The Stress Inducer Arsenite Activates Mitogen-activated Protein Kinases Extracellular Signal-regulated Kinases 1 and 2 via a MAPK Kinase 6/p38-dependent Pathway*

(Received for publication, June 19, 1997, and in revised form, October 3, 1997)

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Cell response to a wide variety of extracellular signals is mediated by either mitogenic activation of the Raf/MEK/ERK kinase cascade or stress-induced activation of the mitogen-activated protein kinase (MAPK) family members c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) or p38. We have examined communications between these stress- and mitogen-induced signaling pathways.

We show here that the stress cascade activator arsenite activates extracellular signal-regulated kinase (ERK) in addition to p38 albeit with different kinetics. Whereas p38 is an early response kinase, ERK activation occurs with delayed time kinetics at 2–4 h. We observed activation of ERK upon arsenite treatment in many different cell lines. ERK activation is strongly enhanced by overexpression of p38 and mitogen-activated protein kinase kinase 6 (MKK6) but is blocked by dominant negative kinase versions of p38 and M KK6 or the specific p38 kinase inhibitor SB203580. Arsenite-induced ERK activation is mediated by Ras, Raf, and MEK but appears to be independent of de novo protein synthesis. These data provide the first evidence for a p38 dependent activation of the mitogenic kinase cascade in stress-stimulated cells.

There are several parallel signal transduction cascades in mammalian cells that connect extracellular stimuli with gene expression in the nucleus. Whereas the classic cytoplasmic cascade consisting of the Raf/MEK/ERK1 kinase module mediates growth and differentiation inducing signals (1, 2), other parallel pathways are involved in response to certain inflammatory cytokines and environmental stress inducers (3–5). One of these stress-activated pathways leads to the activation of the MAPK family member p38 by the upstream dual specificity kinase MKK6 (MAPK kinase 6), the other parallel cascade recruits the “stress-activated protein kinases” (SAPKs, also termed c-Jun N-terminal kinases, JNKs) through the MEK/SAPK module (MEK kinase, SAPK/ERK kinase). In contrast to the detailed knowledge of cellular responses triggered by the Raf/MEK/ERK1 kinase cascade, the physiological consequence of stress cascade activation is an open issue. Both cascades were reported to be involved in stress response, cell cycle delay or apoptosis as well as in the activation of immune reactive cells (reviewed in Ref. 3). Recent findings indicate that stress- and mitogen-induced kinase cascades are interconnected at several levels. Besides cascade convergence at the level of transcription factors (6–8), signals also converge at different MAPK-activated protein kinases, namely 3pK (9) and Mnk1/2 (10, 11). Other examples include kinases such as Tpl-2, which activates both ERK and JNK/SAPKs (12). A more indirect connection involves induction of an autocrine loop. An oncogenic form of Raf activates the SEK/SAPK pathway, by inducing transcription and release of an autocrine factor, heparin-binding epidermal growth factor (13, 14). This factor subsequently activates SAPKs by binding to the epidermal growth factor receptor.

Although extensive literature documents cross-talk between signaling cascades, there are no reports on activation of the mitogenic cascade by elements of stress-induced kinase cascades. We have now identified such a connection, which is initiated by the stress inducer, arsenite. Arsenite is highly carcinogenic (15, 16) and an effective activator of p38 (17). Since the compound exhibits no detectable mutagenic activity (16), it is more plausible that it acts as a tumor promoter. However, the molecular mechanism of this tumor-promoting activity is unknown.

On a molecular level, arsenite acts as a sulfhydryl reagent which binds to free thiol (–SH) groups of proteins (15). All protein tyrosine phosphatases contain such –SH groups (18) and it was recently shown that arsenite inhibits a JNK phosphatase, presumably via this mechanism (19). Additionally, arsenite activates the MKK6/p38 pathway (17) as well as the SEK/SAPK pathway. In previous work, no effect on ERK has been reported, suggesting that arsenite is purely a stress-inducing reagent (17, 19).

We report here that ERK is activated by arsenite in a delayed fashion with different kinetics than ERK activation by growth factors. The activation mechanism involves the MKK6/p38 pathway and the Ras/Raf/MEK-signaling cassette. De novo protein synthesis appears not to play a role, indicating that ERK activation is controlled by a novel intracellular mechanism, connecting stress and mitogenic signaling.

**Experimental Procedures**

Cell Lines, Antibodies, and Inhibitors—The human embryonic kidney cell line HEK293 was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) (heat inactivated
at 56 °C for 30 min) at 37 °C in humidified air with 5% CO₂. After transfection, cells were starved in 0.3% FCS containing Dulbecco's modified Eagle's medium 48 h prior to stimulation. The human promyelocytic cell line HL60 was maintained in RPMI 1640 medium supplemented with 10% FCS. $3 \times 10^5$ cells/well (2-cm diameter) were seeded in 6-well plates and were starved in RPMI 1640 with 0.3% FCS 24 h prior to stimulation. Human promonocytic U937 cells and the A3.01 T-lymphoma cell line were cultured in RPMI 1640 with 10% FCS. $3 \times 10^5$ cells/ml of RPMI containing 10% FCS were seeded one day prior to stimulation. The primary human umbilical vein endothelial cells were cultured in endothelial growth medium containing 2% FCS, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 50 μg/ml gentamycin, 50 μg/ml amphotericin B, and 4 μl/ml bovine brain extract. Specific antibodies to ERK1 (C14), ERK2 (C16), and p38 (C20) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The monoclonal anti-HA antibody 12CA5 and rabbit polyclonal anti-c-Raf antibody were produced at the Institut für Medizinische Strahlenkunde und Zellforschung. Anti-active ERK antibodies were obtained from New England Biolabs, Beverly, MA. The specific p38 inhibitor SB203580 (Calbiochem) was used at a concentration of 5 μM f r o m a 20 mM stock solution in Me₂SO. If cells were cotransfected with different DNAs, DNA content was normalized with an appropriate empty expression vector. Cells were starved in Dulbecco's modified Eagle's medium, 0.3% FCS 48 h prior to stimulation. Cells were stimulated with 0.5 mM sodium-meta-arsenite (Sigma), 100 μg/ml methyl meth-

FIG. 1. ERK is activated by arsenite in different cells. Cells were maintained as described under “Experimental Procedures” and stimulated with arsenite for the indicated times (minutes) (A). After cell lysis ERK activity was assayed by immunoprecipitation with an anti-ERK antiserum in immunocomplex kinase assays with MBP as substrate. p38 and ERK activities were determined from the same cell lysates in immunocomplex kinase assays with the substrates 3pK K-M or MBP, respectively, (B and C).

DNA Constructs and Transfection Procedures—p38 wild-type and p38(AF) mutant were cloned into the pCDNA3 expression vector and tagged with the flag epitope. ERK1 and ERK2 cDNAs were HA-tagged and cloned into KRSPA or pCDNA3 expression vectors, respectively. pCDNA3flagMKK6 wt and mutants were a kind gift from Dr. R. Davis, Worcester, MA. Dominant negative forms of Raf (Raf-C4B) and Ras (Ras N17) were cloned into KRSPA expression vectors described in Flory et al. (20). For transfection of HEK293 cells, $5 \times 10^5$ cells were seeded in a 10-cm diameter dish and grown 24 h in Dulbecco's modified Eagle's medium, 10% FCS prior to transfection. Transfections were performed by a calcium phosphate coprecipitation method using 5–10 μg of DNA unless otherwise indicated, according to a modified Stratagene transfection protocol (20). If cells were cotransfected with different DNAs, DNA content was normalized with an appropriate empty expression vector. Cells were starved in Dulbecco's modified Eagle's medium, 0.3% FCS 48 h prior to stimulation. Cells were stimulated with 0.5 mM sodium-meta-arsenite (Sigma), 100 μg/ml methyl meth-

FIG. 2. Increased ERK activation by arsenite after cotransfection of p38 or MKK6. HEK293s were either left untransfected (A) or transfected with HA-ERK1 and an empty expression vector (B), HA-ERK1 and p38 wt (C and E), HA-ERK2 and p38 WT (F), and HA-ERK1 together with MKK6 WT (D). DNAs were cotransfected in a 1:1 ratio. Cells were starved for 48 h and subsequently stimulated with arsenite for the indicated times. ERK activities were determined in immunocomplex kinase assays as described in Fig. 1 using either an anti-ERK1 antiserum (A) or an anti-HA monoclonal antibody (B-F). Corresponding Western blots are shown for every experiment (A-D, lanes 5–8, E and F, below).
arsenite, 1% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM pefabloc, 1 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM pefabloc, 1 mM sodium orthovanadate, 5 mM benzamidine, 5 mM benzamidine, 5 mM benzamidine, 5 mM benzamidine, 1 mM dithiothreitol, and 1 mM sodium vanadate), and washed extensively with high salt TLB buffer (20 mM Tris, pH 7.4, 4 °C. The immunocomplexes were precipitated with protein A-agarose and detected by a Bio Imaging Analyzer BAS 2000 (Fuji). Every experiment was repeated at least twice.

Immunoprecipitated kinases were washed twice, both in high salt TLB and kinase buffer (10 mM MgCl₂, 25 mM β-glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamidine, 0.5 mM dithiothreitol, and 1 mM sodium vanadate) and then assayed in the same buffer supplemented with 5 μCi [γ-32P]ATP, 0.1 mM ATP, and substrate proteins at 30 °C for 15 min. ERK activity was assayed with MBP (Sigma), p38 with (p38) and 0.8 μM of purified glutathione S-transferase-MEK1 and 0.8 μg of ERK1 in the same buffer supplemented with 0.1 mM ATP in a volume of 20 μl at 30 °C for 30 min. Subsequently 2 μg of MBP and 5 μCi [γ-32P]ATP was added and incubated for a further 15 min at 30 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene fluoride membranes (Millipore) and detected by a Bio Imaging Analyzer BAS 2000 (Fuji). Equal amounts of immunoprecipitated protein was determined by anti-c-Raf specific immunoblot.

**FIG. 3.** Arsenite activates Raf, and ERK activation by arsenite is blocked by dominant negative forms of MKK6, p38, Raf, Ras, and kinase inhibitors SB203580 and PD98059 specific for p38 and MEK, respectively. HEK293 cells were either left untransfected (panel F) or cotransfected with HA-ERK1 and either an empty expression vector (panels A, B, and E, lanes 1–4), MKK6 (A) (panel A, lanes 5–8), p38 (AF) (panel B, lanes 5–8), Raf-C4B (panel E, lanes 5–8), RasN17 (panel E, lanes 9–12), or p38 WT (panels D, C, B, and G). Cells were treated with arsenite for the indicated times or pretreated with SB203580 (panel C, lanes 5–8) or PD98059 (panels D and G, lanes 5–8) for 30 min prior to arsenite stimulation. ERK activities were determined in immunocomplex kinase assays as described in Fig. 1 using either an anti-ERK1 antisemur (panel F) or an anti-HA monoclonal antibody (panels A–C, E, and G). Raf activities were determined in a coupled immunocomplex kinase assay including recombinant MEK, ERK, and MBP as described under “Experimental Procedures” (panel D). As a control (panel D, lane 1) only MEK, ERK, and MBP were subjected to the kinase assay. Panels A–C show a representative autoradiography (left) and quantification data of three independent experiments (right), representing relative kinase activities compared with that of the corresponding unstimulated cells.

**Immunoprecipitation and Western Blot**—Cells were lysed in a modified radioimmune precipitation buffer (25 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 10% (v/v) glycerol, 0.1% SDS, 0.5% (v/v) deoxycholate, 1% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM pefabloc, 1 mM sodium vanadate, 5 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml aprotinin, 5 μg/ml leupeptin) on ice for 30 min. Cell debris was removed by centrifugation at 15,000 rpm for 10 min. Supernatants were then incubated with anti-p38, anti-ERK1/2, or anti-c-Raf antisera or anti-HA antibody for 2 h at 4 °C. The immunocomplexes were precipitated with protein A-agarose and washed extensively with high salt TLB buffer (20 mM Tris, pH 7.4, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 500 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM pefabloc, 1 mM sodium orthovanadate, 5 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin). Immunoprecipitates were used for immunocomplex kinase assays.

**For protein detection in Western blots the immunocomplexes were suspended in electrophoresis sample buffer and heated to 100 °C for 3 min.** For detection with antiactive ERK antibodies, cells were lysed directly in electrophoresis sample buffer. After SDS-polyacrylamide gel electrophoresis, gels were electroblotted onto polyvinylidene fluoride membranes (Millipore) and subjected to immuno-detection using the appropriate primary antibody. Proteins were visualized using a horse-radish peroxidase-conjugated protein A (Amersham) and a standard ECL reaction (Amersham). Detection of proteins by Western blotting was routinely done in every experiment, however, only some blots are shown.

**Immunocomplex Kinase Assays with ERK and p38**—Immunoprecipitated kinases were washed twice, both in high salt TLB and kinase buffer (10 mM MgCl₂, 25 mM β-glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamidine, 0.5 mM dithiothreitol, and 1 mM sodium vanadate) and then assayed in the same buffer supplemented with 5 μCi [γ-32P]ATP, 0.1 mM ATP, and substrate proteins at 30 °C for 15 min. ERK activity was assayed with MBP (Sigma), p38 with (p38) and 0.8 μM of purified glutathione S-transferase-MEK1 and 0.8 μg of ERK1 in the same buffer supplemented with 0.1 mM ATP in a volume of 20 μl at 30 °C for 15 min. Subsequently 2 μg of MBP and 5 μCi [γ-32P]ATP was added and incubated for a further 15 min at 30 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene fluoride membranes (Millipore) and detected by a Bio Imaging Analyzer BAS 2000 (Fuji). Equal amounts of immunoprecipitated protein was determined by anti-c-Raf specific immunoblot.
**ERK Is Activated By Arsenite in Various Cell Lines**—We have monitored ERK activity for up to 6 h after arsenite stimulation. ERK activation was determined in various human cell lines including the T-cell line A3.01, the promyelocytic leukemia cell lines HL60, the promonocytic cell line U937, the human embryonic kidney cell line HEK293, as well as in primary human endothelial cells (HUVEC). Kinase activity was either examined in immunocomplex kinase assays with MBP as substrate or monitored with phospho-ERK specific antisera. Data are not shown), p38 was activated in a strong sustained fashion by arsenite (data not shown), p38 was activated in a strong sustained fashion by arsenite (21), whereas inactive p38 is created by replacement of two activating phosphorylation sites with alanine (A) and phenylalanine (F) (p38 (AF)), coexpression of ERK with the inactive kinases partially blocked the activation of ERK by arsenite (Fig. 3, A and B). These data were confirmed by the use of a specific p38 inhibitor, SB203580, which partially blocked ERK activation even in p38 cotransfected cells (Fig. 3C). These results strongly suggest an involvement of the MKK6/p38 pathway in ERK activation by arsenite.

**Kinase-inactive Mutants of MKK6 and p38 As Well As By the Specific p38 Inhibitor SB203580**—We further analyzed MKK6 and p38 in arsenite-induced ERK activation by cotransfection of HA-ERK1 with kinase-inactive mutants of MKK6 and p38. MKK6 (A) has replaced the conserved lysine in the ATP binding site by alanine (A) (21), whereas inactive p38 is strongly stimulated p38 WT (panels A and C). Cells were either treated with arsenite (panels A-C, lanes 1–4), anisomycin (panel A, lanes 5–8), arsenite in combination with anisomycin (panel A, lanes 9–12), or arsenite in combination with 100 μg/ml cycloheximide (panels B and C, lanes 5–8) for the times indicated (in minutes). ERK activities were determined in immunocomplex kinase assays as described in Fig. 1 using an anti-HA monoclonal antibody.

RESULTS

**ERK is activated by arsenite in a strong sustained fashion, whereas activation by anisomycin is transient.** Cells were cotransfected with HA-ERK1 and Flag-p38 WT and treated either with arsenite (A) or anisomycin (B) for the times indicated (in minutes). p38 and ERK activities were determined in immunocomplex kinase assays as described in Fig. 1 using an anti-Flag or anti-HA monoclonal antibody. Although ERK was activated by arsenite but not by anisomycin (data not shown), p38 was activated in a strong sustained fashion by arsenite (A) and in a transient fashion by anisomycin (B).

**De Novo Protein Synthesis Is Not Required for ERK Activation By Arsenite**—Activation of ERK is commonly mediated by the Ras/Raf/MEK pathway. We tested whether this is also the case after arsenite treatment of cells. Therefore, endogenous Raf activity was measured after arsenite treatment of HEK293 cells. Significantly elevated Raf activity was determined after 180 min of arsenite treatment (Fig. 3D). Moreover, HA-ERK1 was cotransfected with either dominant negative Raf (Raf C4B) or Ras (Ras N17) (Fig. 3E). Both mutants inhibited arsenite-induced ERK activation indicating an involvement of the mitogenic cascade rather than a direct activation by p38. Consistent with these results a specific inhibitor of MEK, PD98059, blocked activation of both endogenous (Fig. 3F) and transfected ERK (Fig. 3G). Thus, up-regulation of ERK activity upon arsenite treatment is mediated by the Ras/Raf/MEK pathway.

**De Novo Protein Synthesis Is Not Required for ERK Activation By Arsenite**—To analyze whether the delayed kinetics of arsenite-induced ERK activation is due to de novo protein synthesis, anisomycin was used to stimulate cells. Anisomycin strongly stimulates p38 activity (9) at concentrations that also block protein synthesis (22). Fig. 4A, lanes 5–8 show that anisomycin has no effect on ERK activation, however, the reagent did not overcome the activating effect of arsenite on ERK.
Overexpression of Constitutively Active MKK6 Results in a Significant Activation of ERK in the Absence of Arsenite—To elucidate whether constitutive activation of the MKK6/p38 pathway gives rise to ERK activation in the absence of extracellular stimuli, MKK6 (EE) was cotransfected with HA-ERK1.

This mutant is constitutively activated by replacement of two activating phosphorylation sites by glutamic acid (E) and thus, leads to a constitutive activation of p38 (21). Cotransfection of MKK6 (EE) resulted in a significant activation of ERK (5-fold compared with vector control), which did not, however, reach the maximal level of activation observed with arsenite and could not be enhanced by increasing doses of MKK6 (EE) (Fig. 6A). However, amplification of the MKK6 (EE) signal by cotransfection of wild-type p38 gave rise to a further elevated ERK activation up to 18-fold (Fig. 6B). This result demonstrates that strong and sustained p38 activity leads to ERK activation. The activation of ERK by MKK6 (EE) overexpression occurs most likely by the same pathway as after arsenite treatment, since it could be blocked by the specific MEK inhibitor PD98059 (Fig. 6C, lane 3). Nevertheless, additional treatment with arsenite strongly increased ERK activation (Fig. 6C, lane 4). This may either be a quantitative effect or due to additional arsenite specific signals.

**DISCUSSION**

We have shown that the stress-inducing agent arsenite activates the mitogen-activated protein kinases ERK 1 and 2 in a delayed fashion, compared with stimulation of cells by mitogens. Employing various active and inactive forms of signaling molecules as well as specific inhibitors, we have demonstrated that the MKK6/p38 pathway as well as the Ras/Raf/MEK-signaling module plays a role in stress-induced ERK activation.

The link between p38 and Ras/Raf is yet unclear. However, de novo protein synthesis appears not to be involved. Since activation of ERK1/2 was not observed with stress-inducing agents such as methyl methanesulfonate (data not shown) and anisomycin, which transiently and less potently activates the MKK6/
p38 pathway, we assume that a strong and sustained p38 activation is required for stimulation of the mitogenic pathway. This assumption is supported by the finding that an active form of MKK6 alone is able to activate the mitogenic cascade. p38 protein amount appears to be rate limiting, since coexpression of p38 with MKK6 (EE) leads to a further increase in ERK activity. However, additional arsenite treatment of constitutively activated MKK6-expressing cells results in a more elevated ERK activation. It might be possible that this is a quantitative effect in that the MKK6 (EE) mutant is not as active as arsenite stimulated wild-type MKK6 and therefore results in a weaker activation of ERK compared with stimulation by arsenite. On the other hand it cannot be ruled out that another arsenite-specific signal may be involved in ERK activation. This secondary signal might either arise from a direct effect on a phosphatase (19) or from activation of yet unidentified pathways that indirectly interfere with ERK activation. Thus, we cannot completely rule out that the differences observed here are qualitative rather than quantitative.

The fact that ERK activation by constitutively active MKK6 could be blocked by the MEK specific inhibitor PD98059 demonstrates that there is a link between the activated MKK6/p38 pathway and the Raf/MEK/ERK cascade mediated by a yet unknown downstream effector of p38. So far p38 is known to regulate gene expression from various promoters (24, 25), presumably via direct phosphorylation of transcription factors. However, since activation of ERK occurs in the presence of protein synthesis inhibitors, transcription factors do not appear to play a role here. Other effectors of p38 are members of the MAPKAP-kinase family, namely MAPKAP-K2, 3pK/MAPKAP-K3 (9) and the recently identified kinases Mnk1 and Mnk2 (10, 11). Nevertheless, none of these kinases have been shown to activate the mitogenic cascade. Thus, a novel effector of p38 could be involved in the process described here. This novel effector may either be an autocrine factor released to the medium when phosphorylated, an upstream component of the Ras/Raf initiated cascade which is directly regulated via phosphorylation by p38, or an indirect adaptor or mediator protein. With respect to this mechanism, Ras might be activated in a growth factor receptor-independent fashion. For example, dominant negative Ras (RasN17) was shown to block the TPA-induced effect of protein kinase C on the downstream cascade (26) indicating that at least some protein kinase C isoforms may act upstream of Ras.

Although we now have some knowledge of signaling pathways involved in ERK activation by arsenite, the biological consequences remain to be elucidated. In addition to the toxic effect of arsenite, the compound exhibits a highly carcinogenic activity (15, 16). Since no mutagenic effects could be demonstrated, arsenite seems to act as a tumor promoter. However, the mechanism of this tumor-promoting activity is still unclear. The novel observation of a relatively weak but sustained activation of the Raf/MEK/ERK cascade may be an explanation for the mode of arsenite action in carcinogenesis.

In summary, we have identified a novel pathway of ERK activation that differs in mechanism from the classic route of activation by mitogens in that MKK6 and p38 play a role here. This is a first example of an involvement of a stress kinase cascade in activation of the Raf/MEK/ERK pathway, which may be important with respect to the quality of the signals mediated by the mitogen-activated kinases ERK1/2.

Acknowledgments—We are very thankful to Manuela Schuler and Renate Metz, Institut für Medizinische Strahlenkunde und Zellforschung, Würzburg, for providing reagents and help with the Raf kinase assays. We also thank R. Davis, Worcester, for providing constructs. Helpful discussion and critical reading of the manuscript by Christoph K. Weber, Josef Slupsky, Bruce Jordan, and Egbert Flory is greatly acknowledged.

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