Communication

The Microtubule-destabilizing Activity of Metablastin (p19) Is Controlled by Phosphorylation*

(Received for publication, December 20, 1996, and in revised form, February 10, 1997)

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Metablastin (also called p19, stathmin, prosolin, p18, Lap18, and oncoprotein 18) is a highly conserved, cytosolic 149-amino acid polypeptide that is expressed in immature vertebrate cells and undergoes extracellular factor- and cell cycle-regulated serine phosphorylation. The protein was shown recently to destabilize microtubules in vitro (Belmont, L., and Mitchison, T. J. (1996) Cell 84, 623–631). Here we demonstrate that microinjection of recombinant metablastin induces a loss of microtubules in Cos-7 cells. This effect is enhanced by serine-to-alanine mutations at several phosphorylation sites and virtually abolished by aspartate substitution at a single site, Ser-63. We also show that stoichiometric amounts of metablastin prevent assembly and promote disassembly of microtubules in vitro. Interestingly, the phosphorylation site mutations of metablastin that have dramatic differential effects in intact cells do not alter the ability of metablastin to block tubulin assembly in vitro. The data suggest that phosphorylation of metablastin controls its microtubule-destabilizing activity in vitro but that this regulation may require additional cellular factors. This control mechanism is poised to play a critical role in the dynamic reorganization of the cellular microtubule network that occurs during morphogenesis and mitosis.

Serine phosphorylation of metablastin is stimulated in mammalian cells by a diverse group of extracellular factors, which include cAMP-linked agonists (1–3), factors known to activate protein kinase C (3, 4), growth factors that initiate signaling through tyrosine kinase receptors (5, 6), heat shock (7), and, in some cells, agents that induce calcium flux (8). Furthermore, the phosphorylation state of metablastin fluctuates during the cell cycle, achieving its highest level in M phase (9–11).

The known phosphorylation sites of metablastin are Ser-16, Ser-25, Ser-38, and Ser-63 (12–14). Although the specific protein kinases that directly phosphorylate metablastin in vivo have not been identified, the available evidence suggests that,

in cells, Ser-63 and, to a lesser extent, Ser-16 are phosphorylated by cAMP-dependent protein kinase (14), Ser-25 and Ser-38 by mitogen-activated protein kinase(s) and cyclin-dependent kinase(s) (14–16), and Ser-16 by Ca2+/calmodulin kinase-GR (8).

To test the microtubule-destabilizing activity of metablastin in intact cells and to explore the potential role of phosphorylation in regulating this activity, we have introduced recombinant metablastin and phosphorylation site mutants into Cos-7 cells by microinjection and assessed changes in the cellular microtubule array by immunocytochemistry.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—Metablastin and mutated forms of the protein were expressed as GST fusion proteins in Escherichia coli. Metablastin cDNA (17) encoding amino acids 6–145 was amplified using the polymerase chain reaction (forward primer, 5’-AGGGATCCTCGAGCTGAGAATTCCA3’; reverse primer, 5’-CAAGAACCCCAGGGACGAGAAG-3’; 30 temperature cycles: 94 °C, 1 min; 60 °C, 45 s; 72 °C, 30 s) and inserted in pGEX-5X (Pharmacia Biotech Inc.) using the restriction sites for BamHI and EcoRI. Fusion proteins were prepared from transfected bacteria as described (18) and extracted from glutathione-Sepharose (Pharmacia) with 10 mM reduced glutathione in 50 mM sodium phosphate buffer, pH 7.4, followed by dialysis against microinjection buffer (see below). The fusion proteins were >90% pure, as assessed on SDS-polyacrylamide gels stained with Coomassie Blue. Protein concentrations were determined fluorometrically using fluorescamine (19) with bovine serum albumin as standard. To quantitate absolute amounts, the original preparation of GST-p19 was purified by reverse-phase high-pressure liquid chromatography using a C4 column and subjected to amino acid analysis, which was performed by the Laboratory for Macromolecular Analysis of the Albert Einstein College of Medicine. Phosphorylation site mutations were introduced using a site-directed mutagenesis kit (Clontech). The sequences of all recombinants used were verified by DNA sequence analysis.

Microinjection of COS-7 Cells and Immunocytochemistry—COS-7 cells were plated on glass coverslips and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 20–24 h at 37 °C (90% humidified air, 7% CO2). For microinjection, the medium was supplemented with 50 mM HEPES. The recombinant protein to be injected was mixed with rabbit IgG in microinjection buffer (75 mM KCl, 10 mM potassium phosphate buffer, 5 mM MgCl2, 0.1 mM dithiothreitol, pH 7.4). Microinjection was carried out at room temperature and utilized an Eppendorf 5171 micromanipulator and an Eppendorf 5242 microinjection mounted on a Nikon Diaphot 300 inverted microscope. The concentration of proteins in the micropipette were: rabbit IgG, 2 mg/ml; recombinant proteins, 15 mg/ml unless otherwise stated. For the nocodazole protocol, cells were injected, followed by incubation in fresh medium at 37 °C for 30 min. Nocodazole (4 mg/ml in Me2SO) was then added (final concentration 4 µg/ml) and the incubation continued for 60 min. The dishes were then rinsed, twice with phosphate-buffered saline (PBS) and twice with Dulbecco’s modified Eagle’s medium, and incubated in fresh, nocodazole-free medium for 1.5 h. The coverslips were rinsed with PBS and immersed in methanol for 6 min at –20 °C. Following rehydration in PBS for 5 min, the coverslips were blocked with 10% normal goat serum for 20 min and incubated for 50 min at 37 °C with a β-tubulin mouse monoclonal antibody (Sigma), diluted 1:100 with 5% normal goat serum in PBS. After rinsing with 0.5% bovine serum albumin in PBS, the secondary antibodies, Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch, 1:250 dilution) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma, 1:50 dilution) were applied for 50 min at room temperature. After rinsing with PBS, the coverslips were mounted with 30% glycerol in PBS containing 1 mg/ml p-phenylenediamine (Sigma) and observed with a

* This work was supported by United States Public Health Service Grants CA39821 (to S. B. H.), NS26333 (to U. K. S.), and 5P30CA133320. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GST, glutathione S-transferase; MES, 2-(N-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline.
Zeiss Axioskop using epifluorescent UV illumination. Photographic images were captured on Kodak Elite-400 film. In the indicated experiments, cells were injected as described above, incubated in fresh medium for 30 min without exposure to nocodazole, and then directly processed for immunocytochemistry. Only viable, morphologically intact interphase cells were counted and scored for the density of interphase microtubules. The data are expressed both as percent of injected cells showing any decrease in the density of the microtubule network and as percent of injected cells exhibiting a marked decrease.

Assembly and Disassembly of Microtubules in Vitro—Tubulin was prepared from calf brain by two cycles of assembly-disassembly as described (20) and stored at −20 °C in MES buffer (0.1 M MES, 1 mM EGTA, 0.5 mM MgCl₂, pH 6.6) containing 6 M glycerol. The purity of the preparation of tubulin was estimated to be 85%. Assembly of tubulin (1 mg/ml tubulin in MES buffer containing 3 M glycerol) was monitored spectrophotometrically (A,630) using temperature-controlled cuvettes (37 °C). To determine the effect of proteins on microtubule assembly, equal volumes of buffer or recombinant proteins were added to the cuvettes 10 min prior to initiating assembly by addition of 1 mM GTP. To assess the effect of recombinant proteins on preassembled microtubules, tubulin polymerization was induced by addition of 1 mM GTP approximately 30 min prior to addition of equal volumes of buffer or recombinant proteins. To confirm assembly and disassembly of microtubules, aliquots removed from the cuvettes were analyzed by electron microscopy using negative staining.

RESULTS AND DISCUSSION

To study the effect of metablastin on microtubules in intact cells, we selected COS-7 cells, which express low levels of endogenous metablastin (data not shown) and spread well as attached monolayers. In the first series of experiments, we tested the effect of recombinant metablastin on the reassembly of interphase microtubules following nocodazole-induced disassembly (21). Loss of microtubules was observed in all cells prior to washout of nocodazole and was not affected by microinjection of recombinant proteins (data not shown). At the time of analysis (1.5 h following nocodazole washout), all noninjected cells had reestablished a network characteristic of interphase microtubules as judged by immunocytochemistry (Fig. 1). However, when recombinant metablastin, which was produced as a glutathione S-transferase fusion protein (GST-p19) in E. coli (see “Experimental Procedures”), was microinjected, the density of microtubules was significantly reduced in a substantial fraction of injected cells. The reduction ranged from moderate to marked, as illustrated by representative examples shown in Fig. 1. Counting the fraction of injected cells that exhibited a moderate or marked reduction in the density of interphase microtubules revealed that nearly half the cells injected with GST-p19 showed an appreciable decrease in microtubules and more than 10% exhibited a more dramatic loss (Table I). This effect was dependent on the concentration of GST-p19 injected (data not shown). When cells were injected with GST, no significant decrease in the density of microtubules was observed (Fig. 1, I and J, and Table I). The data demonstrate that metablastin, when introduced into COS-7 cells, causes a loss of microtubules, consistent with the microtubule-destabilizing effect of metablastin observed in vitro (22).

We considered the possibility that the recombinant protein may undergo phosphorylation following injection into cells, which could potentially alter its effects on microtubule stability. To test this idea, we compared cells injected either with unmodified (wild-type) GST-p19 or with GST-p19 bearing phosphorylation site mutations. We first used recombinant metablastin that carried alanine-for-serine substitutions at all four known phosphorylation sites. This mutant (S16/25/38/63A) was significantly more potent in reducing microtubule density than the wild-type protein (Table I). Because the alanine substitutions would have rendered this protein resistant to phosphorylation at these sites, the data suggest that it is the nonphosphorylated form of metablastin that promotes micro-
tubule destabilization. Interestingly, introducing alanine substitutions at only two of the four serines, Ser-25 and Ser-38 (S25/38A), increased the microtubule-destabilizing activity of metablastin to nearly the same extent as mutating all four sites (Table I). The data demonstrate that serine phosphorylation of metablastin may block its microtubule-destabilizing activity on the interphase microtubule array in COS-7 cells (for details, see “Experimental Procedures”). A–F, I, and J, microinjection using the nocodazole protocol; G and H, microinjection without nocodazole treatment. A, C, E, G, and I, fluorescein isothiocyanate fluorescence images to reveal injected cells by following rabbit IgG; B, D, F, H, and J, Cy3 fluorescence images showing network of interphase microtubules reacted with β-tubulin antibody. The injected fusion proteins were: GST-p19 (A and B), S25/38A mutant (C and D), S16/25/38/63A mutant (E and F), GST-p19 (G and H), and GST (I and J). The density of the microtubule network of the injected cells was judged to be decreased in B, D, F, H, and J and markedly decreased in F. Scale bar = 10 μm.
is necessary and may be sufficient to completely abolish the microtubule-destabilizing activity of metablastin. To explore whether the loss of microtubules observed in these experiments required the prior disassembly of microtubules by nocodazole, we also analyzed COS-7 cells 30 min after microinjection without nocodazole treatment. Using this protocol, we observed loss of microtubules that was indistinguishable from that seen after nocodazole treatment (Fig. 1, G and H). Again, the S16/25/38/63A mutant was more active than wild-type, and the S16/25/38/63D mutant was without effect on the microtubule array. Moreover, the magnitude of the effects of GST-p19 and of the phosphorylation site mutants was comparable in nocodazole-pretreated and nonpretreated cells (data not shown). The data support the hypothesis that recombinant metablastin, in its nonphosphorylated form, when introduced into COS-7 cells, promotes disassembly of interphase microtubules and that this effect is abolished by phosphorylation.

Utilizing an in vitro assay (25), we have explored whether the differential effects of phosphorylation site mutations of metablastin, observed in intact cells, were direct or indirect. When added to tubulin prior to GTP-induced assembly, GST-p19 inhibited tubulin polymerization in a concentration-dependent manner (Fig. 2A). Conversely, when GST-p19 was added following GTP-induced tubulin polymerization, a concentration-dependent disassembly of microtubules was observed (Fig. 2B). Assembly and disassembly of microtubules under these conditions were confirmed by electron microscopy (data not shown). GST had no effect on either assembly or disassembly (Fig. 2, A and B). The concentration of GST-p19 needed to completely prevent microtubule assembly and promote disassembly corresponds to a molar ratio of GST-p19:αβ-tubulin heterodimer approaching one (Fig. 2, A and B). Considering both the concentration-dependent decrease in the steady-state polymer level that is observed in the presence of metablastin, and the stoichiometric concentrations needed, the data are consistent with a direct interaction of metablastin with tubulin dimers, as reported (22). It should be noted, however, that Belmont and Mitchison (22) reported complete inhibition of tubulin polymerization at stoichiometric concentrations of metablastin. It is possible that the activity of the recombinant GST-metablastin fusion protein used in our studies is not identical to that of the native protein.

Next we tested the phosphorylation site mutants of metablastin in the in vitro assembly assay. In contrast to the striking differential effects observed with these mutants upon microinjection of COS-7 cells (Table I), neither alanine nor aspartate substitution of all four serine residues affected the ability of GST-p19 to inhibit tubulin assembly in vitro. The activity of both the S16/25/38/63A and S16/25/38/63D mutants showed a concentration dependence indistinguishable from that of wild-type GST-p19 (Fig. 2C). The data demonstrate that introducing an acidic charge at the four known phosphorylation sites of metablastin, which may be expected to have effects on the conformation of the protein similar to those induced by phosphorylation at these sites, does not alter its inhibitory effect on microtubule assembly in vitro, suggesting that the differential effects observed with these mutants in intact cells may be indirect. One possibility is that phosphorylation at Ser-63 promotes binding of metablastin to another cellular protein, thereby preventing the interaction of metablastin with
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tubulin dimers. It is likely, however, that the regulation is more complex. For example, our observation that the S25/38A mutant was nearly as potent as the S1625/438/63A mutant in promoting loss of microtubules, which is in keeping with the findings of Gullberg and co-workers (23, 24), suggests that phosphorylation at Ser-25 or Ser-38 or both may be required to render Ser-63 accessible to the kinase that phosphorylates this site in cells. It is likely, therefore, that the cellular control of the dynamics of microtubule assembly/disassembly requires interaction of multiple factors. Our data support the proposal that metablastin is one of these and suggest that phosphorylation at several sites controls the activity of this protein, possibly by modulating its interaction with factors that remain to be identified.

We have shown that microinjection of COS-7 cells with recombinant metablastin provides a convenient experimental approach for studying its activity on microtubules in intact cells. It should be noted, however, that these studies do not prove a physiological role of metablastin in regulating the dynamics of interphase microtubules. The cellular events that are controlled by the microtubule-stabilizing activity of endogenous metablastin remain to be identified. Metablastin is highly abundant in immature, postmitotic neurons and oligodendrocytes in the developing brain (26). Therefore, it is likely that it participates in the regulation of the dynamic restructuring of microtubules that is a prominent feature of the morphogenesis of neurons and glia. Consistent with this proposal is a recent report that inhibition of metablastin expression with antisense oligonucleotides prevents nerve growth factor-induced neurite outgrowth in PC12 cells (27).

Abundant expression of metablastin also occurs in male germ cells prior to and during meiosis (28) and in B and T lymphocytes following mitogenic stimulation (29–31). In addition, many transformed cells, particularly those of neuroendocrine and hematopoietic origin, express high levels of this protein (29, 32, 33). This evidence suggests that metablastin plays a role in mitosis by participating in the control of the dynamic instability of the mitotic spindle, as was proposed recently (22). Experimental support for a role of metablastin in cell replication has been reported (11, 23, 24), and immunodepletion experiments on mitotic Xenopus egg extracts described by Belmont and Mitchison (22) strongly suggest that metablastin is required for regulation of the mitotic spindle. However, the lack of expression of metablastin in neuroblasts in vivo and in actively dividing fibroblasts and astrocytes in primary culture (26, 31) would appear not to be consistent with a general role for metablastin in cell division. It is interesting, in this regard, that metablastin knockout mice develop normally and have no overt phenotype (34). However, it is possible that these mice have a compensation in the expression of one or more genes that have not yet been identified.

This report demonstrates that metablastin can have profound effects on microtubules in intact cells, consistent with the recently described activity of metablastin on the dynamic instability of microtubules in vitro (22). An important finding of our study is that aspartate substitution of a single serine residue, Ser-63, completely abolishes the effect of metablastin on cellular microtubules. The data suggest that phosphorylation of this residue plays a critical role in controlling the function of metablastin in vivo.

Acknowledgments—We thank Dr. G. A. Orr for helpful discussions and Dr. Daxin Chen for help with microinjections.

Addendum—It was reported recently that overexpression of both wild-type metablastin and of metablastin carrying alanine substitutions at Ser-25 and Ser-38 induced depolymerization of microtubules in K562 erythroleukemia cells (35). These findings are fully consistent with the results reported here.

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