A PHOSPHATIDYLINOSITOL 3-KINASE REGULATED AKT INDEPENDENT SIGNALING PROMOTES CIGARETTE SMOKE INDUCED FRA-1 EXPRESSION

Qin Zhang1, Pavan Adiseshaiah1, Dhananjaya V. Kalvakolanu2, Sekhar P Reddy1,3,*

1Department of Environmental Health Sciences, Bloomberg School of Public Health,
3Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, Baltimore, MD 21205;
2University of Maryland Greenbaum Cancer Center, Maryland, MD 21201

Address correspondence to: Sekhar P Reddy, The Johns Hopkins University, Department of Environmental Health Sciences, Bloomberg School of Public Health, RM. E7610, 615 North Wolfe Street, Baltimore, MD 21205. Tel.: 410-614-5442; Fax: 410-955-0299; E-mail: sreddy@jhsph.edu

Running Title: PI3K regulates FRA-1 induction independent of Akt

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Summary
The FRA-1 protooncogene is overexpressed in a variety of human tumors and is known to upregulate the expression of genes involved in tumor progression and invasion. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is also known to regulate these cellular processes. More importantly, respiratory toxicants and carcinogens activate both the PI3K-Akt pathway and FRA-1 expression in human bronchial epithelial (HBE) cells. In this report we investigated a potential link between the PI3K-Akt pathway and the CS-stimulated epidermal growth factor receptor (EGFR)-mediated FRA-1 induction in non-oncogenic HBE cells. Treatment of cells with LY294002, an inhibitor of the PI3K-Akt pathway, completely blocked CS-induced FRA-1 expression. Surprisingly, pharmacological inhibition of Akt had no significant effect on CS-induced FRA-1 expression. Likewise, the inhibition of PKCζ, which is known downstream effector of PI3K, did not alter FRA-1 expression. We found that the PI3K through p21-activated kinase 1 (PAK1) regulates FRA-1 proto-oncogene induction by CS and the subsequent activation of the Elk1 and CREB transcription factors that are bound to the promoter in HBE cells.

INTRODUCTION
Exposure to cigarette smoke (CS) has been linked to the development of various respiratory diseases, including lung cancer (1). Furthermore, CS aggravates the incidence of other toxicant-induced respiratory disease (2). Although the molecular mechanisms underlying the CS-promoted respiratory pathogenesis remain unclear, emerging data suggest a role for phosphatidylinositol 3-kinase (PI3K) signaling in this process. PI3K regulates a number of diverse cellular responses, which include cell survival, motility and invasiveness (3). PI3K stimulates the activation of phosphoinositide-dependent kinases-1/2 (PDK1/2), which then phosphorylate Akt at two critical threonine and serine residues. Phosphorylation of these residues is required for Akt activation (4). Activated Akt phosphorylates various effector molecules. In addition to Akt, PI3K dependent activation of MAP kinases, such as ERK1/2, and of certain PKC isoenzymes, such as PKCζ and PKCδ, has also been documented in various cell types (3).

CS and its individual components, such as NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and nicotine, have been shown to activate PI3K-Akt pathway in vitro and in vivo (5,6). Moreover, elevated level of phospho-specific form of AKT (Ser473) has been found in pulmonary metaplasias and dysplasias (7), NNK-induced lung tumors (5), and in human lung tumors derived from smokers.
These data suggest a potential role for PI3K-Akt pathway in CS-induced respiratory pathogenesis. Consistent with this observation, an enhanced activity of Akt, a major downstream effector of PI3K, has been found in various cancer cell lines/tumor tissues (3,9). However, the downstream mechanisms of CS-induced PI3K-Akt pathway are unclear.

The activator protein 1 (AP1) family of transcription factors (Jun, JunB, JunD, c-Fos, FosB, FRA-1, and FRA-2) regulate several cellular responses induced by various oxidants and toxicants (10). These proteins play key roles in the initiation and progression of tumors (11). However, the links between PI3K-Akt signaling and CS-induced AP-1 activation have not been defined in HBE cells. A high level of FRA-1 expression has been detected in various tumors and lung cancer cells (12-15), and some known carcinogens, such as CS and asbestos, persistently activate FRA-1 expression in lung epithelial cells (16). Several observations point out that FRA-1 may play a key role in carcinogen-induced lung epithelial cell proliferation and transformation. For example, FRA-1 is required for asbestos-induced mesothelial cell transformation (17). It up-regulates the expression of genes coding for airway squamous cell metaplastic marker (18), matrix metalloproteinase-9 (19), and CD44 and c-met protooncogene (20), which are implicated in toxin-induced respiratory pathogenesis. FRA-1 controls cell motility, invasion, and maintenance and progression of the transformed state in several cell types (reviewed in (11,21). Recently, several studies demonstrated a critical role for this transcription factor in controlling both cell motility and invasiveness of several tumorigenic cell lines (22-24).

Mutational induction of FRA-1 gene in tumor tissues or cell lines is not known to date. Thus, an abnormal signaling and subsequent transcription factor activation may contribute to high level expression of FRA-1. We have recently shown that metalloproteinase (MMP)-epidermal growth factor receptor (EGFR)-mediated MAP kinase signaling plays an obligatory role in controlling CS-stimulated FRA-1 proto-oncogene induction in HBE cells (25). Although the activation of PI3K-Akt pathway by cigarette smoke and NNK has been reported earlier by others (5-7), none of these studies showed the upstream signals that activate PI3K pathway by CS and the subsequent downstream effectors that control AP-1 protooncogene induction. Here, we show, for the first time, that EGFR dependent PI3K- regulated CS-stimulated FRA-1 expression occurs through a PAK1-c-Raf-MEK1/2-ERK1/2 pathway that culminates in the activation of transcription factors Elk1 and CREB, independently of Akt.

**MATERIALS AND METHODS**

**Reagents and plasmids:** Research-grade 1R4F cigarettes (9 mg tar and 0.8 mg nicotine/cigarette) without filters were obtained from the University of Kentucky Tobacco Research Institute. LY294002, AG1478, Akt inhibitor II (Cat # 124011), Akt inhibitor IV (Cat # 124008), rapamycin, rotterlin, and PKCζ-myristoylated pseudosubstrate Inhibitor (Cat # 539624), were from Calbiochem (La Jolla, CA). GW5074 (Cat # G6416) was from Sigma-Aldrich (St. Louis, MO). c-Jun (SC-45X), FRA-1 (SC-605X), and p-Elk1 (sc-8406) antibodies were all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Native and phosphospecific antibodies for c-Jun (Ser73), PDK1 (Ser241), Akt (Ser473), EGFR (Tyr845), CREB (Ser133), MEK1 (Ser217/221), c-Raf (Ser338), Pak1 (Thr423/402) and PKCζ (Thr410/403) were obtained from Cell Signaling Technology (Beverly, MA). The 379-Luc construct was generated by cloning the -379 to +32 bp human FRA-1 upstream of the reporter firefly luciferase (Luc) gene. Mutations in the SRE (CArG mutant) and ATF binding sites in the context of 379-Luc were generated as described previously (26). A GST-tagged PAK1 vector (83-149), which acts as genetic mutant (27) was provided by Rakesh Kumar (M.D. Anderson Cancer Center, Texas).

**Cell culture and cigarette smoke (CS) exposure:** The 1HAEo cell line, an SV40-transformed nonmalignant HBE cell line (28), was maintained in MEM containing 10% fetal calf serum. Cells were grown in 25 cm² flasks (Becton Dickinson) to 90% confluence, serum-starved for 14 h and then exposed to mainstream CS as described previously (25). In brief, CS was drawn into a syringe-driven device and then delivered via the tube into cell...
culture flasks that were inverted to expose cells directly to smoke for 2 min. After that the flasks were restored to their original orientation, so that the cells were covered with culture medium, and the flasks were placed in the 37°C incubator. Throughout this study, we used CS at a dose of 2 cc/25-cm³ flask. No cell cytotoxicity was observed under the conditions of CS exposure.

**Kinase immunoblot analysis:** Cells were exposed to CS for various time points, washed three times with chilled PBS containing 1 mM Na₃VO₄ and then lysed in a MAP kinase lysis buffer (20 mM Tris, pH 7.5, with 150 mM NaCl, 0.2 mM DTT, 0.5mM PMSF, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM Na₃VO₄, 5 mM β-glycerolphosphate and 1 µg/ml leupeptin). Cell lysates were separated on an SDS-PAGE gel and transferred to a PVDF membrane. The membranes were blocked with 5% nonfat dry milk. Blots were then incubated overnight at 4°C with primary antibodies as indicated in the results and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with ECL reagent (Amersham Biosciences, NJ). Band intensities were quantified by densitometry. Kinase phosphorylation was normalized to that of total kinase protein levels and then expressed as a percentage of that untreated control.

**Immunoprecipitation of PAK1 and c-Raf:** Cells were exposed to CS for various time points, and then harvested in ice-cold cell lysis buffer. Lysates (~200 µg) were incubated with 5 µl of anti-PAK1 or anti-c-Raf antibodies with gentle rocking overnight at 4°C. The immunocomplex was precipitated using protein A agarose beads (20 µl of 50% bead slurry) with gentle rocking for 3 h at 4°C. After centrifugation, the beads were washed with cell lysis buffer five times, followed by addition of 20 µl of 3 x SDS sample buffer. Beads were boiled for 5 min, then centrifuged to remove agarose beads prior to electrophoresis, and analyze sample by Western blotting with phospho-specific antibodies for anti-PAK1 and anti-c-Raf.

**Gene expression analysis:** After CS exposure, cells were washed extensively with cold PBS containing 1 mM sodium orthovanadate (Na₃VO₄) and harvested into a MAP kinase lysis buffer. Protein samples (~ 40 µg) were resolved on a 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked overnight at 4°C in Tris buffer containing 0.1% Tween and 5% nonfat milk and incubated at room temperature for 1 h with FRA-1 antibody. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and immunoreactive bands were detected using the ECL reagent (Amersham Bioscience, NJ). Blots were stripped and probed with β-actin antibodies to ensure a comparable loading of extracts. For Northern blot analysis, after exposing to CS, total RNA was isolated from cells using the Trizol reagent. RNA (~15 µg) was separated on a 1.2% agarose gel, blotted onto a Nytran membrane and sequentially hybridized with 32P-labeled FRA-1 and β-actin cDNAs as probes as described previously (25). The total amount of FRA-1 mRNA and protein in each sample was quantified with a Gel Doc 2000 system (Bio-Rad Laboratories, CA) and normalized to that of β-actin band. For transient transfections, cells were transfected with 100 ng of the -379 to + 32 bp FRA-1 promoter reporter luciferase construct [hereafter abbreviated as 379-Luc, see ref (29) for details] along with 5 ng of the Renilla luciferase plasmid (pRL-TK, Promega Corp). After overnight incubation, cells were serum starved for 16 h and then exposed to CS for 5 h, and luciferase activity was analyzed as described previously (25).

**Electrophoretic mobility shift assay (EMSA):** Serum-starved cells were exposed to CS, nuclear extracts were prepared as described previously (26). Briefly, the EMSAs were performed using 2-3 µg of nuclear extract in a 20 µl of binding buffer on ice for 10 min prior to the addition of the appropriate 32P-labeled double-stranded oligo probe. After incubating for 30 min at room temperature, samples were resolved on a 4% polyacrylamide gel containing 2% glycerol. To demonstrate the presence of a specific protein in the complexes, nuclear extracts were mixed with 1-2 µg of anti- CREB (Cat # 06-519, Upstate Cell
Signaling) antibodies and incubated on ice for 2 h prior to adding the probe.

**Chromatin Immunoprecipitation (ChIP) Assays:** ChIP assays were carried out as described earlier (26). Briefly, cells (~1x10^7) were exposed to filter air or CS for 60 min, and ChIP was performed using a commercially available kit (Upstate Biotechnology Inc., NY). Chromatin was cross-linked by adding formaldehyde (1%) to the tissue culture medium for 10 min at 37°C. A fraction of the soluble chromatin (1%) was saved for measurement of total chromatin input. Precleared chromatin was incubated with specific antibodies for 18 h at 4°C. DNA recovered from the immunoprecipitated products was employed as a template for PCR with *FRA-1* promoter-specific primers (26).

**Statistical Analysis:** Data are expressed as the mean ± S.E. Statistical significance was determined using “t” test and accepted at p < 0.05. All assays were performed in two or three (n= 2-3) independent samples, and each experiment was repeated at least two times.

**RESULTS**  
**PI3K-mediated signaling is required for CS-stimulated *FRA-1* expression.** To determine if the PI3K-mediated signaling is necessary for CS-induced *FRA-1* expression, HBE cells were treated with LY294002 for 40 min and then exposed to CS for 3 h, and *FRA-1* mRNA and protein expression were analyzed by Northern and Western blot analyses, respectively (Fig. 1). Consistent with our previous results (25), CS significantly (~3-fold) stimulated the expression of *FRA-1* mRNA (Fig. 1A) and protein levels (Fig. 1B), as compared to the cells exposed to filtered air. However, pretreatment of cells with PI3K inhibitor LY294002 completely suppressed CS-induced *FRA-1* mRNA expression. Previously, we have shown that CS stimulates FRA-1 induction mainly at the transcription level and the -379 bp promoter contains sufficient information to mediate such induction (26).  
To verify that PI3K-signaling regulates *FRA-1* transcription, cells were transfected with the 379-Luc promoter reporter construct along with the pRK-TL reference plasmid. Promoter activity in response to CS was analyzed in the absence or presence of LY294002 (Fig. 1C). As expected, CS significantly elevated luciferase activity, which was abrogated in the presence of LY294002. No effect of CS on the basal promoter activity was observed. Collectively, these observations strongly support a prominent role for PI3K-dependent signaling in mediating the CS-stimulated *FRA-1* transcription in pulmonary epithelial cells.

**CS activates PI3K signaling in HBE cells.** To determine whether CS activates PI3K signaling, we have monitored the activation of PDK1 (Fig. 2A) and its downstream effector Akt (Fig. 2B). Cells exposed to CS for 0-60 min, an equal amount of total lysates from duplicate samples were separated on PAGE and immunoblotted using phospho-specific PDK1 (Ser241) and Akt (Ser473) antibodies. As shown in Fig. 2A, CS significantly stimulated the phosphorylation of PDK1 as early as 15 min of exposure (lanes 3 and 4), which remained above the basal level through 60 min (lanes 7 and 8) compared to control group (lanes 1 and 2). Consistent with this result, CS markedly stimulated the phosphorylation of the Ser473 residue of Akt (Fig. 2B) as early as 30 min (lane 2), which stayed elevated above the basal level through 60 min (lane 3). A quantification of phosphorylated Ser473 signals on these blots revealed a 147% and 128% increase over the basal level at 30 and 60 min post CS-exposure, respectively. We also found that CS stimulates phosphorylation of Ser473 in another HBE cell line and in the lung tissues of mice exposed to CS (data not shown). Collectively, these data suggest that CS stimulates the PI3K/Akt signaling, consistent with this results reported by others in the lung cell types (5,6).

**CS activates PI3K-Akt pathway through EGFR.** Recently, we have shown that the activation of EGFR is essential for CS-stimulated *FRA-1* induction (25). Given the observation that PI3K inhibitor completely blocked *FRA-1* induction and EGFR-mediated signaling activates the PI3K-Akt pathway in response to growth factors in other cell types (30,31), we next examined whether the activation of PI3K-Akt pathway mediated through EGFR. Cells were treated with AG1478 prior to the exposure and...
CS-stimulated Akt phosphorylation was analyzed by immunoblot analysis using phospho-Ser473 antibodies (Fig. 2C). AG1478 (lanes 7 and 8) completely inhibited CS-stimulated (lanes 3 and 4) Akt phosphorylation. Interestingly, inhibition of EGFR activity with AG1478 significantly stimulated basal levels Akt phosphorylation (lanes 5 and 6) indicating a negative cross-talk between EGFR and Akt signaling in the unstimulated state. We have shown earlier that metalloproteinases (MMP) activity is required for CS-stimulated EGFR mediated signaling in the lung. Consistent with this result, treatment of cells with MMP inhibitor GM6001 markedly blocked (Fig. 2D, lanes 7 and 8) CS-induced Akt phosphorylation (Fig. 2D, lanes 3 and 4). Collectively, these results suggest that signaling pathways involving EGFR and MMP regulate the activation of PI3K-Akt pathway in response to CS.

Akt-mediated signaling is not required for CS-stimulated FRA-1 induction. Enhanced Akt activity has been linked to tobacco–induced cellular responses (5,6) and tumorigenesis (3). Since both Akt and FRA-1 regulate cellular processes such as cell motility and invasiveness, we sought to determine whether Akt-dependent signaling was required for PI3K-mediated CS-stimulated FRA-1 induction. To examine this aspect, we have used two pharmacological inhibitors of Akt, Akt inhibitor II and Akt inhibitor IV. Akt inhibitor II specifically inhibits the activation of Akt without affecting other upstream or downstream kinases (32). Akt inhibitor IV inhibits phosphorylation/activation of Akt by targeting its immediate upstream kinase, PDK-1, but not PI3K (33). We examined first the effects of these inhibitors on Akt-mediated signaling under our experimental conditions by monitoring the activation of GSK, a major downstream target of Akt, in response to EGF. As shown in Fig. 3A, pretreatment of cells with either Akt inhibitor II (top panel, lanes 4 and 5) or Akt inhibitor IV (bottom panel, lanes 4-6) markedly suppressed EGF-stimulated GSK phosphorylation (lanes 2 and 3). To confirm the specificities of these inhibitors, these membranes were stripped and probed with phosphospecific ERK1/2 antibodies. As anticipated, both Akt inhibitor II and Akt inhibitor IV had no significant effect on EGF-induced ERK1/2 activation (Fig. 3A). To determine whether Akt-mediated signaling is required for FRA-1 induction, cells were exposed to CS in the presence or absence of either Akt inhibitor II or Akt inhibitor IV, RNA was isolated, and Northern blot analysis was performed. Neither Akt inhibitor II (top panel, lanes 4-6) nor Akt inhibitor IV (bottom panel, lanes 4-6) had an appreciable inhibitory effect on the CS-induced expression of FRA-1 mRNA (Fig. 3B, lanes 2 and 3). Thus, although CS-activates Akt, it is not required for regulating FRA-1 expression in response to CS.

We next examined whether PKCζ which is a known downstream targets of PI3K (3), is involved in this process using the PKCζ myristoylated pseudosubstrate Inhibitor (Fig. 3C). This inhibitor had no effect on CS-stimulated FRA-1 expression. To determine whether or not CS-activates PKCζ, we have monitored the levels of phosphorylation of this kinase using phospho-specific antibodies. As shown in Fig. 3D, CS stimulated the phosphorylation of PKCζ in HBE cells as early as 5 min (lane 2), compared to unstimulated cells (lane 1), which remained above the basal level through 60 min (lane 5). However, pre-treatment of cells with PI3K inhibitor markedly suppressed both basal (Fig. 3E, compare lane 1 and lane 3) and CS-stimulated PKCζ phosphorylation (Fig. 3E, compare lane 2 and lane 4).

The PI3K regulates CS-induced FRA-1 expression via the MEK1/2-ERK1/2 pathway. Above results indicate that PI3K regulates FRA-1 induction independently of Akt and PKCζ. Recently, we have shown a critical role for ERK1/2 dependent signaling in controlling CS-stimulated FRA-1 expression in HBE cells (25). Since PI3K regulates ERK1/2 kinases in response to growth factors in several cell types (3), and the fact that EGFR activity is critical for CS-stimulated FRA-1 induction, we next examined whether PI3K induces FRA-1 through ERK1/2 signaling. Cells were treated with LY294002 prior to CS exposure and CS-induced ERK1/2 kinase activation was assayed by immunoblot analysis (Fig. 4A). PI3K inhibitor completely blocked CS-stimulated ERK1/2 activation. To further confirm...
that PI3K regulates ERK1/2 through the activation of MEK1/2, but not via a cross-talk between Akt and ERK, we have analyzed the effect of PI3K inhibitor on CS-stimulated MEK1 activation using phosphospecific MEK1 antibodies (Fig. 4B). Indeed, LY294002 robustly blocked CS-induced MEK1 phosphorylation (compare lanes 3 and 4, and lanes 7 and 8) indicating that PI3K regulates ERK signaling by activating MEK1, the upstream known activator of ERK1/2.

PAK1 via c-Raf mediates CS-activated PI3K-MEK-ERK pathway. Both PAK1 and/or Raf kinases have been shown to mediate the cross-talk between the PI3K and ERK pathways (34). To examine whether PI3K activates MEK-ERK signaling via PAK1 or Raf, cells were exposed to CS for 0, 15, and 30 min, and CS-induced PAK1 and c-Raf kinase activation was assessed by immunoprecipitation and Western blot analysis using respective total and phosphospecific antibodies (Fig. 5). As shown in Fig. 5A, CS stimulated both PAK1 (top panel) and c-Raf phosphorylation (bottom panel). Also, pre-treatment of cells with PI3K inhibitor LY 294002 markedly suppressed CS-stimulated PAK1 and c-Raf phosphorylation (compare lanes 2 and 4, Fig. 5B). The basal level c-Raf phosphorylation was high in the presence of LY294002 (lane 3, Fig. 5B). To determine the role of PAK1 in CS-stimulated MEK-ERK1/2 pathway, cells were transfected with a GST-tagged dominant mutant PAK1 expression vector encoding 83-149 amino acids of PAK1 (hereafter referred to as GST-PAK1). When expressed mutant GST-PAK1 inhibits PAK1 kinase activity and blocks PAK1 translocation to the centrosomes during mitosis (27). Cells were then exposed to CS and the activation of c-Raf was monitored by immunoprecipitation and Western blot analysis (Fig. 6A). Overexpression of GST-PAK1 (lanes 3 and 4) significantly inhibited CS-stimulated c-Raf phosphorylation (Fig. 6A) as compared with an empty vector transfected CS exposed cells (lanes 1 and 2). Likewise, expression of GST-PAK1 markedly suppressed CS-stimulated MEK1/2 phosphorylation (Fig. 6B). To determine whether c-Raf activation is necessary for CS-stimulated MEK-ERK pathway, cells were treated with c-Raf specific inhibitor GW5074 prior to CS exposure and MEK-ERK activation was monitored (Fig. 6C). CS stimulated MEK1/2 and ERK1/2 activation was significantly suppressed by c-Raf inhibitor (lane 4) as compared with vehicle treated CS exposed cells (lane 2). Collectively, these results indicate an important role for PAK1 in mediating a cross-talk between the PI3K and Raf-MEK-ERK1/2 signaling pathways.

The inhibition of PI3K or ERK pathways suppresses CS-induced Elk1 and CREB phosphorylation. To determine other pathways activated by the PI3K, we examined the effect of LY294002 on Elk1 and CREB protein phosphorylation (Fig. 7A). These two transcription factors are downstream targets of ERK signaling and are known to regulate the induction of c-FOS (35) and FRA-1 (26) in response to tumor promoters and mitogens. We have also used the MEK-ERK pathway inhibitor PD98059 (Fig. 7B) to compare to the results obtained with the PI3K inhibitor LY294002. As shown in Fig. 7A, CS stimulated the phosphorylation of Elk1 and CREB (lanes 3 and 4) compared to filtered air exposed samples (lanes 1 and 2). Both LY294002 (Fig. 7A, lanes 7 and 8) and PD98059 (Fig. 7B, lanes 7 and 8) completely blocked such phosphorylation. Together, these results show that PI3K/ERK pathway controls the activation of transcription factors Elk1 and CREB in response to CS. To further confirm the role of Elk1 and ATF/CREB proteins in the transcriptional upregulation of FRA-1 by CS, cells were transfected with the wildtype 379-Luc promoter-reporter construct or with 379-Luc constructs bearing mutations in the serum response element (SRE) or in the ATF binding site (Fig. 7C). The SRE has been shown to be critically required for an efficient binding of Elk1 to the SRE (35). After overnight incubation, cells were exposed to CS for 5 h, and reporter expression was analyzed. Consistent with our previous results (25), CS significantly stimulated luciferase activity driven by the wildtype FRA-1 promoter. However, mutations either in the SRE or ATF site significantly reduced CS-stimulated FRA-1 promoter activity. This result implies that protein binding to these sites is required for the induction of FRA-1 transcription by in HBE cells.
CS exposure does not alter Elk1 protein binding at the FRA-1 promoter in vivo. We have recently reported that the -276 to -239 bp segment composed of SRE (containing a TCF site and the CArG box), and a putative ATF site, plays a critical role in mitogen-induced FRA-1 transcription in lung epithelial cells (26). Furthermore, we have shown that specific knockdown of Elk1 transcription factor significantly attenuated tumor promoter-stimulated FRA-1 transcription (26). To examine whether PI3K signaling altered the engagement of Elk1 with the endogenous FRA-1 promoter, we performed a ChIP assay (Fig. 8). Cells were treated with DMSO (lanes 1-4), LY294002 (lanes 5-8) or PD98059 (lanes 9-12) prior to CS exposure. After 60 min incubation with CS, DNA-protein complexes were cross-linked by formaldehyde and soluble chromatin was immunoprecipitated using the anti-Elk1 antibodies and non-immune IgG and DNA fragments recovered from the IP products were used as templates for PCR with FRA-1 promoter-specific primers (Fig. 8A). As expected, ChIP assays with the non-immune IgG showed no amplification of the FRA-1 promoter (data not shown). Elk1 was constitutively engaged with the promoter irrespective of CS exposure (lanes 1-4). Treatment of cells either with PI3K inhibitor (lanes 5-8) or ERK inhibitor (lanes 9-12) did not significantly alter the binding of Elk1 to the promoter.

Inhibition of PI3K and ERK signaling did not affect CREB binding to the FRA-1 promoter. Unlike Elk1, we were unable to amplify the FRA-1 promoter to detectable levels in ChIP assays using CREB antibodies, even at greater than 50 PCR cycles. We therefore performed EMSA assays to examine whether CS enhanced the recruitment of CREB to the ATF site located at position -248 of the FRA-1 promoter (Fig. 9A); and if so PI3K and ERK inhibitors affected such binding. Cells were exposed to CS for 60 min in the presence or absence of LY294002 or ERK inhibitor PD98059, nuclear extracts were prepared, and EMSA was performed using the ATF site of FRA-1 promoter as a probe. Incubation of both filtered air-exposed and CS exposure nuclear extracts with the ATF probe yielded a single specific complex (indicated with an arrow in Fig. 9B). There was no difference in the binding of CREB to ATF site. When incubated with the CREB antibodies (lane 3 and 4) this complex was supershifted. CREB antibodies used in the present study do not cross-react with other ATFs. Collectively, these results (Fig. 8 and 9) indicate that both Elk1 and CREB bind to the SRE and the ATF sites of the FRA-1 promoter in un-stimulated cells, and CS stimulation has a little effect on their steady-state DNA binding.

DISCUSSION

The present study, for the first time, demonstrates a potential link between the EGFR-activated PI3K-dependent ERK MAP kinase signaling and CS-induced FRA-1 protooncogene expression (Fig. 1). We have shown that the inhibition of EGFR kinase activity markedly suppresses both CS-induced PI3K-Akt signaling (Fig. 2C) and FRA-1 induction (25). Consistent with this result, a general inhibitor of MMP activity, which is essential for CS-induced EGFR activation (36-38), also had a similar effects on CS-induced PI3K-Akt signaling (Fig. 2D) and FRA-1 expression (25). Thus, PI3K appears to be one of the downstream effectors of CS-induced MMP-EGFR signaling. The Akt pathway has been shown to regulate HBE cell survival and transformation promoted by NNK (5,6), a potent carcinogen present in the CS. However, results obtained with Akt-specific pharmacological inhibitors in this study indicate that Akt has no significant effect on CS-induced transcriptional activation of FRA-1. Previous studies have shown that Akt both negatively and positively regulates expression of AP-1 proteins. For example, Akt inhibits the expression of c-FOS, a close relative of FRA-1, by down regulating the ERK MAP kinase pathway; and high level of Akt activity is inversely correlated with c-Fos expression in glioblastoma cells (39). In contrast, enhanced Akt activity is required for a high level expression of FRA-1 in prostate cancer cells (40). A different study showed Akt dependent stimulation of c-FOS occurs through its SRE in HeLa cells (41). Thus, it appears that Akt distinctly regulates the expression of FOS family members in a cell type- and signal-specific manner.
Our data indicate that PI3K signals activate ERKs (Fig. 4A), which are essential for high level induction of FRA-1 by CS (25), tumor promoters, and mitogens in HBE cells (data not shown). Consistent with this, a prominent role for ERK signaling in mitogen-inducible FRA-1 mRNA expression has been shown in other cell types (17,42-44). Although a requirement for PI3K pathway in mitogen-induced ERK activation has been suggested in other cells, the present study goes further to demonstrate a link between CS induced PI3K signaling and ERK activation for the first time in HBE cells (Fig. 4A). The activation of ERKs occurs via MEK1 in PI3K dependent manner. Consistent with our studies, the inhibition of PI3K pathway blocked PDGF- and EGF stimulated c-FOS expression via ERK signaling (45,46). The PI3K inhibitor blocked CS-induced ERK1/2 activation (Fig. 4A). Supporting this observation, several studies have shown that PI3K modulates MEK-ERK pathway at the level of PAK1 and/or Raf (47-49). PAK1 is a member of serine/threonine kinnases family which is known to control various cellular processes including cytoskeletal reorganization, cell migration, and MAP kinase signaling (50). PAK1 has been shown to facilitate the cross-talk between the PI3K and ERK signaling pathways (34). For example, PI3K may act through either Raf-1, or PAK1, to phosphorylate MEK1 (47-49), an upstream activator of ERK1/2 kinases. Furthermore, the activation of PAK1 by benzo(a)pyrene, a carcinogen found in CS, in 293T and HeLa cells has been reported (51). Our studies demonstrated that inhibition of PI3K pathway blocked the activation of PAK1 (Fig. 5), and the ectopic expression of a PIK1 mutant inhibited CS activated c-Raf and MEK1 phosphorylation (Fig. 6). Our novel findings indicate that PAK1 mediates the cross-talk between the PI3K and ERK pathways by activating c-Raf and MEK1 kinases in response to CS in HBE cells.

We have recently demonstrated that the -379 to +32 bp genomic fragment, devoid of introns, contains sufficient information to drive FRA-1 transcription in response to tumor promoters and CS exposure in pulmonary epithelial cells (25,26,29). A -379/-237 bp bipartite enhancer comprises of the -318 TRE, SRE (276/-237), and an ATF sites which are necessary for a high level induction of FRA-1 (26,29) in response to TPA. The recruitment of c-Jun to the -318 TRE and the binding of the Elk1, SRF and CREB/ATF-1 proteins to the respective TCF, CArG, and ATF sites was necessary for FRA-1 expression (26,29). In the present study, we found that CS stimulates the phosphorylation of Elk1 and CREB transcription factors, while inhibitors of PI3K or ERK1/2 blocked such activation. The inhibition of PI3K did not block CS-stimulated p38 phosphorylation (data not shown) suggesting that ERK1/2 alone may integrate CS-induced PI3K-dependent signals into Elk1.

Since Elk1 and CREB are also known to regulate the induction of c-FOS and FOS-B (52,53), it is likely that Elk1 and CREB act as downstream targets of CS-induced ERK1/2 signaling and regulates FRA-1 induction. Consistent with this notion, CS failed to significantly stimulate transcriptional activation from the FRA-1 promoter bearing mutations in CArG box and ATF sites suggesting a critical requirement of Elk1 and CREB protein binding at these sites for the induction of FRA-1 by CS (Fig. 7C). However, ChIP (Fig. 8) and EMSA (Fig. 9) analyses showed that CS did not significantly alter the constitutive binding of both Elk1 and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1). These results are consistent with our previous ChIP analyses that demonstrated the constitutive binding of Elk1, SRF, and CREB proteins to the FRA-1 promoter. Although inhibitors of PI3K and ERK signaling did not inhibit DNA binding of Elk1 and CREB, inhibitors markedly attenuated their CS-inducible phosphorylation status (Fig. 7A and 7B) and FRA-1 mRNA expression (Fig. 1).
been shown to facilitate the recruitment of the transcriptional co-activator p300 (56).

In summary, based on above observations, we propose the following model for the CS-induced expression of the \textit{FRA-1} (Fig. 10). In the unstimulated state, the Elk1 and CREB proteins are constitutively bound to the \textit{FRA-1} promoter. Upon CS stimulation, EGFR mediated signaling activates PI3K pathway leading to both Akt and PAK1 activation (step 1). PAK1 then stimulates the phosphorylation of c-Raf/MEK/ERK1/2 (step 2). ERKs translocate into the nucleus and phosphorylate Elk1 and CREB proteins bound to the promoter (step 3) resulting an enhanced \textit{FRA-1} transcription (step 4).

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Abbreviations
AP-1, activator protein-1; ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; FRA-1 or FOSL-1, fos-related antigen 1; Luc, luciferase; PAK1, p21 (CDKN1A)-activated kinase 1; TCF, ternary complex factor; TRE, TPA response element; SRF, serum response factor; SRE, serum response element.

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Figure 1: Effect of PI3K inhibitor on CS-induced FRA-1 expression. Cells were treated with vehicle (DMSO) or PI3K inhibitor LY294002 (20 µM) for 30 min and exposed to CS. After 3 h incubation, cells were lysed in Trizol reagent or in a MAP kinase lysis buffer to analyze FRA-1 mRNA (A) and protein (B) expression by Northern blot (NB) and Western blot (WB) analyses, respectively. Results shown are a representative blot of two independent experiments. (C) Cells were transfected with 100 ng 379-Luc along with the pRL-TK (5 ng). After overnight incubation, cells were serum starved for 16 h, treated with DMSO or PI3K inhibitor LY29402 for 30 minutes and then exposed to filter air (open bars) or CS (filled bars) for 5 h, and luciferase activity was assayed using a dual luciferase kit. The percent increase in CS-stimulated luciferase activity over the filter-air exposed samples in the presence of DMSO was considered equal to 100 (bar 1). Promoter activity in response to CS in the presence of LY294002 is minimal (bar 2). Values represent mean ± SE (n = 3) of a representative experiment. The experiment was repeated twice.

Figure 2: CS stimulates PI3K/Akt signaling and EGFR and MMP activity is required for this activation in HBE cells. Cells were exposed to filter air (-) or CS (+) for 0-60 min and harvested in a MAP kinase lysis buffer. Lysates (~40 µg) were separated on SDS-PAGE and immunoblotted with phospho-specific PDK1 (A) and Akt^{S473} (B) antibodies. The membranes were subsequently probed with anti-PDK1 or anti-Akt antibodies, respectively. Bands were quantified and normalized with the respective total protein content (bottom panels). The level of kinase phosphorylation of filter air exposed (“0” min) control group was designated as one arbitrary unit (AU). Quantification of a representative blot of two independent experiments is shown; Means ± SD. * P <0.05 compared with filter air-exposed samples. (C) Cells were treated with AG1478 (AG, 2 µM) for 40 min and then exposed to CS for 30 min. Akt phosphorylation of was analyzed using phospho-specific Akt^{S473} antibodies. (D) Cells were treated with 25 µM GM6001 for 30 min and then exposed to CS. The total lysates were immunoblotted using phospho-specific Akt^{S473} antibodies. Quantification of CS-stimulated Akt activation was performed as in Fig. 2. Quantification of a representative blot of two independent experiments is shown; Means ± SD. *P<0.001 compared with filter air exposed (- CS) samples, $P<0.05$ compared with DMSO-treated CS-exposed samples, and $ compared with DMSO-treated filter air exposed samples.

Figure 3: The effect of Akt inhibitors on CS-stimulated FRA-1 mRNA expression.
(A) Cells were treated with the Akt-specific pharmacological inhibitors II (Akt inh-II, 50 µM) and IV (Akt inh-IV, 10 µM) for 30 min. DMSO was used as a vehicle control. Cells were treated without (-) or with (+) EGF (20 ng/ml), and immunoblotted with phospho-specific antibodies as indicated. (B) Cells were treated with Akt inhibitors, Akt inh-II and Akt inh-IV, for 30 min and exposed to filter air (-) or CS (+) for 3 hours. Northern blot analysis was carried out under conditions identical to Fig.1. (C) The effect of PKCζ pseudosubstrate inhibitor on CS-stimulated FRA-1 mRNA expression. (D) Analysis of PKCζ phosphorylation in response to CS exposure. Cells were treated with CS for 0-60 min and lysates were immunoblotted with phospho-specific PKCζ antibodies. (E) The effect of PI3K inhibitor on CS-stimulated PKCζ phosphorylation. Cells were treated with DMSO (lanes 1 and 2) or with 20 µM LY294002 (LY, lanes 3 and 4) prior to CS (+) or filter air (-) exposure. A representative autoradiogram of two independent experiments is shown.

**Figure 4: The effect of PI3K inhibitor on CS induced MAP kinase activation. (A)** Cells were treated with DMSO or LY294002 (20 µM) for 30 min prior to CS (+) or to filter air (-) exposure. After 30 min post-exposure, cells were lysed and MAP kinase activation was analyzed using phospho-specific anti-ERK1/2 antibodies. The membranes were stripped and probed with native antibodies (B) Cell lysates were immunoblotted with phospho-specific MEK1/2 antibodies. The β-actin band was used as a loading control. Data shown in C and D are results from a representative experiment, which was repeated twice.

**Figure 5: Effect of PI3K inhibitor on CS-stimulated PAK1 and c-Raf activation. (A)** Cells were exposed to filter air (-) or CS (+) for different points as indicated. Cell lysates were immunoprecipitated (IP) with anti-PAK1 or anti-c-Raf antibodies overnight. The immunocomplex was precipitated using protein A Sepharose beads. Western blot (WB) analysis was performed using the phospho-specific antibodies for PAK1 (pPAK1) and c-Raf (pc-Raf). Lysates (~40 µg protein) was separated and immunoblotted with β-actin antibodies to ensure equal amount protein present in the IP. (B) Cells were treated with DMSO or LY294002 (20 µM) for 30 min prior to CS (+) or to filter air (-) exposure. After 30 min post-exposure, cell extracts were prepared and precipitated with anti-PAK1 or anti-c-Raf antibodies. Western blot analysis was performed using the phospho-specific antibodies for PAK1 and c-Raf. Lysates (40 µg protein) were separately immunoblotted with β-actin or c-Raf antibodies. Each experiment was repeated four times (n=4).

**Figure 6: The PI3K regulates MEK-ERK signaling through PAK1/c-Raf activation.** Cells were transfected with the GST-PAK1 mutant construct for 48 hrs. An empty GST-vector was used as control. Cells were exposed to CS for 30 min, whole cell lysates were harvested. (A) Cell lysates were immunoprecipitated with anti-c-Raf antibody and then immunoblotted with anti-phosphospecific c-Raf antibody. *P <0.03 compared with filter air exposed (-CS) samples, # P <0.05 compared with DMSO-treated CS-exposed samples (n =3). (B) Cell lysates as in panel A were immunoblotted with phosphospecific MEK1 antibody. Membrane was subsequently probed with ERK2 antibody (C) Cells were treated with DMSO or GW5074 (10 µM) for 40 min prior...
to CS (+) or to filter air (-) exposure. After 15 min post-exposure, cells were lysed and MEK1/2 and ERK1/2 phosphorylation was analyzed. The membranes were stripped and probed with the respective total antibodies. Each experiment of panels B and C was repeated at least twice to obtain reproducible results.

**Figure 7:** PI3K and ERK pathway specific inhibitors suppress CS-stimulated Elk1 and CREB phosphorylation. (A) Cells were serum-starved for 14 hours and then exposed to filter air (-) or CS (+) in the presence and absence of LY294002 (20 µM) under conditions identical to Fig. 1. CS-induced Elk1 and CREB activation was analyzed using their phospho-specific antibodies. The β-actin band was used for loading control. Results shown are from a representative experiment that was repeated at least twice. (B) The effect of ERK inhibitor PD98059 (25 µM) on CS-stimulated Elk1 and CREB phosphorylation. (C) Cells were transfected with the wildtype 379-Luc promoter-reporter construct or with mutant 379-Luc constructs bearing mutations in SRE or ATF binding site. pRL-TK was used to monitor transfection efficiency. After overnight incubation, cells were exposed to CS for 5 h, and reporter expression was analyzed. Data are expressed as -fold over room air-exposed (Ctr) samples. The data represent the values of six independent samples. *P <0.01 compared with Ctr samples. $ and # are P <0.05 and P <0.04, respectively, as compared with DMSO-treated CS-exposed samples.

**Figure 8:** The effect of pharmacologic inhibition of PI3K and ERK signaling on Elk1 binding to the endogenous FRA-1 promoter in vivo. (A) The positions of the functional serum response element (SRE) and ATF functional motifs of FRA-1 promoter are shown. Arrows indicate the position of forward and reverse primers used in ChIP assays to amplify a 143 bp promoter fragment. (B) Cells were treated with vehicle, LY294002 or PD98059 for 30 min and then exposed to filter air (-) or CS (+). After 60 min post-exposure, DNA-proteins were cross-linked by formaldehyde at room temperature. The purified nucleoprotein complexes were immunoprecipitated with the anti-Elk1 antibodies. Non-immune IgG was used as a negative control. The ChIP assays were performed with two independent samples at least twice to obtain reproducible results. Lane 13 represents PCR amplification of plasmid DNA containing human FRA-1 promoter (+ template).

**Figure 9:** The effect of CS on CREB protein binding to the FRA-1 promoter. (A) DNA sequence of the -248 ATF site of the FRA-1 promoter (26). (B) Nuclear extracts isolated from filter air (-) or CS (+) stimulated cells in the presence and absence of LY294002 or PD98059, while DMSO used as control. Nuclear extracts (2 µg) were incubated with either IgG or CREB-antibodies followed by incubation with the 32P-labeled ATF probe. The position of the CREB-based DNA-protein complex is indicated by the arrow. Asterisk indicates the position of non-specific (NS) band, which is not reproducible appear between EMSAs. SS indicates the position of supershifted complex. A representative autoradiogram from two independent experiments is shown.

**Figure 10:** Model depicting CS-induced EGFR-activated PI3K-mediated MEK-ERK signaling converging at the FRA-1 promoter in HBE cells. Shown are the positions of EGFR and PI3K in the proposed pathway, in which their stimulation by CS activates FRA-1 induction via PAK1/Raf/MEK1/2-ERK1/2-Elk1/CREB.
signaling. Filled circles with number indicate the steps of activation by CS (see discussion).
Figure 1

A

|       | DMSO | LY294002 |
|-------|------|-----------|
| NB    | -    | +         |
|       | -    | +         |

B

|       | β-actin | FRA-1 |
|-------|---------|-------|
| NB    |         |       |
| WB    |         |       |

C

CS-stimulated promoter activity (% increase over control)

0 50 100 150

DMSO  LY294002

CS
Figure 2

A

0  15  30  60

p-PDK1

PDK1

0  15  30  60

p-Akt

p-PDK1 (AU)

p-Akt (AU)

0  15  30  60

CS(min)

B

0  30  60

p-Akt

Akt

0  30  60

CS(min)
Figure 2

C

DMSO  |  AG1478
-     |  -     |  +     |  +     |  ← CS

\[\text{Akt} \]

\[\text{p-Akt} \]

1  |  2  |  3  |  4  |  5  |  6  |  7  |  8

\[\text{DMSO} \]  |  \[\text{AG1478} \]

\[\text{Bar graph} \]

\[\text{p-Akt (AU)} \]

\[\text{DMSO} \]  |  \[\text{AG1478} \]

\[\text{*} \]  |  \[\text{#} \]  |  \[\text{$} \]

D

DMSO  |  GM6001
-     |  -     |  +     |  +     |  ← CS

\[\text{Akt} \]

\[\text{p-Akt} \]

1  |  2  |  3  |  4  |  5  |  6  |  7  |  8

\[\text{DMSO} \]  |  \[\text{GM6001} \]

\[\text{Bar graph} \]

\[\text{p-Akt (AU)} \]

\[\text{DMSO} \]  |  \[\text{GM6001} \]

\[\text{*} \]  |  \[\text{#} \]
Figure 4

A

DMSO

LY294002

- - + +

- - + +

p-ERK1/2

ERK2

p-MEK1

β-actin

1 2 3 4 5 6 7 8

B

DMSO

LY294002

- - + +

- - + +

p-ERK1/2

ERK2

p-MEK1

β-actin

1 2 3 4 5 6 7 8

CS

CS
Figure 5

(A) CS−→ CS+     CS
p-PAK1
β-actin
c-Raf
pc-Raf
DMSO LY294002

0              15            30    (min)

IP: PAK1
WB: pPAK1
IP: c-Raf
WB: pc-Raf
WB

1  2  3

(B) DMSO    LY294002

IP: PAK1
WB: pPAK1
IP: c-Raf
WB: pc-Raf
WB

1  2  3  4

β-actin
pc-Raf
c-Raf
β-actin
Figure 6

A

CS :
vector GST-PAK1

IP: Raf
WB: pc-Raf

pc-Raf
IgG

1 2 3 4

p-cRaf (AU)

- CS + CS

vector GST-PAK1

* #

B

vector GST-PAK1

+ CS

pc-Raf
pMEK1
ERK2

1 2 3 4

C

DMSO GW5074

+ CS

pMEK1
ERK2

1 2 3 4

p-ERK1 p-ERK2
ERK2
Figure 7

A

|          | DMSO   | LY294002 |
|----------|--------|----------|
| CS       | -      | +        |
| p-Elk1   |        |          |
| p-CREB   |        |          |
| β-actin  |        |          |
| 1        | 2      | 3        | 4      | 5      | 6      | 7      | 8      |

B

|          | DMSO   | PD98059  |
|----------|--------|----------|
| CS       | -      | +        |
| p-Elk1   |        |          |
| p-CREB   |        |          |
| β-actin  |        |          |
| 1        | 2      | 3        | 4      | 5      | 6      | 7      | 8      |

C

ELK     SRE     CREB     ATF

FRA-1

ELK-274 CREB-248

Luciferase activity

-379WT SRE-mt ATF-mt

Ctr CS

* $ #
**Figure 8**

**A**

-379 → SRE → ATFS → -248 → FRA-1 promoter → -237

143 bp

**B**

| CS | DMSO | LY294002 | PD98059 |
|----|------|----------|---------|
|    | -    | -        | -       |
|    | +    | +        | +       |

ChIP antibodies

Elk1

IgG

input

1  2  3  4  5  6  7  8  9  10  11  12  13
Figure 10

CS → EGFR → PI3K → Akt

Akt

PI3K → PAK-1 → c-Raf → MEK1/2 → ERK1/2

ERK1/2

SRE

-274/-254

CREB

ATF

-248

Elk

FRA-1

1, 2, 3, 4
A phosphatidylinositol 3-kinase regulated Akt independent signaling promotes cigarette smoke induced FRA-1 expression
Qin Zhang, Pavan Adiseshaiah, Dhananjaya V. Kalvakolanu and Sekhar P. Reddy

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