DEP-induced fra-1 expression correlates with a distinct activation of AP-1-dependent gene transcription in the lung

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Submitted 10 July 2003; accepted in final form 12 October 2003

Zhang, Qin, Steven R. Kleeberger, and Sekhar P. Reddy. DEP-induced fra-1 expression correlates with a distinct activation of AP-1-dependent gene transcription in the lung. Am J Physiol Lung Cell Mol Physiol 286: L427–L436, 2004.—Recent studies indicate a potential role for Fra-1, a heterodimeric partner of activator protein (AP)-1, in toxicant-induced epithelial injury, repair, and cellular transformation. Here we have investigated the effects of diesel exhaust particles (DEP) on fra-1 expression in C10 cells, a murine lung epithelial cell line. DEP markedly upregulated fra-1, but not fra-2, expression. The increase in fra-1 mRNA expression correlated well with its protein- and DNA-binding activity. DNA-binding assays also revealed a predominant presence of Jun-B and Jun-D in the AP-1 complex. Interestingly, DEP did not alter Jun-B and Jun-D protein levels. Transcriptional analysis revealed that fra-1 induction is regulated in part at the transcriptional level. The −379 to +32 bp 5′-flanking region mediated this induction. Furthermore, inhibitors of ERK1/2, JNK1, and p38 mitogen-activated protein kinases (MAPKs) significantly suppressed DEP-stimulated fra-1 transcription, suggesting their involvement in the induction process. Consistent with this finding, DEP stimulated phosphorylation of ERK1/2, JNK1, and p38 MAPKs with a distinct activation pattern. Overexpression of Fra-1 downregulated c-Jun and Nrf2 enhanced AP-1- and ARE-mediated reporter gene expression, respectively. In contrast, Fra-1 had the opposite effect on mitogen-activated protein kinase (MAPK) signal transduction pathways activated by toxic or mitogenic stimuli. Upon activation, AP-1 binds to DNA sequences, such as 5′-TGAG/CTCA-3′ [TPA response element (TRE) or AP-1 site], and influences the outcome of biological responses (14). A critical part of such regulation is maintenance of optimal intracellular levels of specific AP-1 proteins. Thus regulation of AP-1 protein levels contributes to the strength of AP-1-dependent gene expression and influences the outcome of biological responses (14). Because TREs are often embedded in the antioxidant response elements commonly found in regulatory regions of several antioxidant enzymes (38). AP-1 proteins have been suggested to play both pro- and anti-regulatory roles in pulmonary defense, injury, and repair (47).

DEP, the most abundant component of ambient air particulate matter, contain many chemical compounds, such as polycyclic and halogenated aromatic hydrocarbons and redox-active quinones (36). Exposure to DEP has deleterious effects on pulmonary function and exacerbates respiratory diseases, such as asthma, chronic bronchitis, and allergic airway disease, as well as causes tumors (7, 15, 24, 27). Several studies have shown activation of MAPK signaling pathways, leading to the activation of transcription factors in cellular responses to DEP.

ABERRANT EXPRESSION OF GENES induced by environmental stress, such as exposure to toxins, oxidants, and particles, can result in an altered phenotype and tissue dysfunction, contributing to respiratory pathogenesis. For instance, exposure to tobacco smoke causes chronic obstructive lung disease, bronchitis, emphysema, and lung cancer (19, 53). Similarly, epidemiological studies have shown a association between elevated particulate matter, especially diesel exhaust particles (DEP), in ambient air and increased incidence of lung diseases, such as asthma, and chronic bronchitis (39). Although the exact mechanisms leading to the development of respiratory pathogenesis remain elusive, it has been documented that various toxins after interacting with epithelial cells initiate a cascade of signaling events that activate multiple transcription factors, which then regulate genes involved in various cellular processes, including pulmonary defense and inflammation (29, 49). However, abnormal expression and/or activation of these transcription factor(s) in response to persistent toxin exposure can deregulate the expression of their downstream target genes, thereby altering normal injury and repair processes, which may lead to the development of pathogenesis. Thus a better understanding of the mechanisms regulating the activation of transcription factors by toxins is critical to develop an effective strategy to modulate various diseases.

The transcription factor activator protein-1 (AP-1) plays a central role in regulating the expression of genes involved in cell proliferation, differentiation, apoptosis, and inflammation and immune responses (2, 5, 26, 54). AP-1 is a dimeric complex composed primarily of Jun (c-Jun, Jun-B, Jun-D) and Fos (c-Fos, Fos-B, Fra-1, Fra-2) leucine zipper transcription factors. These transcription factors act as converging points to mitogen-activated protein kinase (MAPK) signal transduction pathways activated by toxic or mitogenic stimuli. Upon activation, AP-1 binds to DNA sequences, such as 5′-TGAG/CTCA-3′ [TPA response element (TRE) or AP-1 site], and regulates the transcription of multiple genes, including themselves, in a context (promoter and cell type)-dependent manner (5). These interactions not only cause differential DNA binding of AP-1 proteins to various promoters but also increase the repertoire of genes controlled by AP-1 proteins in a given cell type (14). A critical part of such regulation is maintenance of optimal intracellular levels of specific AP-1 proteins. Thus regulation of AP-1 protein levels contributes to the strength of AP-1-dependent gene expression and influences the outcome of biological responses (14). Because TREs are often embedded in the antioxidant response elements commonly found in regulatory regions of several antioxidant enzymes (38). AP-1 proteins have been suggested to play both pro- and anti-regulatory roles in pulmonary defense, injury, and repair (47).

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For example, activation of NF-κB by DEP has been shown to regulate IL-8 and granulocyte-macrophage colony-stimulating factor expression in bronchial epithelial cells (9, 57). Similarly, activation of Nrf2 transcription factor, which plays a critical role in the regulation of antioxidant enzyme expression (38), has been correlated with the induction of phase II detoxifying enzymes in bronchial epithelial cells in response to DEP (6). The activation of NF-κB and Nrf2 in part is mediated by oxidative stress and/or reactive oxygen species generated by DEP. However, both the mechanisms of regulation and the role of the AP-1 family of transcription factors in DEP-induced respiratory pathogenesis remain elusive. Members of the AP-1 family are early response proto-oncogenes, and their expression is variably regulated by toxic and mitogenic stimuli in numerous cell types (47). We hypothesized that DEP modulates AP-1 family member expression in airway epithelial cells, thereby altering protein-protein interactions between themselves and other members of the leucine zipper superfamily of transcription factors, such as activation transcription factors, Nrf/Mafs, and CCAAT/enhancer-binding proteins. These alterations may lead to deregulation of gene expression involved in epithelial injury and repair processes, culminating in respiratory pathogenesis, including cellular transformation.

Emerging evidence indicates a potential role for Fra-1 in perturbations of toxicant-induced gene expression (reviewed in Ref. 47). Therefore, we have investigated the effects of DEP on fra-1 expression in murine alveolar epithelial cells. Because matrix metalloproteinases (MMPs) contain functional TREs of AP-1 family are early response proto-oncogenes, and their expression as the means ± SE of six to nine independent samples. Statistical significance of the differences between groups was determined using Student’s t-test.

**Electrophoretic mobility shift assay.** Nuclear extracts from control and DEP-treated cells were prepared, and electrophoretic mobility shift assay (EMSA) was performed as described previously (43, 62). The binding was performed in a 20-μl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, 0.25 mg/ml poly(dI-dC), and 0.5 μg salmon sperm DNA. Nuclear extracts (2–3 μg) were incubated with the binding buffer on ice for 10 min before the addition of 32P-labeled double-stranded consensus TRE or AP-1 site (5'-GGACCTTACTACGGAGAA-3') cloned upstream of mouse heme oxygenase-1 minimal promoter (–35 to +72 bp) was kindly provided by Javed Alam (Ochsner Clinic Foundation, New Orleans, LA). The factor detection kit was generously provided by Michael Birrer (National Cancer Institute) and Eugene Tulchinsky (Houston, TX) and Motoharu Seiki (University of Tokyo, Tokyo, Japan). TEF-Luc bearing seven copies of consensus AP-1 recognition sites (5', 5'-ATGACTAAG-3') upstream of Luc was obtained from Statragen (La Jolla, CA), ARE-Luc containing three copies of Nrf2 binding sequences (ARE, 5'-CGGACCTTACTACGGAGAA-3') cloned upstream of mouse Nrf2 promoter minimal promoter (–35 to +72 bp) was kindly provided by Javed Alam. Cells were transfected with 100 ng of promoter reporter constructs in the presence of pRL-TK plasmid (1 ng). pRL-TK plasmid contains the herpes simplex virus thymidine kinase promoter to provide low to moderate levels of Renilla luciferase expression in cotransfected mammalian cells. After 18–24 h, cells were treated with DEP (5 or 25 μg/ml) for 5 h, and luciferase activities were measured using a commercially available kit as per the manufacturer’s protocol (Promega). Firefly luciferase activity of individual samples was normalized to that of Renilla luciferase, as described in our earlier publications (43, 62). All assays were performed, and data are expressed as the means ± SE of six to nine independent samples. Statistical significance of the differences between groups was determined using Student’s t-test.

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**Kinase immunoblot analysis.** Cells were treated with or without DEP (25 μg/ml) for various time periods, washed three times with chilled PBS containing 1 mM Na₃VO₄, and then lysed in kinase buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 5 mM β-glycerolphosphate, and 1 μg/ml leupeptin. Lysates were sonicated for 15 s and centrifuged for 10 min at 10,000 g at 4°C to remove cellular debris. Cell lysates were separated on SDS-PAGE gel and
DEP induces fra-1 but not fra-2 expression in alveolar epithelial cells. To understand the regulation of fra-1 expression, C10 cells were treated with DEP at 5 or 25 μg/ml for 60–360 min, and Northern analysis was performed. DEP had no significant effect on cell viability, as measured by the trypan blue exclusion method (data not shown). DEP at a concentration of 5 μg/ml caused no appreciable effect on fra-1 mRNA expression (Fig. 1A). However, 25 μg/ml DEP markedly enhanced fra-1 mRNA expression (lanes 5–7) compared with control (lane 1). Induction was seen as early as 1 h, reached a maximum at 3 h (∼3-fold), and remained elevated at 6 h. Although DEP modestly stimulated fra-1 mRNA expression, the induction was relatively weak when compared with TPA treatment (data not shown). In both cases, two alternatively spliced mRNA transcripts of fra-1 (3.3 and 1.7 kb; see Ref. 35) were similarly induced. In contrast, 5 or 25 μg/ml DEP failed to stimulate fra-2 mRNA expression (data not shown). Induction of fra-1 mRNA expression by DEP was also correlated with an increase in its protein levels (Fig. 1C). Consistent with mRNA data, DEP did not have any significant effect on Fra-2 protein levels (Fig. 1C). Interestingly, in contrast to mRNA expression, Fra-1 protein levels were significantly higher after DEP stimulation at 30 min (Fig. 1C). This result suggests the involvement of posttranslational modifications of Fra-1 by DEP.

We next examined whether fra-1 induction by DEP correlates with its DNA binding activity. Nuclear extracts were isolated from cells treated with or without DEP, and protein-DNA binding was evaluated by EMSA using 32P-labeled consensus TRE as probe. Incubation of the labeled probe with nuclear extracts revealed the formation of AP-1 protein complex (Fig. 2A). Specificity of the complex was determined by incubation of nuclear extracts with a 50-fold molar excess of unlabeled TRE or GATA-1 sequence. The TRE (lane 5) but not GATA-1 sequence (lane 6) completely blocked complex formation, indicating the presence of AP-1 dimeric proteins. We next characterized binding of Fra-1 and Fra-2 in the AP-1 complex using specific antibodies. Addition of anti-Fra-1 antibody caused a supershift with nuclear extracts from control and DEP- and TPA-treated cells (Fig. 2B). Although the intensity of the supershifted band observed with nuclear extracts from DEP-treated cells (lanes 5–8) was stronger than untreated cells (lanes 5 and 6), the supershifted band observed with nuclear extracts isolated from TPA-treated cells (lanes 1 and 2) was markedly higher. We next examined the presence of members of Jun (c-Jun, Jun-B, and Jun-D) and other Fos (c-Fos and Fos-B) family proteins in the complex using their specific antibodies. Incubation with anti-Jun-B or -Jun-D antibodies caused a supershift of the complex, whereas c-Jun, c-Fos, Fra-2, and Fos-B did not (Fig. 2D). In contrast to Fra-1, the intensity of supershifted complex by anti-Jun-B and -Jun-D antibodies remained unchanged after DEP treatment. Consistent with this, Western analysis of total or nuclear extracts revealed no change in the Jun-B and Jun-D proteins levels after DEP treatment (data not shown). Collectively, the data presented in Figs. 1 and 2 indicate that DEP activates fra-1 expression in C10 cells.

DEP regulates fra-1 mRNA expression mainly at the transcriptional level. We next examined whether DEP upregulates fra-1 induction by increasing the transcription or the stability of its mRNA. Cells were treated with actinomycin D (AD, 10 μg/ml) or TPA (20 ng/ml) for 30–360 min. A: total RNA (15 μg/lane) was isolated, blotted, and hybridized with either 32P-labeled mouse fra-1 or β-actin cDNA probe. C, control. B: quantification of DEP-induced fra-1 mRNA expression. The intensity of fra-1 mRNA transcripts in each blot was quantitated using a densitometric scanner, and the values were normalized to their respective β-actin mRNAs. Each bar represents the mean ± SE of normalized ratio of 3 independent samples (n = 3). C: Western analysis was performed using anti-Fra-1 or anti-Fra-2 antibodies. Blots were stripped and subsequently probed with β-actin antibodies. Results shown are a representative blot of 4 independent samples. D: Western blots were analyzed, and each bar represents the mean ± SE of the normalized Fra-1-to-β-actin ratio of 4 independent samples. Note: one representative sample of TPA induction was shown as a positive control.
μg/ml), an inhibitor of RNA synthesis, for 30 min before DEP treatment. After 3 h of DEP exposure, RNA was isolated, and fra-1 mRNA levels were determined by Northern blot analysis. Pretreatment of cells with AD completely suppressed both basal and DEP-inducible fra-1 mRNA expression (Fig. 3A). This finding suggests that both basal and DEP-enhanced fra-1 mRNA levels are regulated at the transcriptional level. To confirm these observations, a fra-1 promoter-reporter construct was transiently transfected into C10 cells, and luciferase activity was measured after DEP treatment. We chose the −379 (−379 to +32) fra-1 promoter fragment, since it contains sufficient information for inducible fra-1 transcription in response to disparate stimuli, such as TPA (1), EGF, H2O2, and TNF-α (unpublished data). DEP significantly (P < 0.05) enhanced reporter gene expression driven by the −379 fra-1 promoter (Fig. 3B). Similar results were obtained with a −861 to +32 bp fra-1 promoter, which contains −379 and +32 promoter plus additional DNA sequences. DEP-stimulated fra-1 expression correlates with an increased level of promoter activity, mRNA, and protein. However, induction was not quite as robust as that of other inducers, such as TPA, EGF, and TNF-α (data not shown). Because DEP contains a mixture of components, such as quinones and polycyclic aromatic hydrocarbons (National Institute of Standards and Technology), it is possible that some of these components have a differential effect on fra-1 promoter activation. Indeed, we have demonstrated that β-naphthoflavone strongly suppresses basal-level fra-1 promoter activity (data not shown). Further experiments are needed to identify the component(s) of DEP that upregulates fra-1 induction in airway epithelial cells.

Role of ERK/JNK/p38 MAPK pathways in regulation of fra-1 expression. ERK/JNK/p38 MAPK signaling pathways play a central role in mediating toxicant-induced responses in a variety of cell types (17, 44). Therefore, we have investigated which of these MAPK pathways is involved in DEP-enhanced fra-1 expression. Before DEP exposure, cells were treated with PD-98059, SP-600125, and SB-202190, specific chemical inhibitors of ERK, JNK, and p38 MAPK, respectively. PD-98059 prevents activation of MKK1/2, thereby inhibiting phosphorylation of downstream ERK1/2 kinases (18). SB-202190

Fig. 2. DEP distinctly stimulates the activator protein-1 (AP-1) family of transcription factors binding to the consensus binding site. A: 32P end-labeled double-stranded consensus TRE was incubated with nuclear extracts (2 μg) isolated from control (C) or DEP-treated (DEP) cells. For competition experiments, 50-fold excess unlabeled TRE (self) or GATA (GA) oligo was used. B: nuclear extracts were incubated with anti-Fra-1 (F1) or nonimmune IgG (Ig). Nuclear extracts from TPA-treated (TPA) cells were used as positive control. C: quantification of Fra-1 DNA binding activity. Fra-1 supershifted band in each lane was analyzed by densitometric scanner using the values from the respective Ig lanes as one. D: nuclear extracts isolated from control and DEP-treated (3 h) cells were incubated with antibodies (2 μg) specific to individual members of the AP-1 family as indicated. The vertical bars indicate the position of supershifted (SS) bands, open and filled triangles indicate nonspecific (NS) and AP-1 protein complex, respectively, whereas open arrows indicate the free probe (F). Results shown are a representative autoradiogram of 2 independent samples.
specifically inhibits the p38 MAPK pathway (32). SP-600125 [anthrax-1,9-cd-pyrazol-6(2H)-one] is a recently identified novel JNK inhibitor that completely inhibited JNK-mediated collagenase gene expression induced by IL-1 (12). The effects of these inhibitors on fra-1 promoter activity and gene expression were monitored by transient transfection assays and Western analysis, respectively (Fig. 4). To analyze the promoter activity, cells were transfected with the 379-Luc construct along with pRK-TL. None of the inhibitors had a significant effect on the basal activity of the fra-1 promoter (Fig. 4A). However, DEP-stimulated fra-1 promoter activity was significantly inhibited by all three MAPK inhibitors (P < 0.05). Inhibition of DEP-inducible fra-1 activity by these inhibitors correlated with decreased protein levels (Fig. 4B). Together these observations indicate that the ERK, JNK, and p38 MAPK pathways play important roles in DEP-inducible fra-1 expression in alveolar epithelial cells.

**DEP stimulates phosphorylation of ERK/JNK/p38 MAPKs in alveolar epithelial cells.** To further confirm the involvement of ERK, JNK, and p38 in fra-1 induction, cells were treated with DEP for 15–180 min, and immunoblot analysis was performed using phospho-specific antibodies. After DEP treatment, the ERK1 and ERK2 phosphorylation was increased within 15 min (Fig. 5A). Phosphorylation was maximal at 60 min (~5-fold) and remained elevated through 3 h, suggesting persistent activation of the ERK pathway by DEP. Immunoblot analysis with JNK antibodies that recognize both JNK1 and JNK2 isoforms revealed that DEP prominently stimulates phosphorylation of JNK1 but not JNK2 (Fig. 5B). DEP modestly (80%) stimulated phosphorylation of JNK1 after 15 min, was maximal at 60 min (~2-fold), and remained elevated above basal at 180 min. In contrast to DEP, ultraviolet light activated both JNK1 and JNK2, indicating the presence of a functional form of the latter isoform in C10 cells. Immunoblot analysis revealed transient activation of p38 MAPK by DEP (Fig. 5C). DEP significantly elevated levels of phosphorylated p38 as early as 15 min, but the activity returned to the control level within 30 min. At 180 min, phosphorylation of p38 was markedly lower in DEP-exposed cells compared with controls. Thus these results suggest that DEP differentially activate ERK, JNK, and p38 MAPKs in alveolar epithelial cells.

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**Fig. 3.** DEP regulates fra-1 induction in part at the transcriptional level. A: cells were treated with actinomycin D (AD, 10 μg/ml) for 30 min and stimulated with DEP (25 μg/ml) for 3 h. RNA was isolated, and Northern analysis was carried out as described in Fig. 1A. Results shown are a representative blot of 2 independent experiments. B: a map of human fra-1 promoter indicating various putative cis-acting elements (GenBank accession no. D14493). ATF, ATF-binding site; CArG, serum response element; GC, GC box; EBS, ETS-binding site; TRE, TPA response element. C: cells were transiently transfected with 100 ng of the indicated fra-1-promoter reporter construct along with 1 ng pRL-TK reference plasmid. After overnight incubation, cells were treated with or without DEP (25 μg/ml) for 5 h. The relative promoter activity was calculated, and data are expressed as degree of change over control. *P < 0.05 compared with controls. Data represent the values of 9 independent samples.

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**Fig. 4.** ERK/JNK/p38 MAPKs mediate DEP-stimulated fra-1 induction. A: cells were transfected with ~379 bp fra-1 promoter-reporter construct along with 1 ng of pRL-TK reference plasmid. After transfection and before DEP stimulation, cells were treated with vehicle (Veh) or MAPK inhibitors, PD-98059 (PD, 30 μM), SB-202190 (SB, 20 μM), or SP-600125 (SP, 20 μM). *P < 0.05 compared with DEP-treated group. Data are representative of 6 independent samples. B: to determine the effects of MAPK inhibitors on fra-1 expression, cells were treated with DEP as described above, and Western analysis on the total lysates was performed using the indicated antibodies. Results shown are a representative blot of 4 independent samples.
Fra-1 and DEP transactivate MMP-9 promoter activity. High-level expression of MMPs, such as MMP-2, -9, and -12, has been implicated in toxin-induced respiratory pathogenesis, including asthma, fibrosis, and chronic obstructive pulmonary disease (42). Because MMPs are the downstream targets of the AP-1 family of transcription factors (42), we analyzed the effects of Fra-1 on MMP-9 promoter activity in alveolar epithelial cells. Two promoter-reporter constructs were chosen, M634-Luc or M73-Luc (Fig. 8A). M634-Luc contains two functional TREs at positions −533 and −79, whereas M73-Luc lacks these sites. Overexpression of Fra-1 markedly enhanced reporter gene expression driven by M634-Luc. In contrast, it had no effect on M73-Luc, which lacks functional TREs. Collectively, these data (Figs. 6–8) suggest that Fra-1 distinctly regulates gene expression in a context-dependent manner. These results also indicate that upregulation of fra-1 expression by DEP may play a role in MMP-9 transcription. To confirm these results, the effects of DEP on MMP-9 promoter activity were analyzed. Cells were transfected with M634-Luc or M73-Luc, and reporter expression in control and DEP-treated cells was analyzed. M634-Luc activity was significantly enhanced by DEP (Fig. 8C). However, it failed to stimulate M73-Luc activity. To ascertain the role of Fra-1 in MMP-9 gene regulation, binding of Fra-1 to the −79 and −533 TREs of MMP-9 promoter was examined by EMSA (Fig. 9). The protein complex formed by the −79 TRE was stronger when compared with −533 TRE (Fig. 9). Importantly, protein binding at −79 TRE was enhanced significantly after DEP stimulation (lane 6) compared with controls (lane 5). Specificity of the protein complex was analyzed by competition assays. DEP strongly enhanced the protein complex formation with −79 TRE that was competed out by unlabeled −79 TRE (Fig. 9B, self, lane 10) or consensus TRE (Fig. 9B, TRE, lane 12). In contrast, the GATA oligo showed no such effect (lane 11). We next examined whether Fra-1 binds to the −79 TRE. In contrast to control cells, DEP strongly induced
binding of Fra-1 to the −79 TRE (Fig. 9D, lane 16). Weak binding of Fra-1 was found in nuclear extracts from 3-h DEP-stimulated cells. However, intensity of the Fra-1-supershifted band was significantly higher (lane 18) compared with controls (lane 14) or nuclear extracts incubated with nonimmune IgG (lanes 13, 15, or 17). Together, these results suggest that Fra-1 may play a role in regulating MMP-9 gene expression in alveolar epithelial cells.

**DISCUSSION**

We showed for the first time that DEP induces fra-1 but not fra-2 expression in murine alveolar type II epithelial cells. Induction of fra-1 by DEP is regulated in part at the transcriptional level. Gene expression analysis indicated that ERK1/2, JNK1, and p38 MAPKs activated by DEP play critical roles in the induction process. Our results are consistent with previous studies that showed sustained activation of fra-1 by various toxins in lung cell types (reviewed in Ref. 47). For example, tobacco smoke markedly enhanced fra-1 mRNA expression in bronchial epithelial cells and mouse lungs (48). Similarly, other studies have shown that silica (55) and asbestos (50, 58) upregulate fra-1 expression. Recently, microarray analysis revealed a high-level fra-1 mRNA expression in BEAS-2B cells after arsenic (4 h) treatment (4). In contrast, fra-2 expression, if any, is weakly activated in most of the above studies.

Interestingly, Western blot analyses revealed a modest increase in Fra-1 protein levels that occurred before induction of its mRNA, after DEP stimulation (Fig. 1, C and D). Therefore, we speculate that both transcriptional and posttranslational mechanisms contribute to the DEP-enhanced fra-1 expression in alveolar epithelial cells. In support of this notion, Casalino et al. (11) have recently demonstrated involvement of both transcriptional autoregulation and MEK-dependent posttranslational protein stabilization of Fra-1 in ras-transformed cells. In addition to transcriptional regulation, posttranslational modifications, such as phosphorylation, also play a role in the stabilization of AP-1 proteins (37). For example, ERK-dependent phosphorylation of c-Fos at its COOH terminus increases...
Fig. 9. Fra-1 binds to −79 TRE of MMP-9 promoter. A: sequence of the −533 and −79 TRES of the MMP-9 promoter. B: nuclear extracts isolated from control (Ctr) and DEP-stimulated cells were incubated with the 32P end-labeled proximal (−79) and distal (−533) TRE. C: nuclear extracts isolated from control (lanes 7) and DEP-stimulated (lanes 8–12) were incubated with the 32P end-labeled −79 TRE. Lanes 10–12, nuclear extracts were incubated with 50-fold molar excess of unlabeled double-stranded −79 TRE (self), GATA (GA) sequence, or consensus colTRE (TRE), respectively. D: supershift analysis of the AP-1 complex binding to the −79 TRE. Nuclear extracts isolated from vehicle (lanes 13 and 14) or 30 min (lanes 15 and 16) or 180 min (lanes 17 and 18) DEP-treated cells were incubated with nonimmune IgG or anti-Fra-1 antibodies. For symbols, see legend for Fig. 2.

*Position of the supershifted bands. Shown is a representative autoradiogram of 2 independent samples.

Its stability (13, 40). In our experimental model, DEP-stimulated phosphorylation of ERK signaling cascades leading to Fra-1 stability cannot be excluded (Fig. 4A). Fra-1 is a known target of ERK signaling (20, 65). Further studies are needed to understand this process.

Distinct activation of MAPK signaling pathways by DEP has been demonstrated in airway epithelial cells. For instance, DEP activates p38 in the BEAS-2B clone BETA-1 (21). This was mainly attributed to reactive oxygen species, as thiol supplementation suppressed p38 activation and subsequent IL-8 and RANTES production by DEP. However, no activation of JNKs and ERK1/2 by DEP was noticed in this cell type (21). In contrast, Bonvallot et al. (10) have shown the phosphorylation of ERK1/2 and p38 by DEP in the human airway epithelial cell line 16HBE14o−. In the present study, we demonstrated that DEP stimulates phosphorylation of ERK1/2 and JNK1 but not JNK2, whereas p38 was weakly activated in C10 cells (Fig. 5).

Recent activation of JNK1 and JNK2 isoforms in both primary and transformed (BEAS-2B) human bronchial epithelial cells by organic DEP extracts was demonstrated (33). It is not clear whether these discrepancies are the result of cell-specific responses to DEP or to subtle variations in experimental conditions. For example, BETA-1 (46) and 16HBE14o−(16) represent human tracheobronchial epithelial cell lines, whereas C10 used in this study is a murine alveolar type II-like epithelial cell line (34).

It is well documented that ERK, JNK, and p38 distinctly activate AP-1 family members in multiple cell types (23, 28). However, upstream and downstream kinase modules that regulate the inducible fra-1 expression in bronchial epithelial cells are not clear. Our data (Fig. 5) obtained with various MAPK inhibitors suggest that modulation of fra-1 by DEP is a complex process. The concurrent requirement of ERK1/2, JNK1, and p38 MAPK pathways for induction indicates the involvement of interaction between different cis-elements and trans-acting factors in the regulation of fra-1 expression. Indeed, we recently demonstrated that TPA-inducible fra-1 expression in the alveolar type II-like epithelial cell line A549 is mediated by complex interactions between factors such as specificity protein-1, E26 transformation specific, and AP-1 family members that occur through multiple cis-elements (1). Numerous studies have demonstrated that these transcription factors are effectors of ERK, JNK, and p38 MAPKs. For example, JNKs and ERKs phosphorylate Jun and Fos proteins, respectively, whereas activating transcription factor-2 and Elk1 are targets of p38 (17, 44). Although transient transfection assays indicated that the −379 bp 5′-flanking region of fra-1 promoter can respond to DEP stimuli, the exact DNA sequences and cognate transcription factors regulating fra-1 expression by DEP need further investigation.

The AP-1 complex containing Fra-1 has been implicated in maintenance and progression of the transformed state in other cell types in vitro and in vivo (60). For instance, Fra-1 activates transcription of tumor progression-associated genes, such as high-mobility group protein 1 (Y), urokinase-type plasminogen activator and its receptor, and plasminogen activator-inhibitor type 1 (3, 31), as well as C-met and CD44 receptors (45). Overexpression of fra-1 in fibroblasts results in anchorage-independent cell growth in vitro as well as tumor formation in nude mice (8, 63). We have shown that Fra-1 positively upregulates gene expression associated with airway epithelial injury (43). The present study also indicated that modulation of fra-1 expression by DEP probably plays a role in differentially regulating genes involved in cellular detoxification, injury, and repair. Intriguingly, Fra-1 suppresses Nrf2-dependent reporter gene expression in transient transfection assays. Nrf2 belongs to the Cap n’Collar/basic leucine zipper (CNC-bZIP) family of proteins, which play a critical role in detoxification of reactive oxygen species (38, 41). DEP-induced translocation of Nrf2 and the increase in this transcription factor binding to the ARE correlated with NQO1 induction in bronchial epithelial cells (6). DEP also markedly upregulated ARE-mediated reporter gene expression (Fig. 7). However, induction of Fra-1 by DEP, which also activates Nrf2, is intriguing. In response to stress, Nrf2 rapidly (in most cases within 30 min) translocates into the nucleus and transactivates promoters containing functional ARE (38). Northern analysis indicated that induction of Fra-1 by DEP is a late event and occurs after 30 min. Therefore, we
speculate that modulation of fra-1 expression by DEP may play a role in downregulation of prolonged activation of Nrf2-dependent genes. Further experiments are needed to determine the mechanism by which Fra-1 suppresses Nrf2-dependent gene expression. However, because TRE are often embedded within ARE (see Fig. 7), it is probable that DEP-enhanced fra-1 expression interferes with Nrf2 binding to its cognate site. Consistent with this notion, downregulation of Nrf2-enhanced ARE-mediated NQO1 gene expression by Fra-1 has been demonstrated in hepatoma cells (61).

The present study demonstrated the involvement of Fra-1 in MMP-9 gene regulation. Fra-1 upregulated the MMP-9 promoter activity in transient transfection assays. Furthermore, EMSA assays revealed an enhanced Fra-1 binding to the functional −79 TRE of the MMP-9 promoter after DEP stimulation. Consistent with this finding, DEP also upregulated the MMP-9 promoter activity. Although these observations strongly suggest a potential role for Fra-1 in DEP-induced MMP-9 gene expression in airway epithelial cells, the involvement of other transcription factors, such as NF-κB, that are known to regulate MMP-9 gene transcription cannot be ruled out. DEP also activates NF-κB in bronchial epithelial cells (9, 57). Because Fra-1-Jun heterodimers are more stable than Jun-Jun homodimers (23, 28), it is possible that DEP-induced Fra-1 expression may play a role in either stabilizing or potentiating the interaction between NF-κB and AP-1 proteins binding at the MMP-9 promoter, thereby enhancing the transcription. In support of this view, the interaction between AP-1 and the NF-κB family of proteins has been documented both in vitro (56) and in vivo (25). Consistent with our findings, Wu et al. (64) demonstrated upregulation of MMP-12 gene expression by AP-1 and Fra-1 bound to the functional TRE in human U937 monocytic cells. Interestingly, cigarette smoke, which upregulates fra-1 expression (2), also induced the expression of MMP-12 gene (22) in the lung. Recently, Tower and colleagues (59) demonstrated a critical role for Fra-1 in maintaining a high-level constitutive MMP-1 gene expression in melanoma cells. Although these observations strongly suggest a potential role for Fra-1 in regulating MMP gene expression, further experiments are required to establish a link between Fra-1 and DEP-induced gene expression in airway epithelial cells.

In conclusion, we demonstrated that multiple MAPK signaling pathways regulate DEP-inducible fra-1 by DEP in the murine alveolar type II epithelial cell line. In contrast, expression of fra-2, a close relative of fra-1, was unchanged. Furthermore, our data suggest that Fra-1 distinctly regulates gene expression in a promoter context-dependent manner. Because overexpression of Fra-1 has been implicated in the progression and invasiveness of different tumor types, current and future findings on this transcription factor expression and regulation may provide new insight into molecular mechanisms of DEP effects on lung disease development.

ACKNOWLEDGMENTS

We thank Alvin Malkinson for providing us with C10 cells used in this study. We also thank Douglas Boyd, Motoharu Seiki, Javed Alam, and Michael Birrer for providing us with various plasmids used in this study.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants ES-09606, ES-11863, EPA-R82672401, HL-58122, and HL-66109 (to S. P. Reddy). We acknowledge the Johns Hopkins Urban Environmental Health Center for use of its core facilities supported by NIH Grant ES-30819.

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