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

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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. First published October 17, 2003; 10.1152/ajplung.00221.2003.—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 matrix metalloproteinase (MMP)-9 promoter activity. In particular, it bound to the functional AP-1 site of the MMP-9 promoter after DEP stimulation. Consistent with this result, DEP also markedly upregulated MMP-9 promoter activity. Collectively, these findings suggest that fra-1 induction by DEP may play a role in selectively regulating gene expression involved in alveolar epithelial cell injury and repair. activator protein-1; mitogen-activated protein kinase; diesel exhaust particles; matrix metalloproteinase-9; fos-related antigen-1

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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 and AP-1 family members distinctly regulate their expression in several cell types (42), we have examined the role of Fra-1 on MMP-9 promoter transactivation in transient transfection and DNA-binding assays. Here we report for the first time that DEP upregulates fra-1 expression but not its close relative, fra-2. Furthermore, we demonstrate that fra-1 induction by DEP probably plays a role in the modulation of gene expression involved in pulmonary defense.

MATERIALS AND METHODS

Cell culture and gene expression analysis. C10 is an immortalized nontumorigenic alveolar type II-like epithelial cell line isolated from adult mice (34). C10 cells were maintained in MEM containing 10% FCS, 1% streptomycin and penicillin, gentamicin (250 ng/ml), and fungizone (125 ng/ml). DEP matter, SRM1650a, a Standard Reference Material, was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). Cells were treated with either DEP (5 or 25 μg/ml) or TPA (20 ng/ml), and total RNA was isolated by Trizol reagent (Invitrogen). RNA (~15 μg) was separated on a 1.2% agarose gel, blotted on a Nytran membrane, and hybridized with [32P]cDNA probes of mouse fra-1 or fra-2, as described previously (43, 62). For Western analysis, total cells treated with or without DEP were washed three to four times with cold PBS containing 1 mM Na2VO4 (sodium orthovanadate). Total protein was extracted in a cell lysis 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-glycerophosphate, and 1 μg/ml leupeptin. A comparable quantity of protein (~30 μg) from each sample was separated on a 10% SDS-PAGE gel and transferred to a Hybond-enhanced chemiluminescence (ECL) nitrocellulose membrane. Membranes were blocked overnight at 4°C in Tris-buffer solution containing 0.1% Tween and 5% nonfat milk and incubated for 1 h at room temperature with polyclonal anti-Fra-1 or anti-Fra-2 antibodies (Santa Cruz Biotechnology). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and immunoreactive bands were detected using ECL reagents (Amersham Bioscience).

Plasmids and transient reporter gene assays. DNA transfections were performed using a FuGENE transfection reagent according to the manufacturer’s protocol (Roche Biochemical). The 861-Luc and 379-Luc contain the −861 to +32 bp and −379 to +32 bp 5’-flanking region of human fra-1, respectively. DNA fragments were cloned upstream of the luciferase (Luc) reporter gene between Sac I and Nhe I sites of pGL3 basic vector (see Ref. 1 for more details). The 634-Luc contains a fragment of MMP-9 promoter luciferase reporter construct containing −634 to +32 bp and −73 to +32 bp 5’-flanking regions of human MMP-9, respectively (see Ref. 51 for more details), were kindly provided by Douglas Boyd (M. D. Anderson Cancer Center, Houston, TX) and Motoharu Seiki (University of Tokyo, Tokyo, Japan). TRE-Luc bearing seven copies of consensus AP-1 recognition sites (TRE, 5'-TGACTAA-3') upstream of Luc was obtained from Stratagene (La Jolla, CA). ARE-Luc containing three copies of Nrf2 binding sequences (ARE, 5'-CGGACCTTGACTCAGCGAGAAA-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 pCMV expression vector bearing the wild-type c-Jun and p35 were generously provided by Micheal Birrer (National Cancer Institute) and Eugene Tulchinsky (National Institute of Cancer Biology), respectively. The pEF/Nfr2 expression vector containing the mouse Nrf2 cDNA (30) under the control of the elongation factor-1α (EF) promoter 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 experiments. 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'-CGGACCTTGACTCAGCGAGAAA-3'). After 30 min of incubation, samples were resolved on a 4–6% polyacrylamide gel containing 2% glycerol. To demonstrate the specificity of the complex formation, nuclear extracts were incubated for 10–15 min with a 50-fold molar excess of the unlabeled double-strand oligo before the addition of labeled probes. To determine the presence of a specific protein in the complex, nuclear extracts were incubated with 1–2 μg of specific antibodies against c-Jun (sc-45X), JunB (sc-46X), JunD (sc-74X), c-Fos (sc-7202X), Fra-1 (sc-605X), and Fra-2 (sc-604X) (all obtained from Santa Cruz Biotechnology) for 1–2 h before addition of labeled DNA probe.

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 Na2VO4 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-glycerophosphate, 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
transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was carried out using the phospho-specific ERK, JNK, and p38 antibodies, as previously described (43, 62). Total ERK, p38, and JNK1 content of the samples were determined by immunoblot analysis using ERK2 (C-14), p38 (C-20), and JNK1 (C-17) antibodies, respectively.

RESULTS

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), 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 to +32 fra-1 promoter fragment, since it contains sufficient information for inducible fra-1 transcription in response to disparate stimuli, such as TPA, 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.

**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.

**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.

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.
role of Fra-1 in DEP-induced cellular responses of alveolar tumor progression in epithelial cells (31). To determine the associated with airway squamous cell metaplasia (43, 62) and atoma cells (61). In contrast, Fra-1 markedly upregulates genes suppresses Nrf2-dependent other Fos and Jun proteins in cells (52). For instance, Fra-1 Several studies have shown that Fra-1, which lacks a 

fraction.

Fig. 5. DEP stimulates activation of ERK/JNK/p38 MAPK pathway in alveolar epithelial cells. C10 cells were treated with DEP (25 μg/ml) for 15–180 min. Control and DEP-stimulated cells were harvested in an MAPK lysis buffer. An equal amount of protein (40 μg) was separated on 10% SDS-PAGE, and immunoblot analysis was performed using phospho-specific (p) anti-ERK (A), anti-JNK (B), or anti-p38 (C) antibodies. Blots were stripped, and the total content of ERK, JNK, and p38 was determined using their respective antibodies. As positive controls, cells were exposed to UV light for 1 min and then incubated at 37°C for 1 h. After 1 h of incubation, cells were harvested in a MAPK lysis buffer and used as above. The degree of change in the phosphorylation of MAPKs, which was displayed at the bottom of the autoradiograph, was determined by phosphor imaging using the band intensity of control (vehicle-exposed) sample as one. Results shown are a representative blot of 4 independent samples.

Fra-1 suppresses both c-Jun- and Nrf2-enhanced transcription. Several studies have shown that Fra-1, which lacks a transactivation domain (8, 63), differentially modulates AP-1 activity, depending on the promoter context and the status of other Fos and Jun proteins in cells (52). For instance, Fra-1 suppresses Nrf2-dependent NQO1 gene expression in rat hepatoma cells (61). In contrast, Fra-1 markedly upregulates genes associated with airway squamous cell metaplasia (43, 62) and tumor progression in epitheloid cells (31). To determine the role of Fra-1 in DEP-induced cellular responses of alveolar epithelial cells, its effect on AP-1 and Nrf2-dependent gene expression was analyzed by transient transfection assays using TRE-Luc (Fig. 6) and ARE-Luc (Fig. 7) reporters. DEP failed to significantly stimulate TRE-Luc activity (Fig. 6A). In contrast, it markedly stimulated ARE-dependent reporter expression (Fig. 7B). Overexpression of Fra-1 significantly and dose-dependently suppressed basal (Fig. 6B) and c-Jun enhanced (Fig. 6C) TRE-driven reporter expression. Similarly, Fra-1 suppressed basal and Nrf2-enhanced ARE-mediated reporter expression (Fig. 7C). Together, these results indicate that fra-1 induction by DEP may play a role in modulation of c-Jun- and Nrf2-dependent gene expression in alveolar epithelial cells.

DEP and Fra-1 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. 7.** Fra-1 suppresses basal and Nrf2 enhanced ARE-mediated reporter gene expression. A: schematic representation of ARE-Luc reporter construct. Only the ARE sequence (boxed) derived from the mouse heme oxygenase-1 enhancer is shown. Thick solid line with double arrow indicates the position of embedded TRE. B and C: transient transfections were performed as described in A and B of Fig. 6 with the exception that ARE-Luc was used instead of TRE-Luc. Values are means ± SE of 6 independent samples. *P < 0.05 compared with DEP-treated samples. **P < 0.05 when compared with empty vector group.

**Fig. 8.** Fra-1 transactivates matrix metalloproteinase (MMP)-9 promoter activity. A: schematic representation of human MMP-9 promoter reporter constructs, M634-Luc and M73-Luc (51). Sp1, specificity protein 1 binding site; EK, NF-κB binding site. B: cells were transfected with 100 ng M634-Luc or M73-Luc reporter along with pRL-TK in the presence of empty vector or 25 or 100 ng pCMV-fra-1 expression vector. Empty vector was included to keep the amount of DNA equal in all samples. After overnight incubation, cells were harvested, and luciferase activity was analyzed. C: cells were transfected with M634-Luc or M73-Luc reporter along with pRL-TK. After 24 h, cells were treated with or without DEP, and promoter activity was analyzed. *P < 0.05 compared with DEP-treated samples. Data represent 6 individual 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 16HBE140−. 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). Recently, 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 16HBE140− (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 transacting 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-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.

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