p38 MAPK mediates acid-induced transcription of PEPCK in LLC-PK1-FBPase+ cells

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Feifel, Elisabeth, Petra Obexer, Manfred Andratsch, Stephan Euler, Lynn Taylor, Aimin Tang, Yu Wei, Herbert Schramek, Norman P. Curthoys, and Gerhard Gstraunthaler. p38 MAPK mediates acid-induced transcription of PEPCK in LLC-PK1-FBPase+ cells. Am J Physiol Renal Physiol 283: F678–F688, 2002; 10.1152/ajprenal.00097.2002.—LLC-PK1-FBPase+ cells are a gluconeogenic and pH-responsive renal proximal tubule-like cell line. On incubation with acidic medium (pH 6.9), LLC-PK1-FBPase+ cells exhibit an increased rate of ammonia production as well as increases in glutaminase and phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels and enzyme activities. The increase in PEPCK mRNA is due to an enhanced rate of transcription that is initiated in response to intracellular acidosis. The involvement of known MAPK activities (ERK1/2, SAPK/JNK, that is initiated in response to intracellular acidosis. The involvement of known MAPK activities (ERK1/2, SAPK/JNK, that is mediated by p38 and ATF-2. SB-203580, a specific inhibitor of p38, inhibits the increase in PEPCK and ATF-2. Moreover, incubation of LLC-PK1-FBPase+ cells with acidic medium (pH 6.9) results in a specific phosphorylation, and thus activation, of p38 and of activating transcription factor-2 (ATF-2), respectively. Anisomycin (AI), a strong p38 activator, increased PEPCK mRNA to levels comparable to those observed with acid stimulation. AI also induced a time-dependent phosphorylation of p38 and ATF-2. SB-203580, a specific p38 inhibitor, blocked both acid- and AI-induced PEPCK mRNA levels. Western blot analyses revealed that the SB-203580-sensitive p38α is a strong expressed. The octanucleotide sequence of the cAMP-response element of the PEPCK promoter is a perfect match to the consensus element for binding ATF-2. The specificity of ATF-2 binding was proven by ELISA. We conclude that the SB-203580-sensitive p38α-ATF-2 signaling pathway is a likely mediator of the pH-responsive induction of PEPCK mRNA levels in renal LLC-PK1-FBPase+ cells.

Systemic metabolic acidosis initiates an array of adaptive responses in tubular cell metabolism and transport along the entire renal nephron (1, 9, 36, 37). The pH-induced changes in cell metabolism, however, are confined to the proximal convoluted tubule. In rats, renal proximal tubular cells respond with an increased extraction and catabolism of glutamine and enhanced rates of ammonium ion excretion and HCO3− ion reabsorption, thereby partially restoring acid-base homeostasis (13, 19). This is accomplished, in part, by increases in phosphate-dependent glutaminase (PDG) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK) activities, which result from increased levels of their respective mRNAs (19). However, the two genes are differentially regulated. Increased levels of PEPCK mRNA result from an enhanced rate of transcription, whereas PDG mRNA levels are increased by decreasing its rate of degradation (9, 19).

The differential regulation of the adaptive response of the two enzymes is reproduced in LLC-PK1-FBPase+ cells (13). These cells are a gluconeogenic strain of the renal epithelial LLC-PK1 cell line (12) that exhibit a number of proximal tubule-specific metabolic properties (17). On incubation with acidic medium (pH 6.9), LLC-PK1-FBPase+ cells respond with an increased rate of ammonia production and two- to threefold increases in PDG and PEPCK mRNA levels and enzyme activities. Furthermore, incubation of LLC-PK1-FBPase+ cells in low-potassium (0.7 mM)-containing media for 24–72 h elicits a decrease in intracellular pH while maintaining normal extracellular pH. The LLC-PK1-FBPase+ cells again respond with increased levels of PDG and PEPCK mRNAs, suggesting that an intracellular acidosis triggers the adaptive responses (13).

The mechanism of sensing cellular pH in renal proximal tubular cells and the associated signal transduction pathway for mediating the pH-responsive adaptations are unknown. In recent years, it has been well established that MAPK signaling cascades are activated by various extracellular stimuli, including hypoxia (2, 21, 22, 39). In mammals, hypotension is unique to the renal medullary interstitium, and similarly to metabolic acidosis, it induces a plethora of cellular responses resulting from specific gene expression (reviewed in Refs. 4 and 14). The p38 stress-
activated MAPK superfamily is activated by changes in extracellular osmolality and is likely to mediate the osmoadaptation of renal distal tubule and collecting duct cells (28, 35, 38). Other cellular stresses that are potent in vivo and in vitro activators of p38 MAPK include inflammatory cytokines, heat shock, and the protein synthesis inhibitor anisomycin (AI). Therefore, decreased intracellular pH might also activate an MAPK in LLC-PK1-FBPase− cells.

The promoter region of the cytosolic PEPCK gene contains a cAMP-response element (CRE-1) (15, 16, 25, 32). The sequence of this element (TTTCGTA) was recently recognized as a perfect match to the consensus sequence reported for activating transcription factor-2 (ATF-2) homodimers (7), a downstream substrate of p38 MAPK. These findings prompted us to define the role of p38 kinase and other MAPK signaling cascades (ERK1/2, SAPK/JNK) in the pH-responsive induction of PEPCK mRNA transcription in LLC-PK1-FBPase− cells (Fig. 1). Here, we show that incubation of LLC-PK1-FBPase− cultures in acidic medium resulted in a biphasic phosphorylation, and thus activation, of p38 kinase and ATF-2. In addition, AI specifically increased PEPCK mRNA to levels similar to those observed in acidic cultures. SB-203580, a specific p38 kinase inhibitor, but not the MAPK ERK (MEK)1/2 inhibitor PD-098059 or the SAPK/JNK inhibitor curcumin, produced a dose-dependent inhibition of the acid- and AI-induced PEPCK mRNA levels. These results suggest that p38/ATF-2 signaling may mediate the pH-responsive induction of PEPCK mRNA levels in LLC-PK1-FBPase− kidney cells.

**MATERIALS AND METHODS**

Culture media and chemicals. Culture media were prepared from DMEM base (D-5030, Sigma) supplemented with 2.2 g/l (26.2 mM) NaHCO₃ and 1.0 g/l (17.8 mM) NaCl to correct for medium osmolality (13). L-Glutamine was added from freshly prepared stock solutions (final concentration 2.0 mM). All media additives were of tissue culture grade and were obtained from Sigma-Aldrich (Vienna, Austria). Biochemicals and buffer chemicals were of the highest analytical grade available and were obtained from Boehringer Mannheim (Vienna, Austria) or from Sigma-Aldrich. MAPK inhibitors were purchased from Calbiochem. All tissue culture plasticware (Falcon Labware) was from Becton Dickinson (Heidelberg, Germany).

Cell culture, adaptation protocol, and cell harvest. Gluconeogenic LLC-PK1-FBPase− cells (12, 13, 17) were cultured in DMEM with 5.5 mM d-glucose, 2 mM L-glutamine, and 26 mM NaHCO₃ (pH 7.4), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Acidic medium (pH 6.9) contained 9 mM NaHCO₃ and was supplemented with the appropriate amount of NaCl to maintain equivalent osmolality. Cultures were incubated at 37°C in a 5% CO₂/95% air atmosphere. Routinely, cultures were fed three times a week. Experimental cultures were always fed 24 h before adaptation. Confluent monolayers were subcultured (split ratio 1:3) with 0.25% trypsin and 0.02% EDTA in Ca²⁺- and Mg²⁺-free buffered saline. Experimental cultures were grown for 10–12 days to produce confluent monolayers in 10-cm plastic tissue culture dishes (Falcon Optiplus 3003) by using 10 ml of culture medium or in six-well plates (Falcon 3046) with 2 ml of medium. Therefore, cultures were adapted to acidic media (pH 6.9) for the indicated times.

For Western blotting, monolayer cultures were harvested in freshly prepared ice-cold lysis buffer [(in mM) 50 Tris·HCl, pH 7.5, 150 sodium chloride, 5 EDTA, 40 β-glycerophosphate, 50 sodium fluoride, and 0.1 phenylmethylsulfonyl fluoride, as well as 1% (vol/vol) Nonidet P-40 (Amresco), 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 200 μM sodium orthovanadate, 1 μg/ml leupeptin, and 1 μM pepstatin A] (34). Cells were lysed on ice for 20 min, followed by the forcing of the lysate through a 21-gauge needle with a sy-
rings. The lysates were then transferred to microcentrifuge tubes and centrifuged at 14,000 g for 15 min at 4°C. Aliquots of supernatants were stored at −80°C. For Northern blot analysis, total RNA was isolated by using the acid guanidinium thiocyanate method as described previously (13, 17). Western blotting and antibodies. SDS-PAGE was performed under standard denaturing conditions using 16 × 16-cm slab gels (SE-400, Hoefer). Equal amounts of protein were loaded onto each lane of 10% polyacrylamide gels. Rainbow Marker (RPN 756, Amersham) was used as a molecular weight standard. Electrophoresis was performed overnight at a constant 80 V. Gels were blotted immediately after electrophoresis onto polyvinylidene fluoride membrane (Immobilon-P, Millipore). Gels were rinsed briefly in transfer buffer [25 mM Tris, 200 mM glycine, pH 8.3, 0.1% (wt/vol) SDS, 20% (vol/vol) methanol], and transfer was carried on a constant current of 400 mA for 3 h at 4°C. For immunodetection, membranes were blocked overnight with 5% (wt/vol) dry milk and 0.1% (vol/vol) Tween 20 in Tris-buffered saline (pH 7.6) at 4°C and processed according to the instructions of the manufacturers of the antibodies. The following antibodies were used: PhosphoPlus Antibody Kits (New England Biolabs) for the detection of phosphorylated forms of ERK1/2 (p44/42 MAP Kinase Thr202/Tyr204 Antibody Kit 9100), SAPK/JNK (Thr183/Tyr185 Antibody Kit 9250), and p38 (Thr180/Tyr182 Antibody Kit 9210), respectively. The state of phosphorylation of transcription factor ATF-2 was determined by using the PhosphoPlus ATF-2 (Thr71) Antibody Kit (model 9220, New England Biolabs). The anti-MAPK kinase (anti-MKK) antibodies (anti-MEK-3, sc-959; anti-MEK-4, sc-964; and anti-MEK-6, sc-1991) and the antibodies against the p38 isoforms (anti-p38, sc-535-G; anti-p388, sc-6176; anti-SAPK4, sc-7585; and anti-ERK 6, sc-2020) were obtained from Santa Cruz Biotechnology.Visualization of blots was carried out with enhanced chemiluminescence by using either the Western Star System (Tropix) for the PhosphoPlus antibodies or the ECL System (Amersham) for all other antibodies. All blots were exposed to Hyperfilm ECL (Amersham).

Northern blot analysis and cDNA probes. Formaldehydeagarose gel electrophoresis of total RNA samples, transfer to GeneScreen Plus membranes (New England Nuclear), and hybridization and posthybridization washings of blots were carried out as described previously (13, 17–19). Blots were exposed to autoradiographic film (Kodak BioMax MS). Quantitation of mRNA levels was accomplished by using a Personal Densitometer SI-Scanner (Molecular Dynamics). Sample integrity and equal loading of 20 μg RNA/ lane were monitored by staining with ethidium bromide after electrophoresis. For probing PEPCK mRNA, a 1.6-kb BglII fragment of pPC1-10 (13, 17), which encodes the rat cytosolic PEPCK, was used. The pPC1-10 plasmid was kindly provided by Dr. R. Hanson (Case Western Reserve) (15, 16).

Isolation of nuclear extract and electrophoretic mobility shift assays. Nuclear extracts of LLC-PK1-FBPase cells were prepared as described recently (25). The CRE-1 probe was synthesized by Macromolecular Resources (Ft. Collins, CO) as complementary oligonucleotides that contained bases −99 to −77 of the PEPCK promoter. The sequence of the sense strand is 5′-GATCCGCGCCCTTACGTAGACGGC-GAG-3′, in which the nucleotides derived from the PEPCK promoter are underlined and the CRE-1 element is in bold. The additional sequence was included to create 5′ BamHI overhangs. The oligonucleotides were annealed in 50 mM NaCl, 66 mM Tris·HCl, and 6.6 mM MgCl2, pH 7.5, by heating to 85°C and cooling to 25°C.

The double-stranded oligonucleotide was 5′-end labeled with [γ-32P]ATP by using T4 polynucleotide kinase (30). The indicated amount of nuclear extract was incubated for 20 min on ice with 200 ng poly[d(I·dC)], 200 ng pUC19, and the labeled oligonucleotide (20 fmol, 20,000–60,000 counts/min). For the supershift analysis, the indicated amount of antibody was preincubated with the nonspecific competitors and the nuclear extract for 45 min on ice. The labeled probe was then added, and the complete sample was incubated for another 20 min on ice. The electrophoresis was performed at 170 V for 1.5 h at 4°C by using a 4% polyacrylamide gel.

Specificity of ATF-2 binding to the CRE-1 element was further assessed with the newly developed Mercury TransFactor Kit (model K2062–1, Clontech Laboratories). In this ELISA-based assay, oligonucleotides containing the CRE-1 consensus binding sequence are immobilized in 96-well plates. When nuclear extracts containing the transcription factors are incubated in the wells, ATF-2 and CRE binding protein (CREB)-1 bind to the consensus sequence. Transcription factor binding is then detected by a specific primary antibody and is quantitated after horseradish peroxidase-conjugated secondary antibody binding and measurement of enzymatic color reaction in a standard microplate reader. Nuclear extracts of control (pH 7.4) and acid-adapted LLC-PK1-FBPase cells (15 h at pH 6.9) were prepared as described above (25), except that 40 mM β-glycerophosphate, 50 mM sodium fluoride, and 200 μM sodium orthovanadate were added as phosphatase inhibitors (34). The state of phosphorylation of ATF-2 in nuclear extract preparations was proven by Western blotting.

Statistical analysis of results was performed using an unpaired Student’s t-test.

RESULTS
Effects of specific MAPK activators and inhibitors on PEPCK mRNA levels in LLC-PK1-FBPase cells. Incubation of LLC-PK1-FBPase cells in acidic media (pH 6.9) results in an increased level of the cytosolic PEPCK mRNA (13, 17), which is due to a pH-responsive induction of transcription (18, 19). The time course of this response is depicted in Fig. 2. Within 6 h after transfer of cells to acidic media, the 2.7-kb cytosolic PEPCK mRNA is increased approximately threefold. The induced level is then sustained for at least 24 h.

To determine a possible involvement of MAPK signaling in the acid-mediated induction of PEPCK mRNA transcription, the effects of specific MAPK activators and inhibitors on PEPCK mRNA levels in LLC-PK1-FBPase cells were examined. A detailed pathways diagram is displayed in Fig. 1.

A representative Northern blot is shown in Fig. 3A, and the series of independent experiments are summarized in Fig. 3B. Stimulation of cells with 5 μM A1 at pH 7.4 for 2 h increased PEPCK mRNA to levels comparable to acid stimulation. The stimulatory effects of AI and acidic conditions (pH 6.9) were additive, suggesting the activation of different targets upstream of MAPKs. Cycloheximide (100 μM), a second eukaryotic protein synthesis inhibitor, had no significant effect on PEPCK mRNA levels (Fig. 3A). The p38 inhibitor SB-203580 (23, 30) inhibited the acid-induced increase in PEPCK mRNA levels without affecting basal mRNA expression at pH 7.4 (Fig. 3, A and B).
SB-203580 produced a dose-dependent inhibition of the acid-stimulated increase in PEPCK mRNA levels (Fig. 4). Complete inhibition was achieved with 10 μM SB-203580 (Fig. 4B). The use of SB-202190, another pyridinyl imidazole inhibitor (23), revealed identical results (data not shown). AI-dependent stimulation of PEPCK mRNA levels under normal (pH 7.4) or acidic conditions (pH 6.9) was also blocked by SB-203580 (Fig. 3, A and B). These data strongly indicate an involvement of the p38 MAPK cascade in mediating the AI-stimulated and the pH-responsive induction of PEPCK mRNA transcription.
Acidic incubation of LLC-PK₁-FBPase<sup>+</sup> cells induces p38 MAPK and ATF-2 activation. On the basis of the preceding results, further experiments focused specifically on the p38 MAPK pathway. Initial experiments tested whether incubation of LLC-PK₁-FBPase<sup>+</sup> cells in acidic media (pH 6.9) per se is sufficient to activate the p38 cascade. As depicted in Fig. 7, this treatment resulted in a biphasic phosphorylation, and thus activation, of p38 MAPK with peaks occurring at 0.5–1 and 9 h. A major downstream target of p38 MAPK is the transcription factor ATF-2 (7, 8, 22). An acid-induced phosphorylation of ATF-2 was also evident and occurred with a slight lag. Phosphorylation of ATF-2 peaked at 3 and 9–15 h after transfer of cells to acidic medium.

The direct activation of ATF-2 by the p38 MAPK was further tested by examining the time courses of phosphorylation of p38 and ATF-2 after stimulation of LLC-PK₁-FBPase<sup>+</sup> cells with AI or hyperosmotic sorbitol (39). The results of a representative series of experiments are summarized in Fig. 8. AI caused a time-dependent increase in phosphorylation of p38, with a peak at 30–60 min after onset of stimulation (Fig. 8A). The phosphorylation of ATF-2 again occurred with a lag compared with p38, with a peak in activation at 60–120 min (Fig. 8B). The AI-induced phosphorylation and activation of ATF-2 was specifically blocked by the p38 inhibitor SB-203580 (Fig. 8C), indicative of signaling through an SB-203580-sensitive isofrom of p38 MAPK (20, 23, 30). Hyperosmotic sorbitol showed a slower time course in activating p38 MAPK and ATF-2 compared with AI. Cycloheximide again did not activate either p38 MAPK or ATF-2 (Fig. 8, A and B),
which may explain the lack of an effect on PEPCK mRNA levels shown by Northern blot analysis in Fig. 3A.

ATF-2 from LLC-PK₁-FBPase⁺ cells binds to the CRE-1 element of the PEPCK promoter. Incubation of nuclear extracts from LLC-PK₁-FBPase⁺ cells with a labeled oligonucleotide containing the CRE-1 element of the PEPCK promoter results in the formation of specific complexes that can be resolved on a nondenaturing polyacrylamide gel (25). As shown in Fig. 9, preincubation of the nuclear extract with increasing amounts of antibodies specific for ATF-2 results in the disappearance of the top band and the appearance of a supershifted band. Quantification of the radioactivity contained in each of the shifted bands indicated that a maximum of ~20% of the complexed oligonucleotide was supershifted with the AFT-2-specific antibodies. The percentage supershifted was not increased when nuclear extracts of acid-adapted LLC-PK₁-FBPase⁺ cells were used (data not shown). Thus ATF-2 is one of the proteins contained in LLC-PK₁-FBPase⁺ cells that bind to the CRE-1 element of the PEPCK promoter.

The specificity of ATF-2 binding to the CRE-1 element was further confirmed using an ELISA-based binding assay (Mercury TransFactor Kit, Clontech Laboratories). As depicted in Fig. 10, a low, but specific, binding of ATF-2 was obtained with nuclear extracts of control (pH 7.4) LLC-PK₁-FBPase⁺ cells. In a parallel series of experiments, nuclear extracts of LLC-PK₁-FBPase⁺ cells that were adapted to acidic pH (6.9) for 15 h were used. The 15 h of acidic adaptation were chosen because at that time the increase in PEPCK mRNA levels was completed (Fig. 2) and ATF-2 phosphorylation was at its second peak (Fig. 7). A marked phosphorylation of ATF-2 was observed in nuclear extracts of 15-h acid-adapted (pH 6.9) LLC-PK₁-FBPase⁺ cells (Fig. 10, inset), but no increase in ATF-2 binding could be detected. Thus these data are consistent with the results of the gel-shift assays described above.

Therefore, in vitro, ATF-2 and phosphorylated ATF-2 bind with equal affinity to the CRE-1 site (3). The TransFactor assay for CREB-1 binding revealed no signals (data not shown), indicating that transcription factor CREB is not present in LLC-PK₁-FBPase⁺ nuclear extracts. Nuclear extracts of PC-12 cells served as positive controls for CREB binding.
LLC-PK₁-FBPase⁺ cells express the α-isoform of p38 MAPK and the upstream MKKs. At present, four distinct isoforms of p38 MAPK have been identified: p38α (SAPK2a), p38β (SAPK2b), p38γ (ERK6 or SAPK3), and p38δ (SAPK4) (20, 22, 30). Thus extracts of LLC-PK₁-FBPase⁺ cells were screened for expression of the four p38 homologs by Western blot analysis using isoform-specific antibodies (Fig. 11A). LLC-PK₁-FBPase⁺ cells strongly express the α-isoform of p38 MAPK, whereas the other isoforms are barely detectable. p38δ was present in very low levels, but p38β and p38γ were barely detectable even when crude cell homogenates were initially immunoprecipitated with isoform-specific antibodies (data not shown). LLC-PK₁ wild-type cells showed an identical pattern of p38 kinase protein expression. HepG2 cells, which have been shown to express all four p38 isoforms (22), served as a positive control. The p38 isoforms are functionally divided into two subgroups: the p38α and p38β isoforms, which are inhibited by the pyridinyl imidazole inhibitor SB-203580, and p38γ and p38δ isoforms, which are resistant to this inhibitor (23, 30). Because SB-203580 inhibited the pH-mediated and AI-stimulated increases in PEPCK mRNA levels (Figs. 3 and 4) and ATF-2 phosphorylation (Fig. 8C), the above-mentioned Western blot analysis suggests that the α-isoform of p38 MAPK mediates the observed responses in LLC-PK₁-FBPase⁺ cells. Further Western blot analysis indicates that all three MKKs upstream of the p38 pathway, MKK3, MKK6, and MKK4, are also present in LLC-PK₁-FBPase⁺ cells (Fig. 11B).

DISCUSSION

The pH-responsive and gluconeogenic LLC-PK₁-FBPase⁺ cell line (12, 13, 17) provides an effective model system to identify the molecular mechanism by which the onset of metabolic acidosis leads to the cell-specific induction of the mitochondrial glutaminase (PDG) and cytosolic PEPCK activities in renal proximal convoluted tubular cells. Recent studies indicated that the pH-responsive increases in PDG and PEPCK mRNAs in LLC-PK₁-FBPase⁺ cells (13) occur by means of the separate mechanisms previously characterized to occur in rat kidney cortex (19). Furthermore, a decrease in intracellular pH was recently identified as the stimulus that initiates both responses (13). However, the associated signal transduction mechanism that mediates the pH-responsive activation of PEPCK mRNA transcription had not been previously characterized.

Specific gene expression, either in a temporal or tissue-specific manner, or in response to extracellular or environmental stimuli, requires the coordinate activation of specific transcription factors. In recent years, the MAPK family has been shown to play a pivotal role in the control of cellular responses to external stimuli...
genes were screened in mouse inner medullary collecting duct-3 cells by subtractive hybridization and cDNA microarray analysis, the induction of 11 of the 12 genes found was also inhibited by SB-203580.

In the present study, the effects of specific activators and inhibitors of the MAPK pathways on basal and acid-induced PEPCK mRNA levels were investigated. The resulting data revealed both activation of the p38 MAPK/ATF-2 cascade when LLC-PK1-FBPase⁺ cells are incubated in acidic culture media (Fig. 7) and inhibition of the acid-mediated increase in PEPCK mRNA by SB-203580 (Figs. 3 and 4). In addition, the protein synthesis inhibitor AI activated p38 MAPK and ATF-2 and increased PEPCK mRNA to levels similar to those observed during acidosis (Figs. 3 and 8). These effects of AI were also blocked by SB-203580 (Figs. 3B and 8C). Interestingly, cycloheximide, a second inhibitor of eukaryotic protein synthesis, was unable to activate p38 and ATF-2 or to induce PEPCK mRNA transcription (Figs. 3A and 8). Thus the effects of AI are unrelated to its ability to inhibit protein synthesis and are specific to the p38/ATF-2/PEPCK cascade. Finally, gel-shift analysis (Fig. 9) and ELISA-based binding assays (Fig. 10) indicated that ATF-2 is at least one of the transcription factors contained in nuclear extracts of LLC-PK1-FBPase⁺ cells that binds to the CRE-1 element of the PEPCK promoter. In total, the reported data strongly support the hypothesis that the pH-responsive induction of PEPCK mRNA transcription is mediated by phosphorylation of p38α MAPK that, in turn, phosphorylates and activates ATF-2.

A Western blot survey revealed that p38α is the major isoform expressed in LLC-PK1-FBPase⁺ cells (Fig. 11A). The upstream MKKs of p38, MMK3 and MMK6 (22), were also detectable in LLC-PK1-FBPase⁺ cells. In addition, MMK4 (39) was expressed in these cells (Fig. 11B). The finding that all three MAP/SAP kinases, ERK1/2, JNK, and p38 (39), can be phosphorylated in LLC-PK1-FBPase⁺ cells (Figs. 5–8) indicates that all of the essential components of each MAPK pathway are present in these cells.

Recent reports showed that incubation of MCT cells, an SV40-transformed mouse proximal tubule cell line, in acidic medium induced a twofold increase in c-Src activity, a soluble tyrosine kinase, which was paralleled by an increased phosphotyrosine content of cytosolic proteins (1, 40). These studies suggest that a decrease in extracellular pH may lead to activation of c-Src or a related tyrosine kinase, causing increased expression of Fos and Jun, which in turn activate transcription of pH-responsive genes (40). However, the tyrosine kinase inhibitors, herbimycin A, genistein, or tyrphostin (Fig. 1), had no effect on PEPCK mRNA levels in LLC-PK1-FBPase⁺ cells (data not shown), confirming earlier findings (18). Taken together, these results indicate that ERK1/2 (Fig. 5), SAPK/JNK (Fig. 6), and tyrosine phosphorylation by c-Src (40) do not play a significant role in pH-induced transcription of cytosolic PEPCK mRNA in renal LLC-PK1-FBPase⁺ cells.

The transcriptional regulation of the gene that encodes the cytosolic isoform of PEPCK has been studied...
The sequence of the promoter for the PEPCK gene from mice, rats, and humans has been remarkably conserved (>95% sequence identity). Thus mechanisms of transcriptional regulation deduced from studies with the rodent PEPCK promoter are likely to be characteristic of the control in most mammalian species. The initial 490 bp of the rat PEPCK gene are very complex. They contain at least 12 separate elements that mediate the hormonal and dietary control of PEPCK gene expression in the liver. However, transcription of PEPCK mRNA in the liver and kidney are differentially regulated. Hepatic gluconeogenesis is primarily involved in the maintenance of blood glucose homeostasis, whereas renal gluconeogenesis is linked to ammoniagenesis and the maintenance of acid-base balance. Thus pH-responsive regulation of PEPCK gene expression occurs only in the kidney (13, 17–19, 36).

Previous studies have shown that binding of hepatocyte nuclear factor-1 to the P2 element (−200 to −164) of the PEPCK promoter is essential for basal expression of PEPCK in the kidneys of transgenic mice (5, 15). In addition, mutation or deletion of the P2 element in PEPCK-reporter gene constructs significantly decreased expression in LLC-PK1-FBPase⁺ cells (18). Furthermore, studies by Cassuto et al. (5) showed that the P2 site may also be required for a full induction of PEPCK activity in response to acidic pH. Similar studies have also implicated the CRE-1 and the P3(II) region as elements that contribute to the pH-responsive induction of the PEPCK gene (18).

The P3(II) region contains a 7-bp sequence, TTA-GTCA, that binds activator protein-1 (AP-1; Jun/Fos heterodimers). This sequence differs from an AP-1 consensus (TGAGTCA) sequence by a single G-to-T substitution. The CRE-1 or cAMP response element within the PEPCK promoter is located (−91 to −84) ~60 bp 5' from the TATA box (−29 to −23) (15, 16). CREs are highly conserved regulatory elements found in numerous cellular genes that are induced by cAMP. They typically consist of an 8-bp palindromic consensus sequence (TGACGTCA) located within 100 nucleotides of the TATA box. Within the promoter of the cytosolic PEPCK gene, the sequence of the CRE-1 element (TTACGTCA) again diverges from the consensus sequence by a single G-to-T substitution (8, 15, 16, 32). Several members of the leucine zipper-containing transcription factors have been shown to bind to the CRE-1 element, including CREB, CCAAT/enhancer binding protein (C/EBP)β, C/EBPα, AP-1, and Jun/Jun heterodimers (8, 15, 25).

Previous studies using dominant-negative forms of CREB and of C/EBP (24, 31) and more recent experiments using GAL4-chimeric constructs (25) revealed that binding of C/EBPβ, and not CREB, to CRE-1 mediates the cAMP-dependent activation of PEPCK mRNA transcription in subconfluent LLC-PK1-FBPase⁺ cells (see Fig. 1). In an earlier gel-shift analysis, the shift produced by incubating the CRE-1 element with a nuclear extract of LLC-PK1-FBPase⁺ cells was super-shifted with an antibody against C/EBPβ but not with an antibody against CREB (24). This is in line with data from an ELISA-based binding assay used in the present study, in which no CREB-1 binding signals could be detected, indicating that CREB is not present in LLC-PK1-FBPase⁺ nuclear extracts.

LLC-PK1-FBPase⁺ cells differ significantly in their responses to either cAMP or an acidic environment, depending on the state of confluence of the cultures and thus on the state of differentiation. The cAMP-responsive induction of PEPCK gene transcription, mediated by C/EBPβ, is maximal in subconfluent cells (24, 25), whereas the pH response, triggered by SB-203580-sensitive p38 MAPK signaling, is maximal in confluent, fully differentiated LLC-PK1-FBPase⁺ monolayer cultures or in LLC-PK1-FBPase⁺ epithelia grown on permeable culture supports (13, 17, 18). The time point in culture duration or state of confluence, respectively, of this transition as well as the molecular mechanisms are unknown at present.

Recently, it was recognized that the PEPCK CRE-1 sequence TTACGTCA is identical to the consensus sequence required for binding of ATF-2 homodimers (7). This study demonstrated that in Fao hepatoma cells, a sodium arsenite-induced activation of PEPCK mRNA transcription was mediated by p38 MAPK transactivation of ATF-2. ATF-2 is a basic-leucine zipper transcription factor that exhibits transcriptional activation after dual phosphorylation on Thr³⁵⁹ and Thr⁷¹. Activated ATF-2 forms a homodimer or heterodimer with c-jun, binds to CREs, and stimulates CRE-dependent transcription of genes (3). These observations provide the basis on which to interpret the data obtained in the present study. They support the concept that binding of C/EBPβ and CREB to the promoter of the PEPCK gene activates transcription of the gene in a pH-dependent manner.
clusion that phosphorylation of ATF-2 by the SB-203580-sensitive α-isofrom of p38 MAPK may mediate the increased transcription of cytotoxic PEPCK mRNA during metabolic acidosis.

On the basis of the existing data, the following model (Fig. 12) was developed as a hypothesis for the mechanism by which PEPCK mRNA transcription is induced in the renal proximal convoluted tubule during metabolic acidosis. During normal acid-base balance, C/EBPβ and ATF-2 are constitutively bound to the CRE-1 site. In addition, the P2 and possibly the P3(II) regions of the PEPCK promoter are occupied with bound transcription factors. Phosphorylation of C/EBPβ by protein kinase A leads to nuclear import (27), and phosphorylation of ATF-2 by p38 MAPK exposes its transcriptional activation domain (26). These observations may explain how C/EBPβ and ATF-2 could act through the same element to mediate different responses. Thus a decrease in intracellular pH leads to activation of the α-isofrom of p38 MAPK that in turn phosphorylates and activates ATF-2. The activated ATF-2 then recruits the “auxiliary” factors and/or coactivators that are necessary for transcriptional activation of the PEPCK gene. Similar studies of tumor necrosis factor receptor 1 signaling (3) demonstrated that autoregulation of TNF-α gene expression is also mediated through the p38-dependent phosphorylation of ATF-2/Jun heterodimers that are bound to a CRE promoter element. The associated gel-shift analysis using nuclear extracts from unstimulated or rhTNF-α-stimulated L929 cells again demonstrated no differences in ATF-2 binding. Thus the binding of ATF-2 to a CRE element is not affected by phosphorylation.

Recent experiments suggest that additional cis-acting elements either upstream (10) or downstream (9) of the proximal promoter of the PEPCK gene may be necessary for the pH-responsive induction of PEPCK mRNA transcription. Thus additional experiments will be required to identify and characterize the additional elements and to further test this hypothesis.


REFERENCES


