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c-Src-dependent Activation of the Epidermal Growth Factor Receptor and Mitogen-activated Protein Kinase Pathway by Arsenic

ROLE IN CARCINOGENESIS*

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Environmental or occupational exposure to arsenic is associated with a greatly increased risk of skin, urinary bladder, and respiratory tract cancers in arseniasis-endemic areas throughout the world. Arsenic shares many properties of tumor promoters by affecting specific cell signal transduction pathways responsible for cell proliferation. The activation of the epidermal growth factor receptor (EGFR)-extracellular signal-regulated protein kinase (ERK) pathway is important in mediating gene expression related to regulation of cellular growth. In the current studies, we demonstrate that arsenic activates EGFR and ERK in a human uroepithelial cell line. The EGFR phosphorylation by arsenic is ligand-independent and does not involve the major autophosphorylation site Tyr^{1173}. c-Src activity is also induced by arsenic and is a prerequisite for the EGFR and ERK activation. Consistent with these *in vitro* observations, exposure of mice to arsenic in drinking water, which has been found previously to be associated with AP-1 activation and epithelial proliferation, induces EGFR and ERK activation in the urinary bladder. This response is also accompanied with an increase in c-Src levels interacting with EGFR. These findings represent a potential pathway for mediating arsenic-induced phenotypic changes in the uroepithelium.

Epidemiological studies have established a strong association between exposure to arsenic (through contaminated drinking water) and an increased incidence of skin or urinary bladder cancer in arseniasis-endemic areas of the world including Taiwan, Mexico, and Chile (1–3). In the last few years, a tendency of increased incidences of urinary bladder transitional cell carcinomas in the United States has been reported (4). Epidemiological studies are underway to investigate whether this phenomenon might be associated with the arsenic levels in drinking water (5).

Arsenic is not a classical carcinogen, and adequate scientific data on the mode of arsenic action, which has yet to be established, will help determine the safe exposure levels. Subsequently, the mechanisms of arsenic carcinogenesis have been under intense investigation, and increasing evidence suggests that arsenic shares many properties of tumor promoters by affecting specific cell signal transduction pathways involved in cell proliferation (reviewed in Ref. 6). Accordingly, arsenic has been demonstrated to activate members of the MAP kinase family, transcription factors such as AP-1, and immediate early genes, including c-*fos*, c-*jun*, and c-*myc*, whose products help regulate the expression of transforming oncoproteins and growth factors (7–10).

The application of certain physical or chemical stimuli, which are considered cellular stressors, such as arsenic, sulfhydryl reagents, UV radiation, or oxidants, has been shown to activate EGFR[1] as a prerequisite of MAPK activation (11). EGF stimulates tyrosine phosphorylation of its receptor by homodimerization of EGFR and activation of receptor tyrosine kinases (12). The stressor-induced tyrosine phosphorylation of EGFR might be caused by the activation of receptor tyrosine kinases (as a result of direct effects on the receptor and its kinases or dephosphorylation events through inactivation of protein tyrosine phosphatases) or alternatively, by non-receptor tyrosine kinases including c-Src. Because phosphotyrosine phosphatases have highly conserved sulfhydryl groups in their catalytic site, they are potential targets for oxidation by UV or sulfhydryl reagents (11). Arsenite has been shown to activate c-Jun N-terminal kinase (JNK) through sulfhydryl-dependent inactivation of JNK phosphatase (8). The role of non-receptor tyrosine kinase c-Src in arsenic-induced EGFR-MAPK activation has not been investigated. c-Src can bind physically to EGFR and induce tyrosine phosphorylation (13). Parallel activation of c-Src and EGFR has been identified in many human cancers (14).

The objectives of this study were to evaluate whether arsenic induces EGFR and ERK phosphorylation in human uroepithelium, a specific target of arsenic carcinogenicity, and to determine whether this involves c-Src activation. We (15) previously found that *in vitro* or *in vivo* arsenic exposure induced persistent AP-1 nuclear translocation and increased expression of genes associated with cell cycle regulation and uroepithelial cell proliferation. As we report here, similar conditions of arsenic exposure induced EGFR and ERK phosphorylation, and this response was dependent on c-Src activation.

EXPERIMENTAL PROCEDURES

Materials—All chemicals including sodium *m*-arsenite (referred to as arsenic) were from Sigma with the exception of PP-1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo{3,4-D}pyramidine)m, which was from Alexis Inc. (San Diego, CA), and recombinant human EGFα, which was from ICN Pharmaceuticals Inc. (Costa Mesa, CA).

Cell Cultures—UROtsa, an SV40 immortalized human urothelium

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; NAC, *N*-acetyl-cysteine; PP-1, (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo{3,4-D}pyramidine)m; RIPA, radioimmune precipitation buffer.

cell line, was obtained from Dr. G. Petzoldt, (University College, London). The cell line does not acquire the characteristics of transformed cells, including growth in soft agar or development of tumors in nude mice (16). The cells were grown at 37 °C/5% CO_2 in RPMI 1640 culture media supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen), referred to as complete media. Mouse epithelial cell lines B82 and B82 permanently transfected with human EGFR were a gift from Dr. Gordon N. Gill, University of California, and these cells were maintained as described previously (12).

Immunoprecipitation and Western Blot Analysis—All cell treatments were performed at 37 °C in serum-free medium. After treatment, monolayers were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μM $NaVO_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for immunoprecipitation under non-denaturing conditions or RIPA/SDS buffer (RIPA buffer containing 0.1% SDS) for immunoprecipitation under denaturing conditions. Cell lysates were disrupted by repeated aspiration through a 21G needle and clarified by centrifugation. Immunoprecipitates were prepared from 1-ml aliquots of lysate by incubating with the appropriate primary antibody plus 20 μl of protein G plus agarose (Oncogene Research Products, Cambridge, MA) for 2 h or overnight at 4 °C under slight agitation. Immune complexes were washed 4 times with ice-cold RIPA buffer, denatured in Laemmli sample buffer, and resolved by SDS-PAGE. EGFR was precipitated using monoclonal anti-EGFR antibody (clone LA1, Upstate Biotechnology; Lake Placid, NY) or in some experiments, using monoclonal anti-phospho-EGF receptor (Tyr^{1173}, Upstate Biotechnology). Tyrosine phosphorylation or the presence of co-precipitated proteins was detected by immunoblotting. Phosphotyrosine was detected using a 1:1000 dilution of horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (PY20, Amersham Biosciences, Inc.). Total EGFR was detected using a 1:5000 dilution of a specific rabbit IgG antibody (Oncogene). Phosphorylated ERK and pp38 or total ERK and p38 were detected by Western blot analysis of cell lysates using specific rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution. c-Src was detected using a 1:400 dilution of a specific rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The detection antibodies were either peroxidase-linked anti-mouse or anti-rabbit IgG (Amersham Biosciences, Inc.) used at a 1:10,000 dilution. Immune complexes were visualized on nitrocellulose by enzyme-linked enhanced chemiluminescence (ECL, Amersham Biosciences, Inc.) and quantified by scanning laser densitometry.

Transient Transfection—UROtsa cells (0.5×10^6) were seeded into 100-mm tissue culture dishes and incubated in culture media for 24 h. A mixture containing 4 μg of transfection-grade eukaryotic expression vector or Src cDNA mutated vector (Upstate Biotechnology), 20 μl of Plus® reagent, and 30 μl of LipofectAMINE® reagents (Invitrogen) was gently added to each culture and incubated at 37 °C at 5% CO_2 for 3 h. The DNA-containing medium was replaced with fresh RPMI culture medium containing 10% fetal bovine serum, and the cells were cultured for an additional 48 h. Transfected monolayers were serum-starved in RPMI without serum for 16–20 h prior to stimulation. The transfection efficiency was measured by Western blot of c-Src expression.

In Vitro Src Kinase Assay—Src activity in UROtsa cells was measured according to the protocol of Feder and Bishop (17) following serum starvation for 48 h and treatment with 50 μM arsenic or 10 ng/ml recombinant human EGFα. c-Src was immunoprecipitated from cell lysates using anti-c-Src-specific antibodies (clone GD-1; Upstate Biotechnology). Enolase phosphorylation was detected by SDS-PAGE gel electrophoresis. Src activity was also measured using a specific Src substrate peptide included in a commercial kit (Upstate Biotechnology) according to the manufacturer's instructions.

In Vivo Studies—Female C57BL/6 mice were obtained from Charles River Breeding Laboratories, Portage, MI. All animals were housed at NIOSH National Institutes of Health facilities in compliance with AALAC-approved guidelines for the humane treatment of laboratory animals. Animals were maintained on a 12-h light/dark cycle and were provided chow and water *ad libitum.* Groups of 8-week-old mice were provided 50 μg/ml arsenic as sodium arsenite (Sigma) in their drinking water for 8 weeks and sacrificed by CO_2 asphyxia. The urinary bladders were collected under aseptic conditions. The tissue samples were homogenized in RIPA buffer.

Statistical Analysis—All experiments were replicated, and representative findings are shown. Statistical significance was determined by one-way analysis of variance.

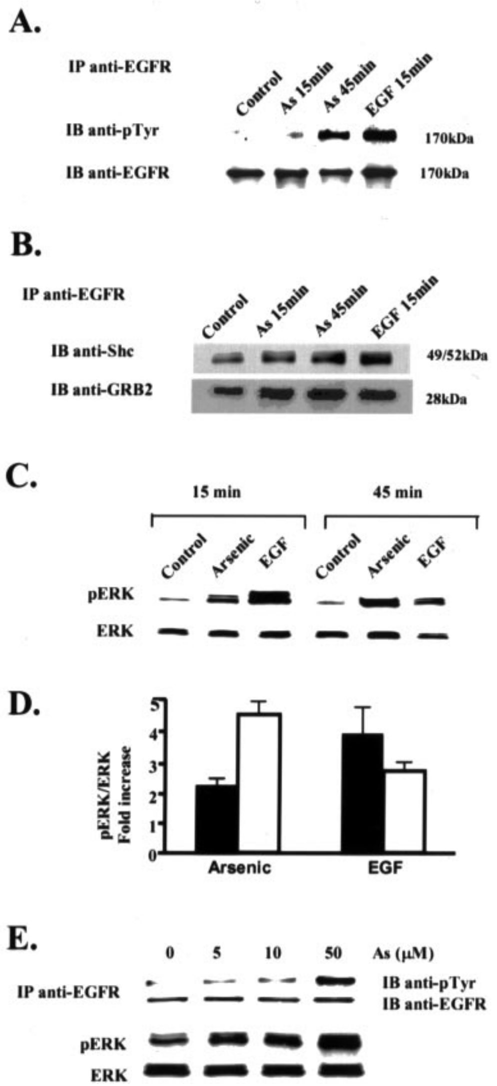

FIG. 1. **Arsenic-induced activation of EGFR and ERK in UROtsa cells.** Serum-starved cells were treated with arsenic as indicated (*A*). The EGFR was immunoprecipitated from cell lysates, and the samples from each lysate were resolved by SDS-PAGE and immunoblotted with either anti-phosphotyrosine antibody, PY20, or anti-EGFR antibody. Cells were treated under the same conditions (*B*). The EGFR was immunoprecipitated from the cell lysates, prepared by non-denaturing RIPA buffer, and analyzed by immunoblotting with anti-Shc or anti-GRB2 antibodies. Total lysates were immunoblotted with antibodies for phosphorylated ERK (*pERK*) or total ERK (*C*). The pERK and ERK bands were quantified by ImageQuaNT analysis of the scanned autoradiographs, and results were presented as fold increase after normalization to total ERK (■, 15 min; □, 45 min) (*D*). Values represent means ±S.E. of three separate experiments. Cells were treated for 45 min with different concentrations of arsenic (*E*). EGFR and ERK phosphorylation was evaluated as described in *panels A* and *C*, respectively.

RESULTS

Arsenic Induces EGFR and ERK Activation in UROtsa Cells—To determine whether arsenic activates EGFR in uroepithelium, UROtsa cells, a human immortalized, nontransformed urothelial cell line, were treated with 50 μM of arsenic, a concentration known to activate AP-1 (15). Arsenic treatment induced EGFR phosphorylation in UROtsa cells as measured by immunoblotting with anti-phosphotyrosine antibody of immunoprecipitated EGFR (Fig. 1*A*). EGFR phosphorylation by arsenic occurred within 15 min with a peak response at 45 min. Ligand-induced autophosphorylation of EGFR causes the recruitment of adaptor proteins, such as Shc and GRB2, to the cytoplasmic domain of the EGFR. As shown on Fig. 1*B*, the treatment of UROtsa cells with either arsenic or EGF resulted in the association of Shc and GRB2 with EGFR, and consistent with the time course of EGFR phosphorylation, the complexes were minimally increased within 15 min and markedly increased within 45 min after arsenic treatment. In parallel with EGFR phosphorylation, EGF and arsenic stimulated ERK phosphorylation in UROtsa cells to similar levels (Fig. 1*C*). However, in contrast to EGF, arsenic-induced ERK activation was higher after 45 min as compared with 15 min (Fig. 1*D*). Arsenic induced EGFR and ERK activation in a dose-dependent manner (Fig. 1*E*). Thus, arsenic appears to increase the phosphorylation of EGFR, which is associated with the recruitment of adaptor proteins including GRB2 and Shc and activation of ERK kinase in UROtsa cells.

Arsenic-induced EGFR Phosphorylation Is Independent of Autocrine EGF, Is Sensitive to N-Acetyl-cysteine (NAC), and Does Not Involve the Major Autophosphorylation Site, Tyr^{1173}—Because arsenic has been shown to induce growth factors from the EGF family (18), it was necessary to determine whether arsenic-induced EGFR phosphorylation is associated with increased EGF levels. Preincubation of UROtsa cells with neutralizing antibodies to EGF did not affect arsenic-induced EGFR phosphorylation, whereas it completely prevented the phosphorylation induced by EGF (Fig. 2*A*). Arsenic is a sulfhydryl-binding metalloid, and many of its effects are altered by glutathione depletion or by the addition of NAC (19). Pretreatment of UROtsa cells with NAC almost completely eliminated arsenic-induced EGFR phosphorylation but had no effect on EGF-induced responses (Fig. 2*B*), suggesting that the sulfhydryl binding properties of arsenic contribute to EGFR stimulation. In ligand-stimulated EGFR autophosphorylation, Tyr^{1173} is one of the specific major autophosphorylation sites (20). To determine whether arsenic phosphorylates Tyr^{1173}, UROtsa cell lysates were immunoprecipitated with antibody specific for Tyr^{1173} and immunoblotted with anti-phosphotyrosine antibody (Fig. 2*C*). In contrast to EGF treatment, tyrosine phosphorylation of the EGFR by arsenic did not include Tyr^{1173}. Therefore, these data demonstrate that arsenic and EGF activate EGFR through distinct mechanisms.

The Inhibitor of Src Activity, PP-1, Inhibits Arsenic-induced EGFR and ERK Phosphorylation—c-Src, a member of the Src non-receptor kinase family, has been implicated in an alternate pathway for EGFR and ERK activation (13). To establish whether Src phosphotyrosine activity is involved in arsenic-induced activation of the EGFR, UROtsa cells were treated with PP-1, a selective Src kinase inhibitor (21). Arsenic-induced EGFR phosphorylation was completely abrogated by PP-1, whereas the response to EGF stimulation was not affected (Fig. 3*A*). Additional experiments were also performed to analyze the phosphorylation of ERK upon Src kinase inhibition. PP-1 dose-dependently inhibited arsenic-induced ERK phosphorylation in UROtsa cells, showing 70% suppression at the highest concentration of PP-1 tested (Fig. 3*B*). At similar concentrations, PP-1 had only a slight effect on EGF-induced ERK phosphorylation. PP-1 alone had no effect on ERK activation. These results suggested that a PP-1 sensitive kinase, such as Src, is a possible upstream mediator of arsenic-induced EGFR and ERK phosphorylation.

The Role of c-Src in Arsenic-induced EGFR as Well as ERK Phosphorylation Was Confirmed by c-Src Kinase Inhibition through Dominant-Negative Src (K297R) Transfection—The dominant-negative c-Src construct transfection allows for the expression of mutated c-Src kinase, which appears without phosphotyrosine activity in the presence of the normal binding activity of the adaptor proteins (22). Consistent with the effect of PP-1, the transfection of the dominant-negative c-Src inhibited arsenic-induced EGFR phosphorylation and ERK activation when compared with cells transfected with the empty vector (Fig. 4, *A* and *B*). The effect of the dominant-negative c-Src transfection was specific for ERK since at the same conditions, arsenic-induced p38 phosphorylation was not affected (Fig. 4*C*).

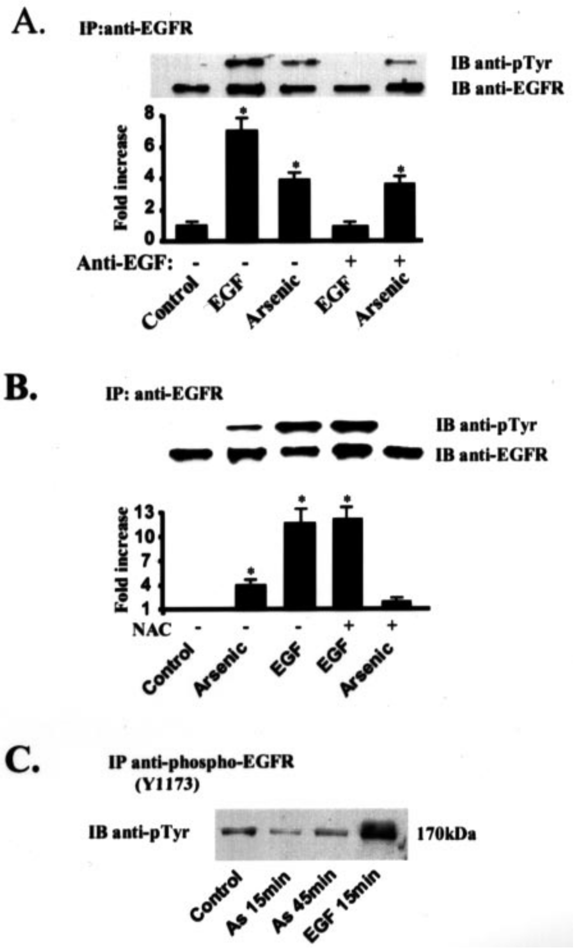

FIG. 2. **Arsenic-induced EGFR phosphorylation is independent of autocrine EGF, is sensitive to NAC, and does not involve Tyr^{1173} in EGFR.** *A*, serum-starved UROtsa cells pretreated for 1 h with anti-EGF neutralizing antibody (10 μg/ml) and treated for 45 min with arsenic (50 μM) or EGF (10 ng/ml) as indicated. EGFR was immunoprecipitated from cell lysates, and samples from each lysate were resolved by SDS-PAGE and immunoblotted with either anti-phosphotyrosine antibody, PY20, or anti-EGF receptor antibody. *B*, serum-starved UROtsa cells were pretreated with NAC (10 mM, pH adjusted to 7.5) for 30 min and processed as discussed in *panel A*. The bands were quantified by ImageQuaNT analysis of the scanned autoradiographs and presented as fold increase after normalization to the total EGFR. Values represent means ±S.E. of three separate experiments; *, $p < 0.05$ *versus* the control. *C*, serum-starved UROtsa cells were treated with arsenic (50 μM) or EGF (10 ng/ml, 45 min). The cell lysates were immunoprecipitated with anti-phospho-EGF receptor (Y1173) antibody and immunoblotted with anti-tyrosine antibody (PY20).

Arsenic Induces c-Src Activity in UROtsa Cells—Further, we examined the ability of arsenic to stimulate c-Src kinase activity. c-Src protein was immunoprecipitated from the lysates of UROtsa cells treated with arsenic or EGF using a specific antibody, and c-Src kinase activity was measured using *in vitro* phosphorylation assays. c-Src kinase activity, measured as enolase phosphorylation, was markedly elevated at 10 and 15 min following treatment with arsenic or EGF (Fig. 5*A*). The ability of arsenic to activate Src was confirmed by measuring the phosphorylation of Src-specific substrate peptide (Fig. 5*B*). Arsenic increased c-Src activity more than 2-fold in 10 min before

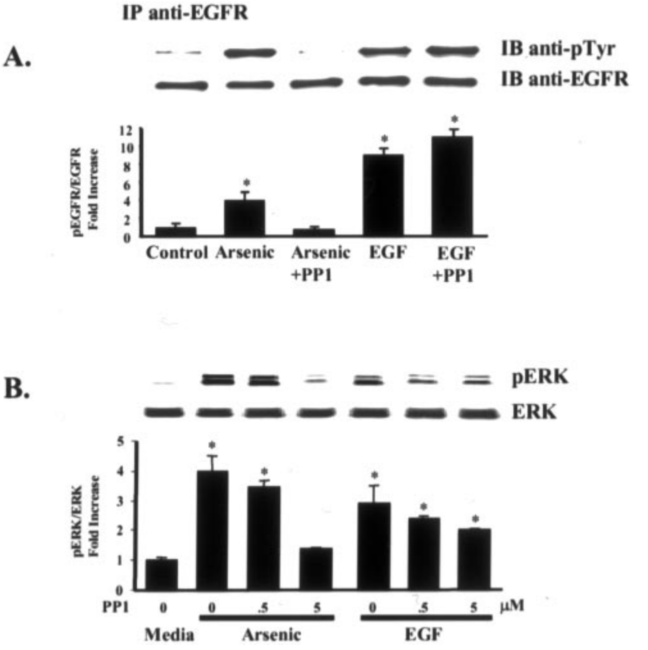

FIG. 3. **Effect of PP-1, an Src inhibitor, on arsenic-induced EGF receptor and ERK phosphorylation.** The serum-starved UROtsa cells were pretreated with PP-1 (5 μM) for 30 min and treated for 45 min with arsenic (50 μM) or EGF (10 ng/ml) (*A*). The bands were quantified by ImageQuaNT analysis of the scanned autoradiographs and presented as fold increase after normalization to the total EGF receptor. The values represent the means ±S.E. for three separate experiments; *, $p < 0.05$ *versus* the control. The serum-starved UROtsa cells were pretreated with PP-1 at the indicated concentrations and exposed to arsenic (50 μM) or EGF (10 ng/ml) for 45 min (*B*). Total lysates were immunoblotted with antibodies for phosphorylated ERK (*pERK*) or total ERK. The values represent the means ±S.E. for three separate experiments; *, $p < 0.05$ *versus* the control.

returning to base-line levels at 30 min. There were no differences in c-Src protein expression observed between lysates obtained from control or stimulated cells as determined by immunoprecipitation and Western blot analysis with anti-c-Src antibody.

Induction of c-Src and EGFR Interactions, EGFR, and ERK Phosphorylation in Urinary Bladder of Mice Exposed to Arsenic in Drinking Water—Previously, we (15) reported that exposure of mice to arsenic in drinking water for 8 weeks induces uroepithelial proliferation and persistent AP-1 activation. To evaluate the involvement of c-Src, EGFR, and ERK in these processes, total lysates were prepared under non-denaturating conditions from the bladders of mice exposed to the same treatment regimes, 50 μg/ml arsenic in drinking water for 8 weeks (Fig. 6). Consistent with the *in vitro* data, exposure of mice to arsenic in the drinking water resulted in increased levels of phosphorylated ERK and EGFR in the bladder tissue. Samples were also immunoprecipitated with anti-EGFR and immunoblotted with anti-c-Src antibody to test for EGFR-c-Src interactions. Arsenic exposure induced an increase in c-Src, which co-immunoprecipitated with the EGFR at equal levels of total EGFR in bladder tissue. Thus, arsenic-induced AP-1 activation and epithelial mitogenic responses in urinary bladder tissue are accompanied by c-Src, EGFR, and ERK activation.

Src-dependent ERK Activation in Arsenic-treated Cells Deficient in EGFR—To evaluate whether c-Src contributes to arsenic-induced ERK activation only as an integral part of the EGFR-ERK pathway, B82 cells that are deficient in EGFR (EGFR$^-$) and B82 cells permanently transfected with human wild-type EGFR (EGFR$^+$) were used. Although EGF induced ERK activation only in EGF$^+$ B82 cells (data not shown), arsenic stimulated ERK in both EGF$^-$ and EGF$^+$ B82 cells. Furthermore, the responses in both cell types were inhibited by PP-1 (Fig. 7). These data indicate that Src activity is also involved in arsenic-induced ERK phosphorylation in the absence of EGFR. Src can activate the ERK pathway either by phosphorylating EGFR or by phosphorylating molecules, such as Shc or FAK, creating binding sites for Grb2, both of which link to the MAPK pathway (13, 23).

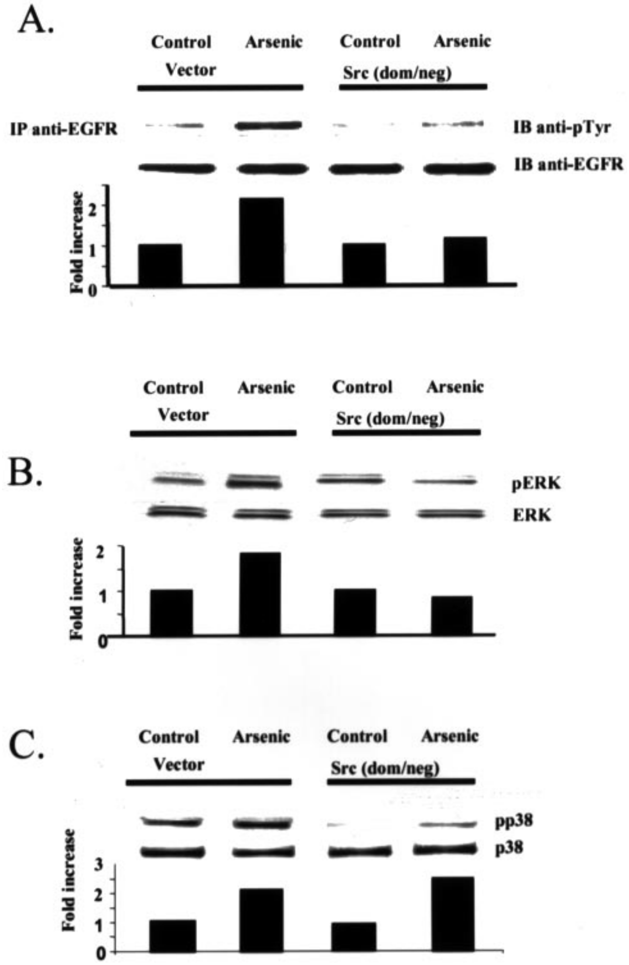

FIG. 4. **Effects of dominant-negative Src transfection on arsenic-induced EGFR and ERK phosphorylation.** The serum-starved UROtsa cells were transiently transfected with c-Src dominant-negative or empty vector by a LipofectAMINE method as described under "Experimental Procedures." The cells were left untreated or treated with arsenic (50 μM) for 45 min. The cell lysates were immunoprecipitated by anti-EGF receptor antibody and immunoblotted with anti-phosphotyrosine antibody (*A*). A representative gel of two experiments is shown. The bands were quantified by ImageQuaNT analysis of the scanned autoradiographs and presented as fold increase after normalization to total EGFR. Total lysates from the same treatment were immunoblotted with antibodies for phosphorylated ERK (*pERK*) or total ERK (*B*). A representative gel of two experiments is shown. The bands were quantified by ImageQuaNT analysis of the scanned autoradiographs and presented as a fold increase after normalization to total ERK. Total lysates from the same treatment were immunoblotted with antibodies for pp38 or total p38 (*C*). The bands were quantified by ImageQuaNT analysis of the scanned autoradiographs and presented as a fold increase after normalization to total pp38.

DISCUSSION

Arsenic exposure is associated with urinary bladder epithelium hyperplasia and a concomitant increased AP-1 activation (15). Additionally, cDNA microarray analysis of the arsenic-exposed human uroepithelial cell line, UROtsa, identified induced genes whose products are involved in cell cycle regula-

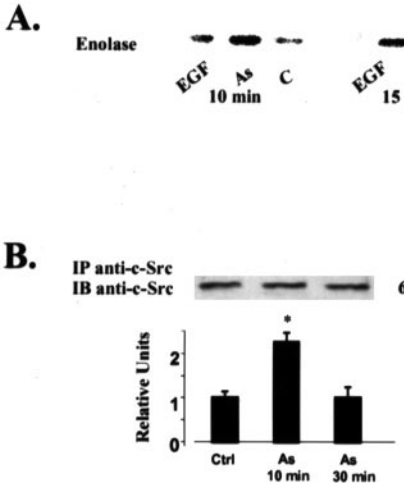

FIG. 5. **Arsenic stimulates c-Src activity in UROtsa cells.** Serum-starved cells were incubated with arsenic (50 μM) or hrEGF (10 ng/ml) for the indicated time (*A*). The c-Src kinase was immunoprecipitated from cell lysates by specific antibody. Immunoprecipitates were incubated with [γ^{32}P]ATP and acid-denatured enolase, a substrate for kinase activity. Enolase phosphorylation was detected following SDS-PAGE and autoradiography. Samples treated and immunoprecipitated as in *panel A* were analyzed by *in vitro* phosphorylation of Src-specific peptide as a substrate (*B*). The indicated values are mean ±S.E. of three experiments; *, $p < 0.05$ *versus* the control. The same immunoprecipitates were immunoblotted with anti-c-Src antibody.

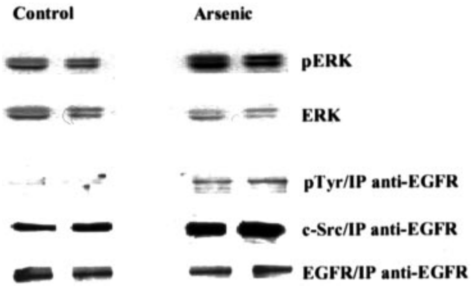

FIG. 6. **Activation of ERK, EGFR and cSrc in urinary bladder of mice exposed to arsenic through drinking water.** Mice were given vehicle or sodium arsenite (50 μg/ml) in their drinking water for 8 weeks. Urinary bladders were lysed in non-denaturating RIPA buffer and immunoprecipitated with anti-EGFR and then immunoblotted with PY20 or anti-c-Src antibody. ERK phosphorylation was measured by Western blot.

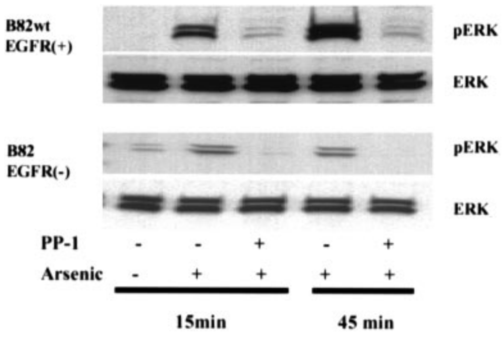

FIG. 7. **Effects of c-Src inhibition on arsenic-induced ERK activation in cells deficient of EGFR.** The serum-starved EGFR-deficient (EGFR$^-$) or human EGFR-transfected (EGFR$^+$) B82 cells were pretreated with PP-1 (1 μM) and exposed to arsenic (50 μM) for 45 min. Total lysates were isolated and after that immunoblotted with antibodies for phosphorylated ERK (*pERK*) or total ERK.

tion and malignancies, such as early growth response gene (EGR)-1, growth arrest and DNA damage (GADD)153, GADD45, and repair-associated protein (RAD) (15). The expression of genes involved in the regulation of cellular growth has been historically related to the effects of growth factors, such as EGF, and their abilities to induce a cascade of events, triggered by binding to the specific receptor and including the activation of receptor tyrosine kinases and phosphorylation of members of the MAPK family. Several studies have suggested that arsenic cellular effects involve MAPK activation (7, 8, 10). For example, the JB6 mouse epidermal cell line exposed to low doses of arsenic demonstrated ERK stimulation associated with cell transformation (24). ERK subtypes, members of the MAPK family, are recognized as key transducers in the signaling cascade mediating the expression of cell growth-related genes (25). Further, a study conducted with PC12 cells, used commonly to explore MAPK activation, has demonstrated that arsenite treatment activates ERK in the EGFR-dependent pathway, and the interaction of arsenic with EGFR vicinal thiols has been supposed as a trigger mechanism in this event (9). Arsenic might activate the EGFR-ERK pathway to induce gene expression and mitogenicity in urinary bladder epithelium. EGFR has been extensively studied as an integral part of human urinary bladder carcinogenesis (26).

In the present study, arsenic was found to induce ligand-independent EGFR phosphorylation and activation in UROtsa cells; however, this response differed from the ligand-induced response in several ways. First, monoclonal antibody, specific for one of the major autophosphorylation sites of EGFR (Tyr^{1173}), discriminated between EGF- and arsenic-induced EGF receptor phosphorylation. Secondly, the ability of arsenic to phosphorylate EGFR and activate ERK was slightly but consistently delayed when compared with the endogenous ligand. Thirdly, inhibition of Src by PP-1 or transfection with a dominant-negative c-Src construct prevented arsenic-induced but not EGF-induced EGFR or ERK phosphorylation in the uroepithelial cell line.

EGF stimulates an intrinsic receptor tyrosine kinase activity, which results in tyrosine autophosphorylation of the receptor including the Tyr^{1173} site (20). The phosphorylation of EGFR through inhibition of tyrosine phosphatase inhibitors, such as sodium orthovanadate, also reflects the activation of the intrinsic tyrosine kinase (27). The involvement of receptor tyrosine kinases by arsenic in UROtsa cells is not likely since the Tyr^{1173} site was not phosphorylated. Additionally, cellular non-receptor kinases, such as c-Src or JAK2, have been demonstrated to phosphorylate EGFR (11). For example, c-Src has been implicated in oxidative stress or lysophosphatidic acid-induced EGFR tyrosine phosphorylation (28). c-Src can physically associate with EGFR, resulting in two unique tyrosine phosphorylations of the receptor (Tyr^{845}, Tyr^{1101}), which are distinct from the autophosphorylation sites (13, 29). The results of the present study indicate that c-Src activity is necessary for arsenic-induced EGFR and ERK activation and that these pathways occur in mouse urinary bladder after arsenic exposure. Alternatively, arsenic can stimulate ERK activation trough Src, at least in the B82 cells, in the absence of EGFR. It has been demonstrated that Src can activate the ERK pathway by phosphorylating molecules, such as Shc or FAK, creating binding sites for Grb2 (13, 23). Taken together, and as summarized in Fig. 8, these data suggest an important role for c-Src in the arsenic-induced signaling of ERK activation and related gene expression. In addition to ERK activation, c-Src can be responsible for the tyrosine phosphorylation of numerous actin-binding proteins, such as cortactin, and can impact the cortical actin assembly (30). The c-Src-dependent mechanisms of cytoskeleton reorganization also might contribute to arsenic-induced pathophysiological processes.

Several lines of evidence have demonstrated that c-Src is associated with the inner cell membrane, particularly in the

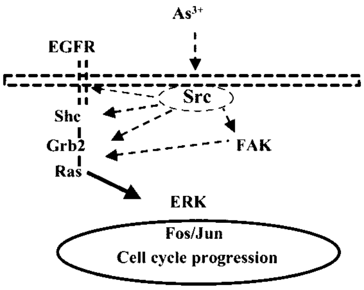

FIG. 8. **Schematic representation showing the role of c-Src in arsenic-induced signaling pathways of growth-related gene expression.**

vicinity of growth factor or integrin clusters (31). c-Src activation involves phosphorylation and dephosphorylation events that can be triggered by diverse stimulants, including growth factors, integrins, or confirmational changes from disulfide bond interactions, which result in the aggregation of c-Src molecules (31). Recently, the latter paradigm has been shown to occur by nitric oxide (32). Arsenic may persuade some of these mechanisms via its reactivity to vicinal sulfhydryl groups. Macromolecules, such as EGFR, integrins, c-Src, or protein phosphatases, contain high numbers of vicinal sulfhydryls and are capable of reacting with arsenic. Alternatively, inorganic arsenic may accumulate in the extracellular matrix bound to keratin or other sulfhydryl-containing molecules in skin or urinary bladder tissue, resulting in cellular integrin rearrangements and c-Src activation. In this respect, we recently demonstrated that inorganic arsenic accumulates in the bladder epithelium following oral exposure (15, 33). Although it is likely that arsenic and other environmental stressors can act like classical tumor promoters and growth factors, identification of unique events in the signal cascades may help provide targets for specific therapeutic or prevention interventions in chemical carcinogenesis.

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