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Stathmin Activity Influences Sarcoma Cell Shape, Motility, and Metastatic Potential

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Submitted September 12, 2007; Revised February 13, 2008; Accepted February 15, 2008

Monitoring Editor: Josephine Adams

The balanced activity of microtubule-stabilizing and -destabilizing proteins determines the extent of microtubule dynamics, which is implicated in many cellular processes, including adhesion, migration, and morphology. Among the destabilizing proteins, stathmin is overexpressed in different human malignancies and has been recently linked to the regulation of cell motility. The observation that stathmin was overexpressed in human recurrent and metastatic sarcomas prompted us to investigate stathmin contribution to tumor local invasiveness and distant dissemination. We found that stathmin stimulated cell motility in and through the extracellular matrix (ECM) in vitro and increased the metastatic potential of sarcoma cells in vivo. On contact with the ECM, stathmin was negatively regulated by phosphorylation. Accordingly, a less phosphorylatable stathmin point mutant impaired ECM-induced microtubule stabilization and conferred a higher invasive potential, inducing a rounded cell shape coupled with amoeboid-like motility in three-dimensional matrices. Our results indicate that stathmin plays a significant role in tumor metastasis formation, a finding that could lead to exploitation of stathmin as a target of new antimetastatic drugs.

INTRODUCTION

Stathmin 1 (also known as OP18 [OncoProtein 18] or metablastin) is a ubiquitously expressed cytosolic phosphoprotein, enriched in neuronal cells and showing a peak of expression at the end of gestation (Koppel et al., 1990). Like other members of its family (stathmin 2, 3, and 4), stathmin 1 (hereafter stathmin) is a microtubule (MT)-destabilizing protein (Curmi et al., 1999), acting either by promoting MT catastrophe (Belmont and Mitchison, 1996) or by sequestering free αβ-tubulin heterodimers (Howell et al., 1999).

Experimental evidence suggests that stathmin MT-depolymerizing activity is negatively regulated by phosphorylation on four serine (S) residues (S16, S25, S38, and S63). Numerous serine-threonine kinases phosphorylate stathmin in vitro and in living cells (Curmi et al., 1999), supporting the hypothesis that stathmin could act as a relay for multiple signal transduction pathways (Sobel et al., 1989). Studies conducted in vivo in mice (Jin et al., 2004) and Drosophila (Ozon et al., 2002; Borghese et al., 2006) and in vitro on cultured cells (Baldassarre et al., 2005; Giampietro et al., 2005; Ng et al., 2006; Watabe-Uchida et al., 2006) demonstrated that stathmin plays a role in several cellular processes, including neuritis formation and cell movement. Moreover, stathmin is overexpressed in several types of human cancers, suggesting that it could have a role in tumor progression (Melhem et al., 1991; Curmi et al., 2000; Price et al., 2000). Recently, a point-mutated stathmin protein (which carries a substitution at Q18) was identified in esophageal cancer. This mutation impairs stathmin phosphorylation, increases its activity, and transforms mouse fibroblasts (Misek et al., 2002).

MT stability influences cell motility in a cell- or context-dependent manner (Etienne-Manneville, 2004), and recent studies found that cell contact with the extracellular matrix (ECM) induces MT stabilization (Etienne-Manneville, 2004; Palazzo et al., 2004), suggesting that cell movement through ECM substrata may also be regulated by the levels and/or activity of the MT-regulating proteins.

Together, these findings support the hypothesis that altering the MT network by deregulation of stathmin could influence cell motility, particularly in cancer cells. In the current study, we addressed this hypothesis by investigating the role of stathmin and its phosphorylation status in regulating cell shape, growth, adhesion, and motility in vitro and in vivo.
MATERIALS AND METHODS

Cell Culture, Transfection, and Treatments
HT-1080 fibrosarcoma cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and transfected using FuGENE 6 (Roche, Indianapolis, IN). Experiments were performed 48 h after transfection. Stable cell clones were obtained by selecting cells in puromycin (2 μg/mL) or hygromycin (400 μg/mL) for FLAG-tagged or untagged vectors, respectively. All experiments were performed on two independent clones derived from each construct. The expression vectors and the adenovirus used in this study are described in the Supplementary Materials and Methods.

Proliferation, Adhesion and Migration Assays and Time-Lapse Microscopy
Proliferation, adhesion and migration assays, and time-lapse microscopy were performed as previously described (Baldassarre et al., 2005, Wolf et al., 2003) and are extensively described in the Supplementary information.

Immunoblotting, Immunoprecipitation, and Immunofluorescence
Western blot analysis was performed as previously described (Baldassarre et al., 2005). Primary antibodies (Abs) were purchased from Sigma (actin, α-tubulin, acetylated α-tubulin, polyglutamylated-tubulin, and FLAG-M1), Santa Cruz Biotechnology (Santa Cruz, CA; β-tubulin, vinculin, AKT, and FAK), Transduction Laboratories (Lexington, KY; stathmin/metastatin), Roche (enhanced green fluorescent protein [EGFP]), Cell Signaling (Beverly, MA; α-, β-, γ-, δ-, and ε-calmodulin, phospho-calmodulin (pCaM), MAPK, pAKT, pFAK, and pJNK), and Chemicon (Tecemula, CA; detyrosinated αI-tubulin).

Secondary HRP-conjugated antibodies were from Amersham (Piscataway, NJ); secondary fluorescein isothiocyanate (FITC)- Texas red– and Alexa Fluor 633–conjugated antibodies were from Jackson Immunoresearch (West Grove, PA) or Molecular Probes (Eugene, OR). For immunofluorescence staining, cells grown in Matrigel or Collagen I drops on coverslips for 24 h were included in Matrigel, injected subcutaneously in nude mice, and allowed to grow for 15 d. Tumors were then excised along with the surrounding mouse tissue and analyzed by H&E staining. For lung metastasis formation, 5 × 104 vector 1 (n = 4) or stathminG107E B9 (n = 4) HT-1080 cells were injected in the mouse tail vein, and lungs were analyzed 30 d later by H&E staining. For the quantification of lung metastasis, 10 sections per lung were analyzed. For small interfering RNA (siRNA) experiments, HT-1080 cells were infected (MOI 300) with adenovirus (Ad-Control siRNA [control] or Ad-stathmin siRNA; 3 d later, the cells (5 × 106) were injected into nude mice (n = 4). After 15 d, intratumoral injection of Ads was repeated, and the mice were killed on day 30 from the initial injection.

RESULTS

Statmin Is Deregulated in Human Sarcoma
Statmin is overexpressed in many tumor types (e.g., leukemias, breast cancer, hepatocellular carcinoma), but its contribution to tumorigenesis is still poorly understood. Sarcomas are a class of mesenchymal tumors characterized by an important invasive potential, responsible for their high rate of local recurrence after surgery and distant metastasis. To determine the role of statmin in tumorigenesis, we evaluated its expression levels, using Western blot (Figure 1) and immunohistochemistry (Supplementary Figure 1, A–D), in a panel of human sarcomas (16 malignant fibrohistiocytomas and 23 leiomyosarcomas) and the normal tumor-free surrounding tissue. More than 80% of the cases displayed overexpression of statmin in tumor specimens compared with adjacent normal tissue (Figure 1A), with an average value for the statmin/actin ratio (n = 10) that was twofold higher, although this difference was not statistically significant (p = 0.187, Student’s t test; Figure 1C). When tumors were sorted as primary or recurrent/metastatic, statmin expression was strongly associated with tumor relapse and distant metastasis formation (Figure 1, B and C). Recurrent (n = 15) and metastatic sarcomas (n = 8) displayed a statmin/actin ratio 20-fold higher than that of the normal tissue, with the difference being highly significant (p = 0.001 and 0.0005, respectively). Furthermore, statmin expression was greatly increased (p = 0.002) between primary and recurrent/metastatic sarcomas, suggesting a role in tumor progression. Because statmin’s biological functions are negatively regulated by phosphorylation, we also checked whether statmin overexpression was paralleled by a variation in its phosphorylation status. Focusing on normal and sarcoma samples expressing comparable levels of total protein, we found a reduction of S16 phosphorylation in neoplastic samples compared with normal ones (Supplementary Figure 1F). Similar conclusions were drawn by studying statmin phosphorylation in seven cases of patients with recurrent diseases (Figure 1D). The expression of pS16, total statmin, and their ratios were evaluated by Western blot and densitometric scanning of the blots. As shown in Figure 1D recurrent diseases showed a decreased pS16/statmin ratio (4/7 cases) or a de novo expression of total statmin protein (two cases). These findings support the hypothesis that an increased statmin expression coupled with an increase in its activity could be relevant in the process of local invasion and distant dissemination.

Statmin Stimulates Cell Motility by Destabilizing MTs
To get deeper insight into the molecular mechanism through which statmin contributes to sarcoma invasiveness, we tested the in vitro ability of statmin to drive fibrosarcoma cell motility. Having previously demonstrated that p27Kip1 overexpression reduces ECM-dependent cell motility by inhibition of statmin tubulin-sequestering activity (Baldassarre et al., 2005), we better characterized the role of statmin in cell motility by separating its tubulin-sequestering activi-
ity from its catastrophe-promoting one. To this purpose, we performed an ECM-dependent motility assay utilizing stathminWT and two deletion mutants: stathmin1-99, retaining the catastrophe-promoting activity, and stathmin25-149, retaining the tubulin-sequestering activity (Howell et al., 1999). In a human fibrosarcoma cell line (HT-1080), stathmin WT overexpression greatly enhanced cell migration through a fibronectin (FN)-coated Transwell. The stathmin25-149 tubulin-sequestering mutant induced a similar effect, whereas stathmin1-99 was unable to do so (Figure 2A), indicating that stathmin tubulin-sequestering activity is sufficient to stimulate cell motility on ECM substrata. Given the fact that stathmin is a MT-destabilizing protein and that MT dynamics play a central role in cell motility, we investigated whether the expression of these stathmin mutants was accompanied by differences in the cellular MT stability. Stable MTs, i.e., MTs with a half-life longer than 15 min (Verhey and Gaertig, 2007), can be distinguished by a variety of posttranslational modifications (Khawaja et al., 1988; Verhey and Gaertig, 2007), such as acetylation, poly-glutamylation, and detyrosination, all recognizable with specific antibodies. We explored the amount of stable MTs in HT-1080 cells overexpressing stathminWT or the deletion mutants after 60 min of FN adhesion, by immunofluorescence and Western blot analysis. Expression of EGFP-stathminWT and EGFP-stathmin25-149 proteins dramatically decreased the amount of cellular acetylated/stable MTs (Figure 2C, left and middle panels), whereas the EGFP-stathmin 1-99 mutant has only minor effects (right panels). Consistently, differential extraction of soluble and polymerized tubulin fractions followed by Western blot analysis also confirmed that the stathmin1-99 mutant displayed a compromised ability to destabilize MTs after cell adhesion to the ECM (Figure 2B).

Collectively, these data support a role for stathmin in ECM-driven cell migration that is achieved through the MT-destabilizing activity of its tubulin-sequestering domain.

Stathmin Is Phosphorylated after Cell Adhesion to ECM Substrata

Stathmin tubulin-sequestering activity is controlled by serine phosphorylation (Curmi et al., 1999) and by association with several proteins, such as p27kip1 (Baldassarre et al., 2005) or STAT3 (Ng et al., 2006). In both cases, these interactions translated into an alteration of cell motility. We
therefore sought to investigate whether stathmin phosphorylation as well was implicated in the regulation of ECM-driven cell motility.

We first analyzed the effect of cell–ECM contact on stathmin phosphorylation in serum-starved HT-1080 cells allowed to adhere to FN for up to 90 min. Using three phosphospecific antibodies recognizing pS16, pS25, and pS38 of stathmin (Gavet et al., 1998), we observed an obvious increase of phosphorylated stathmin at S16 and S38 after 60 min of adhesion to FN (Figure 3, A and B). Endogenous pS25 was not detectable, and total stathmin levels were not affected by cell adhesion under these conditions (Figure 3B). Time-course analysis of cells transiently transfected with a FLAG-tagged stathmin vector confirmed that phosphorylation of S16, S38, and also S25 increased significantly after 30–90 min of adhesion to FN (Figure 3, A and B). Endogenous pS25 was not detectable, and total stathmin levels were not affected by cell adhesion under these conditions (Figure 3B). Time-course analysis of cells transiently transfected with a FLAG-tagged stathmin vector confirmed that phosphorylation of S16, S38, and also S25 increased significantly after 30–90 min of adhesion, when FN (Figure 3C) and collagen I (Coll I; Supplementary Figure 5B and data not shown) were used as substrata.

To test whether these phosphorylations had any effect on stathmin promigratory activity, we generated a less phosphorylatable stathmin mutant carrying the Q18E substitution. The stathminQ18E mutant was originally identified in human cancers (Misek et al., 2002) and was shown to have impaired phosphorylation on S16 (and partially on S25 and S38), to increase MT-depolymerization activity and to induce cell transformation (Misek et al., 2002). We therefore chose to use this more “physiological” mutant for our experiments, rather than the “classical” serine-alanine substitution mutants. Transiently transfected HT-1080 cells were serum starved and then adhered to FN for 60 min. Under these conditions, a marked reduction of S16 phosphorylation was observed in stathminQ18E-expressing cells, compared with stathminWT, whereas no significant difference in the levels of pS25 and pS38 was found (Figure 3C). Similar results were obtained by immunofluorescence analysis of both endogenous and ectopically transfected stathmin in HT-1080 cells adhered to FN (Figure 3D and Supplementary Figure 2). These experiments highlight an FN-dependent signaling pathway leading to stathmin phosphorylation and to the regulation of its MT-stabilizing properties. Additional experiments will be required to identify the specific kinase(s) participating in this pathway that leads to the ECM-driven regulation of stathmin.

**Generation and Characterization of Stathmin Stable Cell Clones**

Several stable HT-1080 cell clones overexpressing the stathminWT or stathminQ18E proteins were generated using either
untagged or FLAG-tagged constructs. The complete characterization in terms of proliferation, survival, and adhesion ability of HT-1080 clone V1 (transfected with the empty vector) and four different stathmin-expressing clones used in this work is extensively described in the Supplementary information. Importantly, we observed that stable stathmin overexpression in HT-1080 cells did not significantly modify cell proliferation, survival, or adhesion properties (Supplementary Figures 3–5). Thus, we continued to focus our attention on the role of stathmin in cell motility.

Stathmin Expression Enhances 3D Migration and Invasion

Parental HT-1080 and stathmin-expressing clones were tested in different types of ECM-driven migration assays, using FN- or Matrigel-coated Transwells or ECM inclusions. Both stathminWT and stathminQ18E proteins promoted cell migration (Figure 4A), invasion (Figure 4B), and evasion (Figure 4C) through and from ECM substrata, with stathminQ18E slightly more active than stathminWT in all types of assays. To exclude the possibility that clonal selection could be responsible, at least partially, for the migratory behavior of stathmin-expressing cells, transiently transfected cells were also used. One day after transfection, cells were plated on top of or below a 1-mm-thick Matrigel layer and allowed to invade the matrix for 3 days. The extent of Matrigel invasion, as evaluated by confocal microscopy, demonstrated that overexpression of EGFP-stathminWT increased the ability of HT-1080 cells to invade Matrigel and that the Q18E substitution further amplified this invasive potential (Figure 4D). Consistently, down-regulation of endogenous stathmin, by means of an adenovirus (Ad) expressing stathmin siRNA, decreased HT-1080 cell migration through FN-coated Transwells (Figure 4E). The concomitant expression of either EGFP-stathminWT or EGFP-stathminQ18E proteins was able to rescue cell migratory ability (Figure 4, F and G). On the whole, these experiments substantiate stathmin involvement in different types of ECM-driven cell motility and the possibility that an altered stathmin phosphorylation pattern, as found in human sarcoma samples (Figure 1F) or in esophageal carcinomas with the Q18E mutation (Misek et al., 2002), increases its promigratory activity.

Stathmin Expression Does Not Enhance Fibrosarcoma Cell Motility in Two-dimensional Assays

Altered MT dynamics have been suggested to exert an inhibitory role on cell motility (Liao et al., 1995; Wittmann et al., 2004). In accordance with this hypothesis, the hyperactivity of stathmin in STAT3-null fibroblasts has been considered the cause of a decreased wound closure (Ng et al., 2006). Importantly, most of these studies were based on two-dimensional (2D) assays, such as wound healing (reviewed in Small et al., 2002; Rodriguez et al., 2003), which substantially differ from motility in 3D matrices or in vivo (Friedl and Brocker, 2000). We thus speculated that stathmin could differently affect cell motility, depending on the type of motility assayed. To verify this hypothesis, HT-1080 clones were tested in 2D experiments. Clones expressing stathminWT or stathminQ18E migrated in a manner similar to that of control cells in a wound-healing assay (Figure 5, A and B), demonstrating that stathmin does not substantially affect fibrosarcoma cell directional motility in 2D, as reported by Ng et al. (2006) for normal fibroblasts. Using time-lapse microscopy to analyze cell motility under 2D or 3D conditions (Supplementary Movies 1–3), we observed that although in wound healing and in 2D random motility on plastic control and stathminQ18E-expressing cells designed very similar trajectories, in 3D Collagen I stathminQ18E cells covered significantly longer distances than control cells did (Figure 5, C–E). Accordingly, stathminQ18E did not enhance HT-single cell velocities in wound healing (Figure 3F) and in 2D random motility assays (data not shown), whereas in 3D assays stathminQ18E cells moved significantly faster (Figure 3G).

Stathmin Expression Influences Adhesion-dependent MT Polymerization

To determine whether increased motility of stathminQ18E-expressing clones corresponded to molecular variations in MT organization, we analyzed the extent of polymerized tubulin in stathminWT and stathminQ18E HT-1080 clones, in suspension and after 1 h of adhesion to FN. Western blot analysis of soluble and polymerized protein fractions showed that adhesion to ECM components leads to an important tubulin reorganization, which was in part counteracted when stathmin was not phosphorylatable (Figure 6A), confirming that stathminQ18E has an enhanced capability to interfere with adhesion-dependent MT polymerization. Moreover, after adhesion stathminQ18E induced a marked reduction in the amount of polymerized MTs, compared with control cells, accompanied by a reduced level of tubulin posttranslational modifications, which are markers of MT polymerization.
stability (Figure 6B). Interference with adhesion-dependent MT polymerization by both stathminWT and stathminQ18E was also demonstrated on transiently transfected HT-1080 cells by Western blot analysis of modified tubulin in polymerized and soluble cellular fractions (Figure 6C). As expected, both endogenous and transfected stathmin resided in the soluble fraction, and stathminQ18E-expressing cells displayed a reduced phosphorylation of S16 but not of S38 (Figure 6C). Likely for the high levels of transiently expressed stathmin proteins in these experiments, only small differences were noticed between stathminWT and stathminQ18E on MT polymerization (Figure 6C, fourth panel). The decrease in MT stability induced by stathminQ18E was further confirmed by performing the tubulin dilution assay on cells adhered to FN for 1 h and subsequently fixed and stained with an anti-α-tubulin antibody. This assay allows visualization of only stable MTs with a half-life longer than 15 min (Khawaja et al., 1988). Results clearly show that HT-1080 V1 cells retain a threefold higher amount of stable MTs compared with stathminQ18E-expressing cells (Figure 6D and data not shown). Importantly, immunofluorescence analysis of acetylated (Figure 6E) and detyrosinated tubulin (data not shown) on the same cells cultured on plastic in 2D did not show any significant difference in the content of stable MTs, supporting the hypothesis that stathmin affects MT polymerization after contact with the ECM and that stathmin ECM-dependent phosphorylation controls this activity.

**Stathmin Activity Influences HT-1080 Cell Morphology in a 3D Matrix**

Different types of cell motility in 3D assays or in vivo have been linked to distinct molecular mechanisms controlling cell morphology (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Wolf et al., 2003). We thus examined the morphology of HT-1080 cell clones up to 24 h after inclusion in 3D matrices, with the shape factor parameter (4A/P², where A is the cell area and P the cell perimeter), which varies from 0 to 1 for elongated to more circular shapes, respectively (Peris et al., 2006), was used to quantify cell morphology differences. Both in Coll I after 6 h (Figure 7A) and 24 h (Figure 7B) of inclusion and in Matrigel after 24 h (Supplementary Figure 6, A and C), stathminQ18E-expressing clones (i.e., B9, F7) presented a more rounded morphology, defined by a high shape factor (Figure 7C at 24 h and Supplementary Figure 6, C and E, at 6 h: p < 0.0001, Student’s t test), compared with the elongated morphology...
of control cells and stathmin\textsuperscript{WT} clones (i.e., vector clone 1 and clone A3, respectively). The absence of an effect on 3D cell morphology of stathmin\textsuperscript{WT} is consistent with the reduced ability of low WT protein levels to interfere with adhesion-dependent MT modification (Figure 6A). Conversely, when cells were simply plated on a plastic dish, no change in cell shape was observed (by shape cell factor calculation and immunofluorescence analysis) between control and stathmin\textsuperscript{Q18E} clones, allowed to move toward a wound (C), randomly as sparse cells in 2D (D) or immersed in a 3D Coll I matrix (E), in serum-free medium. (F and G) Statistical evaluation (Mann-Whitney U test) of cell speed from experiments described in C and E. Median speeds were 0.07 and 0.09 \(\mu\text{m}/\text{min}\) for HT-1080 V1 and stathmin\textsuperscript{Q18E} B9, respectively, in the wound-healing assay (\(p = 0.7\), Mann-Whitney U test) and 0.03 and 0.11 \(\mu\text{m}/\text{min}\) for HT-1080 V1 and stathmin\textsuperscript{Q18E} B9, respectively, in the 3D Coll I assay (\(p < 0.0001\), Mann-Whitney U test).

Stathmin Expression Enhances the Metastatic Phenotype of HT-1080 Cells

To verify whether stathmin effects on cell motility observed in vitro could be recapitulated in vivo, nude mice were subcutaneously injected with 1 \(\times\) 10\textsuperscript{6} parental HT-1080 cells or HT-1080 V1 (\(n = 8\)), stathmin\textsuperscript{WT} A3 (\(n = 6\)), and stathmin\textsuperscript{Q18E} B9 or F7 stable cell clones (\(n = 8\)). After 20 d, mice were killed, and the growth and metastatic ability of each cell clone were evaluated. The analysis of the explanted tumor masses revealed that the local tumor growth was not markedly influenced by stathmin overexpression (Figure 8A). Distant localization of tumor cells was evaluated by RT-PCR on RNA extracted from blood, lung, liver, or spleen, using vector-specific primers (Figure 8, B and C). As shown in Figure 8, B and C, tumor cells were found in distant organs and in the blood of seven of eight mice injected with HT-1080 stathmin\textsuperscript{Q18E} compared with only two of six mice injected with HT-1080 stathmin\textsuperscript{WT} and none of six mice injected with control HT-1080 cells. Stathmin effects on local cell invasion were evaluated instead by subcutaneously injecting into mice (\(n = 4/\text{clone}\)) parental HT-1080 or stathmin\textsuperscript{Q18E} B9 cells previously included in Matrigel. After 15 d, mice were killed and the tumor borders were examined. Although parental HT-1080 cells grew within the Matrigel borders, with only few cells invading the surrounding tissues, cells stably expressing stathmin\textsuperscript{Q18E} aggressively invaded the surrounding host muscular tissues (Figure 8D). We also tested the ability of stathmin\textsuperscript{Q18E}-expressing cells to extravasate and localize to distant organs, in particular to
the lungs. To this aim, we injected HT-1080 clone V1 or stathminQ18E clone F7 cells in the tail vein of nude mice (n = 4/clone); 30 d later, we examined the mouse lungs for the presence of metastases (Figure 8E). Pathological analysis demonstrated that stathminQ18E-expressing cells (clone F7) readily colonized the lungs, whereas HT-1080 V1 cells did not. When we looked at the levels of polymerized acetylated tubulin in explanted tumor lysates, we observed that they correlated with the metastatic potential of sarcoma cells, because polymerized MTs were strongly diminished in tumors derived from the stathminQ18E-expressing cells (Figure 8F). The results of the in vivo experiments prove that stathminQ18E expression strongly increases the metastatic potential of HT-1080 cells, thus substantiating all the in vitro data from motility evasion/invasion assays (Figure 4). Together, the data reported here reinforce the hypothesis that the invasive phenotype of stathmin-expressing cells could be directly related to its activity on MTs and suggest that indeed altered MT stability could also play a role in cell invasiveness in vivo.

**DISCUSSION**

The present work investigates the role of the MT-destabilizing protein stathmin in sarcoma cell growth and motility and highlights several new aspects regarding the role and regulation of stathmin in human tumorigenesis and the formation of metastases.

Our data point to the tubulin-sequestering activity as the main effector of stathmin in the control of adhesion-dependent MT polymerization and of cell movement. This is in agreement with previous observations suggesting that the catastrophe-promoting N-terminal domain plays a more prominent role in controlling the spindle formation during mitosis (Holmfeldt et al., 2001).

We demonstrated that stathmin is phosphorylated after cell adhesion to ECM on at least three different serine residues, suggesting that adhesion to ECM contributes to the regulation of stathmin activity through the modification of its phosphorylation status. The finding that stathminQ18E, unable to undergo S16 phosphorylation, displays a gain of function in stimulating sarcoma cell motility suggests that S16 phosphorylation could represent an important event in the regulation of cell motility by stathmin. Accordingly, recent data demonstrated that stathmin is phosphorylated on S16 in developing neurons adherent to laminin and that this phosphorylation is necessary for proper neuritis outgrowth (Watabe-Uchida et al., 2006). The role of adhesion-dependent S25 and S38 phosphorylations in cell motility remains less clear, because both these residues are readily phosphorylated in the stathminQ18E protein. On the contrary, stathminQ18E did not stimulate HT-1080 cells growth.
This observation is in line with the work of Misek et al. (2002) that showed minor effects on normal fibroblasts growth in 2D assays after expression of ectopic stathminQ18E protein. These authors showed indeed that stathmin Q18E had transforming properties and increased cell growth in soft agar assay, an effect that could not be appreciated in our model because we used the already transformed HT-1080 cells. Importantly, our results demonstrate that stathmin is able to promote sarcoma cell motility through or within the ECM in vitro and stimulate local invasion and/or metastasis formation in vivo. These activities are associated with a change in cell morphology and a decrease in stable MT content, when cells are included in a 3D matrix or grown in vivo. Both cytoskeletal dynamics and cell shape dictate migration mode and efficiency in a 3D context (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Wolf et al., 2003). It has been proposed that motility of rounded cells is characterized by reduced cell elongation, relatively weak cell–matrix interactions, and the ability of the cells to squeeze their body to pass through the ECM fibers, using small ruffles or bleb-like propulsive translocation. This mode of cellular migration has been defined as “amoeboid” motility (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Wolf et al., 2003). It is then conceivable that a more dynamic and flexible MT network (due to increased stathmin activity) could cause a switch in the migration mode, inducing an “amoeboid”-like motility both in vitro and in vivo. This might not only promote tumor cell evasion from the primary site but also result in a more rapid and distant dissemination, as proposed also by others (Friedl and Wolf, 2003). The importance of assaying cell flexibility in response to increased microtubule turnover in a 3D context is underscored by the observation that overexpression of stathminWT or stathminQ18E did not increase cell motility in 2D assays (Figure 5 and Ng et al., 2006). It is important to note that the behavior of control and stathmin-overexpressing clones in random motility substantially differs when 2D versus 3D conditions are analyzed. This allows us to hypothesize that 3D signaling from the matrix is specifically relevant for the enhancement of motility induced by stathmin. Our data suggest that phosphorylation of stathmin after cell adhesion to the ECM could contribute at least in part to the stabilization of the MT network.
network and that impairing this pathway may result in a switch versus an amoeboid type of motility. Our data also confirm that the physicochemical requirements in the cell for 2D and 3D migration are distinct. A clarifying example is given by the studies of Marshall and colleagues, on the role of the small GTPase RhoA in cell motility. These authors demonstrated that hyperactive RhoA decreases wound-healing motility (Vial et al., 2003) but increases or does not affect (depending on the cell type) cell invasion in a 3D context (Sahai and Marshall, 2003). Moreover, recent data suggest that increased RhoA activity at the cell periphery by Smurf1-silencing results in decreased 2D motility, but increased 3D invasive potential, in different tumor cell types (Sahai et al., 2007). These data further confirm that the ability of cells to move on a 2D substrata does not always parallel the invasiveness of cancer cells. Interestingly, increasing evidence underscores the existence of a tight relationship between MT dynamics and small GTPase activity, because MT stability can regulate GTPase activity (Xu et al., 2005) and, in turn, small GTPases can affect adhesion-dependent MT stabilization (Palazzo et al., 2004), at least in part acting on stathmin (Watabe-Uchida et al., 2006).

It is interesting to note that stathmin down-regulation in vivo in mice and Drosophila results in decreased motility of proliferating neurons (Jin et al., 2004) and germ and border cells (Ozon et al., 2002; Borghese et al., 2006), strongly supporting a promigratory role for this protein. Moreover, the fact that the MT-stabilizing protein MAP2 is down-regulated in metastatic melanomas with respect to primary tumors (Soltani et al., 2005)—whereas stathmin is overexpressed in metastatic versus clinically localized prostate carcinomas (Varambally et al., 2005), invasive recurrent hepatocarcinomas (Yuan et al., 2006), advanced mammary carcinomas (Van’t Veer et al., 2002), and recurrent/metastatic sarcomas (our work)—suggests that decreased MT stability could be a general feature in the process of metastasis formation. Our findings indicate that increased stathmin expression and/or activity stimulates sarcoma cell migration in vitro, enhances local and distant dissemination in mice, and correlates with sarcoma recurrence and metastasis formation in human disease. Altogether, these data strongly point to stathmin as a potential prognostic factor and a promising target for biological therapies in refractory diseases, at least in soft tissue sarcomas.

ACKNOWLEDGMENTS

We are grateful to Dr. Andre´ Sobel (INSERM, Paris, France) for providing the anti-phospho-stathmin antibodies and to Dr. Greg G. Gundersen (Columbia University, New York, NY) for providing the anti-Glu-Tubulin Ab. We thank...
Stathmin in Cell Motility and Invasion


