.

**Stathmin Folding Hypotheses**—Delimitation of structured stathmin domains gives a new insight of stathmin folding and helpful data for the construction of stathmin folding models. Fig. 9 presents two stathmin folding hypotheses matching the existence of these domains. We have imagined that stathmin could adopt schematically an extended or a hairpin turn conformation that in both cases agrees with stathmin being an asymmetrically shaped monomer (16, 39). In the two hypotheses, we propose either that the two halves of domain I are packed over each other or that the first half presents a compact structure similar to that proposed for domain IV. In hypothesis A, domains II and III are presented end to end with domain I, whereas in hypothesis B, domains II and III are bent back toward the α-helix of domain I in an antiparallel fashion. Computer analysis of stathmin sequence revealed the existence of an internal repeated sequence with 40% identity between amino acid residues 48–82 and 99–134 (46). Interestingly, both regions have a high probability for coiled-coil formation that may be involved in internal interaction as represented in hypothesis B or for stathmin partner recognition. Finally, it is known that phosphorylation may regulate the secondary, tertiary, and even quaternary structure of proteins (47, 48). Thus we propose that phosphorylation of domain I may promote localized changes in the folding of this domain, which may modify the stathmin ability to interact with protein partners such as tubulin.

**A Stathmin Core Extended on Either Its N- or C-Side Is Required for Interaction with Tubulin**—In order to understand better the molecular mechanisms by which stathmin interacts with tubulin, we examined which of the smallest stathmin fragments can retain the capacity to interact with tubulin. The strategy used to solve the stathmin interaction regions with tubulin combined proteolysis of stathmin, selection of fragments that retain affinity for tubulin by size exclusion chromatography, and their identification by MALDI-TOF mass spectrometry.

In the present work we have found that a minimal stathmin consensus region from amino acid residues 42 to 126, referred to as the "core," is necessary but not sufficient for interaction with tubulin. Interestingly this core corresponds to the entire predicted α-helix of domains I, II, and III, previously predicted to be the core of the interaction domain of stathmin with its functional targets and/or partners (10). For its interaction with tubulin, we have found that this core must possess an extension at least of its N- or C-terminal region, the lengths of which are not yet precisely determined. One possibility is that to interact significantly with tubulin, the core needs to be extended with a few additional amino acids on either or both extremities. A more seducing hypothesis could be that tubulin binding to stathmin may occur in two different ways, an "N way" and a "C way," in which either an N- or C-terminal extension of the core would allow binding of stathmin to tubulin. The N way and the C way may thus reflect the existence on stathmin of two binding sites for tubulin, which could be related to the existence of a stathmin internal repeat (46) and which could account for the stoichiometry of the stathmin-tubulin complex of one stathmin molecule for two tubulins (β-β-heterodimers) (16, 26). It should be noted that each of the folding hypotheses presented above displays a good accessibility of the core region for the interaction with tubulin, and each is compatible with the binding of two tubulin dimers per stathmin. The necessity of either an additional N- or C-terminal extension for the binding of the core to tubulin may also indicate the existence of a symmetry in the folding of part Ia and domain IV. Although we were not able in the present work to access directly the stathmin fragment to tubulin stoichiometry for all the interacting fragments, results obtained with specific stathmin fragments on tubulin polymerization suggest that it
Hypotheses about stathmin folding. A schematic diagram of stathmin derived from domain delimitation by limited proteolysis combined with secondary structure predictions is used to present alternative hypotheses about stathmin domain folding. Predicted α-helices are represented as cylinders. The four stathmin structured domains (see Fig. 4) are indicated in roman numerals, and cleavage positions observed with limited proteolysis are numbered. In hypothesis A, domains II and III are presented in line with domain I, whereas in hypothesis B, domains II and III are bent back toward the domain I α-helix in an antiparallel manner. Regarding domain I folding, its protection toward proteolysis may result from the packing of its two halves (Ia and Ib) over each other or from the presence of a compact structure of region Iα symmetrical to that predicted for domain IV (light gray drawing).

REFERENCES
1. Sobel, A., Bouttier, M. C., Beretta, L., Chneiweiss, H., Doye, V., and Peyro-Saint-Paul, H. (1989) J. Biol. Chem. 264, 3765–3772
2. Hailat, N., Strahler, J. R., Melhem, R. F., Zhu, X. X., Brodeur, G., Seeger, R. C., Reynolds, C. P., and Hanash, S. M. (1990) Oncogene 5, 1615–1618
3. Sobel, A. (1991) Trends Biochem. Sci. 16, 501–505
4. Beretta, L., Bouttier, M. C., Doye, V., and Sobel, A. (1989) Endocrinology 125, 1358–1364
5. Sobel, A., and Tashjian, A. H., Jr. (1983) J. Biol. Chem. 258, 10312–10324
6. Beretta, L., Bouttier, M. C., and Sobel, A. (1988) Endocrinology 122, 45–51
7. Doye, V., Bouttier, M. C., and Sobel, A. (1990) J. Biol. Chem. 265, 11650–11655
8. Larsson, N., Manceau, V., Campbell, D. G., Cohen, P., and Sobel, A. (1993) Mol. Cell. Biochem. 127, 151–156
9. Chneiweiss, H., Cordier, J., and Sobel, A. (1992) J. Neurochem. 58, 282–289
10. Mauauer, C., Camonis, J. H., and Sobel, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3100–3104
11. Manceau, V., Gavet, O., Curmi, P., and Sobel, A. (1999) Electrophoresis 20, 409–417
12. Li, L., and Cohen, S. (1996) Cell 85, 319–329
13. Li, L., Li, X., Francke, U., and Cohen, S. N. (1997) Cell 88, 143–154
14. Mauauer, C., Onoz, S., Manceau, V., Gavet, O., Lawler, S., Curmi, P., and Sobel, A. (1997) J. Biol. Chem. 272, 23151–23156
15. Belmont, L. D., and Mitchison, T. J. (1996) Cell 84, 623–631
16. Curmi, P., Andersen, S. S. L., Lachkar, S., Gavet, O., Karsenti, E., Knossow, M., and Sobel, A. (1997) J. Biol. Chem. 272, 25029–25036
17. Onoz, S., Mauauer, A., and Sobel, A. (1997) Eur. J. Biochem. 248, 794–806
18. Gavet, O., Onoz, S., Manceau, V., Lawler, S., Curmi, P., and Sobel, A. (1998) J. Cell Sci. 111, 3333–3346
19. Marklund, U., Larsson, N., Melander Gradin, H., Brattsand, G., and Gullberg, M. (1996) EMBO J. 15, 5290–5298
20. Horwitz, S. B., Shen, H.-J., He, L., Dittmar, P., Neif, R., Chen, J., and Schubart, U. K. (1997) J. Biol. Chem. 272, 8129–8132
21. Di Paolo, G., Antonsson, B., Kassel, D., Riederer, B. M., and Grenningloh, G. (1997) FEBS Lett. 416, 149–152
22. Melander Gradin, H., Larsson, N., Marklund, U., and Gullberg, M. (1998) J. Cell Biol. 140, 1–11
23. Melander Gradin, H., Marklund, U., Larsson, N., Chatila, T. A., and Gullberg, M. (1997) Mol. Cell. Biol. 17, 3459–3467
24. Riederer, B. M., Pellier, V., Antonsson, B., Di Paolo, G., Stimpson, S. A., Lutjens, R., Catopecas, S., and Grenningloh, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 745–747
25. Antonsson, B., Kassel, D., Di Paolo, G., Lutjens, R., Riederer, B. M., and Grenningloh, G. (1998) J. Biol. Chem. 273, 8439–8446
26. Jourdain, L., Curmi, P., Sobel, A., Pantaloni, D., and Carlier, M. F. (1997) Biochemistry 36, 10817–10821
27. Howell, B., Larsson, N., Gullberg, M., and Cassimeris, L. (1999) Mol. Biol. Cell 10, 105–115
28. Cohen, S. L., Ferre-D’Amare, A. R., Burley, S. K., and Chait, B. T. (1997) Protein Sci. 6, 1088–1099
29. Kwirwacki, R. W., Wu, J., Tennant, L., Wright, P. E., and Sizdak, G. (1997) J. Chromatogr. A 777, 23–30
30. Curmi, P., Mauauer, A., Asselin, S., Lecourtois, M., Schmitter, A., Schmitter, J. M., and Sobel, A. (1994) Biochem. J. 300, 331–338
31. Klopp, J., Bouttier, M. C., Doye, V., Peyro-Saint-Paul, H., and Sobel, A. (1990) J. Biol. Chem. 265, 3703–3707
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
34. Mitchison, T. J., and Kirschner, M. (1984) Nature 312, 237–242
35. Chou, P. Y., and Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251–276
36. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) J. Mol. Biol. 120, 97–120
37. Levin, J. M. (1997) Protein Eng. 10, 771–776
38. Geurjon, C., and Deleage, G. (1995) Comput. Appl. Biosci. 11, 681–684
39. Schubart, U. K., Alago, W., Jr., and Danoff, A. (1987) J. Biol. Chem. 262, 4765–4768.
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40. Morrice, N. A., and Carrey, E. A. (1998) in Protein Structure. A Practical Approach (Creighton, T. E., ed) pp. 117–149, IRL Press at Oxford University Press, Oxford
41. Fruton, J. S. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. B., and Shaw, E., eds) pp. 33–50, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
42. Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M., and Zambonin, M. (1986) Biochemistry 25, 1847–1851
43. Hubbard, S. J., Eisenmenger, F., and Thornton, J. M. (1994) Protein Sci. 3, 757–768
44. Kriwacki, R. W., and Siuzdak, G. (1998) J. Biomol. Tech. 9, 5–15
45. Melhem, R. F., Zhu, X. X., Hailat, N., Strahler, J. R., and Hanash, S. M. (1991) J. Biol. Chem. 266, 17747–17753
46. Maucuer, A., Doye, V., and Sobel, A. (1990) FEBS Lett. 264, 275–278
47. Johnson, L. N. (1992) FASEB J. 6, 2274–2282
48. Antz, C., Bauer, T., Kalbacher, H., Frank, R., Covarrubias, M., Kalbitzer, H. R., Ruppersberg, J. P., Baukrowitz, T., and Fakler, B. (1999) Nat. Struct. Biol 6, 146–150
49. Larsson, N., Segerman, B., Howell, B., Fridell, K., Cassimeris, L., and Gullberg, M. (1999) J. Cell Biol. 146, 1289–1302