Mitotic Raf-1 Is Stimulated Independently of Ras and Is Active in the Cytoplasm*

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Raf-1 is a Ser/Thr protein kinase that is involved in regulation of proliferation, differentiation, and apoptosis. Recently, we and others showed that Raf-1 is not only activated in mitogenic pathways leading to cell cycle entry but also during mitosis. Transient expression studies in COS cells now demonstrate that, in contrast to growth factor-dependent activation of Raf-1, mitotic activation of Raf-1 is Ras-independent. Dominant negative RasS17N does not interfere with mitotic activation of Raf-1, whereas epidermal growth factor-dependent stimulation of Raf-1 is inhibited. In addition, the Raf-1 mutant Raf1T89L, which cannot bind to activated Ras, is still stimulated in mitotic cells. Mitotic activation of Raf-1 seems to be partially dependent on tyrosine phosphorylation since the kinase activity of the Raf-1 mutant Raf1T89L, which can no longer be activated by Src, is reduced in mitotic cells. Surprisingly, cell fractionation experiments showed that mitotic-activated Raf-1 is predominantly located in the cytoplasm in contrast to the mitogen-activated Raf-1 that is bound to the plasma membrane. In addition, mitotic activation of Raf-1 does not lead to stimulation of the mitogen-activated protein kinase kinase (MAPKK or MEK) and the extracellular signal-regulated protein kinase (ERK). These data demonstrate that in mitotic cells a Ras-independent mechanism results in a cytoplasmic active Raf-1 kinase which does not signal via the MEK/ERK pathway. These data demonstrate that in mitotic cells a Ras-independent mechanism results in a cytoplasmic active Raf-1 kinase which does not signal via the MEK/ERK pathway.

The Ser/Thr protein kinase Raf-1 is a key regulator in transmission of mitogenic and developmental signals through growth factor receptor tyrosine kinases and non-receptor tyrosine kinases (1,2). Activation of receptor tyrosine kinases stimulates the small GTP-binding protein Ras, which results in activation of a protein kinase cascade headed by Raf-1 (3,4). Cytoplasmic inactive Raf-1 directly interacts with activated Ras and is thereby recruited to the plasma membrane where further activation steps take place (5–7). Activated Raf-1 phosphorylates and stimulates MEK, which in turn activates ERK1 and ERK2, members of the family of MAP kinases. ERKs are involved in regulation of gene expression and protein biosynthesis (8). Under certain conditions Raf-1 can stimulate AP-1 activity without measurable activation of ERKs (9). Recently, we were able to demonstrate that the avian homologue of Raf-1, c-Mil, can directly interact with c-Jun and phosphorylate c-Jun independently of MAPKs (10).

Raf-1 activity is regulated by protein-protein interactions and by phosphorylation events. Direct interaction between activated Ras and two N-terminal regions of Raf-1 leads to translocation of the cytoplasmic inactive Raf-1 to the plasma membrane (11–13). Proteins of the 14-3-3 family interact with different regions of Raf-1 (14,15). 14-3-3 binds to the N terminus of Raf-1 as a negative regulator and is displaced by activated Ras (16,17). The interaction between 14-3-3 and the C terminus of Raf-1 is independent of the activation status of Raf-1, and mutations at the C terminus of Raf-1, which lead to loss of binding of 14-3-3, abolish its kinase activity (18). Besides protein-protein interactions Raf-1 activity is regulated by phosphorylation events. Growth factor-dependent activation of Raf-1 results in an increase of phosphorylation mainly at Ser and Thr residues of Raf-1 and in stimulation of Raf-1 activity (19). Tyrosine kinases of the Src family can interact with Raf-1 and can phosphorylate two neighboring tyrosine residues at the C terminus of Raf-1, which results in activation of Raf-1 (20,21). Tyrosine phosphorylation of Raf-1 by non-receptor tyrosine kinases is a major event in stimulation of T cells with cytokines such as interleukin-2 (22). Tyrosine phosphorylation also seems to play an essential role in the growth factor-dependent activation of Raf-1, although in this case phosphorylated tyrosine residues are difficult to detect (23). In addition, it has been shown that oncogenic forms of Ras and Src can synergize in phosphorylation and activation of Raf-1 (24–26). Raf-1 kinase activity is also under the control of feedback mechanisms. Feedback regulation of Raf-1 involves MEK and ERK, but while the negative feedback mechanism depends on Ras (27,28) the positive one is independent of Ras and Src (29).

Recently, we and others could demonstrate that Raf-1 is not only stimulated upon mitogenic activation of cells leading to cell cycle entry but also during mitosis (30–32). In fibroblast cells this mitotic activation of Raf-1 correlates with an increase in phosphorylation mainly at Ser residues (31,32). Moreover, it has been shown that in mitotically arrested T cells the Src-like kinase Lck can bind to Raf-1 and can increase its kinase activity (33). In the present study we further analyzed the mechanism of mitotic activation of Raf-1 in fibroblast cells. The data presented here demonstrate that mitotic activation of Raf-1 is independent of Ras. Furthermore, mitotic activation of Raf-1 results in a predominantly cytoplasmic active Raf-1. Moreover, activated Raf-1 does not lead to stimulation of the MEK/ERK pathway in mitotic cells, indicating that mitotic Raf-1 uses another signaling pathway.

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1 The abbreviations used are: MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; EGFr, epidermal growth factor; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RIPA, radiolmmunoprecipitation buffer; FACS, fluorescein-activated cell sorter.
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EXPERIMENTAL PROCEDURES

DNA Constructs and Antibodies—The plasmids pcDNA3-FLAG-Rafwt, pcDNA3FLAG-RafR89L (exchange of Arg89 to Leu) and pcDNA3FLAG-RafY340/341FP (exchange of Tyr340/341 to Phe) have been described recently (29). The cDNAs coding for oncogenic RasG12V (exchange of Gln12 to Val) and for dominant negative RasS17N (exchange of Ser17 to Asn) were a gift from Dr. A. Wittinghofer (34, 35) and were subcloned into pCIus (16). pGEX-MEK K97A (exchange of Lys97 to Ala) coding for a kinase-inactive MEK was obtained from Dr. R. L. Erikson and pSLX-SrcY527F (exchange of Tyr527 to Phe) coding for activated Src was kindly supplied by Dr. T. Hunter (36). Anti-FLAG monoclonal antibody M2 was obtained from Eastman Kodak Co. and anti-active ERK antibody from Promega.

Transient Transfection and Extract Preparation—COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin at 37 °C and 5% CO2. Cells were transiently transfected using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s protocol. 8–10 h after transfection the medium was changed to normal cultivation medium. After an additional 12 h cells were split 1:3 and incubated for a further 8 h. Then cells were treated with 0.1 µg/ml nocodazole (mitotic cells) without nocodazole (exponentially growing cells) for 16 h. Exponentially growing cells were washed two times with ice-cold PBS and lysed in RIPA (10% glycerol, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM dithiorethiol) with a mixture of protease and phosphatase inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate). Mitotic cells were collected by gentle shake-off from nocodazole-treated plates, washed with PBS, and lysed in RIPA.

In Vitro Raf Kinase Assay—Protein concentration of RIPA lysates was determined by Bradford assay (Bio-Rad), and equal protein amounts were incubated with anti-FLAG antibody to immunoprecipitate Raf-GST-tagged Raf proteins. Immunoprecipitates were washed seven times with RIPA and three times with kinase buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MnCl2, 2 mM dithiorethiol, 25 mM β-glycerophosphate, 0.1 mM Na3VO4, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride). Kinase assay was performed in a total reaction volume of 50 µl containing 10 µl nonradioiodinated ATP, 10 µl of [γ-32P]ATP (Amersham Pharmacia Biotech) and 1 µg of recombinant kinase-inactive GST-MEK K97A for 20 min at 30 °C. The reaction was stopped by addition of 4X SDS sample buffer, boiled, and resolved by SDS-polyacrylamide gel electrophoresis (8%). Proteins were blotted on nitrocellulose membranes that were dried, and the radioactivity incorporated into the substrate was evaluated by a PhosphorImager (Molecular Dynamics). The amount of immunoprecipitated Raf proteins was compared by probing the membranes with anti-FLAG antibody followed by detection with detection phosphorimaging systems (Molecular Dynamics). The purification of the recombinant kinase-inactive GST-MEK K97A used as substrate for Raf-1 has been described elsewhere (29).

Cell Fractionation—Cells were washed with PBS and lysed in hypotonic lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM MgCl2, 1 mM dithiorethiol, 0.2 mM EDTA) supplemented with a mixture of phosphatase and protease inhibitors as described above. After incubation for 30 min on ice cells were homogenized with 80 strokes in a tight fitting Dounce homogenizer. Nuclei and cellular debris were removed by two low speed centrifugation steps (1200 × g for 10 min and 5000 × g for 10 min). After the second centrifugation step, the post-nuclear supernatants were adjusted to the same amount of protein followed by high speed centrifugation (100,000 × g for 30 min). The P100 fraction, which is obtained after ultracentrifugation, corresponds to whole cell membranes. If we would take for the Raf immunoprecipitation the same amount of protein from the cytosolic fraction S100 this would correspond to a small part of whole cell cytosol. Therefore, we decided to measure the Raf amount and activity in whole cell membranes and the whole cytosol. The high speed supernatant representing the cytoplasmic fraction S110 was adjusted to 1:1 RIPA and the pellet corresponding to the fraction P100 was resuspended in 1:1 RIPA. Both fractions were precleared with protein G-Sepharose beads, and immunoprecipitations were performed for 2 h at 4 °C with anti-FLAG antibody.

FACS Analysis—Cell cycle distribution experiments were performed with FACSCalibur flow cytometer (Becton Dickinson). A total 5 × 106 to 1 × 107 cells were pelleted by low speed centrifugation and resuspended in 200 µl of PBS. 2 ml of ice-cold 70% ethanol, 30% PBS solution were added. The cell suspension was incubated overnight at 4 °C. The fixed cells were harvested by centrifugation, resuspended in 800 µl of PBS, and checked microscopically. The cell suspension was mixed with 100 µl of RNase A (1 mg/ml) and 100 µl of propidium iodide (400 µg/ml), and incubated in the dark at 37 °C for 30 min. The ethanol-fixed and propidium iodide-stained cells were analyzed by FACS for their DNA content.

Detection of Activated ERK by Immunoblotting—Transfected cells were split and incubated for 8 h. They were then treated without or with nocodazole for 16 h or were serum-starved and stimulated with 100 ng/ml EGF (Sigma). Cells were lysed in RIPA, subjected to SDS-polyacrylamide gel electrophoresis (10%), and blotted to nitrocellulose filters. Blots were probed with the antibodies anti-ACTIVE ERK (Promega) to detect the activated forms of ERK or with anti-ERK C-14 (Santa Cruz Biotechnology) to detect the total amount of ERK. Then, the ERK proteins were visualized using the ECL detection system (Amersham).

RESULTS

Raf-1 Is Activated in Mitotic Cells—In order to analyze the mechanism of Raf-1 activation in mitosis, COS7 cells were arrested in the G2/M phase of the cell cycle by treatment with nocodazole. For control of synchronization the DNA content of mitotically arrested cells that detach from the cell culture dish was compared with that of exponentially growing untreated cells by flow cytometry analysis. Treatment of cells with nocodazole for 16 h resulted in an enrichment of cells in G2/M phase to approximately 90% compared with 15% in exponentially growing cells (Fig. 1, a and b). Removal of nocodazole resulted in release of the reversible G2/M block, and as early as 2 h after a change to medium lacking nocodazole the cells showed a cell cycle distribution of exponentially growing cells (Fig. 1d). To test whether ectopic expression of Raf-1 has an effect on cell cycle distribution, a Raf-1 cell line that expresses Raf-1 under a tetracycline-inducible promoter was analyzed. The induction of Raf-1 wild-type expression did not result in a significant difference in the cell cycle distribution of exponentially growing cells or mitotically arrested cells compared with uninduced cells (data not shown).

In order to compare Raf-1 kinase activity of mitotic cells and exponentially growing cells a FLAG-tagged Raf-1 protein was expressed in COS7 cells. Transient transfection resulted in an at least a 10-fold overexpression of FLAG-Raf-1 compared with exponentially growing Raf-1 (data not shown). Raf-1 was immunoprecipitated with αFLAG antibody, and in vitro kinase assays were performed using recombinant kinase-defective GST-MEK as a Raf-specific substrate (Fig. 2, upper panel). The amount of Raf-1 bound to the antibody was controlled by immunoblotting with the αFLAG antibody (Fig. 2, middle panel). Treatment of cells expressing Raf-1 wild-type (Rafwt) with nocodazole resulted in hyperphosphorylation as indicated by the reduced electrophoretic mobility of Raf-1 and in a 6-fold increase of its kinase activity compared with that of exponentially growing untreated cells (Fig. 2, lanes 1 and 2). As expected the kinase-defective Raf mutant RafK97S was used as a control showed no phosphorylation of GST-MEK (lanes 3 and 4). Interestingly, as early as 1 h after removal of nocodazole the Raf-1 kinase activity was reduced to the level of untreated control cells, even though an electrophoretic mobility shift of Raf-1 was still visible (lane 5). Thus Raf-1 kinase activity and hyperphosphorylation did not correlate under these conditions. Similar results for mitotic activation of Raf-1 were also obtained using Raf-1 cells that inducibly expressed Raf-1 (data not shown).

To demonstrate that mitotically activated Raf-1 did not only result in phosphorylation of MEK but also in its activation in vitro, coupled kinase assays were performed (37, 38). Immunecomplexed Raf-1 was incubated with wild-type GST-MEK and kinase-defective His-tagged ERK2 as a substrate specific for MEK. Also in this assay treatment of cells with nocodazole...
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Raf-1 was reduced by expression of RafNT (Fig. 3, lane 12) (Fig. 3, activation, did not interfere with the mitotic activation of Raf-1. In the growth factor-dependent pathway, Mitotic Activation—

mechanisms are involved in mitotic activation of Raf-1. Interaction between Ras and Raf-1 nor other Ras-dependent

Taken together these data demonstrate that neither direct

interaction with the cytoplasmic inactive Raf-1 protein, which thereby is translocated to the plasma membrane where further activation steps take place. To study the role of Ras in mitotic activation of Raf-1 the effect of the dominant negative Ras mutant RasS17N on Raf-1 kinase activity was analyzed. Fold activation was determined by correction for the amount of Raf-1 protein present in the different immune complexes. RasS17N did not interfere with mitotic activation of Raf-1, whereas it blocked EGF-induced stimulation of Raf-1 (Fig. 3, compare lanes 3 and 6 and lanes 2 and 5). A further evidence that Raf/Ras interaction does not play a role in mitotic activation of Raf-1 was obtained by using the Raf mutant RafR89L, which cannot physically interact with activated Ras. The kinase activity of the Raf mutant RafR89L was elevated in mitotic cells to similar levels like the Raf-1 wild-type and this nase activity of the Raf mutant RafR89L was elevated in mitotic cells prepared by treatment of cells with 0.1 μg/ml nocodazole for 16 h and collected by mechanical shake-off from the plate; c and d, cells were prepared as in b except that mitotic cells were washed and replated in drug-free medium for 1 and 2 h, respectively. Cells were stained with propidium iodide, and the cellular DNA content was analyzed by laser flow cytometry (see “Experimental Procedures”). Abscissa, DNA content; ordinate, cell number.

resulted in a 6–8-fold activation of Raf-1 compared with Raf-1 obtained from interphase cells (data not shown).

Mitotic Activation of Raf-1 Is Independent of Ras—The best characterized upstream activator of Raf-1 is Ras. Activated Ras directly interacts with the cytoplasmic inactive Raf-1 protein, which thereby is translocated to the plasma membrane where further activation steps take place. To study the role of Ras in mitotic activation of Raf-1 the effect of the dominant negative Ras mutant RasS17N on Raf-1 kinase activity was analyzed. Fold activation was determined by correction for the amount of Raf-1 protein present in the different immune complexes. RasS17N did not interfere with mitotic activation of Raf-1, whereas it blocked EGF-induced stimulation of Raf-1 (Fig. 3, compare lanes 3 and 6 and lanes 2 and 5). A further evidence that Raf/Ras interaction does not play a role in mitotic activation of Raf-1 was obtained by using the Raf mutant RafR89L, which cannot physically interact with activated Ras. The kinase activity of the Raf mutant RafR89L was elevated in mitotic cells to similar levels like the Raf-1 wild-type and this nase activity of the Raf mutant RafR89L, which cannot physically interact with activated Ras. The kinase activity of the Raf mutant RafR89L was elevated in mitotic cells to similar levels like the Raf-1 wild-type and this activation was not inhibited by coexpressed RasS17N (Fig. 3, lanes 3, 5 and 10). Furthermore, coexpression of the N terminus of Raf-1 (RafNT), which blocks the Ras-dependent Raf-1 activation, did not interfere with the mitotic activation of Raf-1 (Fig. 3, lane 13). In contrast, the EGF-dependent activation of Raf-1 was reduced by expression of RafNT (Fig. 3, lane 12). Taken together these data demonstrate that neither direct interaction between Ras and Raf-1 nor other Ras-dependent mechanisms are involved in mitotic activation of Raf-1.

Tyrosine Residues 340/341 of Raf-1 May Play a Role in Its Mitotic Activation—In the growth factor-dependent pathway Ras synergizes with Src and Src-like kinases in activation of Raf. Recently it has been published that in mitotic T cells the tyrosine protein kinase Lck can activate Raf-1 (33). Therefore we tested whether tyrosine phosphorylation also plays a role in mitotic activation of Raf-1 in the fibroblast-like COS7 cells used in this study. The Raf mutant RafYY340/341FF in which the two neighboring tyrosine residues are changed to phenylalanines cannot be activated by tyrosine kinases of the Src family (20) (Fig. 4, compare lanes 5 and 7). RafYY340/341FF showed a reduced kinase activity in mitotic cells compared with Raf-1 wild-type (Fig. 4, lanes 2 and 6). Therefore, these two tyrosine residues may play some role in mitotic activation of Raf-1. However, the weak but significant mitotic activation of RafYY340/341FF suggests that a mechanism independent of the tyrosine residues also seems to be involved (Fig. 4, lanes 5 and 6). As expected coexpression of activated Src led to further activation of Raf wild-type but not of RafYY340/341YY, because Src-dependent activation of Raf-1 depends on the tyrosine residues 340/341 (Fig. 4, compare lane 2 with 4 and lane 6 with 8). To detect tyrosine phosphorylation of mitotic-activated Raf-1, phosphoamino acid analyses were performed. However, only serine phosphorylation and not tyrosine phosphorylation could be detected in mitotic activated Raf-1 (data not shown). This suggests that although the two tyrosine residues 340 and 341 seem to be involved in mitotic activation of Raf-1 only a very small subpopulation of Raf-1 is phosphorylated on tyrosine residues, which is lower than the detection limit. Another explanation would be that phosphorylation of these tyrosine residues are essential for initial activation of Raf-1 but not for the maintenance of Raf-1 activity in mitotic cells.

Mitotic Raf-1 Is Active in the Cytoplasm—Most mechanisms known to be responsible for activation of Raf-1 involve translocation of the cytoplasmic inactive Raf-1 to the plasma membrane and result in a membrane-bound active Raf-1 (8). Deactivation of Raf-1 leads to the return of the inactive Raf-1 protein to the cytoplasm (39).

Since mitotic activation of Raf-1 was independent of Ras, the cellular localization of active Raf-1 in mitotic cells was investigated. For this, cell fractionation experiments were performed. Cells were lysed in hypotonic buffer and low and high speed centrifugation was performed to obtain a membrane fraction (P100) and a cytoplasmic fraction (S100). Protein
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**FIG. 2. Raf-1 is activated in mitotic cells.** COS7 cells transfected with FLAG-Rafwt or the kinase defective mutant FLAG-RafK375E were either treated without (-) or with (+) 0.1 μg/ml nocodazole. In one case mitotically arrested cells were washed and replated in nocodazole-free medium for 1 h (-1h). In vitro Raf-1 kinase activity was measured as described under “Experimental Procedures” using kinase inactive recombinant GST-MEK as a substrate (upper panel). The amount of immunoprecipitated Raf protein was detected by Western blotting (middle panel). Raf-dependent GST-MEK phosphorylation was normalized for immunoprecipitated Raf-1 protein content and quantified with PhosphorImager and Image Quant software. Values are means ± S.D.

amounts of the lysates from cells untreated and treated with nocodazole were matched before high speed centrifugation. The cytoplasmic fraction and the membrane fraction were immunoprecipitated with an αFLAG antibody, and the amount of immunoprecipitated Raf-1 was detected by immunoblotting (Fig. 5, lower panel). The corresponding membrane and cytoplasmic fractions of nocodazole-treated and untreated cells contain similar amounts of Raf-1 protein (e.g. Fig. 5, lanes 1 and 3 and lanes 2 and 4). Raf-1 kinase activity was determined by in vitro kinase assay using GST-MEK as substrate (Fig. 5, upper panel). In cells overexpressing Raf-1 wild-type the amount of Raf-1 protein was about three times higher in the cytoplasmic versus the membrane fraction (Fig. 5, lower panel, lanes 2 and 4 versus lanes 1 and 3). However, the mitotic Raf-1 kinase activity was 11-fold increased in the cytoplasmic fraction and 6-fold increased in the membrane fraction compared with the corresponding Raf-1 kinase activity in exponentially growing cells (Fig. 5, upper panel, compare lanes 2 and 4 and lanes 1 and 3). As expected coexpression of Raf wild-type with oncogenic RasG12V resulted in translocation of Raf-1 to the plasma membrane correlating with a strong increase of Raf-1 kinase activity predominantly in the membrane fraction of exponentially growing cells (lanes 5 and 6). Overexpression of activated RasG12V in nocodazole-treated cells also resulted in membrane-bound active Raf-1 (lanes 7 and 8), implying that the activated Ras is overriding the otherwise Ras-independent mitotic activation of Raf-1. Next, the cellular localization of activated Raf-1 upon a Ras-dependent stimulation mechanism without overexpression of Ras was analyzed. For this, COS7 cells were treated with EGF, which activates Raf-1 in a Ras-dependent manner. EGF-mediated stimulation of Raf-1 resulted in an activated Raf-1 predominantly located at the plasma membrane (compare lanes 9 and 10), which clearly differed from the predominantly cytoplasmic location of mitotically activated Raf-1 (lane 4). Further evidence that mitotic activation of Raf-1 is independent of Raf-1 and resulted in a cytoplasmic active Raf-1 was obtained using the Raf mutant RafR89L. This Raf-1 mutant, which cannot interact with activated Ras, behaved similarly to Raf-1 wild-type and resulted in a cytoplasmic active mitotic Raf-1 (lane 14). Coexpression of RasG12V did not interfere with cellular distribution and activation of RafR89L in mitotic cells as well as in exponentially growing cells (lanes 15–18). Taken together, these data demonstrate that, in contrast to the mitogenic activation mechanisms of Raf-1, mitotic activation of Raf-1 is independent of Ras and results in a cytoplasmic active Raf-1.

**Activation of Raf-1 in Mitotic Cells Does Not Correlate with ERK Activity**—As shown in Fig. 2 the increase in Raf-1 kinase activity was similar after treatment of cells with nocodazole or EGF. In the mitogenic pathway Raf-1 signals through MEK to ERK. To test whether the Raf/MEK/ERK pathway was also stimulated in mitotic cells, ERK activity was analyzed in nocodazole-treated cells by immunoblotting using an ERK antibody that detects only the activated forms of ERK1 and ERK2. The total amount of ERK was similar in the different samples as controlled with an antibody recognizing the active as well as the inactive forms of ERK1 and ERK2 (data not shown). In mitotic cells overexpressing Rafwt no activated form of ERK was detectable (Fig. 6, lane 4). In contrast, in cells stimulated with EGF, ERK was strongly activated (lane 2), and even in starved and exponentially growing cells a low level of activated ERK was visible (lanes 1 and 3). Similar results were obtained with untransfected cells (data not shown). Ectopic expression of an epitope-tagged ERK2 resulted in a further increase of ERK activity in EGF-stimulated cells (lane 6), but also under these conditions no active ERK was detectable in mitotic cells (lane 8). In addition, no MEK1 activity could be detected in mitotic cells, whereas MEK1 activity was strongly increased after stimulation of cells with EGF (data not shown). These data demonstrate that mitotic activation of Raf-1 does not correlate with activation of the MEK/ERK pathway, suggesting that Raf-1 may use substrates other than MEK in mitotic cells.

To test whether in mitotic cells there is a general block in Raf-1-dependent activation of ERK, COS7 cells were transfected with constitutively activated Raf-1 mutants, RafCT corresponding to the oncogenic v-Raf, and RafY340D mimicking Src-dependent activation. Expression of a constitutively activated Raf-1 resulted in activation of ERK as well as in nocodazole-treated cells, indicating that in this case the effect on down-regulation of ERK was overcome (data not shown).

**DISCUSSION**

Raf-1, which was originally discovered as an oncogenic Ser/Thr protein kinase in retroviruses (1), is activated by mitogens and is involved in signal transduction in many cell systems. Raf-1 is activated by upstream regulators such as Ras and non-receptor tyrosine kinases. Upon growth factor stimulation activated Ras binds to Raf-1 and translocates it to the plasma membrane where additional factors lead to full activation of Raf-1. Growth factor-activated Raf-1 is mainly phosphorylated on Ser and Thr in addition to weak phosphorylation on Tyr (19, 23). In cytokine-dependent activation, where the key regulators for Raf-1 are the non-receptor tyrosine protein kinases of the Src and Jak family, tyrosine phosphorylation of Raf-1 is the major event (22, 40). The synergism between Ras and Src for
Raf-1 activation is essential in many systems (8), but there are also exceptions. Upon stimulation of the CD4 receptor in lymphocytes Raf-1 is activated by the Src-like kinase Lck independently of Ras (41). We have recently described a positive feedback mechanism for Raf-1, which is independent of Ras, Src, and tyrosine phosphorylation (29), while a negative feedback is Ras-dependent (27, 28). Thus Raf-1 activity can be fine-tuned by an interplay of a number of various signals.

In the present study we describe that mitotic activation of Raf-1 is independent of Ras in COS7 cells. Similar results were also obtained with a Rat-1 cell line inducibly expressing Raf-1 (data not shown). Ras-independent stimulation of Raf-1 is shown by several lines of evidence. The use of dominant negative RasS17N and dominant negative RafNT does not interfere with mitotic activation of Raf-1, whereas EGF-dependent stimulation of Raf-1 is inhibited. Furthermore, the Raf-1 mutant deficient for its binding ability to Ras, RafR89L, is still activated in mitotic cells. In contrast to most known activation mechanisms of Raf-1 that take place at the plasma membrane and lead to a membrane-bound active Raf-1, mitotic activation results in an activated Raf-1 predominantly located in the cytoplasm. This cytoplasmic localization of activated Raf-1 is consistent with its Ras-independent activation in mitotic cells. Although the fractionation experiments performed here cannot answer the question of where the initial activation of Raf-1 takes place, the observation of a cytoplasmic active Raf-1 implies some so far unknown biological relevance for mitotic Raf-1.

In the present study the role of tyrosine phosphorylation of Raf-1 during mitosis was also analyzed. Kinases of the Src family that are able to directly phosphorylate Raf-1 can be activated in mitotic cells (42). Recently a Lck-dependent activation of Raf-1 has been shown in mitotic T cells (33). In fibroblast cells used in our study, phosphorylation of Raf-1 only on Ser and Thr but not on Tyr has been detected (31, 32). The finding that the Raf mutant RafYY340/341FF that cannot be stimulated by Src showed a reduced activity in mitotic cells compared with Raf-1 wild-type indicates that these two tyrosine residues and therefore tyrosine phosphorylation may play a role in mitotic activation of Raf-1. However, the modest activation of RafYY340/341FF implies that an additional activation step for Raf-1 must exist in mitotic cells that is independent of the tyrosine residues 340/341.

The level of Raf-1 activation during mitosis is similar to that of growth factor-dependent activation. Surprisingly, the usual substrate of Raf-1, MEK, is not activated in mitotic cells as is the case in mitogen-stimulated cells (42) (data not shown). The ERK activity is also down-regulated in mitotic cells (43) (Fig. 6). Therefore, regulation mechanisms that involve mitotic active Raf-1 are independent of the MEK/ERK pathway. The mechanism of how MEK activation is uncoupled from Raf-1 activation is unknown. In mitotic cells MEK1 but not MEK2 can be phosphorylated and inhibited by the mitosis-specific Cdc2/cyclin B complex (42). In addition phosphatases may be induced during mitosis which specifically inactivate MEK1/2 and ERK1/2. It is of interest that overexpression of constitutively activated mutants of Raf-1 can overcome the effect of down-regulation of ERK in mitotic cells. Therefore, it seems
that there exists a fine-tuned balance between activation and inactivation mechanisms for MEK/ERK. Nocodazole can mediate activation of phosphatases specific for MEK or ERK only if Raf-1 is still not activated.

Recently, it has been shown that in detached cells growth factors can activate Ras and Raf-1 but not MEK and ERK (44). Growth factor activation of MEK and ERK additionally requires cell adhesion. But also in this system the mechanisms that are involved in uncoupling of Ras/Raf-1 and MEK/ERK activation are unknown.

Raf-1 can influence the cell cycle machinery at different points. In G<sub>0</sub> cells Raf-1 is activated upon mitogenic stimulation of the cells and is involved via the MEK/ERK pathway in regulation of gene expression. During the G<sub>1</sub>/S transition the cell cycle Raf-1 may play a role since it can bind via 14-3-3 to the dual specificity phosphatase Cdc25A and stimulate its phosphatase activity (45, 46). Cdc25A can stimulate the Cdk2/cyclin E complex which leads to progression of the cell cycle. Recently, it has been shown that dependent on the strength of its kinase activity Raf-1 can induce cell proliferation or a growth arrest at G<sub>1</sub> (47-49). While low Raf-1 kinase activity induces expression of the G<sub>1</sub>-specific cyclin D leading to cell proliferation, high Raf-1 kinase activity results in expression of the cell cycle inhibitor p21CIP and thereby to growth arrest in G<sub>1</sub>. This G<sub>1</sub> arrest depends at least in part on the active form of the tumor suppressor p53. COS cells and Raf-1 cells used in the present study did not show growth arrest after ectopic expression of oncogenic RasG12V. This can be explained by the presence of the SV40 large T antigen and polyoma T antigen, which were used for immortalization of these cell lines and can interact and inactivate p53.

The function of Raf-1 at the G<sub>2</sub>/M transition and during mitosis is still not known. It has been discussed that mitotic phosphorylation of transcription factors is involved in repression of transcription observed in mitotic cells (42). c-Myc and Ets-1, which are downstream targets of Raf-1 in growth factor-dependent signaling, are hyperphosphorylated specifically during mitosis. It would be of interest to determine whether mitotic Raf-1 is also involved in this mitotic phosphorylation of transcription factors.

Interestingly, the presence of a cytoplasmic active Raf-1 in mitotic cells as shown in this study strongly suggests that under these conditions Raf-1 can interact with a different subset of substrates compared with the membrane-bound Raf-1 obtained after growth factor stimulation. It has been shown that the kinase activity of MEK1/2 and ERK1/2 is down-regulated in mitotic cells and stimulated after removal of the mitotic block (42, 43). Therefore, mitotic Raf-1 must signal independently of the MEK/ERK pathway. It would be of interest to know whether the positive regulator of mitosis, Cdc25C, can be stimulated by Raf-1, such as Cdc25A at the G<sub>2</sub>/S transition. Cdc25C stimulates the Cdk2/cyclin B complex, which is a mitotic activator of Src. Thereby Raf-1 could be indirectly involved in mitotic activation of Src. Another aspect is that signal transduction pathways are involved in the regulation of microtubule dynamics. Mitotic phosphorylation of the oncoprotein 18 by Cdc2 and other still unknown kinases results in down-regulation of its activity, which is essential for the passage through mitosis (50). In this process mitotic Raf-1 also may play a role.

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FIG. 5. Mitotic Raf-1 is active in the cytoplasm. COS7 cells were transfected with the plasmids as indicated and treated without (-) or with (+) nocodazole. For comparison, two samples were stimulated with EGF (lanes 9 and 10). Cells were lysed in hypotonic lysis buffer. Equal amounts of cell lysates were ultracentrifuged, and P100 and S100 fractions representing the membrane (P) and cytoplasmic (S) fractions, respectively, were adjusted to 1× RIPA. Raf-1 proteins were immunoprecipitated with anti-FLAG antibody, and in vitro kinase assays were performed with GST-MEK as substrate as described in the legend to Fig. 2 (upper panel). The protein concentration was adjusted before the ultracentrifugation step, therefore P100 and S100 fractions contain different amounts of immunoprecipitated Raf protein, but the protein contents of the different P100 and S100 fractions are similar.

FIG. 6. ERK activity is down-regulated in mitotic cells. COS7 cells were transfected with FLAG-Rafwt or hemagglutinin-tagged ERK2. Cells were subsequently left untreated or were treated with 0.1 μg/ml nocodazole for 16 h or 100 ng/ml EGF for 8 min. Cells were lysed and analyzed by SDS-polyacrylamide gel electrophoresis (10% gel) followed by immunoblotting with the anti-ACTIVE ERK antibody as described under "Experimental Procedures." This antibody can detect the activated forms of ERK2, ERK1, and hemagglutinin-ERK2 indicated by the lower, middle and upper arrowhead. Activities of both endogenous and transfected ERK are down-regulated in mitotic cells.
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