Activated Raf Induces the Hyperphosphorylation of Stathmin and the Reorganization of the Microtubule Network*

(Received for publication, March 31, 1998, and in revised form, May 28, 1998)

Josip Lovrić‡, Sascha Dammeier, Arnd Kieser, Harald Mischak§, and Walter Kolch¶

From the Institut für Klinische Molekularbiologie und Tumorgenetik der GSF, Marchioninistraße 25, D-81377 Munich, Germany

Raf kinases are regulators of cellular proliferation, transformation, differentiation, and apoptosis. To identify downstream targets of Raf-1 in vivo, we used NIH 3T3 fibroblasts expressing a Raf-1 kinase domain-estrogen receptor fusion protein (BXB-ER), whose activity can be acutely regulated by estrogen. Proteins differentially phosphorylated 20 min after BXB-ER activation in living cells were displayed by two-dimensional electrophoresis. The protein with the most prominent newly induced phosphorylation was identified as stathmin, a phosphorylation-sensitive regulator of microtubule dynamics. Stathmin is rapidly phosphorylated on two ERK phosphorylation sites (serines 25 and 38) upon BXB-ER activation. The mitogen-activated protein kinase/extracellular signal-regulated kinase-kinase (MEK) inhibitor PD98059 abolished this phosphorylation, demonstrating that stathmin is targeted by BXB-ER via the MEK/ERK pathway. Prolonged BXB-ER activation resulted in the accumulation of a stathmin phosphosomier with impaired microtubule-destabilizing activity. The appearance of this phosphorsomer after BXB-ER activation correlated with rearrangements in the microtubule network, resulting in the formation of long bundled microtubules extending toward the rim of the cells. Our results identify stathmin as a main target of the Raf/MEK/ERK kinase cascade in vivo and strongly suggest that ERK-mediated stathmin phosphorylation plays an important role for the microtubule reorganization induced by acute activation of Raf-1.

* This work was supported by Deutsche Forschungsgemeinschaft Grant MI 489/1-1 (to H. M.) and Grant KO 1492/3-1 (to W. K.). 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 1754 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-89-7099-517; Fax: 49-89-7099-500; E-mail: Lovric@gsf.de.
§ Present address: CRC-Beatson Laboratories, The Beatson Institute for Cancer Research, Garscube Estate, Glasgow G61 1BD, United Kingdom.
¶ Present address: Franz-Volhard Klinik at the Max-Delbrück-Center for Molecular Medicine, Böckergürtel 50, D-13125 Berlin, Germany.

† The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase-kinase; ERK, extracellular signal regulated kinase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MS, mass spectrometry; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
BXB-ER activation. Here, we describe the identification of stathmin as the most prominent immediate target of Raf activation in mouse fibroblasts. Stathmin destabilizes microtubules in living cells and its hyperphosphorylation is known to inhibit this destabilizing activity. BXB-ER activation immediately initiates the phosphorylation of stathmin on serine 25 and serine 38 via the activation of ERKs. This initial phosphorylation results in the appearance of stathmin forms phosphorylated to a higher stoichiometry, known to have an impaired microtubule-destabilizing activity. We further show that the appearance of hyperphosphorylated stathmin following BXB-ER activation correlates with the rearrangement of microtubular networks and the appearance of long bundled microtubules. These findings for the first time link activation of Raf kinase to specific changes in the cytoskeleton and identify stathmin as the responsible target.

MATERIALS AND METHODS

Cell Culture and Generation of Stable Lines—Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 5% bovine serum in humidified atmosphere with 5% CO2. Cells were a Raf-1 mutant rendered transforming by deletion of amino acids 26–303 (1). The BXB-ER expression vector was constructed following a similar strategy as described by Samuels et al. (21). To facilitate detection of the BXB-ER fusion protein, an oligonucleotide encoding the epitope of the influenza virus hemagglutinin-specific 12CA5 antibody plus a stop codon was added to the 3’-end of the estrogen receptor portion. The construct was cloned into the pBabe-puro expression vector and transfected into NIH 3T3 cells (ATCC). Several of the more than 100 puromycin (Sigma)-resistant cell clones were tested for the expression of BXB-ER by immunoprecipitation with a Raf-1 antibody followed by Western blotting with the 12CA5 monoclonal antibody. Seven clones with equal expression levels were pooled to yield 3T3BXB-ER cells, which were cultured in the presence of 4 µg/ml puromycin.

Immunoprecipitation, Immunocomplex Kinase Assays, and Western Blotting—Exponentially growing 3T3BXB-ER cells were stimulated with 5 µM estrogen with or without pretreatment for 30 min with 50 µM PD98059 (Life Technologies, Inc.) as indicated in the figure legend. For each time point, approximately 4 × 106 cells were lysed exactly as described previously (22), adjusted to equal protein levels, and immunoprecipitated exactly as described previously (22) with the following antibodies: 12CA5 for BXB-ER, anti-MEK-1 sc436 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and a 1:1 mixture of anti-ERK1 sc436 and anti-ERK2 sc093 (Santa Cruz Biotechnology). Kinase assays were performed as described by Hafen et al. (22) using as substrates 50 ng of recombinant histidine-tagged MEK-1 (His-MEK1) and 150 ng of kinases-inactive recombinant ERK1 (His-ERK1) (23) for BXB-ER, MEK-1 kinase assays, and 2 µg of MRJ (Life Technologies, Inc.) for ERK1/2 kinase assays. Kinase reactions were resolved by SDS-PAGE and Western blotting on polyvinylidene difluoride membranes (Millipore Corp.) as described (24). Western blots were routinely checked for equal amounts of immunoprecipitated kinases with a c-Raf-specific antibody (22) or with the antibodies used for immunoprecipitation prior to exposition on a phosphor imager (Fuji). Western blots were performed as described by Kolch et al. (24) using 10 mM CAPS (Sigma), 10% methanol, adjusted to pH 11 as blotting buffer and the ECL detection system (Amer sham Pharmacia Biotech).

Metabolic Labeling—Approximately 4 × 106 cells in 10-cm culture dishes were washed twice in medium lacking either phosphate or methionine and cysteine and further incubated for 2 h in this medium. Cells were labeled with 250 µCi [32P]orthophosphoric acid (A mer sham Pharmacia Biotech) or [35S]methionine/cysteine (ProMix, Amer sham Pharmacia Biotech) for a total of 40 min (32P label) or 3 h (35S label). Where indicated in the figure legends, cells were treated during the last 30 min of the labeling period with 50 µM PD98059 or carrier (MeSO4) and for the last 20 min with 5 µM estrogen or carrier (ethanol).

Two-dimensional Electrophoresis—Two-dimensional gel electrophoresis was performed as described by Gorg et al. (25) with the modifications introduced by Bjellqvist and co-workers (26). Briefly, cells were trypsinized and washed twice with phosphate-buffered saline and once in 4 mM Na3HPO4, 0.75 mM KH2PO4, 70 mM NaCl, 1.5 mM KCl, before lysis in 100 µl of sample buffer (11 mM urea, 4% CHAPS, 40 mM Tris, 1% dithioerythritol, 2.5 mM EDTA, 2.5 mM EGTA) per 10-µl cellular pellet, corresponding to approximately 2 × 106 cells. For preparative purposes, 100 µl of sample buffer were used per 40 µl of cellular pellet, and urea was additionally added to a final concentration of 10 M. DNA was sheared using QIAshredders (Qiagen) and removed by centrifugation at 100,000 × g for 50 min. Resolyte7M, pH 4–8 (BDH), was added to the supernatant to a final concentration of 0.5%, and isoelectric focusing was performed using the Phorma gel DryStrap kit (A mer sham Pharmacia Biotech). 100-µl samples were applied to the acidic and basic part of Immobiline strips with a nonlinear gradient pH 3.5–10 (A mer sham Pharmacia Biotech) using sample cup holders. Strips were reswollen in 10 mM urea, 0.15% dithioerythritol, 2% CHAPS, 2.5 mM EDTA, EGTA, 1% Resolyte7M, pH 4–8. A total of 100 kV-h was applied with several stepwise increases in voltage up to 3500 V. Second dimension was a standard SDS-PAGE using 12% gels (26). Apparent molecular weight and isoelectric point (pl) were determined by marker proteins (Bio-Rad, Sigma) separated on parallel processed gels.

Protein Analysis by HPLC-MS—Proteins were cut out from the gels and digested with sequencing grade trypsin (Promega) as recommended by the manufacturer. Resulting peptides were dissolved in buffer A and separated on a 300-µm inner diameter 25-cm length µRPC C2/C18 column (LC PACKINGS) with a flow rate of 5 µl/min. The gradient was 0–50% B for 0–180 min, 50–100% B for 180–270 min (A: 1.5 mM ammonium acetate, 0.15% formic acid; B: 1.5 mM ammonium acetate, 0.15% formic acid, 70% acetonitril). The HPLC was coupled via an ion spray inlet to an API 100 quadrupole mass spectrometer (Perkin-Elmer). Masses were determined in 0.1 steps over the m/z (mass/charge) range from 400 to 1500 atomic mass units in the positive charge detection modus with an orifice voltage of 40 V.

Immunofluorescence—Cells were seeded on Lab-Tak (Nunc) chamber slides 2 days prior to analysis. Indirect immunofluorescence was performed essentially as described (27) with some minor modifications. Cells were fixed in methanol at –20 °C. For blocking and all washing, phosphate-buffered saline containing 10% serum and 0.1% Triton X-100 was used. Cells were incubated with primary antibodies (anti-actin, anti-ß-tubulin; Boehringer Mannheim) at a concentration of 1 µg/ml for 1 h at room temperature. Secondary antibody was used in a 1:50 dilution (fluorescein isothiocyanate-conjugated goat anti-mouse IgG; DAKO).

RESULTS

Analysis of Targets for Activated Raf—The deletion of the regulatory domain of Raf-1 results in a constitutively activated kinase, BXB, with transforming properties (1, 28). The fusion of BXB to the hormone binding domain of the estrogen receptor renders the kinase activity hormone-dependent (21). Expression plasmids encoding such a fusion protein, termed BXB-ER, were stably introduced into NIH 3T3 cells. To avoid artifacts due to clonal variation, seven independent stable clones expressing equal levels of the fusion protein were pooled, and the resulting cells, 3T3BXB-ER, were used in all further experiments. These cells showed a robust induction of the BXB-ER kinase activity within minutes after the addition of estrogen, which was stable for several hours and slightly declined after 9 h but was still higher than in untreated proliferating cells (Fig. 1). The activity of MEK-1 and ERK1/2 followed the activity of BXB-ER throughout the time course. The rapid activation of MEK-1 and ERK1/2 could be reduced by adding the MEK inhibitor PD98059 (15, 16), whereas BXB-ER activity was not affected. BXB-ER activation resulted in the previously described effects such as morphological transformation (elongated shape, higher refractility) and block of the cell cycle in G1 phase (6, 7). These effects were detectable within 6–9 h of hormone addition and fully established after 16–20 h (data not shown). The parental NIH 3T3 cells showed no detectable changes in any of the tested parameters in response to estrogen (data not shown).

To detect targets of activated Raf, NIH 3T3 and 3T3BXB-ER cells were serum-starved overnight and metabolically labeled with [32P]orthophosphoric acid for 20 min prior to the addition of estrogen and harvested after an additional 20 min. Lysates were separated by two-dimensional electrophoresis, and phosphoprotein patterns were analyzed. From the nearly 2000 proteins detected by silver stain, more than 300 were phosphoryl-
shown are phosphor imager exposures of the blots from a representative labeled with \(^{32}\text{P}\)orthophosphoric acid for 20 min, and then treated with estrogen (cated on the described under “Materials and Methods,” with relevant substrates indicated on the kinase assays with the appropriate substrates were performed as de-

were split in three equal aliquots, which were immunoprecipitated (with or without prior addition of the MEK-1 inhibitor PD98059) and 

Exponentially growing 3T3BXB-ER cells were stimulated with estrogen

upon stimulation of BXB-ER only eight phosphoproteins showed reproducible increases in their intensities, ranging from 2- to 8-fold. In control experiments, additional assays were performed in which the MEK activation was inhibited by PD98059 prior to hormone addition. In these assays, only one phosphoprotein showed MEK-independent alterations after BXB-ER activation (data not shown). Hormone addition did not change the phosphoprotein patterns of NIH 3T3 control cells, demonstrating the specificity of the system. In this study, we will focus on the analysis of the Raf-regulated phosphoproteins RRPP2 and RRPP8, while the other BXB-ER targets are still under investigation.

Fig. 2 shows the results from one representative out of six independent experiments for two of the BXB-ER-regulated phosphoproteins designated RRPP2 and RRPP8. The phosphorylation of RRPP2 was increased by a factor of 8 within 20 min of BXB-ER activation in both serum-starved and exponentially growing cells (Fig. 2 and data not shown). RRPP8 could not be detected in NIH 3T3 cells and was exclusively observed in estrogen-stimulated 3T3BXB-ER cells. Since PD98059 completely blocked the BXB-ER induced hyperphosphorylation of RRPP2 and RRPP8, we conclude that they are not phosphorylated by BXB-ER directly, but rather as a result of the activation of MEKs or ERKs (compare Fig. 1). RRPP2 showed the strongest increase in intensity of all proteins analyzed and was therefore chosen for further analysis. Protein staining indicated that the amount of RRPP2 was less than 0.001% of the total cellular protein, while the RRPP8 protein was below the detection limit (data not shown).

Identification of RRPP2 as Stathmin—To identify RRPP2, eight preparative two-dimensional gels were run from a total of \(1 \times 10^{6}\) serum-starved 3T3BXB-ER cells stimulated for 20 min with estrogen. A total of approximately 800 ng of Coomassie-stained RRPP2 was excised from the gels and digested with trypsin. The resulting peptides were separated by HPLC and injected on-line into an electrospray mass spectrometer to determine their exact masses. The peptide masses were used to search for matching peptide mass fingerprints in the European Bioinformatics Institute nonredundant protein data base using the PeptideSearch software. The search identified several stathmin sequences from different species, with Pr22 (mouse stathmin) being the best candidate. Mouse, rat, and human stathmins yielded 10–13 matching peptides, while the next best scores of other unrelated proteins were 5 matches (data not shown). The identification was verified by the analysis of metastable fragment ions. These derive from predictable fragmentations of the peptides during ionization and measurement (29). Since fragmentations preferentially proceed from either the N terminus (y-fragments) or the C terminus (b-fragments), they allow considerable sequence determination of peptides (30). Fig. 3 shows a representative metastable ion analysis of two such peptides. A series of observed masses could be exactly matched to the masses expected from the staggered y fragmentation of peptides corresponding to the stathmin sequence. Several other peptides were analyzed in the same way (data not shown) to prove unambiguously that RRPP2 is indeed stathmin.

Analysis of the Phosphorylation State of RRPP2/Stathmin—Stathmin, also known as p19, Op18, prosolin, and oncprotein 18, is a cytosolic phosphoprotein that can be phosphorylated on four different sites \(\text{in vivo}\), namely Ser\(^{18}\), Ser\(^{25}\), Ser\(^{38}\), and Ser\(^{63}\) (31, 32). Therefore, it was of interest to determine which sites are phosphorylated following BXB-ER activation. This was addressed by searching for phosphorylated tryptic peptides in the mass spectrum of RRPP2. We found the masses of the single phosphorylated tryptic peptides encompassing residues 15–27 and 29–40 containing the phosphorylation sites Ser\(^{18}\)/25 and Ser\(^{38}\), respectively (Fig. 4). The phosphorylated peptides displayed the characteristic increase in mass due to the addition of a single phosphate group. No other phosphopeptides could be detected (data not shown).

To distinguish which of the possible \(\text{in vivo}\) phosphorylation...
sites on peptide 15–27 are actually phosphorylated, we analyzed the metastable fragment ions of the peptide. We could not find a single y-fragment ion mass that would correspond to the phosphorylation of serine 16 but found eight y-fragment ion masses that could only be generated when serine 25 was phosphorylated (Table I). These data clearly show that serine 25 and serine 38 of stathmin are phosphorylated in RRPP2.

To confirm and extend our mass spectrometrical analysis of the stathmin phosphorylation, several experiments with a stathmin-specific antibody were performed. Two-dimensional gels of 3T3BXB-ER cells metabolically labeled with [35S]methionine/cysteine were blotted and probed with an anti-stathmin antibody (33). The signals from the anti-stathmin antibody were overlaid and carefully aligned with the autoradiograph of the same blot (Fig. 5A). Three forms of stathmin could be detected by the stathmin antibody and assigned to [35S]methionine/cysteine-labeled proteins. The observed migration pattern of stathmin in the two-dimensional gel is similar to the pattern described previously (34). It is characteristic for several protein forms distinguished by the extent of phosphorylation. The least phosphorylated form occupies the most basic position and displays the lowest apparent molecular weight. Each phosphorylation causes a shift to the acidic part of the gel and reduces the electrophoretic mobility in the SDS-PAGE, resulting in an increased apparent molecular weight. In addition, the Western blot was aligned with a gel also prepared from [35S]methionine/cysteine-labeled cells, which was silver-stained, dried, and autoradiographed. This comparison allowed the assignment of the stathmin signals from the Western blot to the autoradiographed proteins in the gel (Fig. 5B, upper panel). Finally, the autoradiograph of the gel was aligned with the silver stain of the same gel to assign the observed stathmin forms to silver-stained proteins (Fig. 5B, lower panel). These experiments confirm the identification of RRPP2 as stathmin by mass spectrometry. In addition, they show that RRPP2 phosphorylated on serine 25 and serine 38 represents the 2-fold phosphorylated P2 form of stathmin, according to the nomenclature of Beretta and co-workers (32). The so-called N1 form corresponds to unphosphorylated stathmin (labeled by an asterisk).

Western blot was aligned with a gel also prepared from [35S]methionine/cysteine-labeled cells, which was silver-stained, dried, and autoradiographed. This comparison allowed the assignment of the stathmin signals from the Western blot to the autoradiographed proteins in the gel (Fig. 5B, upper panel). Finally, the autoradiograph of the gel was aligned with the silver stain of the same gel to assign the observed stathmin forms to silver-stained proteins (Fig. 5B, lower panel). These experiments confirm the identification of RRPP2 as stathmin by mass spectrometry. In addition, they show that RRPP2 phosphorylated on serine 25 and serine 38 represents the 2-fold phosphorylated P2 form of stathmin, according to the nomenclature of Beretta and co-workers (32). The so-called N1 form corresponds to unphosphorylated stathmin (labeled by an asterisk).

**Table I**

| y-fragments | expected fragment masses |
|-------------|-------------------------|
| GQAFAELLSPR | 1230.67                 |
| QAFAELLSPR  | 1173.95                 |
| AFELLSPR   | 1045.59                 |
| FELLSPR    | 974.55                  |
| EUILSPR    | 827.48                  |
| LILSPR     | 698.44                  |
| ILSPR      | 585.36                  |
| LSPPR      | 472.27                  |
| SPPR       | 359.19                  |

* Found as (2H<sup>+</sup>) ion.
** Found as y9 (−17) ion due to the loss of ammonia from the carboxy-terminal arginine (29).
for 3 h with \[^{35}\text{S}]\text{methionine}\) and stimulated with estrogen for the last 20 min. After lysis and two-dimensional separation, the gel was silver-stained, dried, and autoradiographed for 3 days. Stathmin forms were identified by aligning the autoradiograms of the blot shown in A with an asterisk of the stathmin forms derived from the Western blot and HPLC-MS analysis (RRP2; compare Fig. 3). For better comparison, the stathmin N1 form is labeled by an asterisk. The P3 form comigrates exactly with RRPP8 identified after in vivo labeling with \[^{32}\text{P}\]orthophosphoric acid (compare Fig. 2), indicating that RRPP8 represents a multiply phosphorylated form of stathmin. This shows that P3 is also present shortly after activation of BXB-ER. However, its abundance is too low to allow detection by Western blot prior to its accumulation 9 h after BXB-ER stimulation.

**Stathmin Hyperphosphorylation Correlates with Rearrangements of Microtubules**—Stathmin has recently been described as a regulator of microtubule dynamics, both in vitro and in vivo (36). Stathmin destabilizes microtubules and this destabilizing activity is reduced in vitro and in vivo by hyperphosphorylation of stathmin (37–41). Hyperphosphorylation of stathmin occurs in vivo prior to the onset of mitosis and is necessary to allow formation of stable microtubules to build up the mitotic spindle. However, rearrangement of the microtubular network also occurs in interphase cells in response to stimuli like serum, epidermal growth factor, insulin, and phorbol esters (42–45). Therefore, we analyzed whether the microtubules are also reorganized following BXB-ER activation.

Unstimulated NIH 3T3 cells and 3T3BXB-ER cells showed a similar fine tangled network of microtubules dispersed throughout the whole cell, excluding only the nucleus (Fig. 7). Estrogen addition resulted in changes of the microtubule organization in 3T3BXB-ER cells but not in control NIH 3T3 cells. The microtubules became concentrated in the middle of the cell and were increasingly bundled upon BXB-ER activation. This consistently resulted in a stronger overall signal in the immunofluorescence showing elongated microtubules. Part of the cytoplasm became consistently devoid of microtubules, and microtubules were organized in a more parallel fashion and not like a network as observed in unstimulated cells. These changes resemble the situation in serum-stimulated cells, where similar changes are observed within minutes after se-
Our results suggest that hyperphosphorylation of stathmin is a prerequisite for the stabilization of long bundled microtubules. Stathmin phosphorylation is a hierarchical process requiring at least two different kinases (47). It can be phosphorylated by Cdc2 and ERKs on serines 25 and 38, by calcium/calmodulin-dependent kinase IV on serine 16 and by cAMP-dependent protein kinase on both serines 16 and 63 (34, 47, 49–53). The phosphorylation of serines 25 and 38 does not seem to affect stathmin function directly, but it seems to be a prerequisite for the phosphorylation of serines 16 and 63 by unknown kinases in mitotic cells (47). Phosphorylation of either serine 16 or serine 63 in combination with serines 25 and 38 inhibits stathmin activity (40). The phosphorylation of both serines 16 and 63 is sufficient to inhibit the microtubule-destabilizing activity in interphase or mitotic cells (37).

Our data suggest that in interphase fibroblasts, Raf-induced ERKs are the physiological kinases that phosphorylate stathmin on serines 25 and 38. Activation of Raf and hence ERKs results in an strong increase of the 2-fold phosphorylated stathmin form P2 (corresponding to spot RRPP2 in Fig. 2). Resembling the situation in mitotic cells, the P2 form is primed for further hyperphosphorylation, leading to the appearance of the multiply phosphorylated P3 form. Autoradiographically, P3 (corresponding to spot RRPP8 in Fig. 2) is already detectable 20 min after BXB-ER activation and further accumulates to become visible on Western blots between 3 and 9 h (Fig. 6). P3 is phosphorylated on serine 25/38 and on either serine 16 or serine 63 (32). As the kinases phosphorylating serine 16 or serine 63 in vivo remain to be identified, it is unclear whether they are also regulated by activated Raf. The accumulation of P3 after BXB-ER activation correlates with a rearrangement of the microtubular network (compare Figs. 6 and 7). Since stathmin acts stoichiometrically (38, 46), it is not surprising that a marked rearrangement of the microtubules only becomes visible when a clearly detectable fraction of stathmin is in the P3 form. The two candidate kinases for serine 16 or serine 63 phosphorylation, cAMP-dependent protein kinase (37) and calcium/calmodulin-dependent kinase IV (49), are unlikely to be responsible for the hyperphosphorylation of the P2 form in our cell system. Overexpression of calcium/calmodulin-dependent kinase IV or cAMP-dependent protein kinase results in an increase in microtubules without changes in the way they are organized (37, 49). In contrast, our data show that activated Raf induces the rearrangement of microtubules, resulting in the transformation of the tangled network to long bundled microtubules. This difference may not only arise from different stoichiometry and kinetics of stathmin phosphorylation, but also from different influences of each kinase on other regulators of microtubular organization, such as microtubule-associated proteins and microtubule-associated motor proteins (54, 55). Microtubule-associated proteins are good substrates for ERKs, and phosphorylation weakens their stabilizing effects on microtubules (56).

Therefore, it is conceivable that ERKs acti-

**DISCUSSION**

In the present study, we have identified stathmin as a prominent downstream target of the Raf/MEK/ERK pathway in living fibroblasts. Since the approach by which stathmin was identified was not biased and detected substrate phosphorylation in intact cells, stathmin is most likely a physiological relevant downstream target of activated Raf.

Stathmin binds to heterodimeric tubulin and regulates microtubules in vitro and in vivo. Stathmin has been suggested to reduce the growth rate of microtubules (38, 46) or, alternatively, to enhance microtubule dynamics by increasing the frequency of catastrophes (i.e. the transition from growth to shrinkage) (36). There is consensus, however, that phosphorylation of stathmin inhibits its microtubule-destabilizing function. Whereas the overexpression of stathmin leads to the destabilization of microtubules in interphase cells, it does not affect the microtubules of mitotic spindles (39, 41). The loss of stathmin function correlates with the phosphorylation of serines 16, 25, 38, and 63 during mitosis (40, 41, 47). Overexpression of stathmin mutants, in which these phosphorylation sites were replaced by alanine in different combinations, results in mitotic arrest and endoreplication cycles, due to the lack of mitotic spindles. It is thus believed that the microtubule-destabilizing effect of endogenous stathmin must be suppressed by hyperphosphorylation in order to allow the formation of normal mitotic spindles and the undisturbed progression through mitosis (40, 41, 47, 48).

Stathmin phosphorylation is a hierarchical process requiring at least two different kinases (47). It can be phosphorylated by Cdc2 and ERKs on serines 25 and 38, by calcium/calmodulin-dependent kinase IV on serine 16 and by cAMP-dependent protein kinase on both serines 16 and 63 (34, 47, 49–53). The phosphorylation of serines 25 and 38 does not seem to affect stathmin function directly, but it seems to be a prerequisite for the phosphorylation of serines 16 and 63 by unknown kinases in mitotic cells (47). Phosphorylation of either serine 16 or serine 63 in combination with serines 25 and 38 inhibits stathmin activity (40). The phosphorylation of both serines 16 and 63 is sufficient to inhibit the microtubule-destabilizing activity in interphase or mitotic cells (37).

Our data suggest that in interphase fibroblasts, Raf-induced ERKs are the physiological kinases that phosphorylate stathmin on serines 25 and 38. Activation of Raf and hence ERKs results in an strong increase of the 2-fold phosphorylated stathmin form P2 (corresponding to spot RRPP2 in Fig. 2). Resembling the situation in mitotic cells, the P2 form is primed for further hyperphosphorylation, leading to the appearance of the multiply phosphorylated P3 form. Autoradiographically, P3 (corresponding to spot RRPP8 in Fig. 2) is already detectable 20 min after BXB-ER activation and further accumulates to become visible on Western blots between 3 and 9 h (Fig. 6). P3 is phosphorylated on serine 25/38 and on either serine 16 or serine 63 (32). As the kinases phosphorylating serine 16 or serine 63 in vivo remain to be identified, it is unclear whether they are also regulated by activated Raf. The accumulation of P3 after BXB-ER activation correlates with a rearrangement of the microtubular network (compare Figs. 6 and 7). Since stathmin acts stoichiometrically (38, 46), it is not surprising that a marked rearrangement of the microtubules only becomes visible when a clearly detectable fraction of stathmin is in the P3 form. The two candidate kinases for serine 16 or serine 63 phosphorylation, cAMP-dependent protein kinase (37) and calcium/calmodulin-dependent kinase IV (49), are unlikely to be responsible for the hyperphosphorylation of the P2 form in our cell system. Overexpression of calcium/calmodulin-dependent kinase IV or cAMP-dependent protein kinase results in an increase in microtubules without changes in the way they are organized (37, 49). In contrast, our data show that activated Raf induces the rearrangement of microtubules, resulting in the transformation of the tangled network to long bundled microtubules. This difference may not only arise from different stoichiometry and kinetics of stathmin phosphorylation, but also from different influences of each kinase on other regulators of microtubular organization, such as microtubule-associated proteins and microtubule-associated motor proteins (54, 55). Microtubule-associated proteins are good substrates for ERKs, and phosphorylation weakens their stabilizing effects on microtubules (56).
vated by oncogenic Raf generate a specific phenotype of microtubule architecture.

Rearrangements of the microtubular network including a transient depolymerization are observed in response to various stimuli such as serum, epidermal growth factor, insulin, and phorbol esters. It has been shown that transient microtubule depolymerization is not only necessary but may also be sufficient to initiate DNA synthesis in quiescent cells. In contrast, inhibition of microtubule depolymerization inhibits the mitogenic effects of thrombin and epidermal growth factor. Under our experimental conditions, BXB-ER induces the morphological transformation of the cells as well as a nearly complete block in the G1 phase of the cell cycle. This phenomenon was also observed by other investigators after strong activation of BXB-ER proteins and was attributed to the induction of the cyclin-dependent kinase inhibitor p21^{waf/cip}.

In our cell system, robust stathmin hyperphosphorylation and the appearance of long bundled microtubules correlate with the cell cycle arrest. Therefore, it is tempting to speculate that the stabilization of microtubules contributes to the G1 arrest induced by acutely activated Raf. This hypothesis is supported by the fact that NIH 3T3 cells transformed by stable overexpression of v-Raf derived from the murine sarcoma virus 3611 retrovirus show neither any significant alterations in their microtubular organization as compared with NIH 3T3 cells nor a growth arrest.

The involvement of microtubule stabilization in the Raf-induced growth arrest appears plausible considering that major ERK targets are linked with the microtubular compartment. ERKs reside within the microtubular network, and about half of the ERK activity induced by serum stimulation is associated with microtubules.

The regulation of microtubules by the Raf/MEK/ERK pathway also may have consequences for progression through mi-
Raf Regulates Microtubules through Stathmin Phosphorylation

We and others have shown that endogenous Raf-1 as well as some transforming forms of Raf are activated during the G2 and M phases of the cell cycle in a Ras-independent manner (61, 62). This could conceivably contribute to proper spindle formation due to stabilization of microtubules by stathmin hyperphosphorylation. Our preliminary results indicate that stathmin is indeed hyperphosphorylated within minutes upon BXR-ER activation in fibroblasts enriched at the S/G2 border. Under these circumstances induction of the P3 form is stronger than in cycling or serum-starved cells (data not shown). This may indicate that the second kinase system responsible for phosphorylation of serine 16/63 is already active in later stages of the cell cycle (47).

In summary, our data identify stathmin as the protein whose phosphorylation was most pronounced in response to acute Raf activation. This suggests that stathmin is an important target of the Raf/MEK/ERK pathway and is fully concordant with an important role for stathmin phosphorylation in the reorganization of microtubules induced by activated Raf.

Acknowledgments—We thank Dr. André Sobel for the stathmin antagonist.

REFERENCES

1. Heidecker, G., Huleihel, M., Cleveland, J. L., Kolch, W., Beck, T. W., Lloyd, P., and Rapp, U. R. (1990) Mol. Cell. Biol. 10, 2503–2512
2. Avruch, J., Zhang, X. P., and Kyriakis, J. M. (1994) Trends Biochem. Sci. 19, 279–283
3. Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U. R. (1991) Nature 349, 426–429
4. Samuels, M. L., and McMahon, M. (1994) Mol. Cell. Biol. 14, 7855–7866
5. Pumiglia, K. M., and Decker, S. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 448–452
6. Sewing, A., Wiseman, B., Lloyd, A. C., and Land, H. (1997) Mol. Cell. Biol. 17, 5588–5597
7. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997) Mol. Cell. Biol. 17, 5598–5611
8. Weisssinger, E. M., Eiesser, G., Grammer, C., Fackler, S., Haefner, B., Yoon, L. S., Liu, S., Bazavov, A., Sediivy, J. M., Mischaik, H., and Kolch, W. (1997) Mol. Cell. Biol. 17, 3229–3244
9. Robinson, J. M., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
10. Cobb, M. H., Xu, S., Hepler, J. E., Hutchison, M., Frost, J., and Robbins, J. D. (1994) Cell Mol. Biol. Res. 20, 253–256
11. Goodnight, J. A., Mischak, H., Kolch, W., and Mushinski, J. F. (1995) EMBO J. 14, 197–198
12. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494
13. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Robbins, J. D. (1994) Oncogene 14, 697–704
14. Weymann, C. M., Ramocki, M. B., Taparowsky, E. J., and Wolfman, A. (1997) Oncogene 14, 4815–4824
15. Gallego, C., Gupta, S. K., Heasley, L. E., Qian, N. X., and Johnson, G. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7355–7359
16. Kuo, W. L., Abe, M., Hibee, J., Eves, E. M., McCarthy, S. A., Yan, M., Templeton, D. J., McMahon, M., and Rosner, M. R. (1996) Mol. Cell. Biol. 16, 1458–1470
Activated Raf Induces the Hyperphosphorylation of Stathmin and the Reorganization of the Microtubule Network
Josip Lovric, Sascha Dammeier, Arnd Kieser, Harald Mischak and Walter Kolch

*J. Biol. Chem. 1998, 273:22848-22855.*