Induction of Stathmin Expression during Erythropoietic Differentiation

Thierry Rabilloud,¹ Roland Berthier, Cecyl Valette, Jerome Garin, and Jean-Jacques Lawrence²


Abstract

Stathmin is a M, 19,000 cytosolic protein proposed to act as a relay for signal-activating pathways regulating cell proliferation and differentiation. In a study of erythropoietic differentiation, stathmin was detected as a protein that is induced during the early stages of differentiation in several cellular model systems. The unphosphorylated form of stathmin was most prominently induced, which suggests that this form does not only play a role in pathways oriented toward cell proliferation, as is the case for lymphocytic systems, but may also play a role in various differentiation pathways.

Introduction

Stathmin, also designated as p19 (1), pp17, prosolin (2), or p18 (3), was identified as an ubiquitous phosphoprotein and was proposed to act as a relay in various pathways for the integration of extracellular signals and the induction of cell proliferation and differentiation. This was based on the fact that stathmin phosphorylation is an early event in the 12-Q-tetrade canoxyphorol-13-acetate-induced differentiation of HL60 cells (2) and in NGF²-induced differentiation of PC12 cells (4). In addition, stathmin was shown to be prominently induced in proliferating lymphocytes such as lymphoma and leukemia cells (3). In normal tissues, stathmin was shown to be highly expressed in the brain and concentrated in neurons (5). The expression varies during development, with the maximum amount at the neonatal stage, followed by a decrease during postnatal growth and a low steady-state level in the adult (5).

Because stathmin is a phosphoprotein, it exists in several forms, from the unphosphorylated form (usually the most abundant), to the mono-, di-, and triphosphorylated forms. Stathmin has been shown to be a substrate for several kinases implicated in growth control or signal transduction, such as p34cdc2 (6) and MAP kinase (7), favoring the notion that stathmin is a relay protein in signal integration. This hypothesis has been strongly supported by recent studies. A gene coding for a Cdc2 target site-deficient stathmin was placed under the control of an inducible promoter and transfected into K562 cells. Expression of this gene resulted in a blocking of the cells in G2 phase or endoreduplication with severe chromosome segregation defects (8). In a second study, antisense oligos against stathmin RNA have been introduced into PC12 cells, and loss of the response to NGF has been observed (9). All these observations have led to a model in which the unphosphorylated form of stathmin is thought to play a role in cell proliferation, while the decrease of the unphosphorylated forms and/or the increase of the phosphorylated forms is thought to orient the cell toward differentiation. This model is supported by data from HL60 and PC12 cells (2, 4) as well as observations in the embryonic carcinoma cells and their differentiated derivatives, in which the stathmin level is reduced (10). It is further substantiated by work carried out in the T-lymphocyte system, in which the expression of the unphosphorylated form of stathmin is correlated with the growth rate (3, 11). Stathmin expression increases when cells proliferate and is very high in leukemia cells (3). It decreases when proliferating lymphocytes cease to divide (11, 12), accompanied by increased phosphorylation in some cases (12). These data suggest that, at least in the mature lymphocyte model, stathmin expression is correlated with the S phase of the cell cycle.

This model may, however, not be true in all systems. For example, stathmin expression is also very high in neurons (5), which are typical postmitotic cells. In the liver regeneration system, stathmin is also induced, but this induction did not appear to be strictly correlated with the cell cycle (13). Last but not least, high levels of stathmin are observed in resting immature lymphocytes (14).

We report here that the unphosphorylated form of stathmin is expressed at very high levels during the early phases of erythroid cell differentiation in several systems, while the cells have a moderate proliferative activity. Furthermore, this high expression is not coupled with cell proliferation, suggesting that the role of the various stathmin forms may vary in different cell types.

Results

Stathmin Expression during Friend Cell Differentiation.

The present work is the result of a research project aimed at the identification of proteins the level of which varies significantly during the early stages of the induced differentiation of Friend murine erythroleukemia cells. Two-dimensional electrophoresis was carried out to separate the proteins of whole-cell extracts prepared from Friend cells at various stages of the induced differentiation process. Fig. 1 shows two-dimensional gels obtained from untreated cells and from cells induced to differentiate with HMBA for 48 h. The spots showing a strong variation were identified by peptide microsequencing. For the spot shown by an arrow on Fig. 1 and displayed on Fig. 2 for the course of the HMBA-induced differentiation, the following peptidic sequences were obtained: EVLQK/HVEEVK/ENREAOQ-

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Fig. 1. Protein expression in Friend cells induced to differentiate or not. Whole-cell extracts obtained from control cells (A) or from Friend cells induced to differentiate for 48 h with HMBA (B) were analyzed by two-dimensional electrophoresis. Some of the spots showing a significant variation are shown with arrowheads. The spot identified as stathmin is shown by an arrow.

Control (393 ppm)  + HMBA 16h (656 ppm)
+ HMBA 48h (1004 ppm)  + HMBA 120h (971 ppm)

Fig. 2. Stathmin expression during Friend cell differentiation. The stathmin zone of the two-dimensional gels is shown, with unphosphorylated stathmin being shown by an arrow and phosphorylated forms by open arrows. The amount of unphosphorylated stathmin in ppm of the sum of all the spots detected on the gels is indicated below each panel. The variation from one experiment to another is less than 20%.

MAAK. This led to the unambiguous identification of the spot as stathmin. This identification was, moreover, in good agreement with the experimental pI and molecular weight of the spot.

To check which form of stathmin was represented in this spot, two dimensional blots were made, stained with colloidal gold, and probed with the anti-stathmin antibody. The results (shown in Fig. 3) demonstrated that the spot identified by microsequencing was the most basic and, therefore, unphosphorylated, form of stathmin. This also allowed us to identify the minor forms on the silver-stained gels (Fig. 2, arrowheads).

The relationship between stathmin induction and the cell cycle during this differentiation process was further investigated. To this purpose, Friend cells were induced to dif-
Stathmin Expression in Normal Lymphoid and Erythroid Cells. To further assess the relevance of the correlation between stathmin expression and erythroid differentiation, an in vitro erythropoiesis was carried out. For this purpose, BFU-E from peripheral blood were induced to proliferate and differentiate into CFU-E by SCF and interleukin 3. These CFU-E were then induced to proliferate and differentiate, first into proerythroblasts and then into erythroblasts, by erythropoietin (18). Whole-cell extracts from proerythroblasts and erythroblasts were analyzed by two-dimension electrophoresis, and the stathmin level was determined. As a control, the lymphocytes from the same donor were also analyzed, either untreated or after 72 h stimulation by PHA. Extracts from Jurkat cells were also analyzed to check the level of stathmin induction observed with silver staining and to compare it with the results obtained with 15S methionine in previous work (3, 11). The results, shown in Fig. 5, demonstrate that the stathmin level in normal proerythroblasts and erythroblasts is much higher than in lymphocytes, even stimulated with PHA.

It may be argued that the high level seen in proerythroblasts could be due to the fact that these cells proliferate. However, proliferating lymphocytes have a much lesser amount of stathmin than proerythroblasts. In addition, erythroblasts also have a high amount of stathmin, while postmitotic orthochromatic erythroblasts are the dominant cell type in our erythroblastic preparations. These results indicate that proliferation is not solely responsible for the high stathmin levels observed in the erythroid cells.

Phosphorylation. Because stathmin is a phosphoprotein, its phosphorylation state may be important for its function. Therefore we tested if any change in the phosphorylated forms occurred during differentiation of the Friend cells. Quantification of the silver stained gels did not reveal any change in the content of the phosphorylated form, which contrasts with what was observed for systems in which stathmin phosphorylation is known to be important (2, 14). This was confirmed by in vivo metabolic labeling of stathmin with 32P and quantification of the phosphorylated forms of stathmin relative to the total phosphoprotein (12). The results, shown in Table 3, demonstrate that the level of the monophosphorylated form of stathmin, which is most precisely detected, varied between 2.8 and 3.7% of the total phosphoprotein, without any correlation with the differentiation process. This variation is, moreover, within the experimental error and is quite far from the 2- to 5-fold increase in monophosphorylated stathmin observed in other systems (2, 12, 14).

Discussion
The ubiquitous phosphoprotein stathmin was originally identified as a protein induced in proliferating cells (3) and by the fact that its phosphorylation increases during differentiation (2, 4, 19). Subsequently, data from several transformed cell systems suggested that the unphosphorylated form of stathmin is, in fact, a marker for cell proliferation: (a)

**Table 1** Level of stathmin and cell cycle parameters during Friend cell differentiation

<table>
<thead>
<tr>
<th>Control</th>
<th>HMBA 16 h</th>
<th>HMBA 48 h</th>
<th>Butyrate 16 h</th>
<th>Butyrate 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stathmin level</td>
<td>592 ppm</td>
<td>527 ppm</td>
<td>2175 ppm</td>
<td>145 ppm</td>
</tr>
<tr>
<td>% cells in G1</td>
<td>19%</td>
<td>34%</td>
<td>21%</td>
<td>19%</td>
</tr>
<tr>
<td>% cells in G2/M</td>
<td>76%</td>
<td>4%</td>
<td>14%</td>
<td>67%</td>
</tr>
</tbody>
</table>

*Expressed as a percentage of the sum of all the spots detected on the gel. The variation of the level was found to be less than 20% from one experiment to another. This range of variation was also found in the experiments described in the Tables 2 and 3 and the figures.

Stathmin Expression in the K562 Cell Model System. To verify that this high stathmin expression during erythroid differentiation was not restricted to the Friend cell system, experiments were carried out with the human erythroleukemia K562 cells. These tumor cells can be induced to differentiate in vitro in the erythroid pathway either with hemin (16) or with butyrate (17), with a concomitant decrease in proliferation. In these cells, stathmin was identified by two-dimensional blotting, as described for Friend cells. The stathmin level was then measured by two-dimensional electrophoresis. The results, shown in Fig. 4 and Table 2, also indicate that there is a correlation between stathmin expression and erythroid differentiation, as measured by heme detection with benzidine. It must be noted that treatment with either hemin or butyrate blocks cell proliferation, showing that stathmin induction can take place in the absence of cell proliferation.

**Table 2** Level of stathmin and extent of differentiation in K562 cells

<table>
<thead>
<tr>
<th>Control</th>
<th>Hemin 48 h</th>
<th>Butyrate 48 h</th>
<th>Butyrate 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stathmin level</td>
<td>592 ppm</td>
<td>527 ppm</td>
<td>2175 ppm</td>
</tr>
<tr>
<td>Benzidine + cells</td>
<td>1.6%</td>
<td>7.4%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Fig. 4. Stathmin expression during K562 cell differentiation. The stathmin zone of the two-dimensional gels is shown, with unphosphorylated stathmin being shown by an arrow and phosphorylated forms by open arrows. The amount of unphosphorylated stathmin in ppm of the sum of all the spots detected on the gels is indicated below each panel.
proliferating, transformed lymphocytes have a much higher stathmin level than normal, resting, lymphocytes (3); (b), treatments that decrease cell proliferation cause an increase in the level of the phosphorylated forms and a diminution of the unphosphorylated form (2, 20); and (c), normal lymphocytes that are induced to proliferate in vitro show an increase of the unphosphorylated form, while proliferating lymphocytes that are forced to stop their proliferation rapidly accumulate the phosphorylated forms (11, 12). This has led others to consider that the unphosphorylated form of stathmin is a marker of cell proliferation and is correlated with the S phase of the cell cycle (11, 12). Work on immature lymphoid cells (14) or nonlymphoid cells (e.g., liver regeneration system; Ref. 13) has however shown that such a correlation is not absolute. Moreover, stathmin is present at high levels in the adult brain (1, 5) and especially in the neurons, which are typical postmitotic cells. In addition, the phosphorylation of stathmin may be induced in processes having no relationship with proliferation, such as secretagogue-induced secretion in the insulinoma cell system (1) and in GH3 cells (21).

Our interest in the unphosphorylated form of stathmin came from the fact that this protein is detected as an early marker of the erythroid differentiation in Friend murine erythroleukemia cells. Because these cells are known to progressively cease their proliferation during induced differentiation (22), this increase in the unphosphorylated stathmin level is related to the erythroid differentiation per se and not to a proliferation effect.

To verify that the increase in the stathmin level was linked to the differentiation process and not to a side effect of the chemical used for the in vitro induction of the differentiation, several control experiments were carried out. We found that the same effect was observed with different inducers of differentiation, which are thought not to act by the same pathways. This was the case in the Friend cell system with butyrate and HMBA, for which the effects as differentiation inducers are not additive, showing that they do not act by the same pathway. These two inducers also differ in the kinetics of the induction of differentiation. Butyrate is able to produce very high differentiation levels (75% of the cells) in 48 h, with a very efficient inhibition of the proliferation. In contrast, the action of HMBA is much slower, since 5 days are required to reach the highest level of differentiated cells. Concomitantly, the inhibition of cell

Table 3: Level of stathmin phosphorylation during HMBA-induced differentiation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HMBA 2 h</th>
<th>HMBA 16 h</th>
<th>HMBA 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monophosphorylated stathmin level*</td>
<td>3%</td>
<td>3.5%</td>
<td>2.8%</td>
<td>3.7%</td>
</tr>
</tbody>
</table>

*Expressed in % of total phosphoprotein quantitated on the gels.

Fig. 5. Stathmin expression in lymphocytes and erythroid cells. The stathmin zone of the two-dimensional gels is shown, with unphosphorylated stathmin being shown by an arrow and phosphorylated forms by open arrows. The amount of unphosphorylated stathmin in ppm of the sum of all the spots detected on the gels is indicated below each panel. Stathmin is not detectable in mature RBC (data not shown; see in Ref. 30).
proliferation is much less pronounced and more transient with HMBA than with butyrate, except for the terminal cessation of proliferation.

Our data show that the increase in the stathmin level does not correlate with any proliferation-related index but rather with differentiation. The fact that similar levels are observed at 48 h of induction for HMBA-induced and butyrate-induced cells suggests that the increase in the stathmin level is not related to terminal differentiation (hemoglobin accumulation) but rather with earlier events in the differentiation process. This is further confirmed by the fact that stathmin does not seem to be present in significant amounts in mature RBC (23).

To further verify that this effect was not a peculiar feature of Friend cells, other experiments were carried out with the human erythroleukemic K562 cells. In this system, a strong correlation between the extent of erythroid differentiation and unphosphorylated stathmin expression is also observed. Here again, there is a strong inhibition of the proliferation of K562 cells when they are induced to differentiate in vitro, suggesting again that the increase in the stathmin level is correlated with the early phases of the erythroid differentiation.

In the case of hemin-treated K562 cells, the level of stathmin is lower than expected from differentiation alone. This could be explained by the fact that there is a true block of proliferation and not an inhibition as for butyrate. This massive block in proliferation could induce a decrease in stathmin expression compensating for the differentiation effect. In this case, the discrepancy between our results (showing a rather stable level of stathmin) and those described by Melhem et al. (24) may be linked either to the variable responsiveness of different K562 clones to hemin in terms of differentiation or to the fact that we measure protein levels in the cells, whereas Melhem et al. (24) assayed mRNA levels.

Further evidence in favor of a correlation between a high expression of the unphosphorylated form of stathmin and the erythroid differentiation process was provided by the in vitro erythropoiesis obtained from normal human blood cells. The levels of unphosphorylated stathmin were dramatically higher in cells engaged in the erythroid differentiation pathway than in mononuclear cells or lymphocytes, even if the latter were induced to proliferate with PHA. The levels reached for proerythroblasts and erythroblasts are close to those reached for transformed lymphocytes such as Jurkat cells. The control levels provided by the lymphocyte (normal, PHA-treated, and Jurkat) were in good agreement with the results described previously in the lymphocyte system with 35S labeling (3). However, it must be kept in mind that stathmin is not detected in mature RBC (23), showing that this increase in the stathmin level is not correlated either with terminal differentiation or hemoglobin accumulation, since the level is highest in proerythroblasts, which do not contain high levels of hemoglobin.

Such an implication for the unphosphorylated form of stathmin seems to be rather uncommon. In the classical model, the unphosphorylated form of stathmin is associated with cell proliferation (3, 11, 25), although this does not seem to be the case with immature T lymphocytes (14). In most of the cellular systems described to date, stathmin has been proposed to act through its phosphorylated forms, to conduct a signal into the cell (4, 14, 21), which may be associated with a shutdown of DNA synthesis (2, 12, 26). This phosphorylation event is generally dramatic, the ratio of the monophosphorylated to the unphosphorylated form climbing from 0.1 to 1.2 in the case of HL60 cells (2), and from 0.3 to 0.8 in the case of thymocytes (14). This massive and sustained phosphorylation is, therefore, easily detected, even with silver staining (14).

This classical pattern of high levels of unphosphorylated stathmin in fast-cycling cells and of massive and sustained phosphorylation is clearly not detected by silver staining in our erythroid differentiation systems. In the Friend cell system, the level of unphosphorylated stathmin increases from ~400 to ~700 ppm, although the cells undergo a transient inhibition of proliferation (at 16 h of induction), then further increases to ~1000 ppm and remains at this level, although the cells stop proliferating. Meanwhile, the level of the monophosphorylated form remains roughly constant at 95 ± 10 ppm.

This situation is also found in the normal erythropoiesis system. Unphosphorylated stathmin slightly decreases from the level observed in proliferating proerythroblasts (1400 ppm), to that in the mitotically-inactive erythroblasts (1000 ppm), but the level of the monophosphorylated forms remains rather constant, going from 120 to 100 ppm.

These observations may be biased by the fact that silver staining reveals steady-state levels of the protein, which may mask transient phosphorylation events. To test this hypothesis, we carried out a phosphorylation experiment with HMBA-induced Friend cells. The induction times were as described for silver-stained gels, and we added a point taken at the very beginning of HMBA induction to test for a very early event of the type detected with HL60 cells (2) or lymphocytes (12). The rather constant level of phosphorylated stathmin observed strongly suggests that such an early event does not take place in the Friend cell system and that the constant amount observed with silver staining is likely to reflect the state of the cell. However, we cannot rule out a very transient, but physiologically important, phosphorylation event at a critical, but still unknown, time in the differentiation process. However, on the whole, our results suggest an implication of the unphosphorylated form of stathmin in erythroid differentiation.

The difference in the levels of the various forms of stathmin between the erythroid lineage and, for example, the lymphoid lineage may be linked to important differences between these two differentiation pathways. In the lymphoid pathway, there are a number of steps at which the cells stop for selection, resulting in either cell death or clonal amplification. These include the rearrangement of the immunoglobulin or T-cell receptor genes, or the elimination of self-reacting clones. Even for mature but resting lymphocytes, such switches between proliferating and resting phases occur, for example, by antigen-driven stimulation. The lymphoid pathway can, therefore, be described as a “stop and go” series. In this case, the fast response provided by phosphorylation can be used by the cell to effect rapid regulation of stathmin functions.

Conversely, the erythroid pathway is a much more continuous process. There is a stimulation in the early phases by interleukin 3 and SCF to reach the CFU-E stage, but the later phases depend only on erythropoietin (27) and seem to be regulated exclusively by this factor. In this model, there is a much reduced need for rapid regulation, so that stathmin phosphorylation might be much less important than in other systems, with more importance being attached to the unphosphorylated form. The fact that both the unphosphorylated form (this work) and the phosphorylated forms (2, 4,
19) of stathmin are correlated with differentiation pathways suggests that stathmin may well be a relay integrating various signal transduction pathways by its phosphorylation as well as by the intrinsic role of its unphosphorylated form. This hypothesis has recently received strong support in the PC 12 system through the use of antisense oligos against stathmin, which block the response of these cells to NGF by decreasing the stathmin level (9). However, this work on PC 12 cells and the work on Cdc2 target site-deficient stathmin (8) put the emphasis on the importance of the phosphorylated forms of stathmin. The role of the unphosphorylated form, which may be important, for example, in the erythroid system, remains to be shown, and this may be accomplished by the use of antisense systems.

Materials and Methods

Cell Culture

Cell Line. Friend murine erythroleukemia cells were cultured in MEM supplemented with 10 mM HEPES buffer, nonessential amino acids, and 10% FCS. Cells were seeded at 10^4 cells/ml and harvested at 2-5 10^5 cells/ml. For induced differentiation, the cells were treated with 1 mM sodium butyrate (Aldrich) or 4 mM HMBA for 3 or 5 days, respectively, with one medium change after 3 days. K562 cells were cultured in RPMI 1640 supplemented with 10% FCS, seeded at 2-5 10^4 cells/ml, and harvested at 2-5 10^5 cells/ml. For induced differentiation, the cells were treated either with 1 mM sodium butyrate or with 0.1 mM hemin (predissolved at 4 mM in 1 N sodium hydroxide). In both cases the extent of erythroid differentiation was measured by the heme-induced benzidine reaction. Briefly, the cells were harvested, then washed and resuspended in PBS. For each volume of cell suspension, an equal volume of a solution containing 2 g/liter benzidine tetrahydrochloride and 1% (v/v) of 30% hydrogen peroxide was added. After 10 min, the cells were counted, and the proportion of blue cells (heme-containing) was recorded.

Normal Cells. Normal human peripheral blood cells collected from cytaphereses were first separated by a Ficoll Hypaque to eliminate RBC and granulocytes. The floating cells were then cultured as described by Fibach et al. (18), to induce erythroid differentiation. Briefly, the cells were first plated overnight in IMDM supplemented with 5% fetal calf serum to remove monocytes. The nonadherent cells were collected and seeded at 5 x 10^5 cells/ml in IMDM supplemented with 10% fetal calf serum, 1 μg/ml cyclosporin A, 2 ng/ml IL3, and 20 ng/ml SCF. Cells were cultured for 5 days in this medium. The nonadherent cells were harvested, washed, and resuspended in the same volume of IMDM supplemented with 30% fetal calf serum, 1 μg/ml cyclosporin A, 10^−6 m dexamethasone, and 1 unit/ml erythropoietin. The cells were cultured for 4 days in this medium. The nonadherent cells were collected and centrifuged into the medium and saved. The cell pellet was suspended in a small volume of culture medium and layered onto a cushion of 45% Percoll in PBS (d = 1.0585) and centrifuged at 2000 x g for 20 min. The upper layer was collected and diluted in PBS to recover the cells by centrifugation. At this stage, examination of the cell population by May-Grünwald-Giemsa gave the following composition: proerythroblasts, 76%; erythroblasts, 7%; lymphocytes, 12.5%; monocytes + macrophages, 3%; megacaryocytes, 1%. These cells were either collected for analysis or returned to the conditioned erythropoietin-containing medium for culture for an additional 3 days. After this time, the cell population had the following composition: proerythroblasts, 2.5%; basophilic erythroblasts, 13%; polychromatophilic erythroblasts, 19.5%; acidophilic erythroblasts, 56%; lymphocytes, 6%; and monocytes + macrophages, 3%. The cells were then collected for subsequent analyses.

The peripheral blood stem cells are composed almost exclusively of BFU-E with very few CFU-GM and virtually no CFU-E. Hence, by using appropriate culture conditions (18), the ordered erythropoietic differentiation steps starting from the BFU-E through CFU-E, proerythroblasts, and ultimate erythroid maturation may be obtained in 12 days by a two-step culture sequence. In the first culture, the BFU-E are induced to proliferate and mature into CFU-E by IL3 and SCF, while cyclosporin prevents the expansion of the lymphocytes and induces them to degenerate slowly. In the secondary culture, CFU-E are induced by erythropoietin to differentiate first into large proerythroblasts (4 days), which can be highly enriched by a Percoll separation step which removes most of the remaining lymphocytes (which still represent more than 75% of the cells prior to the Percoll separation). The additional 3 days of culture of the proerythroblast-enriched fraction in the presence of erythropoietin allows the final erythroid differentiation through basophilic, polychromatophilic, and acidophilic stages, the latter being postmitotic cells.

The lymphocytes from the same donors were collected from a small aliquot of the Ficoll-separated cells. Monocytes and macrophages were removed by adherence to plastic, and the lymphocytes were cultured in IMDM supplemented with 10% FCS. An aliquot of these cells was treated with 10 μg/ml phytohemagglutinin A for 3 days. At this stage, the cells were collected for further analyses.

Cell Cycle Analysis

The separation of the cells into the different phases of the cell cycle was analyzed by a double-labeling technique (15). The cells were incubated for 5 min with 5 μM BrdUrd, and the BrdUrd incorporated into the DNA was detected by an indirect immunofluorescence assay with a primary anti-BrdUrd antibody and a fluorescein-labeled secondary antibody. The total amount of DNA in the cell was detected by Hoechst 33258. Analysis of the cells according to these two fluorescences (fluorescein and Hoechst 33258) was carried out on a FACStar+ (Beckton Dickinson). The results were analyzed with the Procyt software, which gave the proportion of the cell population in the different phases of the cycle.

Two-dimensional Electrophoresis

The cell pellets, washed in PBS, were lysed in 9 mM urea, 3% 3-[3-(cholamidopropyl)dimethylamino]-1-propanesulfonate, 20 mM spermine base, and 40 mM DTT (~400 μl for a ~50-μl cell pellet). After lysis for 1 h at room temperature, nucleic acids were eliminated by ultracentrifugation (200,000 x g for 1 h at 20°C in a Kontron TFF 80.4 rotor). The concentration of protein was determined by a modified dye binding assay (28), using BSA dissolved in lysis buffer as a standard.

Two-dimensional electrophoresis was performed as described by Rabilloud et al. (29). Isoelectric focusing was performed using immobilized 4 to 8 pH gradients (IPG), and the protein was absorbed by the IPG strip during the rehy-
Chemiluminescent three water hydration buffer. A dose-response curve was constructed for stathmin by analyzing serial dilutions of the same extract separated by two-dimensional electrophoresis and silver stained in the same experiment. A linear response with a coefficient of 0.8 was found over the range encountered during the analytical experiments with the different cells and conditions tested. These experiments were carried out in triplicate for the cell lines and in duplicate for the normal cells, always starting from different cultures. The variation in the relative amounts of stathmin from one experiment to another was less than 10%.

For micropreparative purposes, 5 to 10 mg of whole-cell extracts from Friend cells were loaded onto the 6-mm wide IPG strip. The two-dimensional electrophoresis was carried out as for the analytical gel, but the gel was fixed for 30 min in 30% ethanol, 0.5% acetic acid, and stained for 30 min by 0.2% Coomassie Brilliant Blue R in the same solution, followed by destaining in repeated changes of 30% ethanol. The spots of interest were excised, further destained with 30% ethanol for 1 h at room temperature, washed twice with 20 ml of 100 mM ammonium hydrogen carbonate in 50% acetonitrile for 15 min at 30°C, washed again twice with 20 ml of the cleavage buffer (25 mM Tris-HCl, 1 mM EDTA, 10% acetonitrile, pH 8.5), and partially dehydrated in a Speed Vac concentrator. The gel slices were then rehydrated with 10 μl of the endoproteinase Lys-C solution (0.1 μg/μl). The digestion was carried out for 36 h at 37°C. Peptides were extracted from the acrylamide matrix in a two-step procedure (1) with 200 μl of a 100 mM ammonium hydrogen carbonate solution containing 10% acetonitrile and 0.1% hydrogenated Triton X-100 (2) with 200 μl of a 100 mM ammonium hydrogen carbonate solution containing 60% acetonitrile and 0.1% hydrogenated Triton X-100 [adapted from Fernandez et al. (31)]. Both extracts were pooled and then partially dehydrated in a Speed Vac concentrator. Peptides were separated by reverse phase microbore HPLC (column VYDAC C4; 2 x 150 mm). The fractions corresponding to the recorded peaks were manually collected, and the peptides present in these peaks were analyzed for amino acid sequence. Edman degradation was performed on an automated Applied Biosystems sequenator (477A) connected with an on-line phenylthiohydantoin-amino acid analyzer (Model 120A).

For blotting experiments, 1 mg protein was loaded onto a 4-mm wide IPG strip. After two-dimensional electrophoresis, the gel was blotted onto a polyvinylidene difluoride membrane in a semidy Randy setup with the aminocaproic acid discontinuous buffer system (32). SDS (0.05%) was added to the cathodic buffer and 30% ethanol, to the anodic buffer. The polyvinylidene difluoride blot was rinsed with water and stained overnight with colloidal gold (33). After three rinses in PBS (5 min each), the blot was saturated for 1 h with polyvinylpyrrolidone and Tween 20 (34), and the zone of interest was excised and probed with anti-stathmin antibody (a generous gift from André Sobel) in an indirect immunoassay. Detection was carried out with an Enhanced Chemiluminescent Assay.

Phosphorylation Experiments
Friend cells, either untreated or induced to differentiate with HMBA for times up to 46 h, were collected by centrifugation and rinsed twice in phosphate-depleted culture medium; an aliquot was incubated for 2 h in this medium supplemented with 10% FCS, 0.5 mM orthophosphate/m and 4 mM (when required). The labeled cells were collected and rinsed twice in Tris-buffered saline. They were then lysed in urea, and the extract was cleared by ultracentrifugation as described above. The 32P-labeled extracts obtained from 2 x 106 cells were loaded on two-dimensional gels together with 200 μg of unlabeled extracts from the same culture, i.e., induced to differentiate for the same time as the labeled extracts. The resulting two-dimensional gels were stained with silver and dried; then the radioactivity was detected and measured with a Molecular Dynamics Phosphorimager. The phosphorylated stathmin spots were identified by their position in the gels by comigration with the unlabeled extracts, for which the position of all the stathmin spots is known by the blotting experiments. The most abundant monophosphorylated form was quantified as a percentage of the total phosphoprotein, as described by Cooper et al. (12).

Acknowledgments
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