

# Effects of Constitutively Active and Dominant Negative MAPK Kinase (MKK) 3 and MKK6 on the pH-responsive Increase in Phosphoenolpyruvate Carboxykinase mRNA\*

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Metabolic acidosis is partially compensated by a pronounced increase in renal catabolism of glutamine. This adaptive response is sustained, in part, through increased expression of phosphoenolpyruvate carboxykinase (PEPCK). Previous inhibitor studies suggested that the pH-responsive increase in PEPCK mRNA in LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells is mediated by a p38 mitogen-activated protein kinase (MAPK). These cells express high levels of the upstream kinase MAPK kinase (MKK) 3 but relatively low levels of the alternative upstream kinase MKK6. To firmly establish the role of the p38 MAPK signaling pathway, clonal lines of LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells that express constitutively active (ca) and dominant negative (dn) forms of MKK3 and MKK6 from a tetracycline-responsive promoter were developed. Western blot analyses confirmed that 0.5  $\mu$ g/ml doxycycline was sufficient to block transcription and that removal of doxycycline led to pronounced and sustained expression of the caMKKs and dnMKKs. Expression of caMKK6 (but not caMKK3) caused an increase in phosphorylation of p38 MAPK and an increase in the level of PEPCK mRNA that closely mimicked the effect of treatment with acidic medium (pH 6.9, 10 mM HCO<sub>3</sub><sup>−</sup>). Only caMKK6 activated transcription of a PEPCK-luciferase reporter construct. Co-expression of both dnMKKs blocked the increases in phosphorylation of p38 MAPK and PEPCK mRNA. The latter effect closely mimicked that of the p38 MAPK inhibitor SB203580. The expression of either dnMKK3 or dnMKK6 was less effective than co-expression of both dnMKKs. Thus, the pH-responsive increase in PEPCK mRNA in the kidney is mediated by the p38 MAPK signaling pathway and involves activation of MKK3 and/or MKK6.

Metabolic acidosis is produced in response to various conditions, including a high protein diet, prolonged starvation, or uncontrolled type I diabetes. It is characterized by an abnormal increase in H<sup>+</sup> ions and a corresponding decrease in HCO<sub>3</sub><sup>−</sup> ions in the blood. This condition is partially compensated by an increased renal catabolism of glutamine that is initiated by the mitochondrial glutaminase and glutamate dehydrogenase (1). The resulting ammonium ions are largely secreted in the urine to facilitate the excretion of titratable acids (2, 3). The further catabolism of the resulting  $\alpha$ -ketoglutarate generates two HCO<sub>3</sub><sup>−</sup> ions

that are added to the blood to partially correct the acidosis (4). In both humans and rats, the remaining carbons from glutamine are largely converted to glucose (5). In rat kidney, this highly coordinated and essential adaptive response is sustained, in part, by a 6-fold increase in the cytosolic phosphoenolpyruvate carboxykinase (PEPCK)<sup>2</sup> (6, 7). This increase is initiated by a rapid and sustained increase in transcription of the *PCK1* gene (6, 8), which occurs solely within the proximal convoluted segment of the nephron (9, 10). Thus, the characterization of the signaling pathway that results in enhanced expression of the *PCK1* gene provides an important paradigm to characterize the mechanism by which these cells sense very slight changes in pH and subsequently activate expression of specific genes (11).

LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells are a gluconeogenic line of porcine renal proximal tubule-like cells (12). They exhibit an increased ammonia synthesis from glutamine and an adaptive increase in PEPCK mRNA and enzyme activity when incubated in acidic (pH 6.9, 10 mM HCO<sub>3</sub><sup>−</sup>) medium (13, 14). Thus, they serve as an excellent model system for *in vitro* studies of the mechanisms that mediate the adaptive responses to metabolic acidosis (11). Mitogen-activated protein kinases (MAPKs) mediate the regulation of gene expression in response to various stresses (15, 16). The involvement of the known MAPK activities (ERK1/2, SAPK/JNK, p38) in the signal transduction pathway that mediates the adaptive response to acidosis was examined by determining the effects of activators and inhibitors of the specific MAPK pathways on the basal and acid-induced levels of PEPCK mRNA in LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells (17). The addition of anisomycin, a potent but nonspecific activator of the p38 MAPK, produced an increase in phosphorylation of p38 MAPK and ATF-2, a downstream transcription factor that binds to the cAMP-response element-1 and activates expression of the *PCK1* gene (18). The activator also produced an increase in PEPCK mRNA levels comparable with that observed following treatment with acidic medium. Further experiments demonstrated that SB203580, an inhibitor of the p38 MAPK, blocked the acid-induced increase in PEPCK mRNA levels, whereas selective inhibitors of the ERK1/2 and JNK pathways had no effect (17). However, more recent studies suggest that SB203580 also acts as a non-competitive antagonist of the CCK1 receptor, a G-protein-coupled receptor that binds cholecystokinin (19). Thus, SB203580 lacks absolute specificity for the p38 MAPK.

MAPKs are phosphorylated and activated by highly selective MAPK kinases (MKKs) (16, 20). MKK3 and MKK6 mediate the activation of p38 MAPK, which then phosphorylates and activates a number of

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<sup>2</sup> The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; ATF-2, activating transcription factor-2; ca, constitutively active; dn, dominant negative; TBS, Tris-buffered saline; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.

downstream kinases and transcription factors. To firmly establish the role of p38 MAPK in the pH-responsive increase in PEPCK mRNA, LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells were stably transfected with plasmids that express constitutively active (ca) or dominant negative (dn) forms of MKK3, MKK6, or both kinases (MKK3/6) (21) from a tetracycline-responsive promoter (22). The constructs encode an N-terminal FLAG tag that was used to select clonal lines of cells that exhibit very low basal expression when grown in the presence of doxycycline and that produce a pronounced and sustained expression of the transgene upon removal of doxycycline. Western and Northern blots were then used to analyze the effects of the caMKKs and the dnMKKs on the pH-dependent phosphorylation of p38 MAPK and the increase in PEPCK mRNA. The resulting data firmly established that the pH-responsive increase in PEPCK mRNA in the kidney is mediated by the p38 MAPK signaling pathway and involves activation of MKK3 and/or MKK6.

## EXPERIMENTAL PROCEDURES

**Materials**—The pGP plasmid containing the entire 3'-untranslated region of the PEPCK cDNA was obtained from Dr. Yaacov Hod (Stony Brook Health Sciences Center). Constitutively active (ca) and dominant negative (dn) constructs of MKK3 and MKK6 were provided by Dr. Roger J. Davis (University of Massachusetts) (21). Anti-phospho-MKK3/6, phospho-p38, phospho-ATF-2, MKK3, p38, and ATF-2 antibodies were purchased from Cell Signaling Technologies. The anti-MKK6 antibody was obtained from Upstate Biotechnology. The anti-FLAG antibody, Dulbecco's modified Eagle's medium/F-12 base medium, doxycycline, and cloning rings were ordered from Sigma. All of the primary antibodies were rabbit polyclonal IgGs. A donkey anti-rabbit IgG horseradish peroxidase-linked antibody from GE Healthcare was used as the secondary antibody. SuperSignal West Dura from Pierce and ECL-Plus from Amersham Biosciences were used for Western blot analysis. The resulting signals were developed using Optimum Brand x-ray films from PerkinElmer Life Sciences. TRIzol<sup>®</sup> reagent was obtained from Invitrogen. Formazol was purchased from Molecular Research Center. [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) was purchased from MP Biomedicals. The oligo-labeling kit was from Ambion. GeneScreen Plus was purchased from PerkinElmer Life Sciences. Geneticin (G418) and hygromycin were obtained from Mediatech. Chemicals for acrylamide gels and Dual Color Precision Plus protein standards were purchased from Bio-Rad. The Dual Luciferase reporter assay Kit was from Promega. Nitrocellulose membranes and Immobilon-FL polyvinylidene fluoride transfer membranes were purchased from Millipore and Fisher Scientific, respectively.

**Stable Transfections of LLC-PK<sub>1</sub>-FBPase<sup>+</sup> Cells**—LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells (12) were grown in a 50:50 mixture of Dulbecco's modified Eagle's and Ham's F-12 medium containing 5 mM glucose, 25 mM NaHCO<sub>3</sub>, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> atmosphere. The ca and dn constructs of MKK3 and MKK6 were cloned into pTRE2, a plasmid in which the promoter contains multiple tetracycline-responsive elements (TRE). The 8C line (23) of LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells that expresses high levels of the tTA protein were co-transfected with 5  $\mu$ g of the various pTRE constructs and 0.5  $\mu$ g of pcDNA3.1/Hygro (Invitrogen) using a calcium phosphate protocol (24). The transfected cells were selected for growth in Dulbecco's modified Eagle's medium/F-12 medium containing 0.2 mg/ml G418, 0.6 mg/ml hygromycin, and 0.5  $\mu$ g/ml doxycycline. The G418 was necessary to maintain the plasmid that expresses the tTA protein, and the doxycycline was added to prevent expression of the caMKK or dnMKK proteins. The cells were allowed to select for approximately 3 weeks. The resulting colonies were separated with

cloning rings, and the clonal lines were expanded in medium containing 0.2 mg/ml G418, 0.2 mg/ml hygromycin, and 50 ng/ml doxycycline.

**Preparation of Cell Lysates**—Prior to harvesting, the medium was removed and the cells were washed twice with phosphate-buffered saline. Cell extracts were prepared with ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 40 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 200  $\mu$ M sodium orthovanadate, and 10% (v/v) protease inhibitor mixture (Sigma). The cells were lysed on ice for 20 min. The lysates were then scraped and transferred to microcentrifuge tubes and centrifuged at 14,000  $\times$  g for 15 min at 4 °C. Aliquots of the supernatants were stored at -80 °C.

**Screening of Cell Lines by Western Blot Analysis**—Western blots were performed using anti-FLAG antibodies to determine the basal and doxycycline-responsive expression of the caMKK and dnMKK proteins in the isolated cell lines. The clonal cells were grown for various times in the presence or absence of 0.5  $\mu$ g/ml doxycycline and then harvested with lysis buffer. The protein concentrations of the lysates were determined (25) using bovine serum albumin as the standard. Thereafter, 15  $\mu$ g of each sample was separated on a 10% SDS-polyacrylamide gel. The samples were transferred to nitrocellulose, and the membrane was blocked for 2 h in 5% milk in Tris-buffered saline containing 0.1% Tween (TBS/Tween). The blot was incubated overnight at 4 °C with a 1:2000 dilution of the anti-FLAG antibody in 5% milk. The membrane was subsequently washed three times for 10 min with TBS/Tween and then incubated for 1 h at room temperature in a 1:10,000 dilution of the anti-rabbit horseradish peroxidase secondary antibody. After washing three times for 10 min with TBS/Tween, the blot was developed with the ECL-Plus detection system. The cell lines that exhibited a low basal expression and large fold induction upon the removal of doxycycline were selected for further Western and Northern blot analyses.

**Western Blot Analyses of Cells Treated with Anisomycin or Acidic Medium**—The cell lines that expressed the dnMKK constructs were grown to confluency in the presence or absence of 0.5  $\mu$ g/ml doxycycline. About 24 h prior to harvest, the cells were incubated in serum-free Dulbecco's modified Eagle's medium to induce quiescence. The cells were then treated for 30 min with 5  $\mu$ M anisomycin, a potent activator of the p38 MAPK pathway, to determine whether expression of the dnMKK construct was sufficient to block the anisomycin-mediated increase in phosphorylation of the p38 MAPK. Alternatively, the confluent cell cultures were treated for various times with acidic medium (pH 6.9) that contained 10 mM bicarbonate to determine when the maximal increase in phosphorylation of p38 MAPK occurred. The Western blots with the phospho-p38 and phospho-ATF-2 antibodies were performed using 60  $\mu$ g of protein for each sample and polyvinylidene fluoride membranes. The two antibodies were diluted 1:500 in 5% bovine serum albumin in TBS/Tween. The blots were developed with the SuperSignal detection solution and exposed to x-ray films. The membranes were stripped by incubating at room temperature for 1–2 h in a solution containing 0.2 M glycine, 0.1% SDS, and 0.1% Tween. The stripped blots were reblocked in 5% milk in TBS/Tween solution for 2–3 h before reprobing with antibodies against total p38 and ATF-2. The p38 and ATF-2 antibodies were diluted 1:1000 in 5% bovine serum albumin in TBS/Tween solution. The total p38 and ATF-2 levels served as a control for protein loading. Western blots of the MKK3 and MKK6 proteins were also performed using antibodies that were diluted 1:1000 in 5% bovine serum albumin in TBS/Tween.

**Northern Blot Analyses**—Cells were grown for 12 days in the presence and absence of 0.5  $\mu$ g/ml doxycycline to ensure confluency. The cells

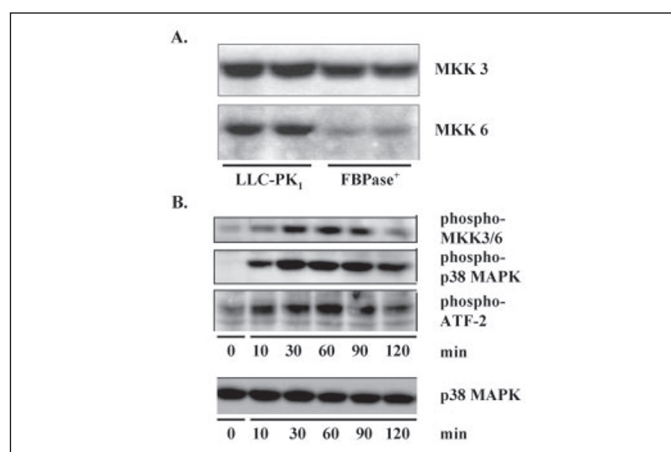
## Role of MKK3 and MKK6 in PEPCK mRNA Expression

were then maintained for 24 h in normal or acidic medium. Total cellular RNA was isolated using the TRIzol® reagent. The RNA concentration was determined by measuring the absorbance at  $A_{260}$ . To probe for PEPCK mRNA, a 1.6-kb BglII fragment of pPCK-10 (26) was used. A 2.0-kb fragment of an 18 S ribosomal RNA cDNA was excised by restricting pAr2 (27) with HindIII and EcoRI. The fragments were separated on a 1% agarose gel, excised, and purified using a GENECLAN kit (Bio101 Systems). The synthesis of oligolabeled cDNA probes and Northern analysis were performed as described previously (28). The blots were exposed to a PhosphorImager screen, and the intensities of the resulting bands were quantified using Molecular Dynamics Software. The level of the PEPCK mRNA was divided by the corresponding level of 18 S rRNA to correct for errors in sample loading. To compare the effectiveness of the dnMKK constructs, cells were grown in the presence of doxycycline and treated for 24 h with 10  $\mu$ M SB203580, an inhibitor of p38 MAPK.

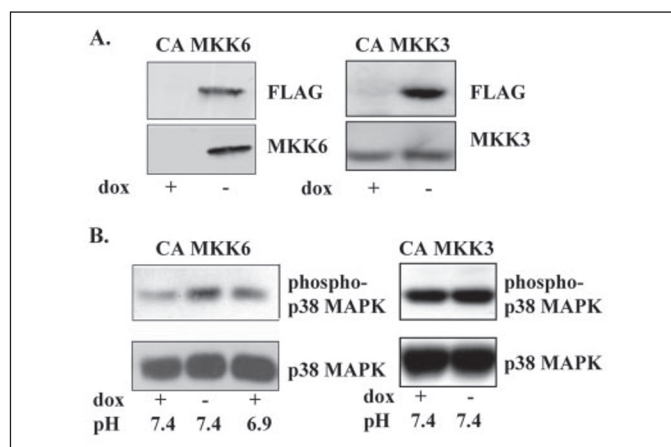
**Luciferase Assays**—The various luciferase constructs were assembled in pGL3-Basic (Promega), which encodes the firefly luciferase gene. The PEPCK<sub>-490</sub>CAT plasmid (29) was restricted with BamHI and BglII to excise the -490 to +73-bp fragment of the rat PEPCK promoter. The purified fragment was then cloned into pGL3-Basic that had been restricted with BglII to produce p490Luc3. A 593-bp fragment that contained the 3'-untranslated region of the PEPCK cDNA (corresponding to nucleotides 2088–2595) was PCR-amplified from pGP using primers that created a 5'-NheI and a 3'-SpeI site. This fragment was cloned into the XbaI site of p490Luc3 to form p490Luc3-PCK3'. LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells were grown for 7 days in 6-well plates to achieve confluence. The cells were transiently co-transfected with 0.6  $\mu$ g of either p490Luc3 or p490Luc3-PCK3' and 0.1  $\mu$ g of pRL-null/well (Promega) by calcium phosphate precipitation (24). The experimental samples were also co-transfected with 0.6  $\mu$ g/well of an expression vector that encodes the caMKK6 construct (21). Approximately 24 h later, the cells were washed twice with 2 ml of phosphate-buffered saline, and then fresh medium was added. The cells were cultured for an additional 24 h and then washed twice with 2 ml of phosphate-buffered saline. Cell extracts were prepared and assayed using the reagents contained in the Dual Luciferase reporter assay. The firefly luciferase activities obtained from the various pLuc3 plasmids were divided by the corresponding *Renilla* luciferase activities to correct for differences in transfection efficiency. The mean of the ratio of luciferase activities measured with p490Luc3 was normalized to a value of 1.

## RESULTS

**Endogenous Levels and Phosphorylation of MKK3 and MKK6**—Western blot analyses indicated that the non-gluconeogenic LLC-PK<sub>1</sub> cells expressed high endogenous levels of both MKK3 and MKK6. By contrast, the derived LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells maintained high levels of MKK3, but expressed relatively low levels of MKK6 (Fig. 1A). To determine in LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells whether MKK3 and/or MKK6 function upstream of p38 MAPK and ATF-2, the cells were treated with serum-free medium for 24 h and then stimulated by the addition of 5  $\mu$ M anisomycin (17). This treatment produced a time-dependent increase in the phosphorylation of MKK3/6 and coordinate increases in phosphorylation of p38 MAPK and ATF-2 that reached a maximum within 30 min after stimulation (Fig. 1B). The changes in phosphorylation of MKK3/6, p38 MAPK, and ATF-2 occurred without changes in the levels of the p38 MAPK protein (Fig. 1B) or the MKK3, MKK6, and ATF-2 proteins (data not shown).



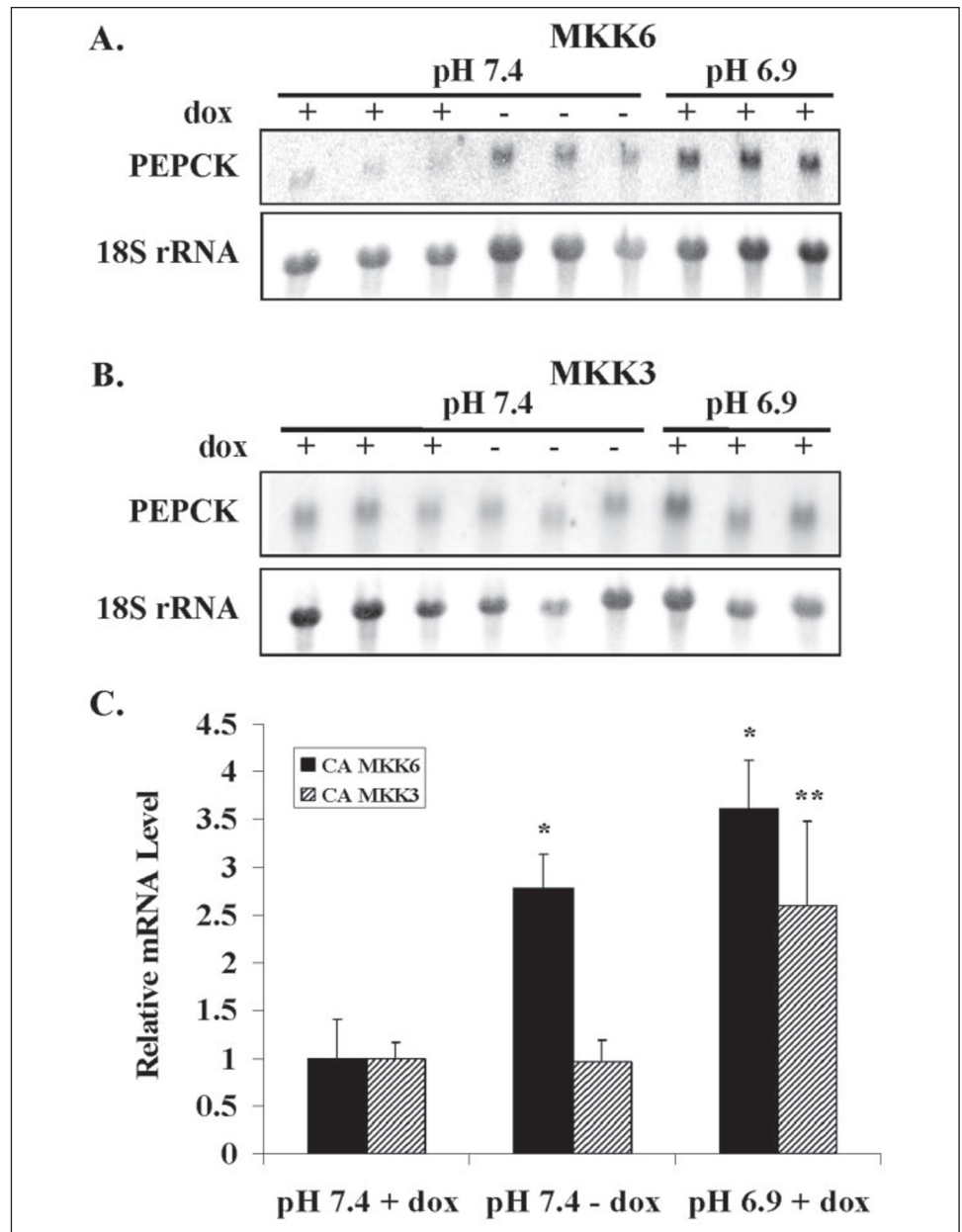
**FIGURE 1. Endogenous expression of MKK3 and MKK6 and anisomycin activation of the p38 MAPK signaling pathway.** A, Western blot analysis of endogenous levels of MKK3 and MKK6 in LLC-PK<sub>1</sub> and LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells. Cell extracts were separated by 10% SDS-PAGE and probed with antibodies that are specific for either MKK3 or MKK6. B, Western blot analysis of the increase in phosphorylation of MKK3/6, p38 MAPK, and ATF-2 in LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells following stimulation with anisomycin. Cell extracts were prepared at various times after the addition of 5  $\mu$ M anisomycin. The samples were separated by 10% SDS-PAGE and probed with antibodies that were specific for the phosphorylated forms of the proteins. The blot for phospho-p38 MAPK was stripped and reprobed with an anti-p38 MAPK antibody.



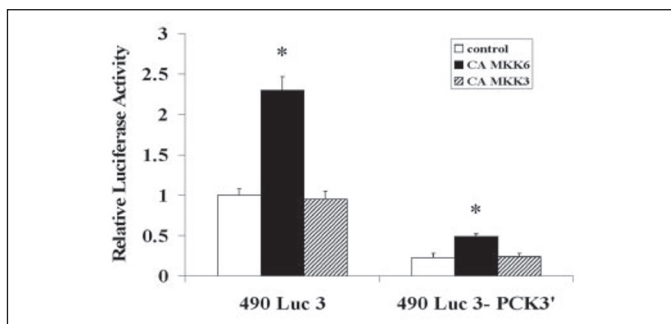
**FIGURE 2. Effect of expression of caMKK6 or caMKK3 on the phosphorylation of p38 MAPK.** A, cells that express either caMKK6 or caMKK3 were grown in the presence (+) or absence (-) of 0.5  $\mu$ g/ml doxycycline (dox). Cell extracts were separated by 10% SDS-PAGE and probed with antibodies that are specific for the FLAG tag and either MKK6 or MKK3. B, cells that express the caMKK6 or caMKK3 were grown to confluency in the presence (+) or absence (-) of doxycycline and where indicated, treated with acidic medium (pH 6.9) for 2 h. Extracts were separated by 10% SDS-PAGE and probed with antibodies that were specific for the phosphorylated p38 MAPK. The blot was stripped and reprobed with an anti-p38 MAPK antibody.

**Expression of caMKK6 or Treatment with Acidic Medium Produces a Similar Increase in Phosphorylation of p38 MAPK**—Cells expressing either the caMKK3 or caMKK6 were cloned and then grown to confluency in the presence or absence of 0.5  $\mu$ g/ml doxycycline. Western blot analysis using the anti-FLAG antibodies established that the addition of doxycycline effectively suppressed expression of the caMKK3 and the caMKK6 (Fig. 2A) and that removal of doxycycline led to a pronounced increase in the levels of the two FLAG-tagged proteins. However, when analyzed with protein-specific antibodies, only the MKK6 protein levels exhibited a pronounced increase upon removal of doxycycline. The inability to detect a similar increase in expression of the caMKK3 protein with the anti-MKK3-specific antibody is probably due to the high levels of the endogenous MKK3 protein. Cells that expressed the caMKK6 were grown in the presence of doxycycline and treated for





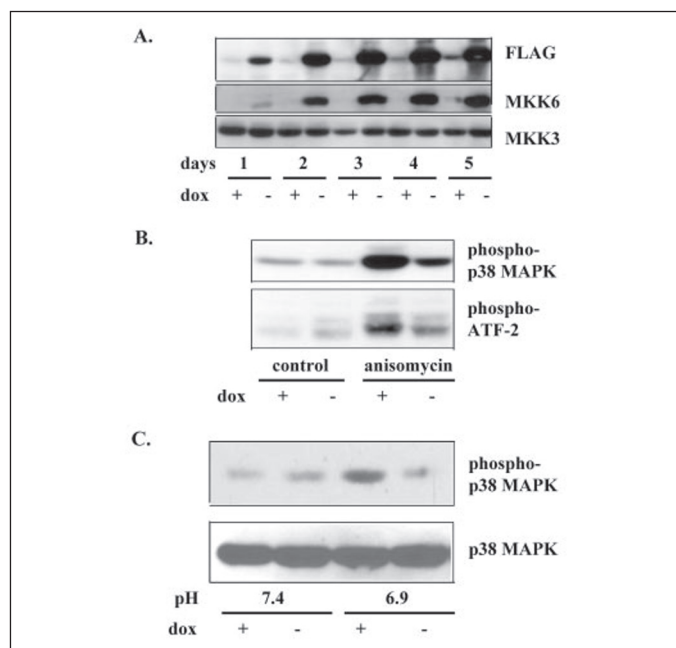
**FIGURE 3. Comparison of the effects of the expression of either caMKK6 or caMKK3 and treatment with acidic medium on PEPCK mRNA levels.** Cells that express either caMKK6 (A) or caMKK3 (B) were grown to confluency in the presence (+) or absence (–) of 0.5  $\mu$ g/ml doxycycline. About 24 h prior to harvest, the indicated cells were treated with acidic medium (pH 6.9). RNA was isolated, and Northern blot analysis was performed to quantify the levels of PEPCK mRNA. The blot was stripped and reprobed for 18S rRNA. C, bar graph of the effect of caMKK6 or caMKK3 expression and acid stimulation on PEPCK mRNA levels. The Northern blots were imaged and quantified using ImageQuant software. The ratio of PEPCK mRNA to 18S rRNA was normalized and plotted as the mean  $\pm$  S.D. of five samples. \*,  $p < 0.005$  and \*\*,  $p < 0.04$ , when compared with RNAs from cells grown in normal medium plus doxycycline (dox).



**FIGURE 4. Effect of co-expression of either caMKK6 or caMKK3 on the activities of the p490Luc3 and p490Luc3-PCK3'.** LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells were transiently transfected with pRL-null and either the p490Luc3 or p490Luc3-PCK3' constructs in the absence (open bars) or presence of an expression plasmid that encodes either caMKK6 (solid bars) or caMKK3 (hatched bars). The resulting firefly luciferase (Luc) activity was standardized against the corresponding *Renilla* luciferase activity and then normalized. The data are the mean  $\pm$  S.D. of six separate transfections ( $n = 6$ ). \*,  $p < 0.001$ .

various times with normal or acidic medium. Within 2 h after transfer to acidic medium, the cells exhibited an increase in phosphorylation of p38 MAPK that closely mimicked the increase caused by removal of doxycycline and expression of the caMKK6 (Fig. 2B). The observed changes in phosphorylation of p38 MAPK occurred without changes in the level of p38 MAPK. By contrast, removal of doxycycline from the cells that expressed the caMKK3 protein had no effect on the level of phosphorylation of the p38 MAPK.

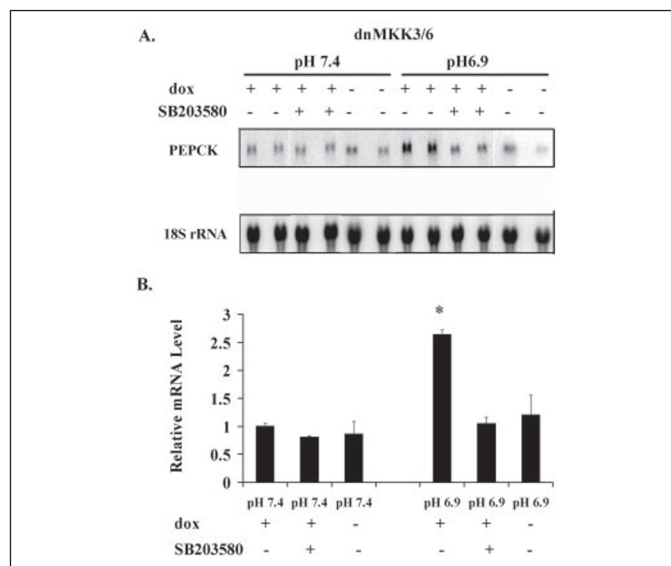
**Expression of caMKK6 Increases PEPCK mRNA Levels**—RNA was isolated from confluent cells carrying the caMKK6 transgene that had been grown in normal medium in the presence or absence of 0.5  $\mu$ g/ml doxycycline and from cells grown in the presence of doxycycline but treated for 24 h with acidic medium. Northern blot analysis indicated that treatment with acidic medium produced a 3.5-fold increase in the level of PEPCK mRNA in cells that were maintained with doxycycline (Fig. 3). The expression of the caMKK6 construct by removal of doxycycline produced a 2.8-fold increase in PEPCK mRNA levels. By contrast, co-expression of the caMKK3 had no effect on the levels of PEPCK



**FIGURE 5. Effect of expression of dnMKK3/6 on anisomycin- and acid-stimulated phosphorylation of p38 MAPK and ATF-2.** A, cells that were stably transfected with both dnMKK3 and dnMKK6 expression vectors (dnMKK3/6) were grown for the indicated numbers of days in the presence (+) or absence (–) of 0.5  $\mu$ g/ml doxycycline (dox). Extracts were separated by 10% SDS-PAGE and probed with antibodies that are specific for the FLAG tag, MKK6, or MKK3. B, confluent cultures of the dnMKK3/6 cells that had been grown with (+) or without (–) doxycycline were transferred to serum-free medium 24 h prior to treatment for 30 min with 5  $\mu$ M anisomycin. Extracts were separated by 10% SDS-PAGE and probed with anti-phospho-p38 MAPK or anti-phospho-ATF-2 antibodies. C, confluent cultures of the dnMKK3/6 cells that had been grown with (+) or without (–) doxycycline (dox) were treated with normal (pH 7.4) or acidic (pH 6.9) medium for 2 h prior to harvest. Western blot analysis was used to detect changes in phosphorylation of p38 MAPK. The blot was stripped and reprobed with an anti-p38 MAPK antibody.

mRNA. Co-expression of caMKK6 also caused a 2.4-fold increase in the luciferase activity expressed from p490Luc3, a construct that contains the –490-bp segment of the rat PEPCK promoter (Fig. 4). However, co-expression of caMKK3 again had no effect on the luciferase activity of this reporter construct. The incorporation of the 3'-untranslated region of the rat PEPCK cDNA into the reporter construct resulted in a significant decrease in basal luciferase activity, consistent with the incorporation of the multiple mRNA instability elements that exist within this segment of the PEPCK mRNA (23). Co-expression of caMKK6 with the 490Luc3-PCK3' produced a similar fold stimulation as observed with p490Luc3. These data demonstrate that activation of the p38 MAPK signaling pathway by the caMKK6 enhances transcription of the *PCK1* gene through elements that are contained in the –490-bp segment of the PEPCK promoter. In contrast, expression of the caMKK3 construct did not significantly affect the level of endogenous PEPCK mRNA and had no effect on the luciferase activity of either construct.

**Effects of Co-expression of the dnMKK3 and dnMKK6 Proteins**—The removal of doxycycline from cells that were transfected with both the dnMKK3 and dnMKK6 constructs (dnMKK3/6) caused a time-dependent increase in expression of the FLAG-tagged proteins that reached a maximum after 4 days (Fig. 5A). Even after 12 days minus doxycycline, the cells still exhibited the same maximal expression of the FLAG-tagged proteins (data not shown), indicating that fully induced expression of the transgenes in the transfected cells is very stable. A similar pattern of expression of MKK6 was observed following removal of doxycycline. However, when the extracts were probed with the MKK3-specific antibody, only slight increases in MKK3 protein were observed

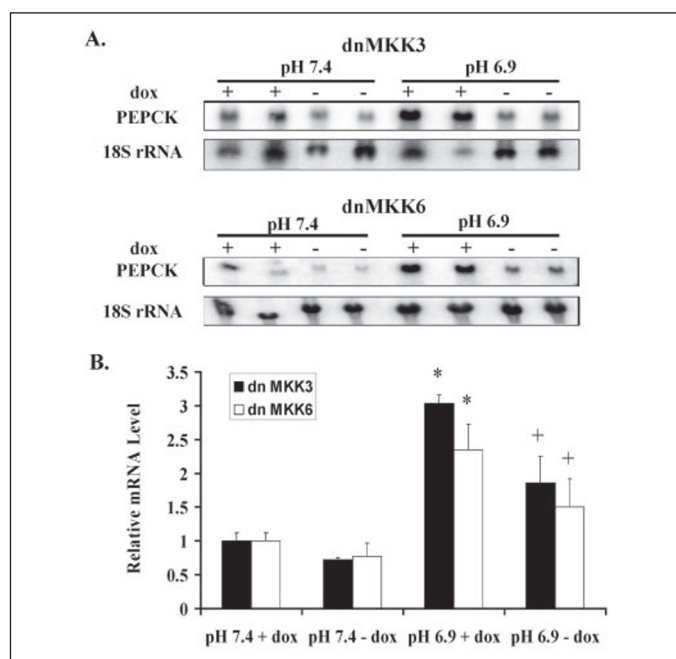


**FIGURE 6. Comparison of the effects of expression of dnMKK3/6 and SB203580 on the pH-responsive increases in PEPCK mRNA.** A, the dnMKK3/6 cells were grown to confluency in the presence (+) or absence (–) of 0.5  $\mu$ g/ml doxycycline (dox). ~24 h prior to harvest, cells were transferred to normal (pH 7.4) or acidic (pH 6.9) medium and, where indicated, 10  $\mu$ M SB203580 was added. RNA was isolated and Northern blot analysis was performed to quantify the levels of PEPCK mRNA. The blot was stripped and reprobed for 18 S RNA. B, bar graph of the effect of dnMKK3/6 expression and acid stimulation on PEPCK mRNA levels. The Northern blots were imaged and quantified using ImageQuant software. The ratio of PEPCK mRNA to 18 S RNA was normalized and plotted as the mean  $\pm$  S.D. of three samples. \*,  $p < 0.001$ , when compared with RNAs from cells grown in normal medium plus doxycycline (dox).

following removal of doxycycline. Again, this may result from the ability of the high endogenous levels of MKK3 to mask an increase in expression of the dnMKK3 protein in cells grown in the absence of doxycycline.

Anisomycin stimulation was initially used to characterize the ability of co-expression of the dnMKK3 and dnMKK6 constructs to block activation of the p38 MAPK. Expression of both dnMKK constructs significantly reduced the anisomycin-stimulated increases in phosphorylation of p38 MAPK and ATF-2 (Fig. 5B). When these cells were grown in the presence of doxycycline and then treated for 2 h with acidic medium, an increase in phosphorylation of the p38 MAPK was observed (Fig. 5C). However, when the dnMKK3/6 cells were grown in the absence of doxycycline, expression of the dnMKK constructs completely inhibited the pH-dependent increase in phosphorylation of p38 MAPK. All of the changes in phosphorylation of the p38 MAPK occurred without changes in the level of the p38 MAPK protein (Fig. 5C).

The ability of the dnMKK3/6 constructs to block the pH-responsive induction of PEPCK mRNA was compared with the effect of treatment with SB203580, an inhibitor of the p38 MAPK. The cells were grown to confluency in the presence or absence of 0.5  $\mu$ g/ml doxycycline. About 24 h prior to harvest, the cells were treated with normal or acidic medium. In addition, cells that were maintained in the presence of doxycycline but treated with normal or acidic medium were also treated for 24 h with 10  $\mu$ M SB203580. Northern blot analyses indicate that co-expression of the two dnMKK constructs or treatment with SB203580 inhibits the pH-dependent increase in PEPCK mRNA levels (Fig. 6). The blots were stripped by incubating for 5 min at 90  $^{\circ}$ C in 1% glycerol and reprobed for 18 S RNA. The quantified and normalized data demonstrate that the co-expression of the dnMKK constructs reproduces the effect observed with the SB inhibitor, in that both treatments completely block the pH-responsive increase in PEPCK mRNA.



**FIGURE 7. Effect of expression of either the dnMKK3 or dnMKK6 construct on the pH-responsive increases in PEPCK mRNA.** A, cells that stably express either the dnMKK3 or dnMKK6 construct were grown for 7 days in the presence of 0.5  $\mu$ g/ml doxycycline (dox) and then grown for 5 days in medium with (+) or without (–) doxycycline. About 24 h prior to harvest, the cells were transferred to normal (pH 7.4) or acidic (pH 6.9) medium. RNA was isolated and Northern blot analysis was performed to quantify the levels of PEPCK mRNA. The blots were stripped and reprobed for 18 S RNA. B, bar graph of the effect of dnMKK3 or dnMKK6 expression and acid stimulation on PEPCK mRNA levels. The Northern blots were imaged and quantified using ImageQuant software. The ratio of PEPCK mRNA to 18 S RNA was normalized and plotted as the mean  $\pm$  S.D. of five samples. \*,  $p < .01$ , when compared with RNAs from cells grown in pH 7.4 medium plus doxycycline. +,  $p = 0.075$  for dnMKK3 and 0.14 for dnMKK6 when compared with RNAs from cells grown in pH 6.9 medium plus doxycycline.

**Effect of dnMKK Expression on PEPCK mRNA Levels**—Cells expressing either the dnMKK3 or dnMKK6 were grown to confluency in the presence or absence of 0.5  $\mu$ g/ml. About 24 h prior to harvest, the cells were treated with normal or acidic medium. Northern analysis demonstrated that the cells exhibit the normal pH-dependent increase in PEPCK mRNA when grown in the presence of doxycycline (Fig. 7A). However, the expression of either construct by growing the cells in the absence of doxycycline was sufficient to partially inhibit the pH-dependent increase in PEPCK mRNA levels. The normalized data (Fig. 7B) clearly indicate that expression of either the dnMKK3 or the dnMKK6 alone is less effective in blocking the increase in PEPCK mRNA than expression of both dnMKK constructs (Fig. 6).

## DISCUSSION

LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells were derived from a non-clonal population of LLC-PK<sub>1</sub> cells by selecting for growth in the absence of glucose (12). The isolated cells exhibit many of the metabolic properties that are characteristic of the renal proximal tubule, including increased levels of fructose 1,6-bisphosphatase (12) and PEPCK (13) and increased rates of ammonia synthesis from glutamine (14). Most importantly, confluent cultures of the LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells exhibit a 3-fold increase in PEPCK mRNA and enzyme activity when transferred to an acidic medium (13), a response that closely models the adaptive increase in PEPCK that occurs in rat kidney following acute onset of acidosis (6). Western blot analysis established that the parental LLC-PK<sub>1</sub> cells contain relatively high levels of both MKK3 and MKK6. By comparison, the LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells exhibit similar levels of MKK3 but clearly reduced levels of MKK6 (Fig. 1A). However, it is unclear whether the

difference in expression of MKK6 contributes to the phenotypic differences between the two cell lines.

The various MKKs are activated in response to different cytokines (30) and stress conditions (15) by dual phosphorylation of a Ser-Xaa-Xaa-Thr motif (21, 31). Activated MKKs subsequently accomplish the dual phosphorylation of a Thr-Xaa-Tyr motif in a downstream MAPK (32). The three primary MAPKs contain distinct activation motifs (ERK, Thr-Glu-Tyr; JNK, Thr-Pro-Tyr; and p38, Thr-Gly-Tyr). Thus, the specificity of the MKKs resides in their ability to recognize the sequence-specific activation domains in the downstream MAPKs. Previous studies demonstrated that MKK6 is a highly specific and potent activator of the multiple isoforms of p38 MAPK (21). In contrast, MKK3 phosphorylates only the  $\alpha$ - and  $\gamma$ -isoforms of p38 MAPK (33) but has a lower specific activity than MKK6 (21).

Previous studies used anisomycin stimulation and inhibition by SB203580 to indicate the potential involvement of p38 MAPK in the pH-dependent increase in PEPCK mRNA in LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells (17). However, both of these compounds affect multiple signaling pathways. In addition, the previous study did not characterize the upstream activator of the p38 MAPK. Thus, initial studies were performed to determine whether anisomycin enhanced phosphorylation of MKK3 and MKK6 in the LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells. The available antibodies against phospho-MKK3/6 detect the phosphorylated forms of both MKK3 and MKK6. Thus, it was not possible to determine the level of phosphorylation of the individual isoforms. However, anisomycin-induced phosphorylation of MKK3/6 occurred with an identical time course as the anisomycin-stimulated phosphorylation of p38 MAPK and ATF-2 (Fig. 1B). Therefore, MKK3 and/or MKK6 are likely to function upstream of p38 MAPK in the LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells.

Constitutively active (ca) and dominant negative (dn) constructs of MKK3 and MKK6 were used to establish the role of the p38 MAPK signaling pathway in the pH-responsive increase in expression of PEPCK mRNA. The caMKK3 (S189E,T193E) and caMKK6 (S207E,T211E) constructs were generated by replacing the sites of phosphorylation with negatively charged glutamate residues (21). In the dnMKK3 construct (S189A,T193A), the phosphorylation sites were converted to alanine residues. In contrast, the dnMKK6 was created by a mutation (L82A) within the ATP binding site. The constructs were cloned downstream of a tetracycline-responsive (tet-off) promoter and stably expressed in 8C cells, a line of LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells that constitutively expresses high levels of the tTA transcription factor. In these cells, the presence of >25 ng/ml doxycycline is sufficient to prevent tTA binding to a tet-responsive promoter and effectively shuts off transcription (34). Clonal lines of cells were generated in the presence of doxycycline to prevent the potential effects of continuous expression of the caMKKs or dnMKKs. Only the cell lines that exhibited at least a 50-fold increase in expression of the FLAG-tagged protein when transferred to medium minus doxycycline were expanded.

Western (Fig. 2) and Northern (Fig. 3) blot analyses indicate that expression of caMKK6 produced an increased phosphorylation of p38 MAPK and an increased level of PEPCK mRNA that are similar to the increases observed with acid stimulation. Experiments with the PEPCK-luciferase constructs indicate that MKK6 activation of the p38 MAPK signaling pathway leads to increased transcription of the PEPCK gene (Fig. 4). Recent experiments demonstrated that the 3'-nontranslated region of the PEPCK mRNA contains multiple instability elements (23) that participate in the cAMP-dependent stabilization of the PEPCK mRNA (35). The inclusion of the 3'-nontranslated region of the PEPCK mRNA into the luciferase reporter construct significantly reduced the measured luciferase activity, consistent with the incorporation of the



mRNA instability elements. However, the latter construct retained a similar fold stimulation when co-expressed with the caMKK6, suggesting that the observed increase in PEPCK mRNA is not due to a p38 MAPK-mediated enhancement of mRNA stability (36).

In contrast, expression of caMKK3 had no effect on the phosphorylation of p38 MAPK (Fig. 2), the level of PEPCK mRNA (Fig. 3), or relative luciferase activity (Fig. 4). A large increase in expression of this construct was evident when monitored with the anti-FLAG antibodies, but not with the MKK3-specific antibodies. These observations may be due to the high endogenous expression of MKK3 and/or the lower specific activity of MKK3 compared with MKK6. Similar results with the caMKK3 construct were observed previously in Chinese hamster ovary cells (21). Co-expression of caMKK3 and p38 MAPK (but not expression of either kinase alone) was able to induce expression of various reporter genes. Thus, the caMKK3 construct was effective only when p38 MAPK was also overexpressed. Therefore, the high endogenous levels of MKK3 in the LLC-PK<sub>1</sub>-FBPase<sup>+</sup> cells may sequester a major fraction of the endogenous p38 MAPK, possibly through interaction with a specific scaffold protein (16) and thereby reduce the effectiveness of the caMKK3. On the other hand, a pronounced increase in expression of the caMKK6 was evident using either the anti-FLAG or MKK6-specific antibodies. Thus, the caMKK6 could effectively compete with endogenous MKK6 for binding to a fraction of the endogenous p38 MAPK, resulting in its phosphorylation and activation.

Although the caMKK3 failed to increase the phosphorylation of p38 MAPK and the level of PEPCK mRNA, expression of the dnMKK3 alone did reduce the pH-responsive induction of PEPCK mRNA (Fig. 7). Expression of only the dnMKK6 also partially blocked the acid-induced increase in PEPCK mRNA. However, expression of both constructs (dnMKK3/6) produced a greater effect (Fig. 6). The dnMKKs may sequester the upstream MAPK kinase kinase (MKKK) that participates either in sensing or mediating the signal produced by a slight decrease in intercellular pH. By sequestering the participating upstream MKKK, either dnMKK construct would block activation of the p38 MAPK signaling pathway. Expression of both dnMKKs also significantly reduced the anisomycin-stimulated increase in phosphorylation of p38 MAPK and ATF-2 (Fig. 5). Furthermore, expression of both dnMKK3 and dnMKK6 is as effective as SB203580 in blocking the pH-responsive induction of PEPCK mRNA. Therefore, the enhanced transcription of PEPCK mRNA in response to acidosis involves activation of MKK3 and/or MKK6 that in turn activate the p38 MAPK signaling pathway. Subsequent phosphorylation and activation of ATF-2, a downstream transcription factor, may contribute to the enhanced transcription of the *PCK1* gene in the kidney (17).

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