Differential Effect of Two Stathmin/Op18 Phosphorylation Mutants on Xenopus Embryo Development*

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Thomas Küntziger†§, Olivier Gavet¶, André Sobel§, and Michel Bornens‡**
From the †Institut Curie, Section Recherche, UMR 144 CNRS, 26 rue d’Ulm, 75248 Paris Cedex 05, France and the ¶INSERM U440, Institut du Fer à Moulin, 17 rue du Fer à Moulin, F-75005 Paris, France

Stathmin/Op18 destabilizes microtubules in vitro and regulates microtubule polymerization in vivo. Both a microtubule catastrophe-promoting activity and a tubulin sequestering activity were demonstrated for stathmin in vitro, and both could contribute to microtubule depolymerization in vivo. Stathmin activity can be turned down by extensive phosphorylation on its four phosphorylatable serines, and down-regulation of stathmin activity by phosphorylation is necessary for cells to proceed through mitosis. We show here that microinjection of a nonphosphorylatable Ser to Ala (4A) quadruple mutant in Xenopus two-cell stage embryos results in cell cleavage arrest in the injected blastomeres and aborted development, whereas injection of a pseudo-phosphorylated Ser to Glu quadruple mutant (4E) does not prevent normal development. Addition of these mutants to mitotic cytostatic factor-arrested extracts in which spindle assembly was induced led to a dramatic reduction of spindle size with 4A stathmin, and to a moderate increase with 4E stathmin, but both localized to spindle poles. Interestingly, the microtubule assembly-dependent phosphorylation of endogenous stathmin was abolished in the presence of 4A stathmin, but not of 4E stathmin. Altogether, this shows that the phosphorylation-mediated regulation of stathmin activity during the cell cycle is essential for early Xenopus embryonic development.

Stathmin/Op18 is a soluble, ubiquitous phosphoprotein originally proposed to act as a relay for diverse intracellular signaling pathway (1). Stathmin was subsequently isolated as a microtubule (MT)1 catastrophe-promoting factor that was shown to interact directly with free tubulin (2). Further work proved that stathmin causes MT destabilization in vitro and in vivo and that its MT-destabilizing activity can be turned off by phosphorylation on its four phosphorylatable sites (3–7).

Stathmin becomes extensively phosphorylated at mitosis (6, 8–10) on its four phosphorylatable serines. These sites are targets both in vitro and in vivo of multiple kinases (for a review, see Ref. 11): Ca2+/calmodulin-dependent kinases II and IV, Gr phosphorylate Ser16, mitogen-activated protein kinases phosphorylate Ser25 and Ser38, cyclin-dependent kinases phosphorylate Ser25 and Ser38, and cAMP-dependent protein kinase phosphorylates Ser16 and Ser63. Overexpression of phosphorylation target site-deficient mutants in mammalian somatic cells revealed that whereas wild-type phosphorylatable stathmin allows spindle formation at mitosis, a stathmin mutant in which the four phosphorylatable serines have been replaced by alanines (4A mutant) prevents formation of a normal spindle and results in a mitotic block of the cells (6, 12, 13). As the 4A mutant mimics the constitutively active form of stathmin, this shows that down-regulation of stathmin MT-destabilizing activity is likely required to allow formation of the mitotic spindle and progression through mitosis. However, it has been recently reported that a 4E pseudo-phosphorylated mutant, in which the four phosphorylatable serines have been replaced by glutamic acid to mimic the constitutively down-regulated form, shows only a limited decrease in tubulin complex formation in vitro and in MT-destabilizing activity in transfected somatic cells (14, 15).

To investigate the in vivo importance of the phosphorylation state of stathmin in an embryonic cell system, we used wild-type human stathmin and the two 4A and 4E mutants. We introduced these recombinants stathmin proteins in two-cell stage embryos and in mitotic egg extracts from Xenopus laevis to examine their effects on embryo development and on the MT network. We then performed immunofluorescence experiments to detect the specific localization of the added forms in the mitotic egg extracts and extended these observations to somatic tissue culture cells.

MATERIALS AND METHODS

Preparation of Extracts and Human Stathmin Expression—Low speed (15,000 × g) Xenopus egg extracts blocked in metaphase II of meiosis (CSF extracts) and permeabilized sperm heads were prepared as described previously (16). Spindles were assembled at 22 °C in the interphase to mitosis pathway as described previously (17) by adding 0.2 mM CaCl2 for 1 h, then adding an equal volume of fresh CSF extract kept on ice to drive the extract back into mitosis. High speed extracts were prepared from CSF extracts spun at 245,000 g for 20 min at 4 °C in a TLS 55 rotor (Beckman) and were complemented with 0.05 volume of Energy Mix (150 mM creatine phosphate, 20 mM ATP, 20 mM MgCl2). Recombinant human wild-type 4A (serine changed to alanine at positions 16, 25, 38, and 63) and 4E (serines substituted with glutamic acid) stathmin were in PBS at 10 mg/ml (18). Briefly, stathmin-expressing bacteria were sonicated, and the extracts were centrifuged at 4 °C for 5 min.
Stathmin/Op18 Phosphorylation Mutants

RESULTS

We microinjected the stathmin 4A and 4E mutants in one blastomere of two-cell stage X. laevis embryos, the uninjected blastomere representing an inner control, to investigate any interference with embryo development. The quantities injected correspond to the double of the endogenous amount of stathmin, estimated to be 110 μg/ml (2). Embryos were photographed (Fig. 1A) and scored for normal development (Fig. 1B) around mid-blastula transition (MBT) 9 h after fertilization, and during late gastrulation 22 h after fertilization. Injection of 4A stathmin resulted in cell cleavage arrest in 95% of the injected blastomeres as observed at MBT, and in exogastrulation or lysis at 22 h (Fig. 1, A and B). All 4A-injected embryos showed aborted development (data not shown). On the contrary injection of the 4E stathmin mutant resulted in no visible phenotype at all observed times and led to normal tadpoles, as was the case for PBS-injected embryos (Fig. 1, A and B). Immunofluorescence analysis using anti-α-tubulin antibody on 4A-injected embryos at the blastula stage revealed the absence of any polymerized tubulin in the injected half, and in particular the absence of mitotic spindles observed in the noninjected area where division and cleavage seemed normal (Fig. 1C). We thus show that injection of heterologous constitutively nonphosphorylated 4A stathmin affects MT dynamics in such a way that cell cleavage and normal embryo development is compromised, whereas the same amount of the constitutively pseudo-phosphorylated 4E form has no effect.

It is known that overexpression in mammalian cells of stathmin with mutated nonphosphorylatable p34cdc2 sites prevents spindle formation in mitosis (12). We therefore wondered if 4A stathmin, but not 4E stathmin, could block embryo development by specifically acting on MT dynamics at mitosis, thus preventing the formation of a functional spindle. We therefore wondered if we could turn this experiment into a real in vitro assay for MT stability. We injected 4A and 4E stathmin mutants (in 3-fold quantity compared with the endogenous amount) to mitotic (CSF) egg extracts in which spindles were isolated from KE37 cells and specifically recognizes a 350-kDa antigen in the pericentriolar material (21). Monoclonal CTR453 was raised against centrosomes isolated from KE37 cells and then layered on top of a BRB80 (80 mM K-Pipes, pH 6.8, 1 mM MgCl2, and 1 mM EGTA/40% glycerol cushion containing 10 μg/ml protease inhibitors: leupeptin, pepstatin, and chymostatin (Boehringer). Centrifugation was at 22 °C for 20 min at 140,000 × g in a TLS 55 rotor (Beckman). A sample from the supernatant was taken and the remaining volume discarded; the pellet washed once with BRB80 and left to dry before an equal volume of Buffer A (24) was added to both supernatant and pellet fractions. An equal fraction in volume of both samples was loaded for 12% SDS-polyacrylamide gel electrophoresis. Western blotting was done as in Ref. 25, except that proteins were further fixed on nitrocellulose with 0.25% glutaraldehyde at room temperature for 20 min. The following primary antibody dilutions used were: serum I, 1:10,000 and anti-α-tubulin, 1:200. Bound antibodies were detected by anti-rabbit coupled to alkaline phosphatase (Promega).

Immunofluorescence Analysis—Spindles assembled in Xenopus egg extracts were fixed with cold methanol after dilution in BRB80, 30% glycerol, 1% Triton X-100 and spun onto coverslips. Coverslips were blocked with PBS-5% bovine serum albumin and incubated for 1 h with the appropriate rhodamine-, fluorescein-, or 7-amino-4-methylcoumarin-3-acetic-acid conjugated antibody (Jackson ImmunoResearch). DNA was stained by a 5-min incubation with DAPI. After three washes, coverslips were mounted with AF1 solution (CitiFluor) and observed with a Leica DMR fluorescence photomicroscope equipped with a Hamamatsu C5985 CCD camera, coupled to a Macintosh computer with Adobe Photoshop software. HeLa cells were fixed with methanol at −20 °C for 6 min, immediately or after a few minutes at room temperature in PBS. A Leica DMRXA fluorescence microscope was used for observation: image stacks (0.2 μm) were recorded using a piezoelectric objective (100 × 1.4 NA) positioning device and a MicroMax CCD camera (Princeton Instruments). All images are maximal intensity projections.

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microtubules length. This phosphorylation of Ser16, which corresponds to an interphase to mitosis path in low speed (27). This phosphorylation of Ser16, which corresponds to an interphase to mitosis path in low speed (27).

Mitotic spindles were assembled from Xenopus sperm heads by the interphase to mitosis path in low speed Xenopus egg extract. PBS (Control) or a 4-fold excess of wild-type or of 4A or 4E human stathmin was added at time 0. Spindles were stained with mAb GT 335, which stains centrioles and microtubules in mitosis. DNA was stained with DAPI. Bar: 10 μm. For each condition in which stathmin was added, spindle length was measured on about 200 spindles from three different experiments and mean and S.D. values were calculated. Although not significantly modified by the presence of wild-type stathmin (see also Ref. 26), spindle size was moderately increased with 4E and dramatically reduced with 4A.

We wondered next whether the significant amounts of human exogenous stathmin added would present a specific localization in the in vitro spindle assembly experiments. We performed therefore immunofluorescence on in vitro spindles formed in Xenopus egg extracts (17). Spindles were assembled from permeabilized Xenopus sperm heads in CSF extracts supplemented with either wild-type, 4A, or 4E recombinant human stathmin. Antibody C specific to human stathmin that does not recognize the endogenous Xenopus stathmin was used (data not shown) or after methanol treatment (Fig. 4A, panel e). Stathmin was then analyzed in supernatants by Western blot with antiserum I, which recognizes both human and Xenopus stathmin (19) (Fig. 3B). Tubulin was also followed by Western blot in pellets to control for its polymerization. MT assembly by Me2SO resulted in the appearance of two additional Xenopus stathmin bands with reduced electrophoretic mobilities (Fig. 3B, lane C, arrows). As expected, the addition of the nonphosphorylatable 4A and 4E human stathmin mutants had distinct effects: 4A prevented the Me2SO-induced hyperphosphorylation of endogenous Xenopus stathmin, whereas 4E did not. As a control, none of the mutants did induce any hyperphosphorylation of endogenous stathmin in the absence of Me2SO. This shows that opposing MT assembly with the 4A mutant prevents hyperphosphorylation of Xenopus stathmin, whereas the pseudo-phosphorylated form does not. Therefore the effect of the 4A stathmin mutant on embryo development could result from both a direct perturbation of MT dynamics in mitosis and from preventing the MT assembly-dependent phosphorylation of endogenous stathmin, which contributes to spindle MTs stabilization (27).

We have recently identified both in Xenopus egg extracts and in somatic cells a new pathway in which it is the assembly of MTs that leads to stathmin hyperphosphorylation. Specifically, we have shown that MT assembly results in the additional phosphorylation, on stathmin Ser16, leading to the appearance of one of two bands with reduced electrophoretic mobilities (27). This phosphorylation of Ser16, which corresponds to an hyperphosphorylation, has been shown to be critical for the down-regulation of stathmin activity (5, 6, 28). This MT assembly-dependent phosphorylation is likely involved in MT stabilization during spindle formation and could contribute to global control of the MT network (see Fig. 3A and Ref. 27). We wondered if MT assembly-dependent phosphorylation of endogenous stathmin in Xenopus egg extracts would be affected by the addition of exogenous recombinant mutant forms of human stathmin. The prediction was that adding 4A human stathmin would limit MT assembly and the resulting MT assembly-dependent phosphorylation on Xenopus stathmin. On the contrary, addition of 4E human stathmin would not significantly affect MT assembly and Xenopus stathmin hyperphosphorylation. Therefore, Me2SO was added to induce MT assembly together with mutant human proteins (in a 3-fold amount compared with the endogenous) to high speed CSF extract for 45 min at 22 °C before MTs were pelleted by ultracentrifugation. Stathmin was then analyzed in supernatants by Western blot with antiserum I, which recognizes both human and Xenopus stathmin (19) (Fig. 3B). Tubulin was also followed by Western blot in pellets to control for its polymerization. MT assembly by Me2SO resulted in the appearance of two additional Xenopus stathmin bands with reduced electrophoretic mobilities (Fig. 3B, lane C, arrows). As expected, the addition of the nonphosphorylatable 4A and 4E human stathmin mutants had distinct effects: 4A prevented the Me2SO-induced hyperphosphorylation of endogenous Xenopus stathmin, whereas 4E did not. As a control, none of the mutants did induce any hyperphosphorylation of endogenous stathmin in the absence of Me2SO. This shows that opposing MT assembly with the 4A mutant prevents hyperphosphorylation of Xenopus stathmin, whereas the pseudo-phosphorylated form does not. Therefore the effect of the 4A stathmin mutant on embryo development could result from both a direct perturbation of MT dynamics in mitosis and from preventing the MT assembly-dependent phosphorylation of endogenous stathmin, which contributes to spindle MTs stabilization (27).

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To extend these observations to somatic cells, HeLa cells were processed for immunofluorescence either after saponin extraction followed by paraformaldehyde/glutaraldehyde fixation (data not shown) or after methanol treatment (Fig. 4B). Cells were then double-stained with antiserum C, together with various anti-centrosome antibodies. Fig. 4B shows in most cells, as expected from a soluble protein, a diffuse cytoplasmic staining for stathmin. However a specific accumulation at mitotic spindle poles in mitosis (Fig. 4B, panel a) and at aster centers in G2 (Fig. 4B, panel a’) could be detected. The specific accumulation at spindle poles (Fig. 4B, panels c and f) was always peripheral to all markers used, as for NuMA (Fig. 4B, panel b), antipericentriolar antigen antibodies (CTR453; Fig. 4B, panel e), or anticentriolar antibodies (GT335; not shown).

There was however a partial overlap of the stathmin staining with NuMA (compare Fig. 4B, panel d with panel g). The staining was sometimes found to be more abundant toward the exterior of the spindle pole and having the appearance of a horseshoe (see Fig. 4B, panels f and g). The stathmin accumulation at spindle poles was no longer observed if antiserum C was preincubated with the peptide C-O (19) against which the antiserum was raised (data not shown).
The phosphorylation state of stathmin, an important physiological regulator of MT dynamics, appears to play an important role in Xenopus egg embryonic development. Our results show dramatic differences in the effects of stathmin mutants with opposed phosphorylation profiles. The uncharged 4A mutant, which mimics constitutively nonphosphorylated stathmin, interferes with several important aspects of normal egg development, including cell cleavage, spindle size, MT assembly, and hence MT-dependent stathmin hyperphosphorylation. In contrast, the 4E mutant, which mimics constitutively phosphorylated stathmin by permanently harboring four negative charges, has no visible effects in this context.

A number of studies performed both in vitro and in vivo have shown that nonphosphorylated stathmin promotes MT disassembly and that this activity is turned off by phosphorylation (see Ref. 11 for a review). Thus, overexpression of 4A stathmin in mammalian somatic cells destabilizes MTs, prevents formation of a normal spindle at mitosis, and blocks the cell cycle (5, 12, 13), whereas a pseudo-phosphorylated mutant has no effect (3, 6). Overexpression of wild-type phosphorylatable stathmin also destabilizes interphase MTs but allows spindle formation and normal progression through the cell cycle, because the kinetochore levels at mitosis are most likely sufficient for the phosphorylation of wild-type stathmin (6, 12). This shows that stathmin inactivation by phosphorylation is required to allow mitotic spindle formation and progression through mitosis. Therefore, the effect of 4A stathmin on Xenopus egg development reported here can be explained by its constitutive MT-depolymerizing activity, resulting in interference with spindle formation and preventing normal division and cleavage.

Furthermore, we have shown in Xenopus egg extracts that MT assembly leads to hyperphosphorylation of stathmin specifically on Ser\textsuperscript{16} (27), the phosphorylation of which is critical for turning down stathmin MT-depolymerizing activity (5, 6). The MT assembly-dependent hyperphosphorylation of stathmin allows MT stabilization around chromatin in mitotic egg extracts (27), a mechanism required in mitotic spindle assembly (26). We show here that the addition of an excess of nonphosphorylatable 4A stathmin prevents Me\textsubscript{2}SO-induced MT assembly and hence phosphorylation of endogenous stathmin on Ser\textsuperscript{16} (see Fig. 3). This could contribute, at least partially, to the impairment of normal spindle assembly and egg development. In contrast, the constitutively pseudo-phosphorylated 4E mutant, which does not prevent MT-dependent hyperphosphorylation of endogenous stathmin, does not interfere with normal Xenopus egg development. Altogether, our results indicate that the MT-dependent phosphorylation of stathmin in Xenopus eggs likely contributes to the regulation of mitotic spindle assembly.

Our observation that 4E stathmin has no apparent effect on egg development apparently contradicts recent overexpression reports in somatic cells showing that the 4E mutant retains significant MT-depolymerizing activity (14, 15). However these experiments have been performed with a 20-fold overexpression level, whereas we performed 2–5-fold addition experiments, which proved to be sufficient with the 4A mutant to elicit effects on egg development. As 4E stathmin retains significant tubulin binding activity in vitro (13, 30–32), and as tubulin sequestering represents a probable mechanism to explain stathmin MT-depolymerizing activity in cells, a large excess of 4E stathmin could result in an effect on MT stability that is not seen at close to physiological levels.

Whereas the majority of stathmin is cytosolic, we observed a...
responding overlays are shown in exterior of the spindle. 

particles (10) or antipericentriolar markers CTR453 (insets). Note the localization of stathmin aside centrioles (c). The asymmetrical accumulation of stathmin at the spindle poles of somatic cells, toward the cell cortex rather than toward the chromosomes, is also striking. It could reflect stathmin interaction with complexes associated to the MT plus ends, that contain proteins such as dynein and dynactin (40, 41), CLIP-170 (42), or EB1 (43).

Altogether, this report demonstrates for the first time in an embryonic development system that the phosphorylation level of stathmin and its variations during the cell cycle have dramatic effects on cell development. We specifically show that the pseudo-phosphorylated form of stathmin does not apparently interfere with normal progression through the cell cycle in early developmental stages, as opposed to the nonphosphorylatable mutant. Furthermore, the fact that the effects of the 4A and 4E stathmin mutants are similar in a control extract, but totally different when tubulin is forced to assemble by Me2SO, shows that the stathmin phosphorylation level becomes critical only in conditions where the free tubulin concentration is very low (when tubulin is assembled into MTs). These observations could suggest that the pool of tubulin dimers ready to assemble in vivo is very low.

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