MAPKAP Kinase 2 Phosphorylates Serum Response Factor in Vitro and in Vivo

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Several growth factor- and calcium-regulated kinases such as pp90^raf or CaM kinase IV can phosphorylate the transcription factor serum response factor (SRF) at serine 103 (Ser-103). However, it is unknown whether stress-regulated kinases can also phosphorylate SRF. We show that treatment of cells with anisomycin, arsenite, sodium fluoride, or tetrafluoroaluminate induces phosphorylation of SRF at Ser-103 in both HeLa and NIH3T3 cells. This phosphorylation is dependent on the kinase p38/SAPK2 and correlates with the activation of MAPKAP kinase 2 (MK2). MK2 phosphorylates SRF in vitro at Ser-103 with similar efficiency as the small heat shock protein Hsp25 and significantly better than CREB. Comparison of wild type murine fibroblasts with those derived from MK2-deficient mice (Mk2^−/−) reveals MK2 as the major SRF kinase induced by arsenite. These results demonstrate that SRF is targeted by several signal transduction pathways within cells and establishes SRF as a nuclear target for MAPKAP kinase 2.

The serum response element (SRE), a growth factor- and stress-regulated promoter sequence, is essential for the expression of immediate early genes such as c-fos and egf-1 (1, 2). SREs are bound by the transcription factor serum response factor (SRF) permitting the recruitment of SRF accessory factors like the Ets-family ternary complex factors (TCFs). SRF binds with its MADS domain as a homodimer to the CaR G box, which has the consensus sequence C2(A/T)6G2 (1). Ternary complex factors, namely Elk-1, Sap-1, and Net-1 can only bind to the c-fos SRE via adjacent GGAT Ets-binding sites in the presence of SRF. In addition, on its 3′-site the c-fos SRF-binding site is flanked by an AP-1-like site (3). In vivo footprinting studies suggest that all three sites of the c-fos SRE element are continuously occupied (4).

The SRE is a converging point for several signal transduction cascades. Particularly well documented is the role of TCFs as nuclear targets in signal transduction. C-terminal TCF phosphorylation by MAP kinases such as ERKs, JNKs, or p38 members results in transcriptional activation (5–11). In contrast, the role of SRF in phosphorylation-regulated gene expression is not well understood. Small GTP-binding proteins of the Rho family regulate c-fos expression via SRF (12, 13). However, it is unclear which kinase cascade transmits the signal toward SRF, and whether SRF is a direct target of phosphorylation, or instead acts primarily as a docking element for another protein. SRF contains several phosphorylation sites (14), although functional consequences, particularly of C-terminal SRF phosphorylation, have not been identified unequivocally. N-terminal phosphorylation including that of Ser-103 immediately N-terminal to the MADS box affects the interaction of SRF with its DNA recognition sequence (14–16).

Two groups of kinases have been shown so far to phosphorylate SRF at Ser-103: pp90^raf, also known as MAPKAP-K1, which is activated by serum via ERKs, as well as CaM kinases II and IV, which are regulated by calcium levels (16, 17). We wondered whether a link existed between activation of gene expression by Rho GTPases and SRF phosphorylation at Ser-103. Here we investigated whether agents known to activate SRF-dependent gene expression, particularly involving activation of or cooperation with Rho GTPases, cause SRF phosphorylation at Ser-103. In addition, we intended to identify kinases phosphorylating SRF. Using in-gel kinase assays we screened for SRF kinases induced by anisomycin, tetrafluoroaluminate, arsenite, and stress factors, and measured the resulting SRF phosphorylation of Ser-103. We show that these factors activate p38-dependent SRF kinases. In particular we identify MAPKAP-K2 (MK2) as an SRF kinase and demonstrate that MK2 contributes to SRF phosphorylation at Ser-103 in vivo. In MK2^−/− fibroblasts arsenite-induced SRF phosphorylation at Ser-103 is greatly reduced. Our data identify SRF as a direct intracellular target of signal transduction cascades activated by tumor-promoting and stress-inducing stimuli (18).

MATERIALS AND METHODS

Cell Culture and Cell Treatment—Cells were kept at 37 °C, 5% CO2 and 92% humidity. NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 mg/ml streptomycin, HeLa cells in minimal essential medium containing 10% FCS, 100 units/ml penicillin, and 100 mg/liter...
The washing solution was removed completely prior to stimulation cells were incubated for 24–48 h in serum-free medium. Cells were stimulated with either 20% FCS, 10 mM sodium fluoride, 100 

For the preparation of cell extracts cells extracts were grown on 10-cm dishes. After preparation, cell culture supernatants were discarded and cell layers were washed with 5 ml of ice-cold phosphate-buffered saline (PBS) containing 50 mM sodium fluoride and 2 mM Na3VO4. The washing solution was removed completely followed by addition of 400 

Ser-103, 0.5 mM oridine and 2 mM Na3VO4. The washing solution was removed completely and pSV40Tag, coding for the SV40 large T antigen, and pREP8 (Invitrogen) precipitated from HeLa cells were incubated with 10

Cell Extractions and Immunoprecipitations—For the preparation of cell extracts cells were collected by scraping, transferred into a 1.5-ml reaction tube, and resuspended in 5 ml of buffer A (6 M guanidine hydrochloride, 50 mM sodium fluoride, 2 mM Na3VO4, 500 

Stress-induced SRF phosphorylation in HeLa cells. Extracts from HeLa cells stimulated for the indicated times with anisomycin (Aniso), sodium fluoride (NaF), or tetrafluoroaluminate (AlF4–) were subjected to EMSA supershift analysis as described under "Materials and Methods. In lanes 2–14, α-phospho-Ser-103-SRF antisera was included in the binding mixtures. SRF + Elk, control with recombinant SRF and 100 

To immunoprecipitate MK2 and JNKs, cell extracts (25 

To express recombinant SRF, a BamHI/EcoRI fragment containing the SRF gene with an N-terminal His-tag was cloned into pLlA503 (21). His-tagged SRF was purified from Escherichia coli BL21-LysS/pILASRF after heat induction. Pelleted cells were re suspended in 5 ml of buffer A (6 M guanidine hydrochloride, 100 mM NaH2PO4, 10 mM Tris, 0.1% Triton X-100, 0.1% Tween 20) for 2 h followed by overnight incubation with affinity purified anti-P-SRF antibody (1:500 diluted in PBST containing 4% bovine serum albumin). Phospho-SRF was visualized by enhanced chemiluminescence.

After blocking with 4% bovine serum albumin in PBST (1.4 

Both aliquots were frozen in liquid nitrogen and stored at −80 °C. To immunoprecipitate MK2 and JNKs, cell extracts (25 

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When phosphorylated at Ser-103 (16, 17). After a 30-min incubation period at room temperature the DNA-protein complexes were separated on a 5% polyacrylamide gel containing 0.5 × Triton borate buffer at 10

Finally, gels were dried and exposed to PhosphorImaging screens.

In Vitro Phosphorylation and Immunoblotting—For in vitro phosphorylation experiments, constitutively active MK2 kinase (MK2ΔRAPC) was incubated in kinase assay buffer (50 mM sodium β-glycerophosphate, pH 7.4, 100 mM NaCl, 10 mM dithiothreitol, 10 mM MgCl2, 90 mM NaH2PO4, 50 

After incubation at 30 °C, the samples were separated by SDS-PAGE followed by either PhosphorImaging analysis or blotting on a PVDF membrane. Immunoblotting analysis, 140 mg/liter substrate protein for 10 min at 30 °C. His-tagged SRF, His
tagged CREB and murine Hsp25 were used as substrates. For immunoblotting analysis, 140 mg/liter SRF were incubated for 30 min at 30 °C. The reaction was stopped by addition of sample buffer. After denaturation at 65 °C for 5 min, the samples were separated by SDS-PAGE followed by either PhosphorImaging analysis or blotting on a PVDF membrane. Immunoblotting was essentially performed as described (17). Blots were blocked with 4% bovine serum albumin in PBST (1.4 

After phosphorylation, SRF was digested by endoproteinase Lys-C (Roche Molecular Biochemicals, Mannheim, Germany) in 25 mM Tris-HCl buffer, pH 8.1, for 14 h at 37 °C. Digestion was terminated by addition of one-fifth volume of 10% trichloroacetic acid. Peptides generated were fractionated by reversed-phase high-performance liquid chromatography (HPLC).

**Peptide Sequencing—** For detection of MK2 phosphorylation sites in SRF, 20 μg of either full-length SRF or core SRF (amino acids 90–244 of SRF) were labeled as described above, with the exception of an extended labeling time of 8 h. 32P-Labeled SRF (0.4 mg/ml) was digested with 0.4 μg of endopeptidase Lys-C (Roche Molecular Biochemicals, Mannheim, Germany) in 25 mM Tris-HCl buffer, pH 8.1, for 14 h at 37 °C. Digestion was terminated by addition of one-fifth volume of 10% trichloroacetic acid. Peptides generated were fractionated by reversed-phase high-performance liquid chromatography (HPLC).
RESULTS

Induction of SRF Phosphorylation at Ser-103 Correlates with the Activation of p38/SAPK2 and MK2—Stimulation of serum-starved NIH3T3 fibroblasts with FCS results in a rapid and transient induction of SRF phosphorylation at Ser-103, which, at least in part, can be attributed to the activity of the protein kinase pp90^{rff} (MAPKAP-K1) (16). However, other downstream kinases of the MAPK cascades, such as MAPKAP-K2 (MK2) (22) may also be involved in SRF phosphorylation. The closely related MAPKAP-K3 (MK3) (23, 24) has recently been shown not to phosphorylate SRF (25). To examine more specifically the role of stress-activated protein kinase (SAPK) pathways of the JNK and p38 classes, we stimulated HeLa cells with anisomycin and tetrafluoroaluminate which have been recently shown to induce immediate early gene expression including c-fos by activating or cooperating with Rho GTPases (12, 19, 13). We analyzed kinase activities and SRF phosphorylation by EMSA supershift assays using an a-phospho-Ser-103-SRF antiserum (Fig. 1), by in-gel kinase assays (Fig. 2) and by directly measuring kinase activities in all cell extracts (Fig. 3). Since tetrafluoroaluminate is formed in solution by mixing aluminum chloride and sodium fluoride, we included these two compounds as controls in our study. In all assays shown in Figs. 1–3, we analyzed the identical extracts to allow a direct comparison of the results.

Using EMSA supershift analysis we first examined the extent of SRF Ser-103 phosphorylation before and after treatment of cells with the different agents. As a DNA-binding probe we used the c-fos SRE sequence. Addition of a phospho-Ser-103-specific a-SRF antiserum to the DNA binding reaction causes a mobility supershift of phospho-SRF-containing protein-DNA complexes (16, 17). In these assays, HeLa cell extracts show two bands (Fig. 1). According to control lanes with recombinant protein (Fig. 1, lane 1), the faster migrating band represents the binary complex containing only SRF homodimer bound to the SRE whereas the slower band represents the ternary complex containing SRE, SRF homodimer, and an additional TCP protein. In extracts of uninduced cells, addition of a-phospho-Ser-103-SRF antiserum causes only a very weak supershift (Fig. 1, lane 2). Therefore, only a minor fraction of SRF is phosphorylated at Ser-103 in uninduced HeLa cells. Treatment of these cells for 15, 30, or 60 min with anisomycin induces SRF phosphorylation at Ser-103, leading to a complete supershift of both SRF containing bands (Fig. 1, lanes 3–5). No obvious decrease in the degree of SRF phosphorylation is observed during 60 min of anisomycin treatment. The tyrosine phosphatase inhibitor sodium fluoride also induces SRF phosphorylation, albeit with slower kinetics compared with anisomycin (Fig. 1, lanes 6–8). No significant SRF phosphorylation can be detected after 15 min, whereas after 30 and 60 min both the binary and the ternary complex bands are completely supershifted by a-phospho-Ser-103-SRF antiserum. Aluminum chloride does not induce SRF phosphorylation (Fig. 1, lanes 9–11). However, tetrafluoroaluminate induces SRF phosphorylation with similar kinetics as sodium fluoride (Fig. 1, lanes 12–14). Indeed, we observed no difference between sodium fluoride and tetrafluoroaluminate in any assay used in this report.

To characterize potential SRF kinases induced by anisomycin, sodium fluoride, and tetrafluoroaluminate, the HeLa cell lysates used in Fig. 1 were analyzed by in-gel kinase assays with copolymerized substrate (SRF, c-Jun, or no substrate) indicated on the right side. All three gels were run using the same extracts, whereby the SRF gel was run on a different day than the c-Jun and the control gels. Panel A, in-gel kinase assay with SRF as copolymerized substrate. The arrow indicates the major induced SRF kinase of 50 kDa. As shown, this kinase activity corresponds to MK2. Panel B, in-gel kinase assay with c-Jun as copolymerized substrate. The arrows indicate induced c-Jun kinases of 46 and 55 kDa, corresponding to JNK1 and -2, respectively. Panel C, in-gel kinase assay without any copolymerized substrate. This gel serves as a control for autophosphorylation. The arrow indicates an autophosphorylating kinase of 50 kDa comigrating with MK2.
MK2 Is a Potential SRF Kinase—Since activation of MK2 parallels SRF phosphorylation at Ser-103, we suspected that MK2 might directly phosphorylate SRF. To test whether MK2 phosphorylates SRF in vitro, we analyzed recombinant constitutively active MK2 and anisomycin-induced HeLa extracts using in-gel kinase assays. Different in-gel substrates were employed (Fig. 4, A–F). Hsp25 is a published MK2 substrate (27) and served as a positive control for renaturation of MK2 employed (Fig. 4, A–F). Hsp25 and SRF (Fig. 4, A–F) or GST-MK2 (Fig. 4, A–F). Recombinant MK2 also phosphorylates c-Jun, whereas endogenous MK2 is probably masked by prominent JNK/SAPK1 activity (Fig. 4C). Endogenous MK2 phosphorylates the MK2 substrate weakly, whereas with recombinant MK2 autophosphorylation is not visible (Fig. 4E). This finding is consistent with the autophosphorylation of the 50-kDa band observed in Fig. 2C. Neither recombinant nor endogenous MK2 phosphorylate the TCF/Ets transcription factor Elk-1 (Fig. 4D) or histone H1 (Fig. 4F). However, Elk-1 (Fig. 4D), as well as c-Jun (Fig. 4C), are efficiently phosphorylated by two 46- and 55-kDa kinases correlating with the molecular masses of JNKs. Taken together, SRF is phosphorylated by MK2 to a similar extent as its well-established substrate Hsp25. In contrast, the SRF-interacting TCF Elk-1 is not a substrate for MK2 under in-gel kinase assay conditions, nor in solution in MK2 enzyme reactions (data not shown).

To verify that MK2 was the SRF kinase observed in Fig. 2A, we immunoprecipitated MK2 from the same extracts using an antisera specific for MK2 and MK3 followed by in-gel kinase analysis of the precipitated kinases. Since MK3 does not phosphorylate SRF in vitro (25), only MK2 should be visible on SRF gels. For these experiments we took again the same extracts as used in Figs. 1–3. In addition, we examined extracts from arsenite-treated HeLa cells, since arsenite is a potent inducer of both JNKs and p38 kinases (29). Whereas in extracts from untreated HeLa cells no SRF kinases are precipitated, anisomycin, sodium fluoride, tetrafluoroaluminate, and arsenite in-

required 30 min (lanes 8–10). With these three stimulators the induction lasts for at least 60 min. Aluminum chloride does not induce any SRF kinase (lanes 11–13). Therefore, the activation kinetics of these SRF kinases parallel the phosphorylation kinetics of SRF at Ser-103 (Fig. 1).

Since the size of the 55-kDa band seen in Fig. 2A agrees with the reported molecular weight of JNK2 (26), we examined JNK/SAPK1 activation in parallel in the same lysates using, instead of SRF, c-Jun as in-gel substrate (Fig. 2B). In c-Jun-containing gels, a different pattern of induced kinases compared with SRF gels is observed. Whereas the 50-kDa kinase is absent, two bands of 46 and 55 kDa are strongly induced. Thus, these two bands very likely reflect activated JNK1 and -2. As already seen for SRF kinases (Fig. 2A), anisomycin (Fig. 2B, lanes 2–4) activates both JNKs with faster kinetics than tetrafluoroaluminate (lanes 5–7) and sodium fluoride (lanes 8–10). Again, no Jun kinase is activated by addition of aluminum chloride (lanes 11–13). The observed increase in intensities is not due to autophosphorylation, as shown by the control gel (Fig. 2C). Therefore, anisomycin, sodium fluoride, and tetrafluoroaluminate apparently induce the same SRF and Jun kinases, respectively, but with different kinetics. Aluminum chloride does not induce any kinase in the in-gel kinase assays used here.

Based on the apparent molecular mass, the 50-kDa band seen in the SRF gel of Fig. 2A may represent human MK2 (26).

To examine directly the possible activation of MK2 and its upstream activating kinase, namely p38/SAPK2, both kinases were immunoprecipitated from the respective HeLa cell extracts followed by analysis of their activities by in vitro phosphorylation assays (Fig. 3). In contrast to aluminum chloride, which does not activate either MK2 (Fig. 3A) or p38/SAPK2 (Fig. 3B), anisomycin, sodium fluoride, and tetrafluoroaluminate lead to activation of both MK2 and p38/SAPK2. Anisomycin activates both kinases within 15 min to maximal extent, whereas maximal activation of MK2 and p38/SAPK2 by sodium fluoride or tetrafluoroaluminate takes more than 30 min. In addition, p38/SAPK2 isolated from sodium fluoride- or tetrafluoroaluminate-treated cells exhibits only 50% of the activity caused by anisomycin treatment. Thus, despite similar extents of MK2 activation, sodium fluoride and tetrafluoroaluminate are less potent p38 inducers than anisomycin. However, anisomycin-, sodium fluoride-, and tetrafluoroaluminate-mediated activation of the p38/MK2 signal transduction pathway correlates with the induction of SRF phosphorylation at Ser-103.

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FIG. 3. Induction of MK2 and p38/SAPK2 in HeLa cells. MK2 (Panel A) or p38/SAPK2 (Panel B) were immunoprecipitated from the identical HeLa extracts used for Figs. 1 and 2 and examined for their activity using either GST-Hsp25 (Panel A) or GST-MK2 (Panel B) as substrates for phosphorylation. The reaction mixtures were separated on SDS-polyacrylamide gels, and the intensities of bands corresponding to 32P-labeled substrates were quantified by PhosphorImaging analysis. Inducers were anisomycin, sodium fluoride, tetrafluoroaluminate, or aluminum chloride. The relative inductions were measured 15 (black bars), 30 (hatched bars), and 60 (gray bars) min after addition of the corresponding inducer.
FIG. 4. **MK2 is an SRF kinase in HeLa extracts.** Panels A–F, characterization of anisomycin-stimulated kinases by in-gel kinase assays. Constitutively active MK2 (GST-MK2ΔPC) or cell extracts from either untreated or anisomycin-stimulated (Aniso) HeLa cells were analyzed by in-gel kinase assays. The copolymerized substrates were Hsp25 (Panel A), SRF (Panel B), c-Jun (Panel C), Elk-1 (Panel D), MK2 (Panel E), or histone H1 (Panel F), as indicated at the bottom. Bands corresponding to recombinant MK2 are indicated on the left. Those bands corresponding to endogenous MK2 or JNK/SAPK1 are indicated at the right. The size of recombinant MK2 is 66 kDa due to its GST tag. Panel G. In-gel kinase assay of immunoprecipitates. JNK/SAPK1s (lanes 3, 6, 9, 12, and 15) and MK2/3 (lanes 4, 7, 10, 13, and 16) were immunoprecipitated from HeLa lysates and analyzed for their activation status by in-gel kinase assays with SRF as copolymerized substrate. The treatment of the cells prior to lysis is indicated at the top of the gels. The same lysates as in Figs. 1–3 were examined. In addition to Fig. 2, lysates of arsenite-treated cells are also included. Cells were lysed 30 min after addition of anisomycin (Aniso) or arsenite, or 60 min after adding sodium fluoride (NaF) or tetrafluoroaluminate (AlF4). α-JNK IP, immunoprecipitates obtained with an α-JNK antibody; α-MK IP, immunoprecipitates obtained with an α-MK2/3 antibody. The length standard is shown on the left. The black arrows indicate MK2 bands, the white arrows JNK/SAPK1 bands. In lane 15, some spillover of lane 16 is noticeable.
cated by a supershift upon addition of
Treatment of serum-starved NIH3T3 cells with serum (FCS) with the binary complex containing only an SRF dimer (31). Net-1 which does not cause a significant retardation compared shown). However, the complex may include the TCF protein binding site does not change the band shift pattern (data not shown). In agreement with these results, mutation of the TCF- the TCF protein ELK-1 is observed (Fig. 5
the binary SRE-SRF complex. No ternary complex containing extracts show in EMSA studies a singular band which contains the top band 103-SRF antiserum. After 10 min the phosphorylation reaches its maxi-
phorylation of SRF at Ser-103 with different kinetics than conclusion, arsenite treatment of NIH3T3 cells causes phos-
phosphorylation to 50% within 45 min is seen (Fig. 5
phosphorylation, a slower but steady increase of SRF phospho-
possible MK2-mediated SRF phosphorylation in NIH3T3 cells. As an inducing agent we chose arsenite because of its efficient and relatively restricted activation of JNK/SAPK1 and p38/ SAPK2 (29).

In contrast to HeLa extracts (Fig. 1, lane 2), NIH3T3 cell extracts show in EMSA studies a singular band which contains the binary SRE-SRF complex. No ternary complex containing the TCF protein ELK-1 is observed (Fig. 5A and data not shown). In agreement with these results, mutation of the TCF-binding site does not change the band shift pattern (data not shown). However, the complex may include the TCF protein Net-1 which does not cause a significant retardation compared with the binary complex containing only an SRF dimer (31). Treatment of serum-starved NIH3T3 cells with serum (FCS) results in a rapid induction of SRF phosphorylation as indicated by a supershift upon addition of α-phospho-Ser-103-SRF antiserum. After 10 min the phosphorylation reaches its maximum, but decreases after 20 min to background levels (Fig. 5A, lanes 1–6). Thus, in agreement with previously published results (16), FCS induces a transient SRF phosphorylation at Ser-103. Addition of arsenite to serum-starved cells also leads to SRF phosphorylation at Ser-103, but different phosphorylation kinetics are observed. Instead of a rapid and transient phosphorylation, a slower but steady increase of SRF phosphorylation to 50% within 45 min is seen (Fig. 5A, lanes 7–12). In conclusion, arsenite treatment of NIH3T3 cells causes phosphorylation of SRF at Ser-103 with different kinetics than serum treatment. This difference is congruent with the slow and long lasting activation of SAPKs by arsenite due to the inhibition of a SAPK specific phosphatase activity (29).

As already mentioned above, arsenite causes activation of both JNK and p38 kinase family members. In addition, recent data suggest an arsenite-dependent activation of the ERK pathway via p38/SAPK2 (32). To distinguish between different MAPK cascades potentially involved in SRF phosphorylation, we induced serum-starved cells with arsenite in the presence of either the p38-specific inhibitor SB202190, a closely related analog of SB203580 (33), or, alternatively, with PD98059, which prevents activation of ERKs due to inhibition of MEK-1 stimulation (34). Pretreatment of cells with 10 or 30 μM SB202190 reduces the extent of arsenite-induced SRF phosphorylation from 80 to 40 and 30%, respectively (Fig. 5B, lanes 2–4). In contrast, PD98059 hardly affects the arsenite-influenced phosphorylation status of SRF at all (lanes 5 and 6). These results suggest the p38 pathway as the primary MAPK cascade involved in arsenite-induced SRF phosphorylation at Ser-103. Other pathways such as the JNK/SAPK1 or ERK cascades contribute only to a minor extent, collectively amounting to not more than 30% of SRF Ser-103 phosphorylation.

Besides activation of MK2 (22), the p38 pathway induces several other downstream kinases such as MK3 (23, 24), MNKs (35, 36), MSKs (37), and PRAK (38) (for reviews, see Refs. 18 and 28). Of all these kinases, only MK2 is exclusively activated by p38/SAPK2 (22). Whereas MK3 does not phosphorylate SRF in vitro (25), other kinases have not been tested yet. Thus, we wondered whether MK2 was the major SRF kinase in NIH3T3, and whether NIH3T3-derived JNKs also phosphorylated SRF, thereby paralleling the results obtained with HeLa cell extracts. To test for this, we performed in-gel kinase assays with SRF as in-gel substrate using the identical NIH3T3 extracts investigated in Fig. 5B. Extracts from serum-starved NIH3T3 cells show a major band at 58 kDa and minor bands at 200 kDa and around 60 kDa (Fig. 6A, lane 1). Since these bands are also visible with similar intensities in control gels (data not shown), they are due to autophosphorylation of unidentified protein.

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kines. NIH3T3 extracts differ from HeLa extracts in the pattern of arsenite-induced SRF kinases (Fig. 6A, lanes 2 and 3). Particularly the 50-kDa band corresponding to human MK2 is missing in NIH3T3 extracts (lane 2). Instead, arsenite treatment of NIH3T3 results in the induction of a kinase band around 45 kDa and in increased intensities of two bands at 55 and 60 kDa (lane 3). All three bands can be also seen weakly in the control gels lacking substrate, but particularly the p55 and p45 bands are significantly stronger in the SRF-containing gels (Fig. 6A, lane 3 and data not shown). Thus, the protein kinases corresponding to 55 and 45 kDa, although capable of autophosphorylation, also phosphorylate SRF in this experiment. The sizes of the two bands correlate well with molecular masses published for two murine MK2 isoforms (26) and, therefore, would correspond precisely to the results obtained with HeLa extracts (lane 2). Interestingly, SB202190 (Fig. 6A, lanes 4–6), but not PD98059 (lanes 7–9), completely inhibits activation of the p45 kinase, whereas the intensity of the p55 band is only partially reduced. It is unlikely that this band is only less susceptible than p45 to SB202190, since increasing the inhibitor concentration to 100 μM does not cause a further reduction of this band (lane 6). Instead, the reason for this partial reduction might be the comigration of the p55 form of MK2 with another SRF kinase of 55 kDa, such as JNK2 which has the same apparent molecular mass (26). A similar band was already observed in HeLa extracts (Fig. 2A). Thus, based on these inhibitor studies, in both HeLa and NIH3T3 cells, arsenite induces MK2 via the p38 kinase cascade. In addition, at least JNK2 may also phosphorylate SRF whereas the Erk pathway apparently does not contribute to arsenite-stimulated SRF phosphorylation. Furthermore, the activation kinetics of these kinases and their inhibition by SB202190 suggest that MK2 phosphorylates SRF at Ser-103.

As already stated above, the sensitivities of p45 and p55 to treatment with SB202190 suggests that they are murine MK2 isoforms (26), which are activated by p38/SAPK2. To further characterize these observed SRF kinase activities, we immunoprecipitated MK2/3 from the cell extracts already used in Figs. 5B and 6A and analyzed the MK2 activation status by in-gel kinase assays using SRF as in-gel substrate (Fig. 6B). The α-MK2/3 antiserum precipitates both the 45- and 55-kDa bands (lane 4). Cell extracts from SB202190-treated cells do not contain active MK2 (lane 6), whereas PD98059 hardly affects their activation (lane 8). Thus, in NIH3T3 as well as in HeLa cells, arsenite activates MK2 via p38/SAPK2 which in turn phosphorylates SRF, suggesting this signal transmission as a common intracellular pathway.

MK2 Phosphorylates SRF at Ser-103 in Vitro—To confirm the data obtained with cell extracts and to identify the SRF site phosphorylated by MK2, we incubated recombinant SRF protein with a constitutively active form of MK2 in the presence of [γ-32P]ATP. This reaction yields a 32P-labeled band of an apparent molecular mass of 67 kDa (Fig. 7A, lane 2). Therefore, MK2 also phosphorylates SRF in vitro. As positive controls, we included again Hsp25 as a well characterized and efficient substrate for MK2 (lane 1), and CREB (lane 3), which has been shown to be accepted by MK2 as a substrate (39). The intensity of the SRF signal compares very favorably with Hsp25 (lanes 1 and 2) suggesting once more that SRF is a very efficient substrate for MK2. In contrast, CREB is only very weakly phosphorylated by MK2 (lane 3). This result agrees with recent findings that MK2 is not a CREB kinase in vitro (37).

The sequence flanking Ser-103 of SRF, i.e., LKRSLS103EM, conforms to the consensus sequence LRXXXXX required for MK2-mediated phosphorylation. To examine if purified MK2 phosphorylates SRF in vitro at Ser-103, SRF was phosphorylated as described in the previous paragraph followed by immunoblotsing with the α-phospho-Ser-103-SRF antiserum. In the absence of ATP and MK2, only a very weak SRF band can be seen (Fig. 7B) due to the low affinity of this antibody for unphosphorylated SRF (16). However, incubation of SRF with ATP and MK2 results in an intense band of 67 kDa indicating that SRF is phosphorylated at Ser-103. Stripping and reprobing of the membrane with an α-SRF antiserum assured equal loading with SRF. Thus, MK2 phosphorylates SRF at Ser-103 in vitro and, given the above MK2 activity profiles in extracts of untreated cells, MK2 is likely to be responsible for arsenite-induced SRF phosphorylation at the same site in vivo.

To further investigate this notion, the major phosphorylation site for MK2 within the SRF substrate was identified empirically. For this purpose, SRF was labeled in vitro by MK2 as described above and was eluted from the gel, digested using endoproteinase Lys-C and the resulting peptides were fractionated by reversed phase-HPLC. In two fractions (asterisks in Fig. 7C) 32P-radioisotope could be detected indicating the existence of phosphopeptides in these samples. Subsequent Edman degradation of these samples revealed two peptides with different lengths both starting with residue Arg-100 of SRF and ending with either Lys-135 or Lys-138, respectively. Detection of 32P release during each Edman degradation cycle (Fig. 7D) demonstrates single phosphorylation of SRF by MK2 at position Ser-103, supporting the data obtained above with the phosphorylation specific antiserum. Taken together, the above results demonstrate that MK2 phosphorylates SRF at Ser-103, and that this is the major site of phosphorylation.

MK2 Contributes to SRF Phosphorylation at Ser-103 in Vivo—The results presented so far demonstrate that inside the cells the kinetics and inhibition profiles of SRF phosphorylation and MK2 activity overlap, and that MK2 can phosphorylate SRF at Ser-103 in vitro. However, the question remained whether MK2 is a true in vivo SRF kinase, and if so, whether it is a major SRF kinase. Other arsenite-induced kinases such as PRAK may also phosphorylate SRF at Ser-103. To test for MK2-dependent SRF phosphorylation in vivo, we used embryonal fibroblasts derived from MK2-deficient mice obtained by homologous recombination. These mice were generated in a separate study. As a control we used fibroblasts derived from wild-type mice. Analysis of SRF phosphorylation at Ser-103 was performed by EMSA supershift studies using extracts of these MK2(--/-) fibroblasts. Arsenite treatments were performed for 60 min. Generally, the EMSA supershift patterns obtained with extracts from MK2(--/-) (Fig. 8A, lanes 1–4) and wild-type (lanes 5–8) fibroblasts were similar to those observed with NIH3T3, with no obvious ternary complex band (Fig. 5). However, the extent of SRF phosphorylation at Ser-103 after arsenite induction differed significantly between knockout and wild-type cells. Whereas with wild-type cell extracts 73% of the SRF-DNA complex was supershifted by the α-phospho SRF antibody (Fig. 8, A, lane 8, and B), only 23% was supershifted with extracts from MK2-deficient cells (Fig. 8, A, lane 4, and B). Therefore, inside living cells, MK2 mediates arsenite-induced SRF phosphorylation at Ser-103 to a major extent in at least this cell system. Kinases other than MK2 contribute only to a minor extent to SRF phosphorylation after arsenite induction.

DISCUSSION

SRF and SRF-containing complexes are important nuclear relays between cellular signaling and gene activity. SRF serves both as a direct and indirect target of signaling cascades. SRF contains multiple phosphorylation sites, of which Ser-103 has

been investigated in more detail (14, 15, 41, 42). Phosphorylation of Ser-103 has been shown previously to be regulated by growth factors or calcium levels and to be mediated, at least in
vitro, by pp90rsk, CaMKII, CaMKIV, and protein kinase A (16, 17, 43). We show in this report that activation of SAPK pathways by agents such as anisomycin, arsenite, sodium fluoride, or tetrafluoroaluminate leads to SRF phosphorylation at Ser-103 (Figs. 1, 5, and 8). Thus, SRF is targeted by several kinase cascades (Fig. 9) and, therefore, is a converging point for both growth factor-regulated and stress-activated signaling cascades as is the case for its interaction partners Elk-1, Net-1, and Sap-1a (6, 8, 10, 11, 44).

Induction of SRF phosphorylation at Ser-103 by anisomycin, arsenite, sodium fluoride, or tetrafluoroaluminate correlates kinetically, and with regard to immunoprecipitation and inhibitor studies, with the activation profiles of MK2 (see Figs. 1–6). Indeed, MK2 phosphorylates SRF at Ser-103 (Fig. 7). A comparison of the substrate properties of several putative MK2 substrates shows that CREB is a poorer substrate than Hsp25 or SRF (Fig. 7A). This result agrees with recent findings that MK2 phosphorylates another small heat shock protein, Hsp27, significantly better than CREB (45, 37). Because of this result,
in combination with inhibition studies, the authors of that study concluded that not MK2 but rather Msk1 mediates the growth factor- and stress-mediated CREB activation (37). In contrast to CREB, SRF is as efficiently phosphorylated by MK2 as Hsp25 (Fig. 7A), which agrees with our observation that MK2 is the major SRF kinase in arsenite- or anisomycin-treated fibroblasts (Fig. 8 and data not shown). Interestingly, MK3 does not phosphorylate SRF in vitro (25) demonstrating for the first time different substrate preferences of these two closely related kinases. Thus, MK2 is the second example of a MAP kinase-activated SRF kinase, the other being p90^Rsk (16). It remains to be examined whether other kinases downstream of MAP kinases, such as PRAK, Msk1, or Mnks, are also able to phosphorylate SRF.

MK2 has both nuclear and cytosolic substrates. SRF belongs to the first group, whereas the small heat-shock proteins belong to the second group. Of interest, MK2 is a nuclear kinase, which translocates to the cytoplasm upon phosphorylation within the nucleus by p38/SAPK2 (27, 46). After some 40 min of anisomycin stimulation of fibroblasts, the majority of MK2 has left the nucleus. These export kinetics correlate with the arsenite-induced phosphorylation kinetics of SRF (Fig. 5A). Thus, MK2 may phosphorylate first its nuclear targets, such as SRF, before being exported to the cytoplasm and phosphorylating cytosolic substrates, such as Hsp25. In contrast, regulation of the intracellular localization of SRF by MK2 seems to be rather unlikely, since SRF localization is not affected by mutating Ser-103 to either alanine or aspartate (43).

Besides MK2-mediated SRF phosphorylation at Ser-103 JNKs may also phosphorylate SRF, albeit at different sites. This is not surprising, since Ser-103 is not neighbored by a proline and therefore does not represent a phosphorylation site for JNKs. We have not identified these sites yet, but initial kinase assays using p54 MAP kinase-α (47) and SRF indicate that JNKs do phosphorylate SRF in vitro and thereby agreeing with the immunoprecipitation results and in-gel kinase assays shown here (Fig. 4G). Interestingly, in NIH3T3 cells only p55/JNK2 is visible by in-gel kinase assays upon arsenite stimulation (Fig. 6). This finding points to differences between the two cell lines HeLa and NIH3T3 regarding the regulation of the JNK/SAPK2 pathway. Future experiments will address the role of this signal transduction pathway in SRF-mediated gene expression.

In NIH3T3 cells FCS induces c-fos expression via SRF by a TCF-independent mechanism. This pathway has been suggested to include heterotrimeric G proteins and Rho GTPases (12). In this context, tetrafluoroaluminate as an inducer of heterotrimeric G proteins has been shown to efficiently activate c-fos expression in transient transfection assays. In contrast, sodium fluoride, a well known phosphatase inhibitor, hardly activated c-fos expression (12). To identify possible downstream kinases the authors also tested for the activation of MAPKs, including JNKs and p38, by these agents. In their hands, neither tetrafluoroaluminate nor sodium fluoride activated MAPKs. We have not identified these sites yet, but initial kinase assays using p54 MAP kinase-α (47) and SRF indicate that JNKs may also phosphorylate SRF, albeit at different sites. This is not surprising, since Ser-103 is not neighbored by a proline and therefore does not represent a phosphorylation site for JNKs. We have not identified these sites yet, but initial kinase assays using p54 MAP kinase-α (47) and SRF indicate that JNKs do phosphorylate SRF in vitro and thereby agreeing with the immunoprecipitation results and in-gel kinase assays shown here (Fig. 4G). Interestingly, in NIH3T3 cells only p55/JNK2 is visible by in-gel kinase assays upon arsenite stimulation (Fig. 6). This finding points to differences between the two cell lines HeLa and NIH3T3 regarding the regulation of the JNK/SAPK2 pathway. Future experiments will address the role of this signal transduction pathway in SRF-mediated gene expression.

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know the exact mechanism, but, in analogy to the SAPK activation by arsenite, sodium fluoride may inhibit SAPK-specific phosphatases leading to an increase in SAPK activity. This model is supported by the slower kinetics of kinase induction by sodium fluoride, which is reminiscent to the SAPK activation kinetics of arsenite.

Thus, SAPKs may possibly be involved in SRF activation controlled by Rho GTPases. In transient transfection assays, Rho GTPases can efficiently activate SRF reporter genes (12), whereas chromosomal SRF reporter genes are only activated by CDC42 and Rac1, but not by RhoA (13). In contrast to RhoA, CDC42 and Rac1 are potent activators of both JNKs and p38 members (48). In agreement with these findings, RhoA can activate chromosomal SRF reporters in the presence of either anisomycin or constitutively active MKK3 (13). It was suggested that MKK3 induces histone acetylation thereby complementing the RhoA signaling onto SRF. However, it could not be excluded that MKK3 may also affect SRF directly. MKK3 is known to activate p38α and p38β (51, 52, 53), which, in contrast to p38γ and p38δ, are efficient MK2 activators. Using MK2 as a phosphorylation substrate we show that anisomycin induces at least two of these p38 forms efficiently (Fig. 3B). Since both MKK3 as well as anisomycin activate MK2, SRF phosphorylation by MK2 may be important for SRF-dependent gene expression. However, addition of the p38 inhibitor SB203580 did not interfere with SRF activation by a combination of RhoA and anisomycin (13). These conflicting results, on the one hand cooperative activity of RhoA with MKK3, on the other no SB203580 effect, remain to be resolved (13).

Clearly, the in vivo significance of SRF phosphorylation at Ser-103 remains an open question. Ser-103 lies in close proximity to the MADS box of SRF. This conserved region is involved in homodimerization and DNA binding. Therefore, phosphorylation of Ser-103 may have functional consequences for the formation of the SRF-DNA complex. In fact, SRF mutants containing an alanine residue at position 103 displayed a reduced cooperativity of RhoA with MKK3, on the other no SB203580 expression. However, addition of the p38 inhibitor SB203580 by MK2 may be important for SRF-dependent gene activation, since SRF has been shown to be essential for mesoderm formation during mouse embryogenesis (57) as well as for the formation of the SRF2-DNA complex. In fact, SRF mutants containing an alanine residue at position 103 display a reduced cooperativity of RhoA with MKK3, on the other no SB203580 expression. However, addition of the p38 inhibitor SB203580 may possibly be involved in SRF activation controlled by Rho GTPases. In transient transfection assays, Rho GTPases can efficiently activate SRF reporter genes (12), whereas chromosomal SRF reporter genes are only activated by CDC42 and Rac1, but not by RhoA (13). In contrast to RhoA, CDC42 and Rac1 are potent activators of both JNKs and p38 members (48). In agreement with these findings, RhoA can activate chromosomal SRF reporters in the presence of either anisomycin or constitutively active MKK3 (13). It was suggested that MKK3 induces histone acetylation thereby complementing the RhoA signaling onto SRF. However, it could not be excluded that MKK3 may also affect SRF directly. MKK3 is known to activate p38α and p38β (51, 52, 53), which, in contrast to p38γ and p38δ, are efficient MK2 activators. Using MK2 as a phosphorylation substrate we show that anisomycin induces at least two of these p38 forms efficiently (Fig. 3B). Since both MKK3 as well as anisomycin activate MK2, SRF phosphorylation by MK2 may be important for SRF-dependent gene expression. However, addition of the p38 inhibitor SB203580 did not interfere with SRF activation by a combination of RhoA and anisomycin (13). These conflicting results, on the one hand cooperative activity of RhoA with MKK3, on the other no SB203580 effect, remain to be resolved (13).
MAPKAP Kinase 2 Phosphorylates Serum Response Factor in Vitro and in Vivo
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