COM Crystals Activate the p38 Mitogen-activated Protein Kinase Signal Transduction Pathway in Renal Epithelial Cells*

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Interaction of calcium oxalate monohydrate (COM) crystals with renal cells has been shown to result in altered gene expression, DNA synthesis, and cell death. In the current study the role of a stress-specific p38 MAP kinase-signaling pathway in mediating these effects of COM crystals was investigated. Exposure of cells to COM crystals (20 μg/cm²) rapidly stimulated strong phosphorylation and activation of p38 mitogen-activated protein kinase (p38 MAP kinase) and re-initiation of DNA synthesis. Inhibition of COM crystal binding to the cells by heparin blocked the effects of COM crystals on p38 MAPK activation. We also show that specific inhibition of p38 MAPK by 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole (SB203580) or by overexpression of a dominant negative mutant of p38 MAP kinase abolishes COM crystal-induced re-initiation of DNA synthesis. The inhibition is dose-dependent and correlates with in situ activity of native p38 MAP kinase, determined as mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2) activity in cell extracts. In summary, inhibiting activation of p38 MAPK pathway abrogated the DNA synthesis in response to COM crystals. These data are the first demonstrations of activation of the p38 MAPK signaling pathway by COM crystals and suggest that, in response to COM crystals, this pathway transduces critical signals governing the re-initiation of DNA synthesis in renal epithelial cells.

Renal tubular fluid is commonly supersaturated with calcium and oxalate ions, which nucleate to form crystals of calcium oxalate monohydrate (COM), the most common constituent of kidney stones. The majority of people do not form renal stones despite crystalluria (1). Up to 1.1 × 10⁷ crystals are excreted daily by normal individuals without any evidence of stone disease (2). It is therefore believed that uncomplicated crystalluria does not indicate kidney stone disease. The mechanisms by which urinary crystals are retained in the kidney and grow into kidney stones are not known. Finlayson (3) calculated that, given the most favorable conditions, it would take 10 h for 1-μm COM crystals to grow large enough to block the duct of Bellini and become the nidus of a urinary stone. Because urinary transit time from glomerulus to the renal pelvis is ~3 min, crystalline particles formed in the urine flowing freely through the renal tubule do not stay in the lumen long enough to attain the dimensions required to block a collecting duct and form a urinary microlith. Therefore, the attachment of crystals to the renal epithelial cells and the cellular responses to crystal interaction are critical in understanding the pathogenesis of renal calcification.

Previous studies (4–6) in several different cell lines of renal tubular origin suggest that COM crystals bind to specific receptors on the cell surface. Furthermore, the receptors that interact with COM crystals in tubular cells may be only minimally exposed under normal circumstances and increase in number under a variety of conditions that lead to cellular stress and injury (4, 7, 8). Nonethess, COM crystal interaction with renal epithelial cells results in a program of events, including alterations in gene expression, initiation of DNA synthesis, cell growth, and death (9–13). However, the specific signaling pathways activated in renal cells following crystal exposure are not delineated.

Signal transduction via mitogen-activated protein (MAP) kinases plays a key role in a variety of cellular responses, including proliferation, differentiation, and cell death (14–18). MAP kinases have provided a focal point for remarkably rapid advances in our understanding of the control of cellular events by growth factors and stresses (19). So far over a dozen MAPK families have been identified. Each cascade consists of no fewer than three enzymes that are activated in a series. These regulatory cascades not only convey information to the target effec tors but also coordinate incoming information from parallel signaling pathways, which allow for signal amplification, generation of a threshold, and a sigmoidal activation profile and is subject to multiple activation mechanisms (20). These pathways include the extracellular signal-regulated kinase (ERK, also known as p42/44 MAP kinase), the c-Jun N-terminal kinase (JNK, also known as SAPK1), and p38 mitogen-activated protein kinase (p38 MAP kinase, also known as SAPK2/reactivating kinase). In general, ERK1 and ERK2 are key transducers of proliferation signals and are often activated by mitogens. In contrast, SAPKs/JNK and p38 are poorly activated by mitogens but strongly activated by cellular stress inducers (21).

The family of p38 MAP kinases is activated in response to diverse extracellular stimuli including osmotic stress, UV irra-

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The abbreviations used are: COM, calcium oxalate monohydrate; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases; MAPKAP, mitogen-activated protein kinase-activated protein; MBP, myelin basic protein; ATF, activating transcription factor; MEF2C, myocyte enhancer factor 2C; CHOP, C/EBP homologous protein; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole; PBS, phosphate-buffered saline; DMEM, Dulbecco-Vogt modified Eagle’s medium; PMSF, phenylmethylsulfonyl fluoride; MAP, mitogen-activated protein.
diation, heat shock, ionizing radiation, high osmotic stress, shear stress, proinflammatory cytokines, thripom, epithelial growth factor, and hemopoietic growth factors with the exception of interleukin-4 (21). The majority, but not all, of these stimuli are associated with cellular stress. The p38 MAP kinase pathway has been shown to mediate signals for the generation of important biological responses, such as phosphorylation of transcription factors involved in transcriptional regulation, platelet aggregation, induction of cytokine production, and apoptosis in neuronal cells and fibroblasts (22–26).

Because of the important role that the p38 family of kinases plays in the generation of biological responses under a variety of stress conditions, we set out to determine the effects of COM crystals on p38 MAP kinase-signaling pathway.

In this study, we investigated the effect of COM crystals on LLC-PK1 cells, a line of renal epithelial cells. We used SB203580, a specific inhibitor of p38 MAP kinase. The kinase-dead dominant negative expression vector pCMV-p38 (AGF), which functions as a dominant inhibitor of p38 MAPK activation (27), was also used to determine the role of p38 MAP kinase pathway in mediating the cellular response to COM crystals. We provide the first evidence that p38 MAP kinase is rapidly and robustly phosphorylated and activated in response to COM crystal interaction with renal epithelial cells. In addition, we demonstrate that p38 MAP kinase activity is essential for the effects of COM crystals on re-initiation of DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco-Vogt modified Eagle’s medium (DMEM), fetal bovine serum, penicillin/streptomycin, and myelin basic protein (MBP) were purchased from Invitrogen. Antibodies against phosphokinase and Roger J. Davis (Howard Hughes Medical Institute, University of Connecticut Biotechnology, Inc. (Lake Placid, NY). Goat anti-rabbit IgGs and antibodies against phosphokinase pathway in mediating the cellular response to COM crystals. Anise-myc was purchased from Calbiochem-Novabiochem. [γ-32P]ATP (4500 Ci/mmol) and [myethyl-3H]thymidine (3 Ci/mmol) were obtained from ICN Radiochemicals, Inc (Costa Mesa, CA). ImmobilON™-P membrane was obtained from Millipore (Bedford, MA). All cell culture reagents were purchased from Invitrogen. The expression vector pCMV-p38 (AGF) (dominant negative mutant of p38 MAPK) was a generous gift from Roger J. Davis (Howard Hughes Medical Institute, University of Connecticut Biotechnology, Inc. (Lake Placid, NY).

**Preparation of Calcium Oxalate Crystals**—Calcium oxalate monohydrate (COM) crystals were prepared as described previously (28) with slight modifications. Briefly, 5 ml each of 10 mM CaCl2 and 10 mM sodium oxalate were mixed simultaneously. Upon mixing the solutions at room temperature, COM crystals formed immediately.

**Culture and Transfection of LLC-PK1 Cells**—LLC-PK1 cells (American Type Culture Collection, Manassas, VA), grown on polystyrene (Corning Glass) T-75 flasks and were used between passages 216 and 224. The cells were serially passaged in low glucose DMEM, supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For transfections, LLC-PK1 cells were grown in 6-well plates to 60% confluency and were transiently transfected with appropriate control vector (pCMV Tag 5) or kinase-dead dominant negative expression vector pCMV-p38 (AGF) using LipofectAMINE transfection reagent (Stratagene), following the manufacturer's instructions. The transfection efficiency was 50–65% as determined by β-galactosidase expression in parallel experiments. For specific treatments, transfected cells were allowed to grow to confluence and serum-starved overnight prior to use in experiments. All culture were maintained at 37 °C under a humidified atmosphere of 85% air, 5% CO2.

**COM Crystal Binding**—[14C]COM crystal binding was carried out as described previously (4). Briefly, LLC-PK1 cells were plated at a high density in 6-well plates and grown to confluence. These cells were serum-starved for 12–18 h prior to COM crystal binding. The cells were exposed to COM crystals (20 μg/cm2) in the presence or absence of hormone-dependent inhibition of COM crystal binding to the cells for 4 h at 37 °C. At the end of the experimental period, media were aspirated and the cells washed twice with ice-cold PBS (2 ml/well/wash). The cells were lysed by the addition of 500 μl of lysis solution (0.1 N NaOH, 1% SDS), and the amount of 14C label associated with the cells was used as an index of the amount of COM crystal binding.

**Activation of p38 MAP Kinase by COM Crystals**—For these assays, the cells were solubilized with ice-cold lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were sonicated for 1 s with a micro-ultrasonic cell disrupter and centrifuged at 14,000 g × 4 °C for 15 min to remove the insoluble material. Samples containing equal amounts of protein (50 μg) were separated on 10% SDS-PAGE and then transferred to an Immobilon-P membrane using standard electroblotting procedures.

**Western blot analysis** was carried out as described previously (30, 31). Briefly, blots were immunodebated overnight at 4 °C with monoclonal antibodies that specifically recognize dual phosphorylation motif at Thr180 and Tyr182 of p38 MAP kinase (1:1500) or with an antibody that equitably recognizes phosphorylated and dephosphorylated p38 MAP kinase (1:3000). Immunoblots were washed with several changes of TBST (100 mM Tris, pH 7.2, 1% Tween 20) before membrane was developed using horseradish peroxidase (Eastman Kodak Co.). Immunoreactivity was detected with enhanced chemiluminescence detection system (Kodak).

**Immunoprecipitation and Immunocomplex Kinase Assays**—For these assays, the cells were solubilized with ice-cold lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 25 mM HEPES, pH 7.2, 1.1 μg/ml aprotinin, 2 μM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and centrifuged at 14,000 × g for 15 min at 4 °C. Immunoprecipitation of p38 MAP kinase was achieved by adding 0.5 μg of anti-p38 MAP kinase antibody, respectively, to cell lysate containing 500 μg of total cellular protein and rocking at 4 °C for 2–4 h. 50 μl of a 10% (w/v) suspension of recombinant protein A-agarose beads was then added, and the reaction slurry was allowed to rock at 4 °C for 5–8 h. The immunoprecipitation complexes were washed twice with 0.5 ml of ice-cold lysis buffer and five times with kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl2, 0.1 mM sodium orthovanadate, and 1 mM dithiothreitol). The kinase assay reactions consisted of kinase buffer supplemented with 20 μM ATP containing 20 μCi of [γ-32P]ATP and 10 μg of MBP in a final volume of 50 μl. The reactions were carried out at 30 °C for 20 min with shaking. Reactions were stopped by 2 min of centrifugation at 14,000 × g, and the supernatant was suspended in 2× Laemmli SDS sample buffer containing β-mercaptoethanol and bromphenol blue. Samples were boiled for 2 min and run on 15% SDS-polyacrylamide gel. Kinase activity was measured as the amount of 32P incorporation into MPB assayed by scintillation counting.

**MAPKAP Kinase-2 Enzyme Assays**—These enzyme assays were carried out as described previously (31). Briefly, cells were harvested by scraping in 0.5 ml of ice-cold non-denaturing lysis buffer A containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 0.1% β-mercaptoethanol, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 10 mM NaF, 0.1 mM pyrophosphate.
Aktivation of p38 MAP Kinase by COM Crystals

RESULTS

COM Crystals Stimulate p38 MAP Kinase Activity—As can be seen from Fig. 1A, exposure to COM crystals progressively induced phospho-p38 MAP kinase immunoreactivity. These blots were then stripped and re-probed with an antibody that equally recognizes phosphorylated as well as unphosphorylated p38 MAP kinase, i.e. total p38 MAP kinase. As shown in Fig. 1B, COM crystal exposure did not alter the total amount of p38 MAP kinase protein. The maximum activation of p38 was reached at 15 min of COM crystal exposure (4–6-fold over control) with an average 3.7-fold increase in p38 MAP kinase phospho-immunoreactivity (Fig. 1C). The COM crystal exposure-induced increase in phospho-p38 MAP kinase immunoreactivity was comparable with anisomycin, a known activator of p38 MAP kinase. These results suggest that COM crystals activate p38 MAP kinase.

To characterize further the effects of COM crystals on p38 MAP kinase enzyme activity, p38 MAP kinase was immunoprecipitated with an antibody that recognizes total p38 MAP kinase (phosphorylated as well as unphosphorylated), and immunocomplex kinase assay was performed as described under “Experimental Procedures.” COM crystals rapidly stimulated p38 MAP kinase activity within 5 min of exposure as shown in Fig. 1D. Densitometric analysis in Fig. 1E showed that the effect of COM crystals on p38 MAP kinase was maximal at 15 min (~9-fold activation over control), whereas anisomycin exposure resulted in about a 5-fold increase in p38 MAP activity as compared with untreated control. These data demonstrate that COM crystal exposure resulted in a rapid and robust increase in p38 MAP kinase activity.

COM Crystals Activate MAPKAP Kinase-2—Some concerns have been raised that in vitro activity of p38 MAP kinase may not reflect its in situ activity. Thus in situ activity of p38 MAP kinase was determined by MAPKAP kinase-2 enzyme activity. It is important to point out here that p38 MAP kinase is the only known activator of MAPKAP kinase-2, and the activity of MAPKAP kinase-2 is dependent on its phosphorylation by p38 MAP kinase (32). Therefore, in vitro activity of immunoprecipitated MAPKAP kinase-2 reflects the in situ activity of p38 MAP kinase. Results presented in Fig. 2 demonstrate that COM crystal-induced MAPKAP kinase-2 activity followed the activation pattern similar to that of p38 MAP kinase, with maximal activation 15 min following COM crystal exposure. These data demonstrate that the rapid and robust activation of p38 MAP kinase by COM crystals correlates with the in situ activity of MAPKAP kinase-2.

COM Crystal Binding to the Cells Is Required for p38 MAP Kinase Activation—Heparin has been shown to be the most effective natural polysaccharide inhibitor of COM crystal binding to renal epithelial cells (13). We evaluated the effect of heparin on COM crystal binding to LLC-PK1 cells and on COM

![Fig. 1. COM crystals activate p38 MAP kinase in LLC-PK1 cells. Confluent, growth-arrested, and serum-starved LLC-PK1 cells were exposed to COM crystals (20 μg/cm²) for various times between 0 and 30 min or exposed to anisomycin (10 μg/ml) for 30 min, as indicated. The cells were solubilized in ice-cold lysis buffer, separated on 10% SDS-PAGE, and blotted on Immobilon-P membrane. Blots were probed with antibodies specific for either total (phospho- and dephosphorylated) or phosphorylated p38 MAP kinase. A, representative Western blot illustrating the effects of COM crystals on phospho-p38 MAP kinase. B, blot shown in A was stripped and reprobed for total p38 (phospho- and dephosphorylated) MAP kinase. The activity of p38 MAP kinase was measured in immunocomplex protein kinase assay using [γ-32P]ATP and MBP as substrate. The cell lysates (500 μg of protein) were immunoprecipitated with 1 μg of anti-p38 antibody and recombinant protein A-agarose complex, and the immune complexes were resolved on 15% SDS-PAGE and visualized by autoradiography. C, densitometric analysis of phosphorylated MBP (p38) shown in D. Data are expressed as average percentage of change from control ± S.D., and each point represents three separate independent experiments. *, p < 0.01; **, p < 0.001 compared with untreated control.](http://www.jbc.org/)

http://www.jbc.org/
Crystal-stimulated p38 MAP kinase activation.

As shown in Fig. 3A, exposure of LLC-PK1 cells to heparin (50 and 100 μg/ml) inhibited COM crystal binding to the LLC-PK1 cells by 65.9 ± 3.5 and 75.8 ± 1.2, respectively. Data presented in Fig. 3C show that inhibition of COM crystal binding by heparin (50 and 100 μg/ml) had no effect on total p38 MAP kinase protein levels, but inhibited COM crystal-stimulated p38 MAP kinase phosphorylation (Fig. 3D) and p38 MAP kinase activation (Fig. 3D). These data suggest that COM crystal-stimulated p38 MAP kinase activation requires COM crystal binding to the renal epithelial cells.

**COM Crystals Induce Re-initiation of the DNA Synthesis—**

Exposure of renal epithelial cells to COM crystals has been shown to result in the initiation of DNA synthesis (12). We evaluated the effect of varying COM crystal concentrations on re-initiation of the DNA synthesis in LLC-PK1 cells. As shown in Fig. 4A, exposure of LLC-PK1 cells to COM crystals resulted in re-initiation of DNA synthesis. COM crystals induced DNA synthesis in a dose-dependent fashion in the range tested (from 2–100 μg/cm² to 10–500 μg/ml). We observed significant stimulation (~2-fold) at 20 μg/cm² to 100 μg/ml COM crystal exposure (p < 0.01). Hence, 20 μg/cm² COM crystal was used in all additional studies. Fig. 4B shows that COM crystal-induced DNA synthesis was completely inhibited by aphidicolin (5 μg/ml), a specific inhibitor of DNA polymerase α. These data demonstrate the requirement of DNA polymerase α for COM crystal-induced re-initiation of the DNA synthesis.

**SB203580 Inhibits COM Crystal-induced DNA Synthesis—**

For these studies, confluent, growth-arrested, and serum-starved LLC-PK1 cells were exposed to COM crystals (20 μg/cm²) for various time points (0 min to 24 h) in the absence or presence of increasing concentrations of SB203580. The DNA synthesis was measured as described under “Experimental Procedures.” Exposure to COM crystals resulted in a 2-fold increase of DNA synthesis above that of untreated (control) cells. Pre-treatment of cells with SB203580 attenuated the COM crystal-induced DNA synthesis in a dose-dependent manner with complete inhibition at 20 μM (Fig. 5A). (Please note that at concentrations above 50 μM, SB203580 was toxic to these cells and inhibition of the DNA synthesis above that of control may reflect that fact.) These data suggest involvement of p38 MAP kinase pathway in COM crystals-induced re-initiation of the DNA synthesis.

It has been shown that the inhibitory effects of imidazole compounds on p38 MAP kinase are reversible (33). This raises the possibility that in vitro activity of p38 MAP kinase may not reflect its native in situ activity following addition of SB203580 to the cells. Thus, additional studies evaluated the specificity of SB203580 on the in situ activity of p38 MAP kinase in our system. As shown in Fig. 5B, SB203580 inhibited COM crystal-activated MAPKAP kinase-2 activity in a dose-dependent manner. The inhibitory effect of SB203580 on COM crystal-activated MAPKAP kinase-2 shows complete inhibition at 20 μM. Thus the inhibitory effects of SB203580 on COM crystal-induced DNA synthesis correlates with inhibitory effects of SB203580 on COM crystal-activated MAPKAP kinase-2 in dose-dependent fashion. These studies indicate that p38 MAP kinase is essential for COM crystal-induced re-initiation of the DNA synthesis.

**Dominant Negative Expression of Kinase-dead Mutant of p38 MAP Kinase Attenuates COM Crystal-induced DNA Synthesis—**

As shown in Fig. 5C, exposure of LLC-PK1 cells to a specific inhibitor of DNA polymerase α (200 μg/ml) inhibited COM crystal binding to the LLC-PK1 cells (20 μg/cm²) for various time points (0 min to 24 h) in the absence (control) or the presence of heparin (50 and 100 μg/ml) for 4 min, and COM crystal binding was evaluated. In parallel experiments, the cells were exposed to COM crystals (20 μg/cm²) in the absence (control) or the presence of heparin (50 and 100 μg/ml) for 20 min. The cells were solubilized in ice-cold lysis buffer, separated on 10% SDS-PAGE, and blotted onto Immobilon-P membrane. Blots were probed with antibodies specific for either total (phospho- and dephosphorylated) or phosphorylated p38 MAP kinase. A, effect of heparin on COM crystal binding to LLC-PK1 cells; data shown are representative of those obtained in three separate experiments. B, representative Western blot illustrating the effects of heparin on COM crystal-induced phosphorylation of p38 MAP kinase. C, blot shown in B was stripped and re-probed for total p38 (phospho- and dephosphorylated) MAP kinase. D, representative blot showing the effect of heparin on the activity of p38 MAP kinase, measured in immunocomplex protein kinase assay using [γ-32P]ATP and MBP as substrate.

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For these studies, confluent, growth-arrested LLC-PK1 cells were exposed to COM crystals (20 μg/cm²) for various time points (0 min to 24 h) in the absence or presence of increasing concentrations of SB203580. The DNA synthesis was measured as described under “Experimental Procedures.” Exposure to COM crystals resulted in a 2-fold increase of DNA synthesis above that of untreated (control) cells. Pre-treatment of cells with SB203580 attenuated the COM crystal-induced DNA synthesis in a dose-dependent manner with complete inhibition at 20 μM (Fig. 5A). (Please note that at concentrations above 50 μM, SB203580 was toxic to these cells and inhibition of the DNA synthesis above that of control may reflect that fact.) These data suggest involvement of p38 MAP kinase pathway in COM crystals-induced re-initiation of the DNA synthesis.

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Activation of p38 MAP Kinase by COM Crystals

 sis—The role of p38 MAP kinase pathway in COM crystal-stimulated DNA synthesis was further confirmed by overexpression of kinase-dead dominant negative mutant of p38 MAP kinase.

In these studies, we first evaluated the effects of transfection of cells with kinase-dead dominant negative expression vector pCMV-p38 (AGF) on COM crystal-stimulated activity of p38 MAP kinase. The expression of the kinase-dead mutant was confirmed by Western blot analysis using anti-M2 monoclonal antibody. As shown in Fig. 6A, only cells transfected with dominant negative expression vector pCMV-p38 (AGF), but not control cells or cells transfected with vector (pCMV Tag 5), showed immunoreactivity to M2 monoclonal antibody. As shown in Fig. 6B and C, transfection of cells with kinase-dead dominant negative expression vector pCMV-p38 (AGF), but not with control vector (pCMV Tag 5), greatly attenuated the effects of COM crystals on p38 MAP kinase activity (COM + pCMV-p38 (AGF) versus pCMV-p38 (AGF), 188.6 ± 19.3 versus 69.1 ± 14.4; COM + control (pCMV Tag 5) vector versus control (pCMV Tag 5) vector, 448.6 ± 16.5 versus 100 ± 6.6).

We also evaluated the effects of transfection of cells with kinase-dead dominant negative expression vector pCMV-p38 (AGF) on the in situ activity of p38 MAP kinase, measured as in vitro MAPKAP kinase 2 activity. We observed that the transfection of cells with kinase-dead dominant negative expression vector pCMV-p38 (AGF), but not with control vector (pCMV Tag 5), greatly attenuated the effects of COM crystals on MAPKAP kinase 2 activity (COM + pCMV-p38 (AGF) versus pCMV-p38 (AGF), 6.74 pmol/min versus 2.5 pmol/min; COM + control (pCMV Tag 5) vector versus control (pCMV Tag 5) vector, 13.78 pmol/min versus 3.47 pmol/min) (Fig. 6C).

Results presented in Fig. 6D show that the exposure of cells transfected with control vector (pCMV Tag 5) to COM crystals resulted in a 2-fold increase in DNA synthesis over untreated cells (195.78 ± 19.58 versus 100 ± 2). These results are similar to the effects of COM crystals on non-transfected cells. The transfection of cells with kinase-dead dominant negative expression vector pCMV-p38 (AGF) greatly attenuated the COM-induced DNA synthesis (COM + pCMV-p38 (AGF) versus pCMV-p38 (AGF), 115 ± 5 versus 73 ± 6.4). The inhibitory effect of kinase-dead dominant negative expression of p38 MAP kinase on COM crystal-induced re-initiation of the DNA syn-

Fig. 4. COM crystals induce re-initiation of the DNA synthesis. A, confluent, growth-arrested, and serum-starved LLC-PK1 cells pre-exposed to DMEM alone or various doses of COM crystals (2, 4, 8, 10, 20, 40, 60, and 100 µg/cm²) for 24 h. During the last 6 h of COM crystal exposure, [3H]thymidine (2–3 µCi) was added per well. The radioactivity retained in the trichloroacetic acid precipitate was measured and used as an index of DNA synthesis. B, confluent, growth-arrested, and serum-starved LLC-PK1 cells pre-exposed to aphidicolin (5 µg/ml) for 1 h prior to addition of COM crystals (20 µg/cm²) for 24 h. During the last 6 h of COM crystal exposure, [3H]thymidine (2–3 µCi) was added per well. Data shown are representative of those obtained in three separate experiments. * (p < 0.01) indicates significant difference from control, and # (p < 0.001) indicates significant difference from COM treatment.

Fig. 5. SB203580 inhibits COM crystal-induced DNA synthesis and abolishes MAPKAP kinase-2 activity. A, confluent, growth-arrested, and serum-starved LLC-PK1 cells were exposed to DMEM alone or various concentrations of SB203580 (1–100 µM) for 1 h prior to the addition of COM crystals (20 µg/cm²) for 24 h. During the last 6 h of COM crystal exposure, [3H]thymidine (2–3 µCi) was added per well. The radioactivity retained in the trichloroacetic acid precipitate was measured and used as an index of DNA synthesis. B, inhibition of p38 kinase in situ by SB203580 as determined by examination of MAPKAP kinase-2 activity. Confluent, growth-arrested, and serum-starved LLC-PK1 cells were exposed to various concentrations of SB203580 (1–30 µM) for 1 h prior to addition of COM crystals (20 µg/cm²). The cells were lysed in ice-cold non-denaturing lysis buffer A, and cell lysates (500 µg of protein) were immunoprecipitated with anti-MAPKAP kinase-2 polyclonal antibody coupled to protein A-agarose beads and tested for the ability to phosphorylate a specific MAPKAP kinase-2 substrate peptide (KKLNRTLSVA) as described in Fig. 2. Data shown are representative of those obtained in three separate experiments. # (p < 0.01) indicates significant difference from control, and * (p < 0.01) indicates significant difference from COM crystal treatment.
expression of dominant negative p38 MAP kinase. The blots were stripped and re-probed with an antibody that recognized β-tubulin to confirm equal protein loading. B, LLC-PK1 cells were transiently transfected either with control vector (pCMV Tag 5) or with dominant negative (DN) p38 expression vector pCMV-p38 (AGF), and cell lysates were analyzed by Western blotting using M2 monoclonal antibody (M-2 Ab) to confirm the expression of dominant negative p38 MAP kinase. The blots were then confirmed the role of p38 MAP kinase signal transduction pathway in the COM crystal-induced DNA synthesis.

**DISCUSSION**

The results demonstrate that COM crystals stimulate phosphorylation and enzymatic activation of p38 MAP kinase (Figs. 1 and 2). Diverse extracellular stimuli have been shown to trigger the p38 MAP kinase pathway (21). The majority but not all of these stimuli are associated with cellular stress. Upon exposure to COM crystals, renal cells have been shown to display a program of events consistent with cellular stress (9–12). These findings suggest involvement of p38 MAP kinase in cellular actions of and/or responses to the COM crystals. Our results demonstrate that COM crystal exposure to renal epithelial cells stimulates enzymatic activation of p38 MAP kinase. The observed correlation of phosphorylation and activation of p38 MAP kinase (Fig. 1, A and D) is consistent with the evidence that phosphorylation of tyrosine at TGY activation motif is required for enzymatic activity (27, 34). The activation of p38 MAP kinase by COM crystals in LLC-PK1 was rapid and robust. Our results also demonstrate that COM crystal exposure resulted in *in situ* activation of p38 MAP kinase. Results presented in Fig. 3 demonstrate that heparin, the most effective natural polysaccharide inhibitor of COM crystal binding to the renal epithelial cells (13), inhibited COM crystal-induced p38 MAP kinase activity. These data suggest that COM crystal binding to the renal epithelial cells is essential for p38 MAP kinase activation. Activation of the p38 pathway results in a plethora of changes in transcription, protein synthesis, cell surface receptor expression, and cytoskeletal structure, ultimately affecting cell survival or leading to programmed cell death (35–38). Thus activation of p38 MAP kinase cascade is suggestive of a functional role of this kinase cascade in mediating cellular actions of COM crystals.

Previous studies (12) have shown that COM crystal exposure to monkey kidney epithelial cells of the nontransformed BSC-1 line and canine kidney epithelial cells (Madin-Darby canine kidney cells) resulted in DNA synthesis. In the present study, we confirmed these effects in LLC-PK1 cells (Fig. 4A). Moreover, the stimulation of DNA synthesis following COM crystal exposure was dose-dependent (Fig. 4A). These results demonstrated that like BSC-1 and Madin-Darby canine kidney cells,
LLC-PK1 cells also responded to COM crystal exposure by re-initiation of the DNA synthesis.

We observed that pretreatment of cells with SB203580 also inhibited COM crystal-stimulated DNA synthesis. The inhibition of COM crystal-stimulated DNA synthesis also showed dose dependence (Fig. 5A). SB203580, a selective and specific inhibitor of p38 MAP kinase, has been used as a versatile agent to investigate the role of p38 MAP kinase pathway in several studies (33, 39–42). It has been demonstrated that SB203580 specifically inhibited p38 MAP kinase and had no effect on ERK1/ERK2 and JNK signaling pathways and several other kinases (41, 43). It has been suggested that, of the known isoforms of p38 MAP kinases, p38 and p38β are the only p38 MAP kinases relevant to the study of kidney cells (42). SB203580 inhibits both p38 and p38β. Thus we evaluated the effects of SB203580 on COM crystal-induced in situ activity of p38 MAP kinase. Results presented in Fig. 4 demonstrate that pre-treatment of cells with SB203580 inhibited COM crystal-stimulated in situ activity of p38 MAP kinase as measured by MAPKAP kinase-2 activity. Moreover, SB203580 inhibited MAPKAP kinase-2 activity in a concentration-dependent manner with complete inhibition at 20 μM, indicating total inhibition of COM crystal-stimulated p38 MAP kinase activation (Fig. 5B). There was a striking similarity between the inhibitions of COM crystal-stimulated p38 MAP kinase activity and COM crystal-stimulated DNA synthesis by SB203580. This suggests the involvement of p38 MAP kinase signal transduction in COM crystal-induced re-initiation of the DNA synthesis in renal epithelial cells.

The role of p38 MAP kinase in COM crystal-stimulated DNA synthesis was confirmed by kinase-dead dominant negative expression of p38 MAP kinase that allowed us to inhibit specifically the activity of p38, thereby enabling us to determine the role of p38 MAP kinase pathway. This was made possible by the availability of kinase-dead dominant negative expression vector pCMV-p38 (AGF) (27), which has been widely used to study the role of p38 MAP kinase (44–46). Results presented in Fig. 6 show that the overexpression of kinase-dead dominant negative mutant of p38 MAP kinase (p38 (AGF)) resulted in attenuation of COM crystal-induced p38 MAP kinase activity as well as COM-induced re-initiation of the DNA synthesis. Taken together these studies clearly establish involvement of the p38 MAP kinase signal transduction pathway in COM crystal-induced DNA synthesis. In Schizosaccharomyces pombe the p38 MAP kinase homologue Spc1 is needed for cell cycle progression under stressful conditions, and overexpression of Ppy-1, a tyrosine phosphatase that inactivates Spc1, results in slowing of growth (47). p38 MAP kinase plays a critical role in DNA synthesis in response to hemopoietic growth factors with the exception of interleukin-4 (48). Similarly in fibroblasts and cultured mesangial cells, hypoxia-associated DNA synthesis but not serum-stimulated DNA synthesis has been shown to be dependent on the activation of p38 MAP kinase pathway (49–51). Moreover, we observed that p38 MAP kinase activation is critical for oxalate induced re-initiation of the DNA synthesis in the renal epithelial cells (31). Taken together these data suggest that p38 MAP kinase activation may play a central role in DNA synthesis associated with cellular stress. Several studies have also implicated p38 MAP kinase in the induction of apoptosis (21). Withdrawal of nerve growth factor from PC-12 cells has been shown to stimulate apoptosis in p38 MAP kinase-dependent manner (52). Similarly p38 MAP kinase-dependent apoptosis has been shown in transforming growth factor-β1-induced apoptosis in murine hepatocytes and in cytokine-induced rat islet cell apoptosis (7, 46). Whether or not p38 MAP kinase plays any role in renal epithelial cell apoptosis following COM crystal exposure has not been evaluated and needs further study.

The mechanisms by which p38 MAP kinase regulates COM crystal-stimulated DNA synthesis are not understood. p38 MAP kinase is known to phosphorylate and activate a number of transcription factors as follows: Elk-1 and ATF2, cAMP-response element-binding protein, and ATF1 through MAPKAP kinase-2 (24, 43, 53). Additionally, a number of substrates of p38 kinase have been identified. These include other kinases such as p38-activated/regulated protein kinase, MAP kinase-interacting kinase 1 and 2 (MK1/2), mitogen, stress-activated kinase 2 (MSK1)/RLPK, ribosomal S6 kinase-B (Rsk-B), transcription factors such as activation transcription factor 2 and 6 (ATF2 and ATF6), myocyte enhancer factor 2C, C/EBP homologous protein, SAP-1P, and cytosolic proteins such as stathmin. These transcription factors control the expression of various genes (54), which have been demonstrated to play a key role in cell growth and cellular homeostasis. These observations suggest that the effects of p38 MAP kinase may be complex. Additional studies are required to investigate these mechanisms.

The mechanisms involved in kidney stone formation remain poorly understood. Renal epithelial cells have been shown to bind COM crystals and display a program of events, including gene expression and re-initiation of the DNA synthesis in response to the crystals (4–6, 9–13, 55). Moreover, interaction of COM crystals with renal epithelium is believed to be a critical step in stone formation (4–6). The present study, by demonstrating the central role of p38 MAP kinase signal transduction in mediating the cellular effects following cell-crystal interactions, may provide an opportunity to identify specific targets in treatment of stone disease.

In summary, these studies demonstrate that COM crystal interaction with renal epithelial cells results in rapid and robust activation of p38 MAP kinase. Furthermore, we demonstrate that p38 MAP kinase activity is essential for the effects of COM crystals on re-initiation of DNA synthesis. Thus, p38 MAP kinase signal transduction pathway, by mediating the effects of COM crystals, could play a key role in nephrolithiasis.

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