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The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase

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Trivalent arsenic (As^{3+}) is highly carcinogenic, but devoid of known mutagenic activity. Therefore, it is likely to act as a tumor promoter. To understand the molecular basis for the tumor-promoting activity of As^{3+}, we examined its effect on transcription factor AP-1, whose activity is stimulated by several other tumor promoters. We found that As^{3+}, but not As^{5+}, which is toxic but not carcinogenic, is a potent stimulator of AP-1 transcriptional activity and an efficient inducer of c-*fos* and c-*jun* gene expression. Induction of c-*jun* and c-*fos* transcription by As^{3+} correlates with activation of Jun kinases (JNKs) and p38/Mpk2, which phosphorylate transcription factors that activate these immediate early genes. No effect on ERK activity was observed. As^{5+}, on the other hand, had a negligible effect on JNK or p38/Mpk2 activity. Biochemical analysis and co-transfection experiments strongly suggest that the primary mechanism by which As^{3+} stimulates JNK activity involves the inhibition of a constitutive dual-specificity JNK phosphatase. This phosphatase activity appears to be responsible for maintaining low basal JNK activity in non-stimulated cells and its inhibition may lead to tumor promotion through induction of proto-oncogenes such as c-*jun* and c-*fos*, and stimulation of AP-1 activity. The same phosphatase may also regulate p38/Mpk2 activity.

Keywords: AP-1/arsenite/JNK phosphatase/tumor-promoting activity

Introduction

Trace metal ions are necessary for the proper function of all organisms, yet when present in high concentrations they can lead to severe toxicity, including cell death and genotoxic damage. Zinc (Zn) and copper (Cu), for example, are essential elements serving as cofactors for many enzymes (Vallee and Wacher, 1970). Cadmium (Cd), mercury (Hg) and arsenic (As), on the other hand, lack obvious biological function and are environmental toxins (Klaassen, 1991). In addition to acute toxicity caused by inhibition of enzymes involved in energy metabolism, protein synthesis and transcription, chronic exposure to chromium (Cr), nickel (Ni), Cd and As can result in carcinogenesis (reviewed by Snow, 1992). Both the free ions and organic derivatives of Cr (Wolf *et al.*, 1989), Ni (Ciccarelli and Wetterhahn, 1985) and Cd (Koizumi and Waalkes, 1990) are tumor initiators that can interact directly with nucleic acids or chromatin, causing genotoxic damage and mutagenesis. The mechanism of As carcinogenesis, however, is not clear and there is suggestive evidence that it acts as a tumor promoter rather than an initiator because it does not cause direct genotoxic damage (Snow, 1992). Exposure to As is generally in the form of either arsenite (As^{3+}) or arsenate (As^{5+}), the former being considerably more carcinogenic than the latter (Snow, 1992). While both As^{3+} and As^{5+} are toxic, they affect different mechanisms; As^{5+} is similar in structure to phosphate and is, therefore, an uncoupler of oxidative phosphorylation, whereas As^{3+} is a sulfhydryl reagent that interacts with thiol groups (Snow, 1992). Treatment of cells with As^{3+} inhibits glutathione reductase and diminishes the intracellular level of reduced glutathione (GSH). Correspondingly, elevated GSH protects against As^{3+} (Snow, 1992). GSH depletion was, therefore, suggested to play an important role in mediating the carcinogenic effect of As^{3+}.

Another plausible mechanism for tumor promotion by As^{3+} involves direct interaction with components of intracellular signaling pathways via free -SH groups on proteins. By affecting intracellular signaling, As^{3+} may function in a manner akin to other well-studied tumor promoters, the phorbol esters, which bind to protein kinase C (PKC) isozymes and activate them (Nishizuka, 1984). PKC activation results in the induction of immediate early genes, such as c-*fos*, c-*jun* and c-*myc*, whose products stimulate cell proliferation (Angel and Karin, 1991). Reasonable targets for As^{3+} that have an important role in cellular signal transduction are protein tyrosine phosphatases (PTPases). All known PTPases are -SH enzymes (Walton and Dixon, 1993) and therefore sensitive to thiol reagents (Tonks *et al.*, 1988). Metal ions that inhibit PTPases, such as Zn^{2+} and As^{3+}, have a high affinity for -SH groups. As^{3+} reacts most avidly with vicinal thiols and less strongly with thiols proximal to hydroxyls (Hannestad and Sorbo, 1980; Brown *et al.*, 1987). It is conceivable that As^{3+} interacts *in vivo* with the cysteine at the catalytic site of PTPases to inhibit their activity. Although the cellular functions of most PTPases are not clear, their ability to dephosphorylate phosphotyrosine residues, together with the known function of tyrosine kinases in mitogenic signaling (Ullrich and Schlessinger, 1990), suggest that at least some of them may function as negative growth regulators (Sun *et al.*, 1993; Hunter, 1995). Indeed, some evidence to that effect was provided recently (Klingmüller *et al.*, 1995). Therefore, inhibition of PTPases may result in activation of mitogenic signaling cascades and thereby lead to tumor promotion.

One transcription factor whose activity is stimulated by

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a diverse array of mitogens and tumor promoters is AP-1, a dimeric, sequence-specific, DNA binding protein composed of *jun* and *fos* proto-oncogene products (reviewed by Angel and Karin, 1991). Activation of AP-1 target genes may mediate many of the biological effects of tumor promoters. We therefore examined the effect of arsenicals on AP-1 activity and the signaling pathways involved in its regulation.

As we describe here, like two other tumor promoters (phorbol esters and UV light; Devary *et al.*, 1991, 1992), treatment of cells with low concentrations of As^{3+}, but not As^{5+}, induces c-*jun* and c-*fos* transcription, and stimulates AP-1 transcriptional activity. As^{3+} also strongly stimulates the activity of the Jun kinases (JNKs), which belong to the mitogen-activated protein kinase (MAPK) family. As^{3+} also had a modest stimulatory effect on p38/Mpk2, but hardly any effect on ERK activity. Previous work indicates that JNK phosphorylates and activates several transcription factors involved in the induction of c-*jun* and c-*fos* synthesis, including c-Jun, ATF2 and Elk-1 (reviewed by Karin, 1995). ATF2 and Elk-1 are also phosphorylated and activated by p38/Mpk2 (Dérijard *et al.*, 1995; R.Treisman, personal communication). The activation of JNK by As^{3+} correlates with inhibition of a constitutive dual-specificity phosphatase activity that acts on JNK and may also affect p38/Mpk2. As discussed, these mechanisms are likely to account for the tumor-promoting activity of As^{3+}. Inhibition of PTPases can result in activation of signaling cascades that are normally activated by mitogenic growth factors and proinflammatory cytokines.

Results

As^{3+} but not As^{5+} stimulates AP-1 activity

The AP-1-responsive –73Col-LUC reporter containing the luciferase gene under the control of a truncated human collagenase promoter (Angel *et al.*, 1987; Deng and Karin, 1992) was used in transient transfection assays to asses the effect of arsenicals on AP-1 activity. Treatment of transiently transfected HeLa cells with 50 μM As^{3+} resulted in a 12-fold increase in luciferase activity (Figure 1). By contrast, exposure to 50 μM As^{5+} was ineffective. Treatment of HeLa cells with TPA (12-*O*-tetradecanoyl phorbol 13-acetate), a tumor promoter and activator of PKC and AP-1 (Angel *et al.*, 1987), led to a 5-fold increase in luciferase activity. Induction of luciferase activity by either As^{3+} or TPA required an intact AP-1 binding site, as a –60Col-LUC reporter lacking that site did not respond to either agent (Figure 1).

Next we examined the effect of As^{3+} on the expression of immediate early genes encoding components of AP-1. Exposure of HeLa cells to 50 μM As^{3+} resulted in induction of c-*jun* and c-*fos* mRNAs, peaking between 60 and 120 min post-treatment (Figure 2A). As^{3+} weakly induced *jun*B expression, but other *jun*/*fos* genes (*jun*D, *fos*B, *fra*I, *fra*II) were either not induced or were expressed at very low levels within the time period examined (data not shown). Dose–response analysis indicated that c-*jun* and c-*fos* mRNAs were induced by similar concentrations of As^{3+} (Figure 2B). However, while c-*fos* induction was gradual, c-*jun* induction was more abrupt. As^{5+}, even at

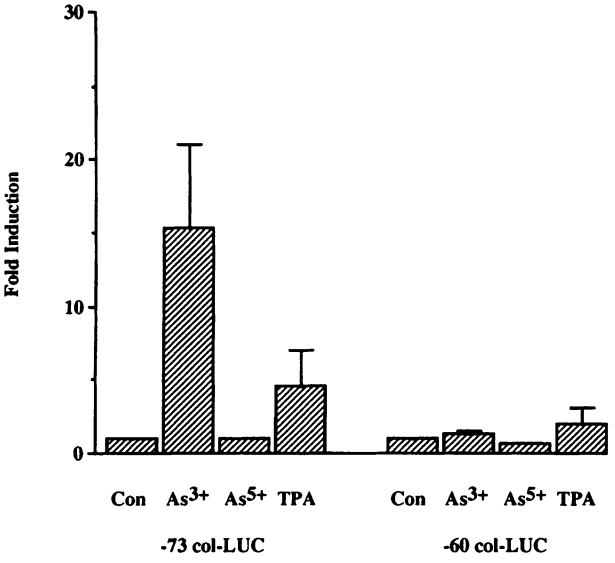

Fig. 1. Stimulation of AP-1 activity by As^{3+}. HeLa cells were transfected with the –73Col-LUC and –60Col-LUC reporters, and 16–20 h later were exposed to either 50 μM As^{3+}, 50 μM As^{5+} or 100 ng/ml TPA. After 16–18 h, the cells were lysed and luciferase activity determined. The results shown are averages ± SD for six (–73Col-LUC) or three (–60Col-LUC) independent transfections done in duplicate.

100 μM, did not induce either c-*jun* or c-*fos* expression, confirming the results of the transient transfection assays.

To determine the effect of As^{3+} and As^{5+} on AP-1 DNA binding activity, gel mobility shift assays were performed using a col-TRE oligonucleotide probe (Angel *et al.*, 1987). While As^{3+} produced little change in AP-1 binding activity within the first hour post-treatment, after 2 h As^{3+}-treated cells exhibited elevated AP-1 binding activity, that peaked at 8 h post-treatment (Figure 3A). As previously described (Devary *et al.*, 1991), TPA treatment increased AP-1 DNA binding activity within 25 min, peaking at ~3 h (data not shown). Later on, this AP-1 binding activity decreased, but was still elevated after 10 h (Figure 3B). As^{5+}, on the other hand, did not stimulate AP-1 binding activity even after 10 h of treatment. The sequence specificity of the As^{3+}-induced AP-1 DNA binding activity is demonstrated by competition with unlabeled col-TRE.

The DNA binding activity of transcription factor NF-κB is induced by a wide variety of stress conditions, including oxidative stress (Schreck *et al.*, 1992). We therefore examined the effect of both As^{3+} and As^{5+} on NF-κB DNA binding activity. Neither As^{3+} nor As^{5+} induced NF-κB DNA binding activity in HeLa cells even after 10 h of treatment (Figure 3C). TPA, as expected, induced NF-κB binding activity, which peaked at 1 h (data not shown), but was still detectable after 10 h. These results indicate that exposure of HeLa cells to 50 μM As^{3+} does not result in indiscriminate activation of all stress-inducible transcription factors and probably does not generate an oxidative stress condition which leads to NF-κB activation (Schmidt *et al.*, 1995).

As^{3+} stimulates JNK and p38/Mpk2 activities

Since As^{3+}-treated cells did not show increased AP-1 DNA binding until after several hours of exposure, it

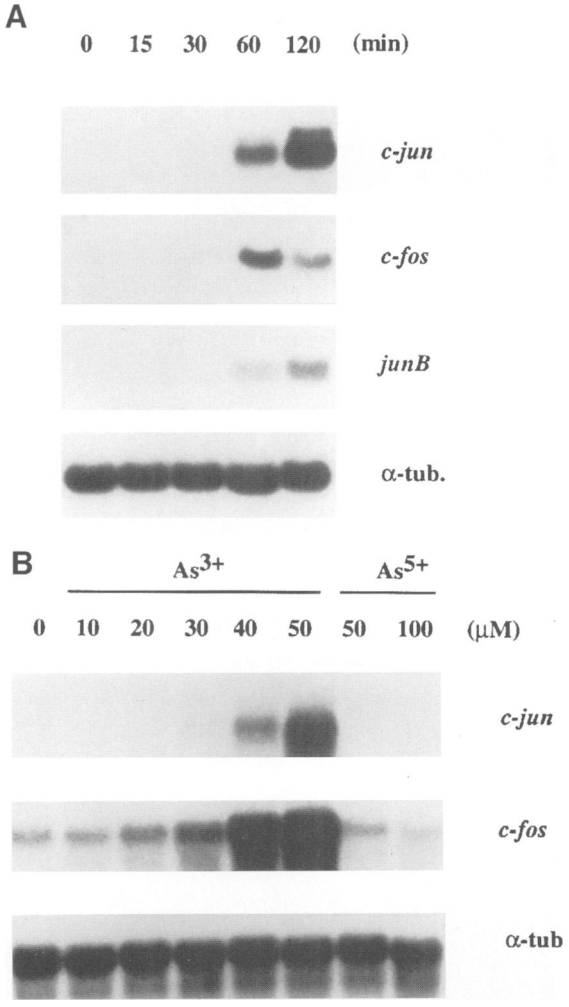

Fig. 2. Induction of *jun* and *fos* gene expression by As^{3+}. (**A**) Serum-deprived HeLa S3 cells were exposed to 50 μM As^{3+} for the indicated times, after which total cytoplasmic RNA was extracted, separated on a denaturing agarose gel, transferred to nitrocellulose and hybridized to c-*jun*, c-*fos*, *jun*B and α-tubulin probes. (**B**) HeLa S3 cells were treated with the indicated concentrations of As^{3+} or As^{5+} for 2 h, after which total cytoplasmic RNA was extracted and analyzed as described above.

is unlikely that As^{3+} causes dephosphorylation of the inhibitory sites at the C-terminus of c-Jun (Boyle *et al.*, 1991; Lin *et al.*, 1992). Most likely, the effect of As^{3+} on AP-1 binding activity is due to increased c-*jun* and c-*fos* transcription. Induction of c-*jun* transcription is well correlated with increased phosphorylation of c-Jun's N-terminal transactivation domain (Binétruy *et al.*, 1991; Smeal *et al.*, 1991; Devary *et al.*, 1992). c-Jun together with ATF2 binds and autoactivates the c-*jun* promoter (Angel *et al.*, 1988; van Dam *et al.*, 1993). The c-Jun N-terminal activation domain is phosphorylated by the JNKs (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994), which also phosphorylate ATF2 and stimulate its transcriptional activity (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). The JNKs can also mediate c-*fos* induction by phosphorylating transcription factor TCF/Elk-1 (Whitmarsh *et al.*, 1995; Cavigelli *et al.*, 1995). We therefore used an in-gel kinase assay (Hibi *et al.*, 1993) to examine the effect of As^{3+} on JNK activity. Lysates of untreated and As^{3+}-treated cells were separated on SDS–polyacrylamide gels that were polymerized in the presence of GST-cJun(1–223). *In situ* renaturation and phosphorylation revealed JNK1 and JNK2 activities migrating at 46 and 55 kDa, respectively (Figure 4A). Both activities were stimulated following exposure to 50 μM As^{3+}. To prove that As^{3+} exposure does indeed activate JNK, a polyclonal antibody raised against JNK1 (Kallunki *et al.*, 1994) was used to measure JNK activity by an immune complex kinase assay. As Figure 4B shows, JNK activity was greatly enhanced in As^{3+}-treated cells and by 1 h the response was similar in magnitude to the UV response. Exposure to As^{5+} did not stimulate JNK1 activity. We also used immune complex kinase assays to determine the response of two other MAPKs involved in stimulation of AP-1 activity: p38/Mpk2 and ERK (Karin, 1995). With antibodies specific to p38/Mpk2 and ATF2 as a substrate (Han *et al.*, 1994; Gupta *et al.*, 1995), we found a modest increase in p38/Mpk2 activity in response to As^{3+}, approximating 25% of the UV activation response. As^{5+} had no effect on p38/Mpk2 activity. ERK2 activity measured with myelin basic protein (MBP) as a substrate, on the other hand, was only marginally stimulated by As^{3+} or UV. Unlike JNK1 or p38/Mpk2, ERK2 activity exhibited the most robust stimulation by TPA.

Modulation of the response to As^{3+} by thiol compounds

As^{3+}-triggered effects are thought to be due to its high affinity towards dithiols (Hannestad and Sorbo, 1980). To examine whether induction of c-*jun* and c-*fos* and JNK activation correlate with the dithiol reactivity of As^{3+}, we used 2,3-dimercaptopropanol (BAL; British Anti-Lewisite) and monothiolglycerol (MTG) as competitors for As^{3+}. HeLa cells were treated with 50 μM As^{3+} for 20 min (to allow a sufficient amount of As^{3+} to enter the cell) followed by the addition of 50 μM BAL or 100 μM MTG (thus maintaining equimolar concentration of -SH groups), and after a further 40 min incubation the levels of c-*jun* and c-*fos* mRNA and JNK activity were determined. Neither MTG nor BAL alone affected c-*jun* mRNA levels or JNK activity (Figure 5A). Addition of BAL abolished induction of c-*jun* and JNK activation by As^{3+}, whereas MTG was not inhibitory. Similar results were obtained when the effect of these compounds on c-*fos* induction was examined (data not shown). BAL was not a general inhibitor of JNK activation as it had no effect on its activation by UV (data not shown). These results are consistent with the notion that vicinal thiol groups are much more likely to be targeted by As^{3+}, than monothiols.

Several reports, however, described that prolonged As^{3+} treatment results in GSH depletion (Snow, 1992). To examine the effect of altered GSH levels on c-*jun* and JNK activation by As^{3+}, HeLa cells were either treated with *N*-acetylcysteine (NAC) or buthionine-sulfoximine (BSO). NAC is readily taken up by cells and serves as a precursor to elevate intracellular GSH (Staal *et al.*, 1990). By contrast, BSO selectively inhibits glutamylcysteine synthetase, resulting in GSH depletion (Meister, 1983). As Figure 5B shows, pre-treatment with NAC inhibited the activation of c-*jun* and JNK by As^{3+}, whereas BSO potentiated these responses. By themselves, these agents had only a minimal effect on c-*jun* expression or JNK activity (data not shown). Thus, even though monothiols

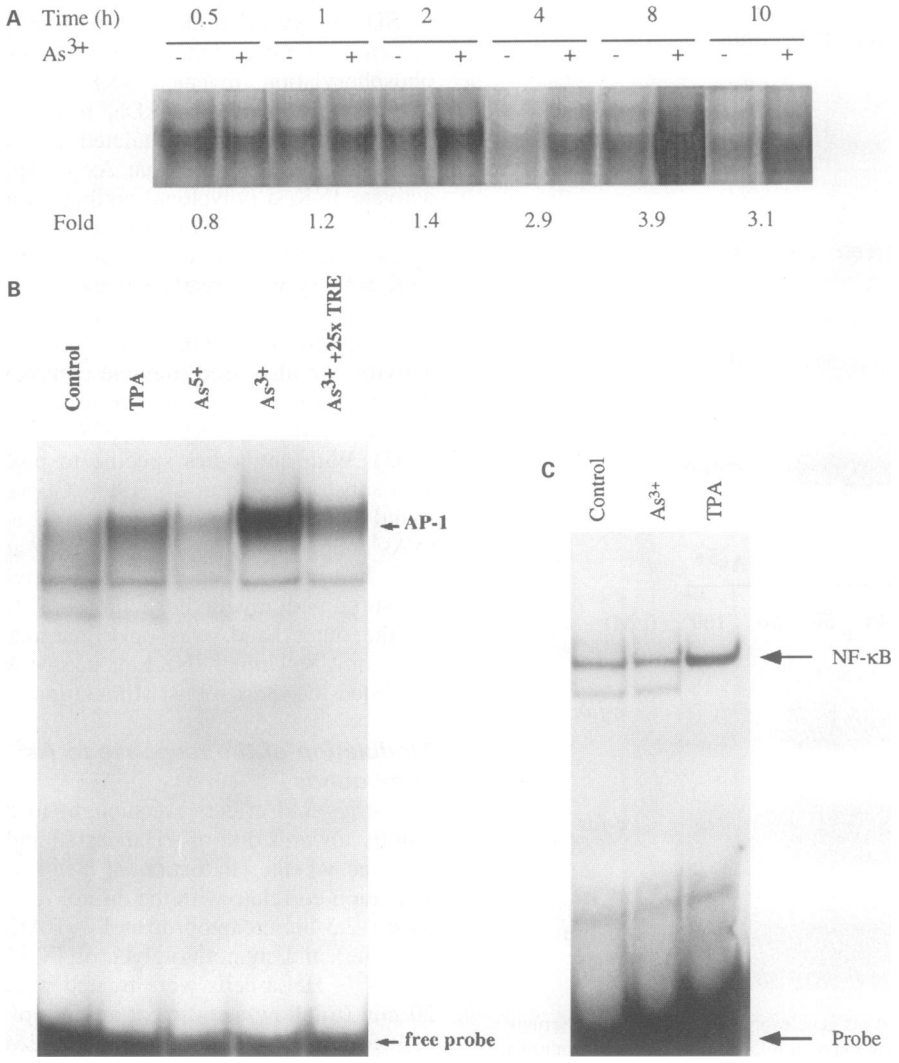

Fig. 3. As^{3+} induces AP-1, but not NF-κB DNA binding activity. (**A**) Serum-starved HeLa S3 cells were incubated either in control medium (–) or in the presence of 50 μM As^{3+} (+). At the indicated time points, whole-cell extracts were prepared and assayed for AP-1 binding activity by incubation with a ^{32}P-labeled col-TRE probe and an electrophoretic mobility shift assay. (**B**) Serum-deprived HeLa S3 cells were incubated with 50 μM As^{3+}, 50 μM As^{5+}, 100 ng/ml TPA or no further additions (control). After 10 h, whole-cell extracts were prepared and assayed for AP-1 binding activity, as described above. The specificity of binding was determined by inclusion of a 25-fold molar excess of unlabeled col-TRE oligonucleotide. (**C**) The same extracts as in (B) were incubated with an NF-κB-specific probe and analyzed by an electrophoretic mobility shift assay.

are not effective direct targets for As^{3+}, modulation of the intracellular level of the major cellular monothiol, GSH, has a strong effect on c-*jun* induction and JNK activation by As^{3+}.

As^{3+} inhibits JNK dephosphorylation

In addition to dithiols, As^{3+} interacts, albeit with lower affinity, with sulfhydryls that are proximal to hydroxyls (Hannestad and Sorbo, 1980). Both arrangements are found at the catalytic sites of several PTPases (Zhang *et al.*, 1995; Muda *et al.*, 1996). JNK and p38/Mpk2 activation requires phosphorylation at Thr and Tyr residues that are conserved among all MAPKs (Dérijard *et al.*, 1994; Han *et al.*, 1994). It is likely that either a mono-specific PTPase or a dual-specificity phosphatase that belongs to the PTPase family may inactivate JNK and p38/Mpk2, as recently demonstrated for the ERKs (Sun *et al.*, 1993) and the yeast homolog of p38/Mpk2, Hog1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995). In fact, the inducible MKP-1 PTPase was recently suggested also to be capable of JNK inactivation when overexpressed (Liu *et al.*, 1995). To examine the effect of As^{3+} on JNK phosphorylation, untreated and UV-treated HeLa cells were lysed in the absence or presence of As^{3+} and JNK activity measured by immune complex kinase assay. This experiment demonstrated that JNK activity in lysates of either non-stimulated or UV-irradiated cells was considerably higher when isolated in the presence of As^{3+} (Figure 6A). To investigate whether this potentiation is due to inactivation of a JNK phosphatase, activated JNK isolated from UV-irradiated HeLa cells was incubated with lysates of untreated cells in the presence or absence of As^{3+} prior to measurement of its activity. The incubation conditions were chosen to prefer phosphatase activity, i.e. absence of divalent ions. The results are shown in Figure 6B (top panel). JNK activity was almost abolished after a 30 min incubation with untreated cell lysates in the absence of As^{3+}. A much smaller decrease was observed when the

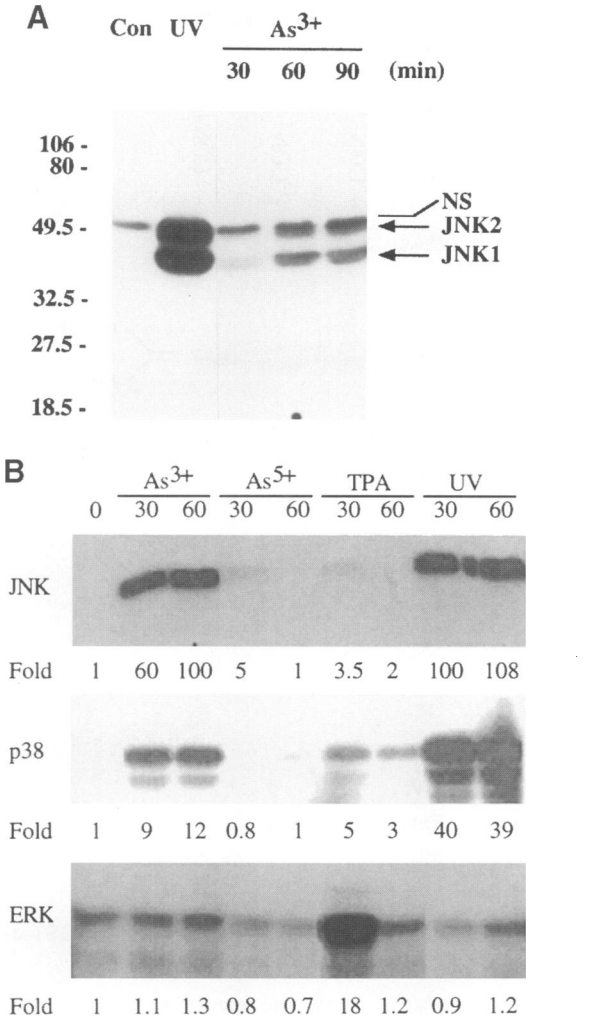

Fig. 4. Activation of MAPKs by As^{3+}. (**A**) Activation of JNK revealed by an in-gel kinase assay. Extracts of non-stimulated (Con), UVC exposed (80 J/m^2) or 50 μM As^{3+}-treated cells prepared at the indicated times or 30 min after irradiation with UVC were separated on SDS–polyacrylamide gels polymerized in the presence of 100 μg/ml GST-cJun(1-223). After electrophoresis, the gel was subjected to renaturation and kinase activity revealed by incubation with 100 μCi/ml [γ^{32}P]ATP. The gel was dried and autoradiographed. The migration positions of molecular weight markers, JNK1, JNK2 and a non-specific kinase band (migrating above JNK2) are indicated. (**B**) HeLa cells were subjected to no further stimulation (Con) or exposed to either 50 μM As^{3+}, 50 μM As^{5+}, 100 ng/ml TPA or 40 J/m^2 UVC. After 30 and 60 min, the cells were lysed and aliquots were immunoprecipitated with antibodies specific to JNK1, p38/Mpk2 or ERK2. The immune complexes were incubated with the respective substrates in kinase buffer in the presence of [γ-^{32}P]ATP for 20 min. The reactions were stopped and separated on 10% SDS–polyacrylamide gels. The dried gels were autoradiographed and quantitated using a phosphoimager. A segment of each autoradiogram depicting the relevant substrate band and the fold stimulation of ^{32}P incorporation over the control values, indicated below the autoradiograms, are shown. Similar results were obtained in two separate experiments.

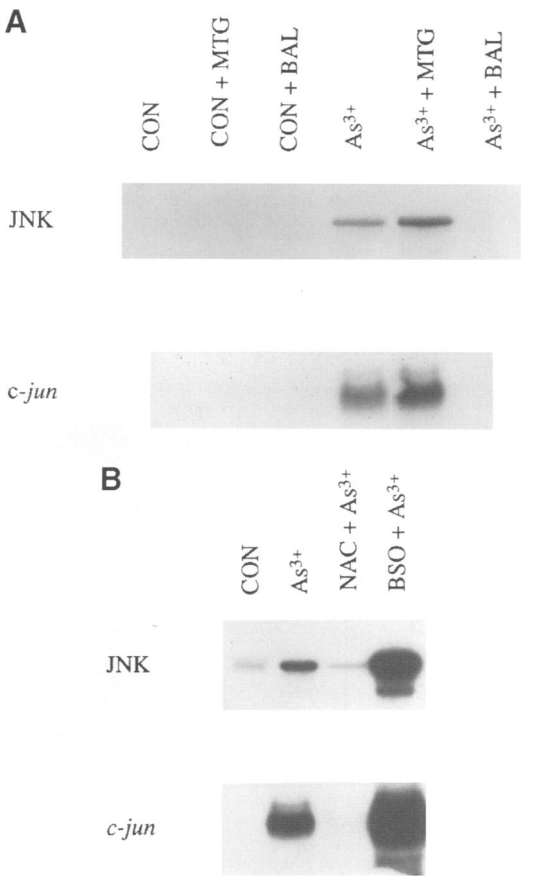

Fig. 5. Effects of thiols on JNK activation by As^{3+}. (**A**) HeLa cells were incubated in either control medium or in the presence of 50 μM As^{3+}. After 20 min, where indicated, 100 μM monothiolglycerol (MTG) or 50 μM 2,3-dimercaptopropanol (British Anti-Lewisite, BAL) were added and the cells were incubated for an additional 40 min, after which cell extracts were prepared. A part of each extract was used to measure JNK activity by immune complex kinase assay, as described in Figure 4 and another part was used to measure c-*jun* and c-*fos* (not shown) induction by Northern blotting, as described in Figure 2. (**B**) HeLa cells were incubated with control medium (CON), 40 mM NAC or 100 μM BSO for 16 h prior to exposure to 50 μM As^{3+}, as indicated. After 1 h, the cells were lysed, and JNK activity and c-*jun* expression were determined, as described above.

same treatment was carried out in the presence of As^{3+} (Figure 6B) or phenylarsine oxide (PAO) (data not shown). Immunoblot analysis of the immunopurified JNK1 revealed that the loss of activity was not due to proteolysis (Figure 6B, middle panel). However, JNK inactivation was associated with increased electrophoretic mobility. Only a partial change in electrophoretic mobility was produced by brief incubation with the extract or a 30 min incubation in the presence of As^{3+}. Previous analyses have shown that JNK1 activation is associated with decreased electrophoretic mobility, most likely caused by its phosphorylation on Thr183 and Tyr185 (Cavigelli *et al.*, 1995).

To examine whether incubation with the cell extract did indeed result in JNK dephosphorylation, ^{32}P-labeled JNK1 was immunopurified from metabolically labeled HeLa cells that were UV irradiated. The purified kinase was subjected to the incubations described above. While 30 min incubation with cell extract in the absence of As^{3+} resulted in a substantial loss of radioactivity, incubation with the same extract in the presence of As^{3+} resulted in only a partial decrease in JNK1 phosphorylation.

Phosphoamino acid analysis of this material indicated that incubation with the cell extract in the absence of As^{3+} resulted in preferential disappearance of phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), while the amount of phosphoserine (P-Ser) was not considerably reduced (Figure 6C). The presence of As^{3+} prevented the preferen-

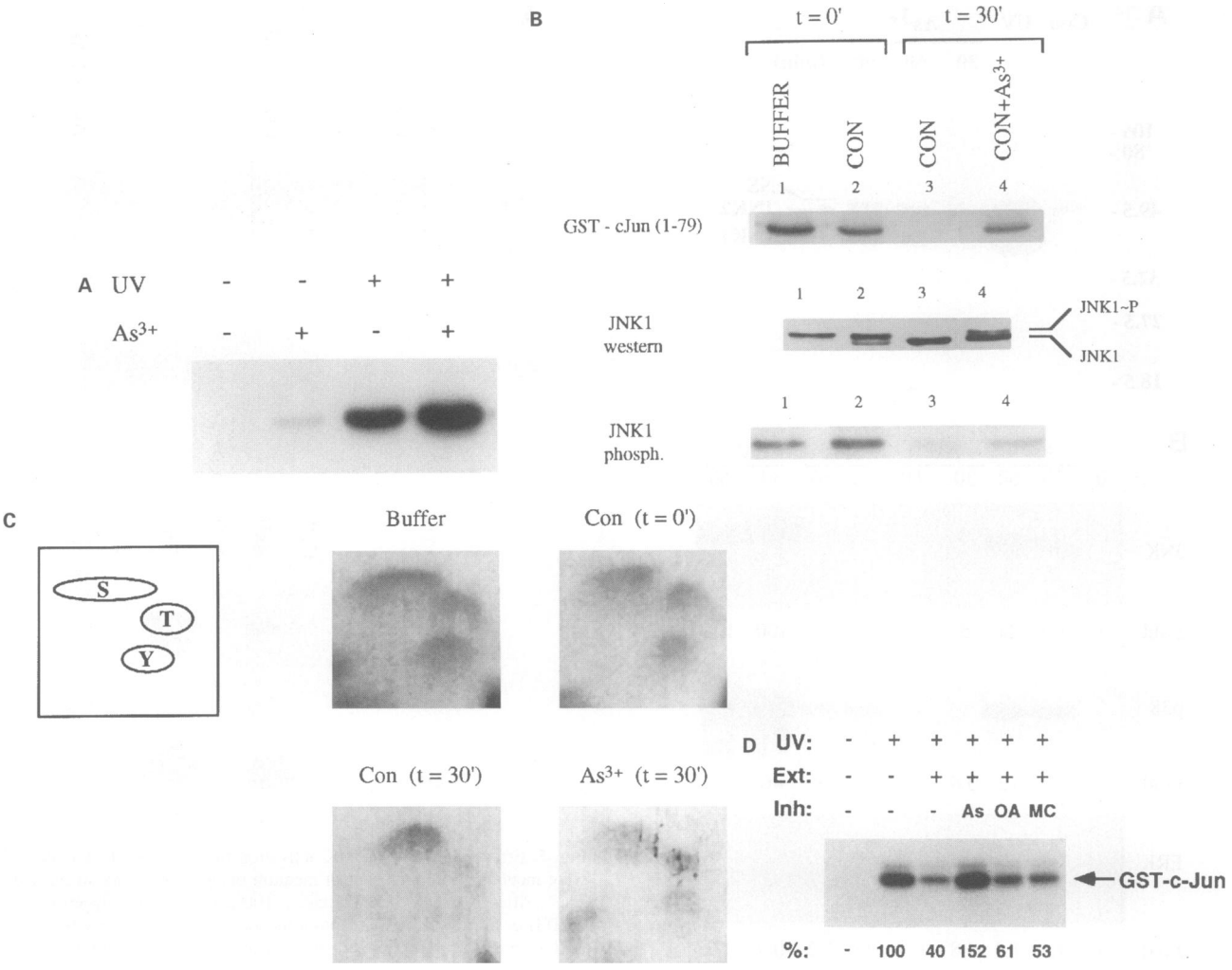

Fig. 6. As^{3+} inhibits a JNK PTPase. (**A**) As^{3+} increases the recovery of JNK activity. JNK1 was immunoprecipitated from lysates of unstimulated or UV-irradiated cells and its activity measured by phosphorylation of GST-cJun(1–223). Where indicated by a + sign, the lysis buffer included 50 μM As^{3+}. (**B**) As^{3+} inhibits a JNK phosphatase. JNK1 was immunopurified from UV-irradiated HeLa cells and incubated for 0 or 30 min with either buffer alone (lane 1) or a cytosolic extract of non-stimulated HeLa cells that were incubated for 30 min prior to harvesting in the absence (CON; lanes 2 and 3) or presence of 50 μM As^{3+} (CON + As^{3+}, lane 4). After this incubation, the immune complexes were isolated and their kinase activity determined by phosphorylation of GST-cJun(1–79) (top panel). A portion of each immune complex was dissociated in SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to a membrane and immunoblotted with JNK1 antibodies (middle panel). JNK1 was also precipitated from ^{32}P-labeled HeLa cells that were UV irradiated. The immune complexes were subjected to the same treatments described above and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography for the extent of JNK1 phosphorylation (bottom panel). (**C**) As^{3+} inhibits a JNK PTPase. The ^{32}P-labeled JNK1 bands from the experiment shown in the bottom panel of Figure 6B were excised and subjected to phosphoamino acid analysis. The autoradiographs of the different plates are shown as well as the migration positions of phosphoamino acid standards. The relative levels of the different phosphoamino acids were quantitated by a phosphoimager to confirm that the JNK phosphatase preferentially affected phosphothreonine and phosphotyrosine, and that this activity was inhibited by As^{3+}. (**D**) The JNK phosphatase is sensitive to As^{3+}, but not okadaic acid or microcystin. JNK1 was isolated from non-stimulated or UV-irradiated HeLa cells by immunoprecipitation and incubated for 1 h at 30°C with either buffer or a cytosolic extract of non-stimulated HeLa cells in the absence or presence of 50 μM As^{3+}, 200 nM okadaic acid (OA) or 200 nM microcystin (MC), as indicated. After these incubations, the immune complexes were reisolated, washed several times and JNK activity determined using GST-cJun(1-79) as a substrate. ^{32}P incorporation was quantitated using a phosphoimager and expressed relative to the level of kinase activity observed after pre-incubation with buffer alone, which was given an arbitrary value of 100%.

tial loss of P-Thr and P-Tyr. These results suggest that untreated HeLa cells contain a constitutive JNK phosphatase that affects both P-Thr and P-Tyr sites.

To examine this phosphatase activity further, we immunopurified JNK1 from UV-irradiated cells (as well as from non-irradiated cells) and incubated it with extracts of non-stimulated cells that were pre-treated with either As^{3+} or the Ser/Thr phosphatase inhibitors okadaic acid and microcystin. As shown in Figure 6D, only As^{3+} inhibited the activity responsible for JNK inactivation. The lack of sensitivity to rather high concentrations of okadaic acid or microcystin indicates that this activity is not protein phosphatase (PP) 1 or PP2A (Holmes and Boland, 1993).

The decay of JNK activity following its stimulation by UV irradiation is likely to be due to phosphatase action. To examine whether the As^{3+}-sensitive JNK phosphatase is responsible for this decay, we irradiated HeLa cells with UV-C and split the cultures into two: one half was left in normal growth medium and the other half incubated in the presence of a suboptimal dose of As^{3+}. At various time points, cell samples were lysed and JNK and p38/Mpk2 activities were measured by immune complex kinase assays. Incubation with As^{3+} had no effect on the initial

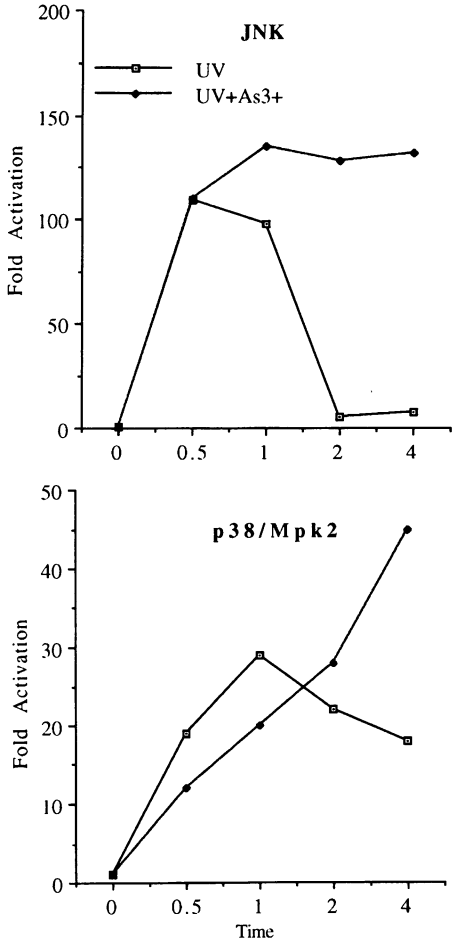

Fig. 7. As^{3+} prolongs JNK and p38/Mpk2 activation. Serum-starved HeLa cells were irradiated with 40 J/m^2 UVC followed by incubation in the absence or presence of As^{3+} (25 μM). At the indicated time points, cells were collected, lysed, and JNK1 and p38/Mpk2 were immunoprecipitated with specific antibodies and their activity measured by immune complex kinase assays. The fold stimulation of kinase activity over the basal activity in non-irradiated serum-starved cells was determined and is plotted as a function of time. Similar results were obtained in two separate experiments.

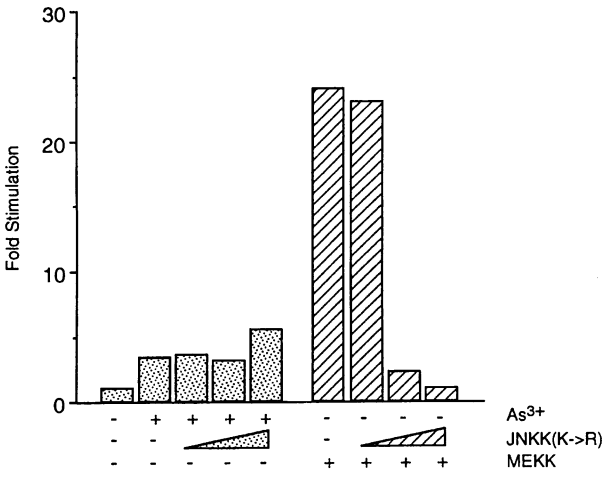

Fig. 8. As^{3+}-stimulated JNK activity is not inhibited by catalytically inactive JNKK. HeLa cells were transiently transfected with pSRα-HA-JNK2 (1 μg/plate) expression vector in the absence or presence of pSRα-JNKK(KR) (1, 2 and 3 μg/plate) and/or pCMV5-MEKK1 (0.2 μg/plate) expression vectors, as indicated. DNA concentration was kept constant by addition of empty pSRα expression vector. After 48 h, HA-JNK2 was isolated from untreated or As^{3+} (50 μM for 1 h) treated cells and its kinase activity determined by phosphorylation of GST-cJun(1–79). Phosphorylation was quantitated using a phosphoimager and converted to fold stimulation above that achieved by incubation with HA-JNK2 isolated from non-stimulated cells. The results are averages of two separate experiments.

rate of JNK or p38/Mpk2 activation (data not shown for early time points; Figure 7). After 30 min, JNK activity began to decay and returned to almost basal levels by 2 h in cells incubated with normal growth medium. However, in cells treated with a suboptimal dose of As^{3+}, JNK activity did not decline and remained elevated for at least 4 h. In the absence of As^{3+}, p38/Mpk2 activity began to decline at 1 h post-UV irradiation. Incubation with As^{3+}, however, prevented this decline and p38/Mpk2 activity continued to rise for at least 4 h (Figure 7).

PTPases can, in principle, affect JNK activity indirectly by inhibiting a tyrosine kinase, such as Src or EGF receptor which lead to JNK activation (Minden *et al.*, 1995). To examine further whether a JNK phosphatase is the major target for As^{3+} that affects JNK activity in intact cells, we used transfection experiments. Epitope-tagged JNK2 (HA-JNK2) expression vector was co-transfected into HeLa cells in the absence or presence of a catalytically inactive JNK kinase [JNKK1(KR)] expression vector. While JNKK1(KR) had no effect on HA-JNK2 activation by As^{3+}, it did inhibit its more efficient activation by co-transfection of an MEKK1 expression vector (Figure 8). The lack of sensitivity to the catalytically inactive JNKK is consistent with As^{3+} affecting JNK activity through inhibition of a JNK phosphatase rather than an upstream component of the JNK signaling cascade.

Discussion

This study was undertaken to identify molecular mechanisms that can explain the carcinogenic activity of trivalent arsenic, As^{3+}. This form of arsenic is much more carcinogenic than the pentavalent form, As^{5+}, a difference that is most likely due to their distinct chemical properties. As^{3+} has a high affinity for dithiols or thiols located next to hydroxyls, whereas As^{5+} is similar in structure to inorganic phosphate and thus interferes with oxidative phosphorylation by formation of unstable As^{5+} esters. Inorganic and organic arsenicals are not mutagenic in either bacterial or mammalian cell systems (Snow, 1992). Since tumor initiation is dependent on mutagenesis or genotoxic damage, trivalent arsenicals are more likely to act as tumor promoters rather than initiators. Indeed, the mode of tumor induction by As^{3+} is mostly consistent with it being a tumor promoter (Snow, 1992). Previous mechanistic analysis of tumor promoters indicates that these agents most likely act by affecting cellular signal transduction pathways, which mimic the actions of mitogens (Weinstein, 1988). For example, phorbol ester tumor promoters, such as TPA, are potent PKC activators (Nishizuka, 1984), while the tumor promoters okadaic acid and microcystin are inhibitors of PP2A and PP1 (Suganuma *et al.*, 1988; Nishiwaki-Matsushima *et al.*, 1992). Both TPA and okadaic acid stimulate AP-1 activity and activate AP-1 target genes (Angel *et al.*, 1987; Kim

et al., 1990; Angel and Karin, 1991). UV light, which, in addition to being a mutagen and a tumor initiator is a tumor promoter, is also a stimulator of AP-1 activity (Stein *et al.*, 1989; Devary *et al.*, 1991). UV stimulates AP-1 activity by activating signaling pathways that result in JNK and p38/Mpk2 activation (Hibi *et al.*, 1993; Dérijard *et al.*, 1994, 1995; Karin, 1995). Thus, the activation of signal transduction pathways that lead to stimulation of AP-1 activity seems to be a common mechanism for diverse types of tumor promoters. The results of this study suggest that As^{3+}, but not As^{5+}, also stimulates AP-1 activity by causing activation of JNK and to a lesser extent p38/Mpk2 activation. However, the mechanism by which As^{3+} stimulates JNK and possibly p38/Mpk2 activity is very different from the mechanism through which UV light triggers the activation of these MAPKs. While UV (either UV-C or UV-B) affects the JNK signaling cascade by activating tyrosine kinases that act upstream of Ras (Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994), the results described above strongly suggest that As^{3+} stimulates JNK activity by inhibition of a constitutively active JNK PTPase. Although not thoroughly explored in respect to p38/Mpk2, the same mechanism may also apply to activation of that MAPK.

As^{3+} induced c-*jun* and c-*fos* expression in a dose- and time-dependent manner. In both cases, the induction requires 30–60 min of incubation with As^{3+}, a considerably slower response in comparison with the UV induction response (Devary *et al.*, 1991; Cavigelli *et al.*, 1995). These slower kinetics, however, are consistent with the kinetics of JNK activation by As^{3+}. While elevated JNK activity can be detected within 5 min of UV exposure, its activation by As^{3+} requires ~30 min. This slow activation is consistent with the kinetics of PTPase inhibition by As^{3+} measured *in vitro*; 50% inhibition of low-molecular-weight PTPase by 50 μM PAO requires ~1 h (Zhang *et al.*, 1992). Good correlation between JNK activation by As^{3+} and c-*jun* and c-*fos* induction is also revealed by comparing the dose–response relationships for all three end points. As previously shown, JNK activation can lead to induction of both c-*jun* and c-*fos* (reviewed by Karin, 1995). By its ability to phosphorylate the stimulatory sites of ATF2 (Dérijard *et al.*, 1995) and TCF/Elk-1 (R.Treisman, personal communication), p38/Mpk2 can also contribute to c-*jun* and c-*fos* induction by As^{3+}. Further support for the role of JNK in the effect of As^{3+} on AP-1 is provided by the tight correlation between the effects of thiols on JNK activity and c-*jun* and c-*fos* induction. While elevated GSH inhibits JNK activation and c-*jun* induction by As^{3+}, both responses are potentiated by lowering the level of intracellular GSH. In addition to supporting a role for JNK activation in c-*jun* and c-*fos* induction, these experiments support the hypothesis, based on chemical considerations, that As^{3+} exerts its biological effects by reacting with SH groups on cellular targets. In fact, As^{3+} does not react with all thiols indiscriminately, but is known to exhibit much higher affinity towards vicinal thiols (Hannestad and Sorbo, 1980). In agreement with these predictions, we find that the dithiol BAL can inhibit c-*jun* induction and JNK activation by As^{3+}, while the monothiol MTG is ineffective. The modulation of both JNK activation and c-*jun* induction by GSH, however, can be explained by the very high intracellular concentration of this monothiol, reaching 10 mM (Kosower, 1989). Since As^{3+} leads to JNK activation at considerably lower concentrations, it is unlikely that its biological activity is simply mediated by sequestration of GSH. This assumption is supported by the finding that inhibition of GSH synthesis using BSO does not lead to JNK activation by itself, although it potentiates its activation by As^{3+}.

What are the cellular thiols whose targeting by As^{3+} results in JNK activation and c-*jun* induction? The simplest interpretation of our results is that As^{3+} inhibits a constitutively active dual-specificity JNK phosphatase. The same phosphatase may also act on p38/Mpk2. We find that inclusion of As^{3+} or PAO in the isolation buffer enhances the recovery of JNK activity. In addition, we find that incubation with suboptimal doses of As^{3+} prevents the attenuation of JNK activity following irradiation. As^{3+} also prolongs the activation of p38/Mpk2, suggesting that it also inhibits a phosphatase involved in the inactivation of this MAPK. This phosphatase was not characterized and may or may not be the same molecule responsible for inactivation of JNK. Most importantly, we find that cytoplasmic extracts of non-stimulated HeLa cells contain an activity that inactivates JNK and removes phosphates from its activating tyrosine and threonine sites, but has no effect on its phosphoserine content. This activity is inhibited by As^{3+}, but not by okadaic acid or microcystin, potent inhibitors of the PP2A and PP1 Ser/Thr phosphatases. As these *in vitro* experiments were carried out under conditions in which protein kinases are inactive (no free divalent ions), it is unlikely that the higher levels of JNK activity obtained after its isolation in the presence of As^{3+} are due to activation of a JNK kinase (JNKK). Indeed, we have not been able to observe *de novo* JNK phosphorylation under these conditions (M.Cavigelli, unpublished results). The existence of an As^{3+}-sensitive JNK phosphatase is further supported by the inability of catalytically inactive JNKK to inhibit the stimulation of JNK activity by As^{3+}. In parallel experiments, this dominant negative mutant blocked JNK activation by MEKK, as expected (Lin *et al.*, 1995).

While identification of the As^{3+}-sensitive PTPase responsible for inactivating the JNKs will require its purification, several predictions pertaining to its identity can be made. So far, all known PTPases are -SH enzymes having a critical cysteine in their active site. However, a subset of these enzymes is probably more sensitive to As^{3+} than the rest because, as discussed above, As^{3+} binds with highest affinity to vicinal thiols and a subgroup of PTPases, the low-M_r PTPases, contain a second cysteine, five residues away from the invariant cysteine common to all PTPases (Zhang *et al.*, 1995). Indeed, low-M_r PTPases were shown to be sensitive to As^{3+} (Zhang *et al.*, 1992). While previously thought to be monospecific PTPases, recent studies indicate that the ubiquitously expressed low-M_r PTPases are actually dual-specificity PTPases (Zhang *et al.*, 1995). The first identified group of dual-specificity phosphatases, which includes MKP1 and several other members (Sun *et al.*, 1993; Muda *et al.*, 1996), is less likely to be involved in As^{3+}-sensitive JNK inactivation. First, members of this group have a serine residue instead of the second cysteine found in the low-M_r PTPases (Muda *et al.*, 1996). As^{3+} is known to react

much more readily with proximal dithiols than with a thiol proximal to an hydroxyl (Hannestad and Sorbo, 1980). Second, many, if not all, members of the MKP group of dual-specificity PTPases are nuclear proteins (Muda *et al.*, 1996), while the As^{3+}-sensitive JNK PTPase is cytosolic. Third, the JNK PTPase is expressed in non-stimulated cells, while most members of the MKP group are inducible and appear to be involved in negative feedback control of ERK rather than JNK activity (Sun *et al.*, 1993; Muda *et al.*, 1996). It should be noted that As^{3+} did not stimulate ERK activity.

Regardless of the exact identity of the As^{3+}-sensitive JNK PTPase, it appears to be an important target for this tumor promoter, whose inhibition results in induction of proto-oncogenes c-*jun* and c-*fos*, and AP-1 activity. Another tumor promoter, okadaic acid, is also a phosphatase inhibitor (Suganuma *et al.*, 1988; Holmes and Boland, 1993) and is also capable of inducing AP-1 activity (Kim *et al.*, 1990). However, the mechanism by which the two phosphatase inhibitors affect AP-1 is different, as okadaic acid is an inhibitor of PP2A. Most likely by inhibiting PP2A, okadaic acid leads to activation of MAPKs, including ERK and JNK. Once these protein kinases are activated AP-1 activity is induced, leading to transcriptional activation of AP-1 target genes, some of which may be required for tumor promotion.

Materials and methods

Cell culture and transfection

HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and transfected as described (Cavigelli *et al.*, 1995). All reporters and expression vectors were described previously (Minden *et al.*, 1994, 1995; Lin *et al.*, 1995). As^{3+} or other treatments were performed for the indicated times after cells had reached confluency and had been serum starved for 24–36 h.

Total cytoplasmic RNA extraction and mobility shift assay

Total cytoplasmic RNA was extracted, electrophoretically separated on 1% formaldehyde–agarose gels and transferred to hybridization transfer membrane (ZetaBind). The membranes were hybridized with ^{32}P-labeled probes specific for c-*jun*, *jun*B, *jun*D, c-*fos*, *fra*-I, *fra*-II, *fos*B and mouse α-tubulin, as described (Cavigelli *et al.*, 1995). To assay AP-1 and NF-κB, whole-cell extracts were prepared as described (Yoshioka *et al.*, 1995). Protein–DNA complexes were formed at 23°C for 20 min in 20 μl reactions containing 10 mM HEPES–KOH (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 100 μg/ml poly(dI-dC) and 0.1 ng of ^{32}P-labeled col-TRE or κB probes (1.0×10^9 c.p.m./μg). Protein–DNA complexes were resolved on 5% non-denaturating polyacrylamide gel containing 0.4×TBE buffer (pH 7.9) at 160–200 V for 1.5–2.0 h at room temperature and visualized by autoradiography.

Protein kinase assays

In-gel kinase assays were performed as described (Hibi *et al.*, 1993) by resolving cell lysates on SDS–polyacrylamide gels containing GST-cJun(1–223) as a substrate. For immune complex kinase assays, cells were lysed for 20 min on ice with a buffer consisting of 20 mM Tris–HCl (pH 7.6), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100 μM Na-vanadate, 0.5% NP-40, 30 μg/ml phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml pepstatin, 5 μg/ml aprotinin and 5 μg/ml leupeptin. After centrifugation for 10 min, a polyclonal antibody generated against human JNK1 (Kallunki *et al.*, 1994) was added and 15 min later protein A beads were also added. After incubating for 2–4 h, the immune complexes were spun down and washed three times with the above buffer complemented with 0.1% SDS and twice with kinase buffer [50 mM HEPES–NaOH (pH 7.6), 10 mM $MgCl_2$]. GSTc-Jun(1–223) was eluted from GSH–agarose beads by adding kinase buffer containing 10 mM GSH and 0.1% β-mercaptoethanol. The kinase reaction was initiated by the addition of 30 μl of kinase buffer containing 10 μM ATP, 0.25 μCi [^{32}P]ATP and 10 μg GSTc-Jun(1–223) per reaction at 30°C for 20 min. The reaction was stopped by addition of 5×SDS sample buffer, boiled and applied to a 10% SDS–polyacrylamide gel. After electrophoresis, the dried gel was autoradiographed and quantitated using a phosphoimager.

In vitro phosphatase assay

Untreated or As^{3+}-treated HeLa S3 cells were washed three times with ice-cold phosphate-buffered saline (PBS), lysed in PTPase buffer [50 mM HEPES–KOH (pH 7.6), 10 mM EDTA, 10 mM EGTA, 0.1% β-mercaptoethanol and protease inhibitors (30 μg/ml PMSF, 5 μg/ml pepstatin, 5 μg/ml aprotinin and 5 μg/ml leupeptin)]. Cells were sonicated (2×15 s), centrifuged for 4°C for 10 min, lysed by freeze–thawing in liquid nitrogen and clarified at 15 000 *g* for 30 s. JNK isolated by immunoprecipitation from UV-irradiated HeLa cells as described above was washed twice in 50 mM HEPES–NaOH (pH 7.6) to remove any divalent ions, and was added to lysates and incubated at 30°C for 30 min. Afterwards, the JNK immune complexes were washed three times with PTPase buffer containing 0.1% SDS, and twice with kinase buffer. The kinase assay was performed as described above.

To examine whether JNK kinase activity can be detected in this buffer, the lysates were incubated at 30°C for 30 min in the presence of 25 μCi [^{32}P]ATP. Equal amounts of the lysates were subjected to SDS–PAGE and autoradiography.

To examine Jun phosphatase activity, GSTc-Jun was subjected to solid-phase kinase assay as described in the presence of [γ-^{32}P]ATP. The beads were washed with kinase buffer containing 20% acetonitrile to remove JNK and twice with the PTPase buffer. The ^{32}P-labeled GSTc-Jun was added to the lysates and incubated at 30°C for 30 min. The beads were pelleted, the proteins were eluted by addition of SDS sample buffer, resolved by SDS–PAGE and autoradiographed.

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