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Freshly Fractured Crystalline Silica Induces Activator Protein-1 Activation through ERKs and p38 MAPK*

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The transcription factor activator protein-1 (AP-1) reportedly plays an important role in the induction of neoplastic transformation and multiple genes involved in cell proliferation, differentiation, and inflammation. To investigate the mechanisms of silica-induced carcinogenesis, AP-1-luciferase reporter transgenic mice were used as an *in vivo* model, whereas the JB6 mouse epidermal cell line and a rat lung epithelial cell line were employed as *in vitro* models to study the effects of silica at the molecular level. Freshly fractured silica caused an 8-fold increase in AP-1 activity in JB6 cells and a 2.5-fold increase in rat lung epithelial cells. The induction of AP-1 activity in cultured cell lines was time- and dose-dependent. Intratracheal administration of silica was also able to induce AP-1 transactivation in transgenic mice. AP-1 activation was first observed at 2 days after silica administration and reached its maximum at 3 days post-exposure of the mice to silica. The signal transduction pathways for AP-1 activation were also investigated using these cell lines. The results demonstrate that freshly fractured silica stimulates mitogen-activated protein kinase (MAPK) family members, as determined by the phosphorylation of p38 MAPK and extracellular signal-regulated protein kinases (ERKs). Inhibition of ERKs with PD98059 or of p38 with SB203580 significantly inhibited silica-induced AP-1 activation. These findings demonstrate for the first time that freshly fractured silica induces AP-1 activation, which may be mediated through p38 MAPK and ERK pathways. Unraveling the complex mechanisms associated with these events may provide insights into the initiation and progression of silica-induced carcinogenesis.

Epidemiologic and pathologic studies have established that occupational exposure to crystalline silica is associated with the development of acute and chronic pulmonary silicosis (1, 2). Acute silicosis usually occurs in individuals who work in occupations where silica is fractured or ground into fine powder by mechanical processes. Acute silicosis becomes clinically apparent within 2–5 years after exposure and frequently results in death due to hypoxia (3). Chronic silicosis occurs in workers with prolonged exposure to silica-containing dusts, with clinically apparent disease developing after 20 or more years of exposure (3). In addition, increasing evidence obtained from epidemiologic and laboratory animal studies in recent years suggests that crystalline silica may be a carcinogen (4, 5). For example, inhalation of silica has been shown to be carcinogenic in rats, whereas intratracheal instillation has been shown to be carcinogenic in other animals (6–9). Intrapleural administration of crystalline silica in rats leads to the induction of localized malignant histiocytic lymphomas. Epidemiologic studies also show that there is an increased lung cancer risk in many, but not all, human subjects with silicosis (4, 5). Based on current evidence obtained from studies on laboratory animals and epidemiologic studies on humans, the International Agency for Research on Cancer has classified crystalline silica as a human class 1 carcinogen (5).

Although silica is now a documented carcinogen, the molecular mechanisms involved in silica-induced carcinogenesis remain poorly understood. Previous studies have shown that silica causes direct DNA damage and mammalian cell transformation, including binding with deoxyribonucleic acid, which has been used as an *in vitro* analog of cancer induction (10, 11). Earlier studies have demonstrated that freshly fractured silica is capable of generating hydroxyl radicals (·OH) upon reaction with aqueous media (12–14). Superoxide anion radicals ($O_2^{\cdot-}$) may also be generated (15, 16). The silicon-based free radicals Si·, SiO·, and SiOO· and the associated generation of H_2O_2 and ·OH might be involved in the lipid peroxidation and membrane damage that lead to the loss of membrane integrity and eventual pulmonary fibrosis (12–14, 17). These radicals are also associated with silica-induced activation of the nuclear transcription factor NF-κB (18) and DNA damage, *e.g.* strand breakage and hydroxylation of dG residues (19).

AP-1[1] is a transcription factor that interacts with regulatory DNA sequences known as TPA response elements or AP-1 sites (20). Many stimuli, including the tumor promoter TPA and reactive oxygen species, regulate AP-1 binding to the DNA of the promoter region of a number of intermediate genes that govern inflammation, proliferation, and apoptosis (21–23). AP-1 and its regulated gene expression have been reported to play important roles in neoplastic transformation, tumor progression, and metastasis (24–27). On the basis of the importance of AP-1 activity in tumor promotion and progression, we hypothesized that the carcinogenic effect of silica may be mediated through the activation of AP-1 activity. To test this hypothesis, we used JB6 P^+ mouse epidermal cells and rat lung epithelial cells as *in vitro* models and AP-1-luciferase trans-

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[1] The abbreviation used are: AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated protein kinase; MEM, minimal essential medium; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; RLE, rat lung epithelial; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase.

genic mice as an *in vivo* model for these studies. The JB6 family of mouse epidermal clonal genetic variants that are P^+ or P^- provides a suitable model for studying critical gene regulation events that occur during carcinogenesis (28, 29). The signal transduction cascades involved in AP-1 activation were also investigated. We demonstrate here that freshly fractured silica induces AP-1 activation in both *in vivo* and *in vitro* systems and that this activation appears to occur through the ERK1, ERK2, and p38 kinase signal transduction pathways.

MATERIALS AND METHODS

Reagents and Plasmids—Eagle's MEM was obtained from Whittaker Biosciences (Walkersville, MD). Fetal bovine serum, gentamicin, and L-glutamine were purchased from Life Technologies, Inc. Luciferase assay substrate was obtained from Promega (Madison, WI). PhosphoPlus MAPK Antibody kits were purchased from New England Biolabs Inc. (Beverly, MA). The rat lung epithelial cell line was obtained from American Type Culture Collection (Manassas, VA). The AP-1-luciferase reporter plasmid (collagenase-luciferase) and the CMV-neo vector plasmid were constructed as reported previously (30, 31). PD98059 and SB203580, which are specific MEK1 and p38 kinase inhibitors (32, 33), respectively, were from Calbiochem.

Preparation of Freshly Fractured Silica—Crystalline silica was obtained from the Pennsylvania State University Genetic Center (State College, PA). The detailed method for preparation of the freshly fractured silica has been described elsewhere (13). Briefly, crystalline silica (0.2–10 mm in diameter) was ground for 30 min using a ball grinder equipped with agate balls. The ground silica was sieved through a 10-μm mesh filter for 20 min before use. Purity was checked using x-ray analysis and morphometric analysis, which indicated that the ground silica had a mean diameter of 3.7 μm.

Generation of Stable Transfectants—RLE cells were transfected with the AP-1-luciferase reporter plasmid and the G418-selecting plasmid pcDNA3-CMV-neo. Fifteen individual clones were selected by ring isolation and cultured in F12K medium containing 10% FBS and G418 (200 μg/ml) for 30 days. Stable transfectants were screened by assaying for luciferase activity following stimulation with TPA or vanadate. A stable transfectant (RLE/AP02) was established and cultured in G418-free medium for the experiments.

Cell Culture—The JB6 P^+ mouse epidermal cell line, which was stably transfected with the AP-1-luciferase reporter plasmid (JB6/AP/κB) (34), was cultured in Eagle's MEM containing 5% FBS2, 2 mM L-glutamine, and 50 μg/ml gentamicin. The RLE cell line, stably transfected with the AP-1-luciferase reporter plasmid (RLE/AP02), was cultured in F12K medium containing 10% fetal calf serum and 50 μg/ml gentamicin. The cells were grown at 37 °C in a 5% CO_2 atmosphere.

Assay of AP-1 Activity in Vitro—A confluent monolayer of JB6/AP/κB cells was trypsinized, and 5×10^4 viable cells (suspended in 1 ml of Eagle's MEM supplemented with 5% FBS) were added to each well of a 24-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Twelve hours later, cells were cultured in Eagle's MEM supplemented with 0.5% FBS for 12–24 h to minimize basal AP-1 activity and then exposed to silica in the same medium to monitor the effects on AP-1 induction. The cells were extracted with 200 μl of 1× lysis buffer provided in the luciferase assay kit by the manufacturer, and the luciferase activity was measured. The results are expressed as relative AP-1 activity compared with controls. The AP-1 activity assay for RLE/AP02 cells was similar to that described for JB6 cells, except that in RLE cells, F12K medium containing 10% fetal calf serum was used.

Animals and Administration of Silica—AP-1-luciferase reporter (2× TPA response element-binding sites) transgenic mice were originally established by Rincon and Flavell (35). A C57BL/6 male mouse carrying the 2× TPA response element-luciferase transgene was crossed with a DBA2 female (SASCO, Omaha, NE) (36). The offspring were screened for the presence of the AP-1-luciferase reporter gene by testing the TPA-induced level of luciferase activity. Males and females were housed separately in solid-bottom polycarbonate cages on ventilated animal racks (four to five mice per cage) under temperature-, humidity-, and yellow light-controlled conditions. Food and water were available *ad libitum*.

The AP-1-luciferase reporter-bearing male and female mice (8–12 weeks old) were randomly divided into six groups consisting of eight mice in each group. Freshly fractured silica was suspended in 0.9% sterile NaCl (70 mg/ml) and was administered at 0.07 ml/mouse (5 mg) with an intratracheal cannula after the animals were anesthetized with sodium pentobarbital (50 mg/kg). Control mice were instilled intratracheally with 0.07 ml of 0.9% sterile NaCl/mouse.

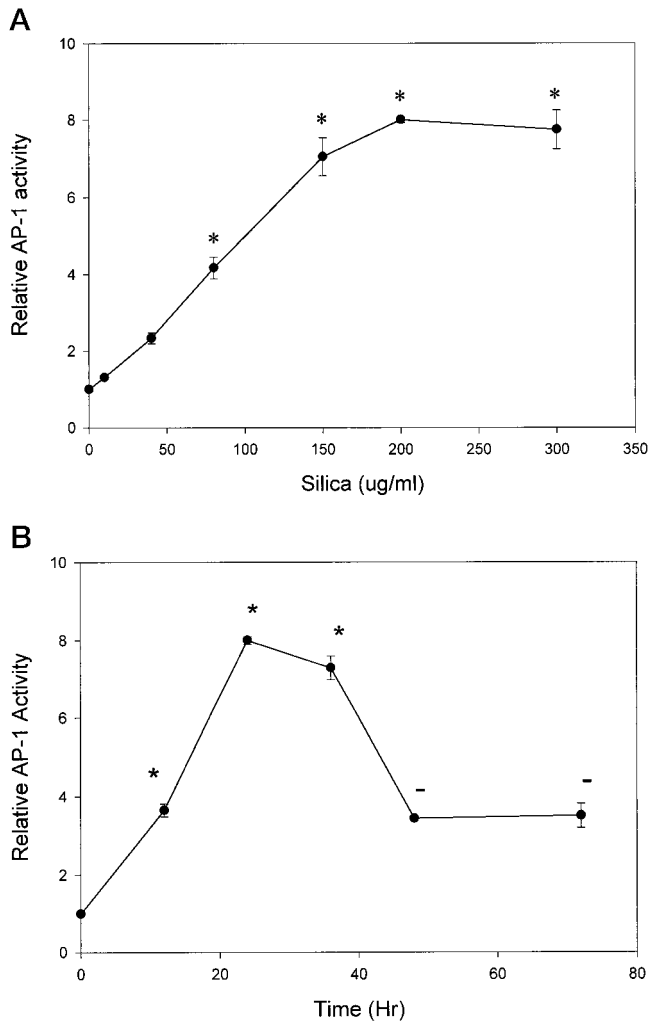

FIG. 1. **Freshly fractured silica-induced AP-1 activation in JB6 P^+ cells.** JB6/AP/κB cells (5×10^4 in 1 ml of MEM with 5% FBS) were seeded into each well of a 24-well plate. After overnight culture at 37 °C, the cells were cultured in MEM plus 0.5% FBS for 12 h. The cells were then treated with various concentrations of silica suspended in the same medium for 24 h (*A*) or with 200 μg/ml silica/well (100 μg/cm^2) for different times as indicated (*B*). AP-1 activity was measured by the luciferase activity assay as described under "Materials and Methods." The results, presented as relative AP-1 induction compared with the untreated control cells, are means ± S.E. of nine assay wells from three independent experiments. *, significant increase from control ($p \leq 0.05$); −, significant decrease from the 24-h value ($p \leq 0.05$).

Assay of AP-1 Activity in Vivo—One to four days after intratracheal instillation of silica, the mice were sacrificed by exsanguination under deep pentobarbital anesthesia. Lung tissue was removed and minced with scissors. Lysis buffer (100 μl/10 mg of tissue) was added, and the tissues were lysed overnight at 4 °C. The luciferase activity of the tissue supernatant obtained after lysis was measured with a luminometer as described previously (36). AP-1 activity is expressed relative to the level of luciferase activity of controls.

Protein Kinase Phosphorylation Assay—Immunoblotting for phosphorylation of ERKs, JNKs, and p38 kinase was carried out as described by the protocol of New England Biolabs Inc. using phospho-specific antibodies against phosphorylated sites of ERKs, JNKs, and p38 kinase, respectively. Non-phospho-specific antibodies against ERKs, JNKs, and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay using the same transferred membrane blot.

Electrophoretic Mobility Shift Assay—Gel shift assays were performed to detect AP-1 binding activity after exposure to silica. The nuclear extracts and the ^{32}P-labeled oligonucleotide were prepared as described previously (34). Briefly, an oligonucleotide containing the

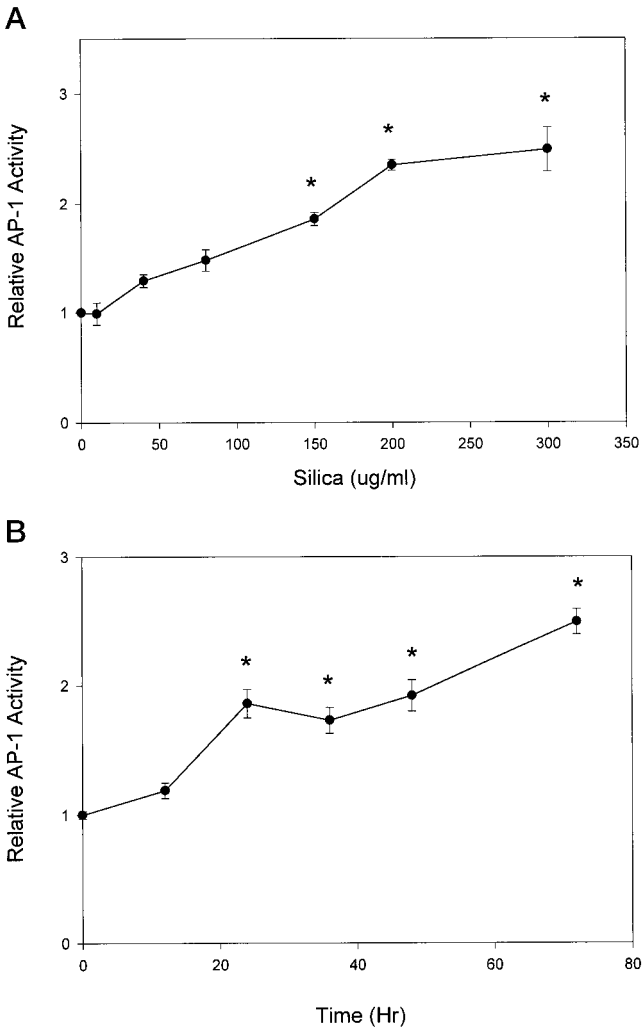

FIG. 2. **Induction of AP-1 activity in RLE cells.** RLE/AP02 cells (5×10^4 cells in 1 ml of F12K medium containing 10% of FBS), stably transfected with the AP-1-luciferase reporter plasmid, were seeded into each well of a 24-well plate. After overnight culture at 37 °C, the cells were cultured in F12K medium plus 0.5% FBS for 12 h. The cells were then exposed to various concentrations of silica suspended in the same medium for 72 h (*A*) or 200 μg/ml silica/well (100 μg/cm^2) for different times as indicated (*B*). Other experimental conditions were the same as those described in the legend to Fig. 1. The results, presented as relative AP-1 induction compared with the untreated control cells, are means ± S.E. of 12 assay wells from three independent experiments. *, significant increase from control ($p \leq 0.05$).

AP-1 binding site (5′-CGCTTGATGAGTCAGCCGGAA-3′) was synthesized and labeled with [^{32}P]dCTP. Nuclear proteins (3–5 μg), extracted from cells exposed to silica, were mixed with the labeled probe and incubated at room temperature for 30 min. The DNA-protein complexes were resolved on a 5% nondenaturing acrylamide gel. The gel was dried and visualized by autoradiography.

Statistical Analysis—The data presented are the means ± S.E. of values compared and analyzed using a one-way analysis of variance. Statistical significance was determined by two-tailed Student's *t* test for paired data and is considered significant at $p \leq 0.05$.

RESULTS

Establishment of RLE AP-1 Reporter Cells—To study the AP-1 induction by silica in pulmonary cells, we established a stable AP-1-luciferase reporter transfectant from the RLE cell line. The stable transfectants were generated by ring selection. After transfecting cells with plasmids and selecting by G418, 15 colonies were isolated. The AP-1 activation of each clone was screened by measuring luciferase activity induced by TPA or vanadate. One of the colonies (REL/AP02), with stable luciferase activity, was maintained for the experimental studies. The AP-1 activity of RLE/AP02 cells was elevated 2- and 10-fold after a 24-h exposure of the cells to 20 ng/ml TPA or 40 μM vanadate, respectively (data not shown). The control cell line transfected with the vector only did not show any luciferase activity after a 24-h exposure of cells to TPA or vanadate (data not shown).

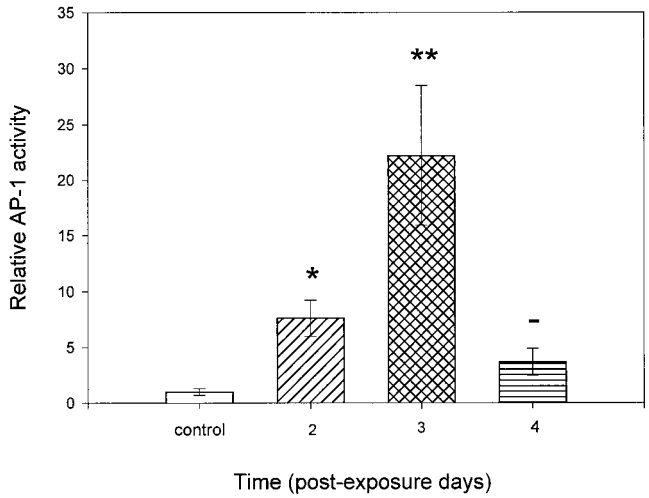

FIG. 3. **Freshly fractured silica induces the transactivation of AP-1 in AP-1-luciferase reporter transgenic mice.** The AP-1-luciferase transgenic mice were intratracheally instilled with 5 mg of freshly fractured silica suspended in 0.07 ml of 0.9% sterile saline. At 2, 3, or 4 days post-exposure, the mice were sacrificed, and the lung tissue was removed. The luciferase activity of the tissue was measured as described under "Materials and Methods." The results, presented relative to the level of luciferase activity of the control groups, are means ± S.E. of eight mice. *, significant increase from controls; **, significant increase from 2 days post-exposure; −, significant decrease from 3 days post-exposure ($p \leq 0.05$).

Freshly Fractured Silica Causes AP-1 Activation in JB6 and RLE/AP02 Cells—To explore the effects of silica on the induction of AP-1 activity, 5×10^4 JB6/AP/κB cells were exposed to varying doses (10~300 μg/ml) of freshly fractured silica for 24 h. Freshly fractured silica caused a significant dose-dependent AP-1 activation in JB6 cells (Fig. 1*A*). The AP-1 activation attained significance at a low silica concentration of 80 μg/ml (≈40 μg/cm^2) and reached its maximum activation at 200 μg/ml (≈100 μg/cm^2). Based on this result, 200 μg/ml silica was selected as the concentration to be used for time course studies. At intervals from 12 to 72 h, the relative AP-1 activity was tested using the luciferase assay. Induction of AP-1 activity was first observed after 12 h of incubation; and thereafter, AP-1 activity increased to a maximum of 8-fold activation at 24 h (Fig. 1*B*). Further incubations of cells with silica for 48 and 72 h resulted in a decrease in AP-1 activation.

Since crystalline silica causes pulmonary epithelial hyperplasia and neoplastic lesions, we next asked whether freshly fractured silica induces AP-1 activation in rat lung epithelial cells. Freshly fractured silica was incubated for 72 h with 5×10^4 RLE/AP02 cells stably transfected with the AP-1-luciferase reporter plasmid. Freshly fractured silica also caused a dose-dependent induction of AP-1 activation in RLE/AP02 cells (Fig. 2*A*). At a silica concentration of 300 μg/ml (≈150 μg/cm^2), RLE cells exhibited AP-1 levels 2.5-fold greater than those observed in control cells. Time course studies in which a silica concentration of 200 μg/ml (≈100 μg/cm^2) was used indicated that the time required for maximal induction of AP-1 activity was 72 h (Fig. 2*B*). The AP-1 induction in RLE/AP02 cells was different from that observed in JB6 cells. In RLE cells, the induction of AP-1 activity in response to silica occurred more slowly and

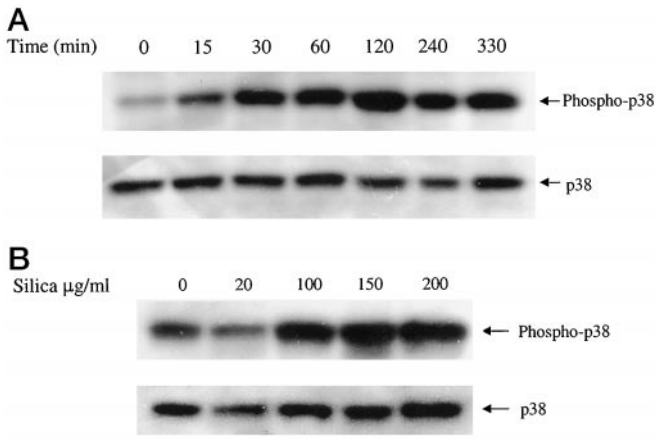

FIG. 4. **Freshly fractured silica stimulates the phosphorylation of p38 MAPK.** JB6 P^+ cells were cultured in MEM containing 5% FBS in six-well (35-mm diameter) plates until 80% confluent and then cultured in MEM containing 0.5% FBS for 24 h. After this time, the cells were exposed to 150 μg/ml silica (47 $\mu g/cm^2$) suspended in the same medium for different times as indicated (*A*) or to various concentrations of silica for 2 h (*B*). The cells were lysed, and phosphorylated and non-phosphorylated p38 kinase proteins were assayed using the PhosphoPlus MAPK kit. The phosphorylated and non-phosphorylated proteins were analyzed using the same transferred membrane blot.

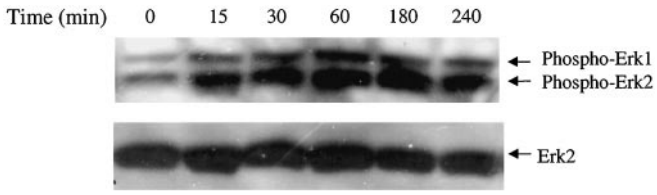

FIG. 5. **Freshly fractured silica induces activation of ERK1 and ERK2.** JB6 P^+ cells were cultured in MEM containing 5% FBS in six-well (35-mm diameter) plates until 80% confluent. The cells were cultured in MEM containing 0.5% FBS for 24 h and then exposed to 150 μg/ml silica (47 $\mu g/cm^2$) suspended in the same medium for different times as indicated. The cells were lysed, and phosphorylated ERK1 and ERK2 proteins and non-phosphorylated ERK2 proteins were assayed using the PhosphoPlus MAPK kit. The phosphorylated and non-phosphorylated proteins were analyzed using the same transferred membrane blot.

persisted for at least 72 h. The level of AP-1 induced by freshly fractured silica was lower in RLE cells than in JB6 cells. Because JB6 cells respond to a greater degree than RLE cells, JB6 cells were chosen for further studies.

Transactivation of AP-1 by Freshly Fractured Silica in AP-1-Luciferase Reporter Transgenic Mice—To investigate whether similar mechanisms exist *in vivo*, we used AP-1-luciferase reporter transgenic mice for these studies. The transgenic mice were exposed to freshly fractured silica (5 mg/mouse) by intratracheal instillation of a silica suspension (70 mg/ml in 0.9% sterile NaCl). At intervals of 1, 2, 3, and 4 days post-exposure, animals were anesthetized with sodium pentobarbital and sacrificed by exsanguination; lungs were removed; and their luciferase activities were measured as described under "Materials and Methods." Elevated AP-1 transactivation was not detected at 1 day post-exposure (data not shown). However, AP-1 activation increased significantly at 2 and 3 days post-exposure and decreased to control levels at 4 days post-exposure (Fig. 3). At day 3 post-exposure, the induction of AP-1 activation in lung tissue by freshly fractured silica was 24 times higher than that in the control group.

Activation of ERKs and p38 Kinase by Freshly Fractured Silica in JB6 Cells—Since mitogen-activated protein kinases, including p38 kinase, ERKs, and JNKs, are the upstream ki-

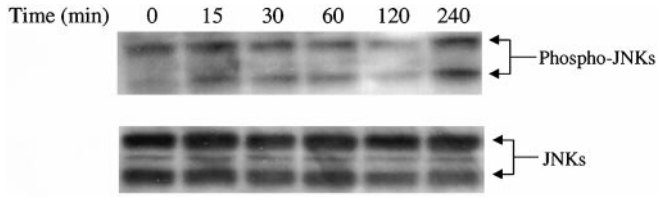

FIG. 6. **Effect of freshly fractured silica on JNK activity.** JB6 P^+ cells were cultured in MEM containing 5% FBS in six-well (35-mm diameter) plates until 80% confluent. The cells were cultured in MEM containing 0.5% FBS for 24 h and then exposed to 150 μg/ml silica (47 $\mu g/cm^2$) suspended in the same medium for different times as indicated. The cells were lysed, and phosphorylated and non-phosphorylated JNK proteins were assayed using the PhosphoPlus MAPK kit.

nases responsible for c-Jun phosphorylation and AP-1 activation (37–40), we tested which class of MAPK is involved in the AP-1 activation by silica. We examined the influences of silica on the phosphorylation of ERK1, ERK2, JNKs, and p38 kinases. Using antibodies specific for the above MAPK family and phospho-specific for the phosphorylated MAPKs, we studied ERK1, ERK2, JNKs, and p38 kinase proteins and the protein phosphorylation of ERK1, ERK2, JNKs, and p38 kinase in JB6 P^+ cells. Exposure to freshly fractured silica significantly stimulated the phosphorylation of p38 kinase and ERKs. The time course of p38 kinase phosphorylation induced by silica (150 μg/ml) is shown in Fig. 4*A*. Phosphorylation of p38 kinase was first apparent at 15 min after exposure to silica. and its maximal activation was obtained at 2 h. To examine the dose dependence of the p38 kinase response in cells exposed to silica, JB6 cells were treated for 2 h with various concentrations of silica. A dose-related increase in p38 kinase phosphorylation was observed in cells treated with increasing concentrations of silica, *i.e.* with prominent increases at 100–200 μg/ml (Fig. 4*B*). Freshly fractured silica (150 μg/ml) also caused phosphorylation of ERK1 and ERK2 in a time-dependent manner (Fig. 5). In contrast, silica did not affect the phosphorylation levels of JNKs (Fig. 6). Similar results were obtained using the RLE cell line (data not shown). These results suggest that ERKs and p38 kinase, but not JNKs, may be involved in silica-induced AP-1 activation in JB6 cells as well as in RLE cells.

Inhibition of ERKs or p38 Kinases by Specific Inhibitors Also Blocks Freshly Fractured Silica-induced AP-1 Activation—To further confirm that activation of AP-1 by silica is mediated through p38- and ERK-dependent signal transduction pathways, we examined the effects of PD98059 and SB203580 on silica-induced AP-1 activation. PD98059 has been shown to act as a highly selective inhibitor of MEK1 activation, whereas SB203580 has been shown to be a specific inhibitor of p38 kinase. MEK1 is an upstream activator of ERKs. Silica-induced AP-1 activation was significantly inhibited by 20 μM PD98059 or 2 μM SB203580 (Fig. 7).

PD98059 and SB203580 Inhibit Freshly Fractured Silica-induced AP-1 DNA Binding Activity—To study the molecular basis of the induction of AP-1 activity by silica and to further confirm the above findings, the AP-1 DNA binding activity was analyzed by gel shift assay. As shown in Fig. 8, silica induced AP-1 DNA binding activity, and PD98059 or SB203580 inhibited silica-induced AP-1 DNA binding activity. These data provide further support that ERKs and p38 kinase are involved in silica-induced AP-1 activation.

DISCUSSION

Occupational exposure to silica is associated with the development of silicosis and lung cancer (1, 4, 5). The molecular mechanisms involved in silica-induced carcinogenesis are unclear. We hypothesize that activation of nuclear transcription factors induced by silica is a primary event in the initiation of

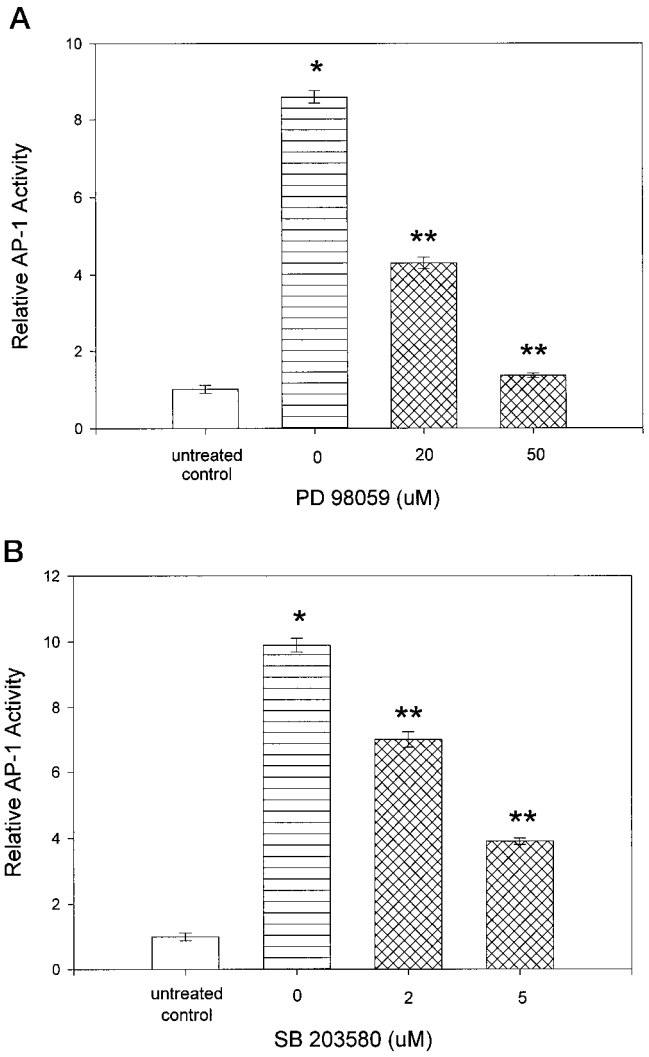

FIG. 7. **Inhibition of silica-induced AP-1 activation by PD98059 and SB203580.** JB6 cells (5×10^4) were seeded into each well of a 24-well plate. After overnight culture at 37 °C, the cells were cultured in MEM plus 0.5% fetal bovine serum for 12 h. The cells were then pretreated with various concentrations of the ERK inhibitor PD98059 (*A*) or the p38 inhibitor SB203580 (*B*) for 2 h and exposed to 150 μg/ml silica in the presence of the inhibitors for 24 h. AP-1 activity was measured by the luciferase activity assay as described under "Materials and Methods." The results, presented as relative AP-1 induction compared with the control cells, are means ± S.E. of 12 assay wells from two independent experiments. *, significant increase from untreated controls; **, significant decrease from silica alone ($p \leq 0.05$).

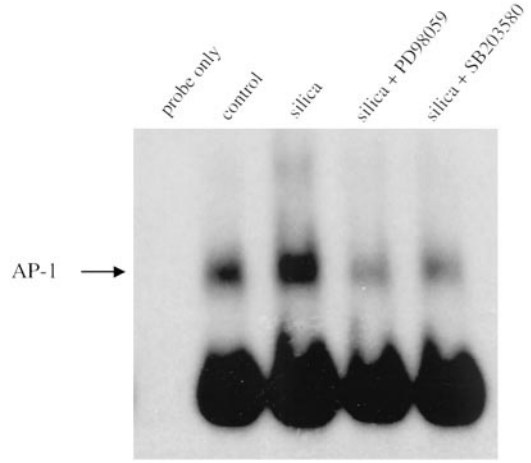

FIG. 8. **Electrophoretic mobility shift assay.** JB6 cells were seeded into each well of a six-well plate until 80% confluent. The cells were then cultured in MEM plus 0.5% fetal bovine serum for 24 h. The cells were pretreated with 20 μM PD98059 or 5 μM SB203580 for 2 h and then exposed to 150 μg/ml silica in the presence of the inhibitors for another 2 h. The AP-1 DNA binding activity was determined by gel shift assay as described under "Materials and Methods."

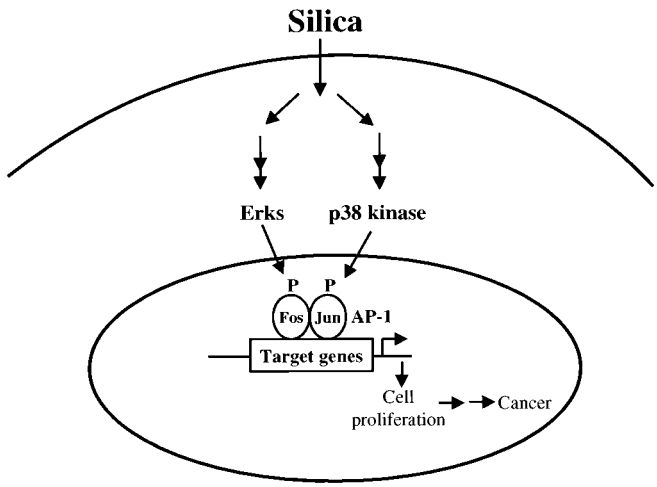

FIG. 9. **Mechanistic schema of events in silica-induced carcinogenesis.**

signal transduction cascades at the cell membrane level leading to the induction of early response genes that are critical in carcinogenesis. In this study, we examined the effect of silica on the activation of AP-1 and the signal transduction pathways involved in AP-1 activation in cell culture models and in transgenic mice. The results show that silica stimulates AP-1 DNA binding activity as well as AP-1 transactivation activity. Silica induced an 8-fold increase in AP-1 activity in JB6 cells and a 2.5-fold increase in RLE cells. Silica also stimulated AP-1 transactivation in pulmonary tissues of transgenic mice. At 3 days after intratracheal instillation of silica, AP-1 activity was elevated 23-fold as compared with the controls. Most important, we found that phosphorylation of ERK1, ERK2, and p38 kinases, which are involved in silica-induced AP-1 activation, was induced by freshly fractured silica. These data demonstrate for the first time that freshly fractured silica induces AP-1 activation through MAPK signal transduction pathways.

Previous studies using different model systems have suggested an important role of AP-1 activation in preneoplastic-to-neoplastic transformation in cell culture and animal models (22, 41–43). AP-1 is a critical mediator of tumor promotion and is involved in a diversity of processes. This transcription factor is able to alter gene expression in response to a number of stimuli, including the tumor promoter TPA, epidermal growth factor, tumor necrosis factor-α, interleukin-1, and UV irradiation (22). Some of the genes regulated by AP-1 are involved in immune and inflammatory responses, tumor promotion, and tumor progression. These include cytokines such as interleukin-1, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, collagenase IV, and stromelysin (44–46). Overexpression of c-*jun* in JB6 P^+ cells causes neoplastic transformation. Inhibition of AP-1 activity by either pharmaceutical agents such as fluocinolone acetonide and retinoic acid or molecular biological inhibitors such as dominant-negative c-*jun* and dominant-negative phosphatidylinositol 3-kinase was found to block tumor promoter-induced neoplastic transformation (25, 31, 34, 41, 42, 47).

AP-1 is a complex protein composed of homodimers and heterodimers of oncogene proteins of the Jun and Fos families. The genes encoding these proteins, c-*jun* and c-*fos*, are induc-

ible in response to a variety of extracellular stimuli and function as intermediary transcriptional regulators in signal transduction processes leading to proliferation and transformation. The activation of AP-1 may trigger downstream signal cascades such as *jun*, *fos*, and other target genes. The members of the Jun and Fos protein families may couple cell signaling events at the cell surface to changes in gene expression that modulate cell responses, including proliferation and changes in phenotype. The important element of this study is that freshly fractured silica stimulates AP-1 activation, which may be one of the critical mechanisms in silica-induced carcinogenesis. The level of AP-1 induction by silica was lower in RLE cells than in JB6 cells. This may due to the following differences between these two cell lines: (*a*) the rate of luciferase gene expression, (*b*) the half-life of luciferase, (c) the basal MAPK or AP-1 activities, or (d) the anti-stress enzyme activity.

The signal transduction pathways leading to transcription factor activation have been extensively studied in the last several years. It is believed that stress-related signals such as UV light or reactive oxygen species induce the activation of MAPK pathways (ERKs, JNKs, and p38). AP-1 is a downstream target of these three MAPK members (48). In this study, the possible role of the MAPK family, including p38 kinase, ERKs, and JNKs, in silica-induced AP-1 activation has been investigated. We found that freshly fractured silica phosphorylated ERKs and p38 kinase, but not JNKs. Pretreatment of cells with the p38 and ERK inhibitors PD98059 and SB203580 inhibited AP-1 transactivation as well as AP-1 DNA binding activity induced by silica. Thus, these results suggest that silica-induced AP-1 activation might be through p38 MAPK and ERK pathways.

The development of AP-1-luciferase transgenic mice makes it possible to study the role of AP-1 activation in tumor promotion *in vivo* (49). The results obtained in this study show that freshly fractured silica is able to cause AP-1 activation in transgenic mice. Maximal AP-1 activation was increased by 23-fold in pulmonary tissues at 3 days after intratracheal instillation of silica. However, the cell types involved in this AP-1 activation response have not yet been identified. Additional studies are required to answer this question.

Our studies present a model for the elucidation of events involved in cell proliferation and carcinogenesis by crystalline silica (Fig. 9). By activating the AP-1 transcription factor through MAPK signal transduction pathways, silica may induce chronic cell proliferation, which subsequently contributes to silicosis and carcinogenesis in the lung. It is possible that activation of AP-1 is a crucial event that initiates cell proliferation and progression through the cell cycle. Biopersistent silica particles may provide prolonged redox signals and growth stimulus during the long latency periods of tumorigenesis and thereby contribute to the eventual fixation of genetic changes caused by silica itself or other agents. Furthermore, the induction of AP-1 activity may affect changes in cell phenotype that contribute to neoplastic transformation.

In summary, using the AP-1-luciferase reporter transgenic mouse and cell culture models, we demonstrated that freshly fractured silica induces AP-1 activation through p38 MAPK and ERK pathways. These studies provide new and important clues regarding molecular mechanisms that may be involved in silica-induced carcinogenesis. Therefore, elucidating the mechanisms involved in silica-induced carcinogenesis in parallel with the manipulation of target signaling could provide insights for the understanding and possible prevention of silica-induced carcinogenesis.

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Freshly Fractured Crystalline Silica Induces Activator Protein-1 Activation through ERKs and p38 MAPK

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