Cloning of Porcine 25-Hydroxyvitamin D₃ 1α-Hydroxylase and Its Regulation by cAMP in LLC-PK₁ Cells

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Abstract. The 25-hydroxyvitamin D₃ 1α-hydroxylase, also referred to as CYP27B1, is a mitochondrial cytochrome P450 enzyme that catalyzes the biosynthesis of 1α, 25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) from 25-hydroxyvitamin D₃ in renal proximal tubular cells. Recently, human, mouse, and rat CYP27B1 cDNA have been cloned, however the gene regulation has not been fully elucidated. In the present study, porcine CYP27B1 cDNA was cloned, and the effects of cAMP and vitamin D₃ on the regulation of CYP27B1 mRNA expression in LLC-PK₁ cells were examined. PCR cloning revealed that porcine CYP27B1 cDNA consisted of 2316 bp, encoding a protein of 504 amino acids. The deduced amino acid sequence showed over 80% identity to the human, mouse, and rat enzyme. LLC-PK₁ cells were incubated with humoral factors, and expression of CYP27B1 mRNA was measured by a quantitative reverse transcription-PCR. At the completion of 3-, 6-, 12-, and 24-h incubations, 500 μmol/L 8-bromo-cAMP had significantly increased CYP27B1 mRNA expression (260 to 340%). The adenylyl cyclase activator forskolin at 50 μmol/L also had a stimulatory effect at 6 h (190%). Moreover, the protein kinase A inhibitor H-89 reduced the cAMP effect. On the other hand, 1α,25(OH)₂D₃ had no effect on CYP27B1 mRNA expression at 10 and 100 nmol/L, whereas expression of 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24) mRNA was markedly increased by 1α,25(OH)₂D₃. These findings suggest that LLC-PK₁ cells express CYP27B1 mRNA, and that cAMP is an upregulating factor of the CYP27B1 gene in vitro.

The physiologically active form of vitamin D₃, 1α, 25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), plays an important role in calcium metabolism, bone growth, and cell differentiation. The biosynthesis of 1α,25(OH)₂D₃ from 25-hydroxyvitamin D₃ (25(OH)D₃) is catalyzed by 25-hydroxyvitamin D₃ 1α-hydroxylase, a mitochondrial cytochrome P450 enzyme in renal proximal tubular cells (1–3). The level of 1α,25(OH)₂D₃ is primarily controlled by this enzyme rather than the other enzymes involved in vitamin D₃ metabolism. In 1997, we and other investigators succeeded in molecular cloning of rat, mouse, and human 25-hydroxyvitamin D₃ 1α-hydroxylase cDNA (4–8), which were later assigned the name CYP27B1 by the Cytochrome P450 Gene Superfamily Nomenclature Committee. Vitamin D dependency rickets type I is an autosomal recessive disorder characterized by early onset of hypocalcemia, secondary hyperparathyroidism, and severe rachitic lesions (9). It is known to be caused by impaired CYP27B1 activity in the kidney (10). Recently, molecular defects in the CYP27B1 gene have been identified in one American, four Japanese, and four French-Canadian patients (8,11,12).

The enzymatic activity of CYP27B1 is regulated by a number of physiologic factors, such as parathyroid hormone (PTH), 1α,25(OH)₂D₃, calcitonin, insulin-like growth factor-I, calcium, and phosphorus (13–19). Numerous reports have demonstrated that PTH is the most potent upregulator of CYP27B1 activity (13,14). PTH exerts its effects through binding to PTH/PTH-related peptide (PTHrP) receptor and then activating the protein kinase A (PKA) and/or protein kinase C pathway (20). It has been demonstrated previously that the effect of PTH on the CYP27B1 activity is due to activation of the PKA pathway (14). On the other hand, 1α,25(OH)₂D₃ is a downregulator of its own synthesis (15). In general, 1α,25(OH)₂D₃ alters transcriptional activity by binding to vitamin D receptor in target cells. However, Brenza et al. (21), who cloned the 5′ flanking region of mouse cyp27b1 gene, reported that 1α,25(OH)₂D₃ did not suppress basal promoter activity of its gene by luciferase assay. The precise regulation of the CYP27B1 gene has not yet been clarified.

In the present study, to investigate the humoral regulation of the CYP27B1 gene in vitro, we first cloned porcine CYP27B1 cDNA in LLC-PK₁ cells. Although LLC-PK₁ cells retained many functional characteristics of renal proximal tubular cells, they were not responsive to PTH because they lack the PTH/PTHrP receptor (22,23). We therefore examined the effects of cAMP, a second messenger of PTH, and 1α,25(OH)₂D₃ on regulation of CYP27B1 mRNA expression in these cells.
Materials and Methods

Reverse Transcription-PCR

Total RNA was isolated from LLC-PK₁ cells (American Type Culture Collection, Rockville, MD) by acid guanidinium thiocyanate-phenol-chloroform extraction (24). Approximately 1 μg of total RNA was reverse-transcribed by random hexamer (GeneAmp RNA PCR Core kit; Applied Biosystems, Foster City, CA). PCR was performed by using several pairs of primers (Table 1 and Figure 1). The resulting PCR products of predicted size were extracted and subcloned.

Isolation of the cDNA Ends

To obtain the 3'-cDNA ends, 1 μg of total RNA from LLC-PK₁ cells was reverse-transcribed with adapter primer using a rapid amplification of 3'-cDNA ends (3'-RACE) system (Life Technologies BRL, Grand Island, NY) according to the protocol. PCR was carried out with 1A-H-52 primer (Table 1 and Figure 1) and universal amplification primer.

Rapid amplification of 5'-cDNA ends (5'-RACE) was performed using a 5'-RACE system (Life Technologies BRL). First-strand cDNA was synthesized using 1A-H-51 primer and 1 μg of total RNA from LLC-PK₁ cells. The 5' end of cDNA was amplified with 1A-H-53 primer and 5'-RACE abridged anchor primer followed by nested PCR with 1A-H-54 primer and abridged universal amplification primer.

DNA Sequencing

The PCR products were ligated into pT7-Blue T vector (Novagen, Madison, WI) and subcloned. Nucleotide sequences were determined with an ABI DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on DNA sequencing systems (model 310; Applied Biosystems). All sequences were confirmed by reading both DNA strands of more than four independent clones.

Cell Culture

LLC-PK₁ cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. When cells were at 70 to 80% confluence, LLC-PK₁ cells were maintained for 12 h in serum-free Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin. Then humoral factors were added to the medium and incubation was continued for 3, 6, 12, and 24 h. The humoral factors used were 5, 50, and 500 μmol/L 8-bromo-cAMP, 50 μmol/L forskolin, 10 μmol/L H-89, 10 and 100 nmol/L 1α,25(OH)₂D₃, 100 nmol/L 25(OH)D₃, and 100 nmol/L 24,25(OH)₂D₃. After treatment with the humoral factors, total RNA was extracted.

Quantitative Reverse Transcription-PCR

Expression of CYP27B1 mRNA was measured by the quantitative reverse transcription (RT)-PCR/Southern blot technique described previously (25). Briefly, first-strand cDNA was synthesized from 1 μg of total RNA using random hexamers. After an initial denaturation for 3 min at 93°C, 26 cycles of PCR were performed for CYP27B1 with 1A-H-61 and 1A-H-62 primers, and 18 cycles for β-actin with β-actin-04 and β-actin-05 primers (Table 1). The preliminary experiments showed linear relationship between PCR products and PCR cycles around these cycles. Each cycle was programmed as follows: 1-min denaturation at 93°C, 2-min annealing at 67°C or 64°C (for CYP27B1 and β-actin, respectively), and 2-min extension at 72°C. No products were detected when RT was performed in the absence of reverse transcriptase.

Table 1. Oligonucleotide primers used in this experiment

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Sense or Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A-H-41</td>
<td>5'-TTCTGCAAGGGGGGGCTGTC-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-42</td>
<td>5'-CAGCTGTGATCTCTGATGGG-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>1A-H-43</td>
<td>5'-GGGGCTCTGGGTTTGTGTC-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-44</td>
<td>5'-AGCTGGACGAAAGAATTTGGCCT-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>1A-H-51</td>
<td>5'-AGCCAGCTGGGCGATCGCATGG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-52</td>
<td>5'-TATGCCACTTCAAGGGACCC-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-53</td>
<td>5'-TGGACACAGTCTCAAGGGG-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>1A-H-54</td>
<td>5'-ACTGCGGAGCCTCTGCAATCTC-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>1A-H-61</td>
<td>5'-TGCCCGCATCTGGGACCAGATG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-62</td>
<td>5'-AGGAGGATGTGTTTGGCACACCG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>1A-H-63</td>
<td>5'-AGCGAGGAAAAGCCTGAGGAG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>β-actin-04</td>
<td>5'-TGGAAGAAGAGCTACAGCGACTG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>β-actin-05</td>
<td>5'-ACGTCACTATGAGTTTGGAAGG-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>β-actin-06</td>
<td>5'-AGGTCACTACCATCGGGCAACC-3'</td>
<td>Sense</td>
</tr>
<tr>
<td>24-H-03</td>
<td>5'-ATGAGAGAGATTGGGCTCCATCC-3'</td>
<td>Sense</td>
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<tr>
<td>24-H-04</td>
<td>5'-GTCACAGAGCGATCATATCCACC-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>24-H-05</td>
<td>5'-ACCTCAACACCAAGGTCTGCGG-3'</td>
<td>Sense</td>
</tr>
</tbody>
</table>
Analysis of the PCR Products

The PCR products specific for CYP27B1 and β-actin were combined and subjected to electrophoresis on a 3% agarose gel. For Southern blot analysis, gels were blotted onto Hybond N+ nylon membranes (Amersham Corp., Arlington Heights, IL), and the membranes were hybridized with 32P-labeled CYP27B1-specific 1A-H-63 probe. After washing, membranes were exposed to imaging plates and analyzed (BAS2000; Fuji Film Institution, Tokyo, Japan). To quantify the mRNA expression for CYP27B1, the membranes were rehybridized with 32P-labeled β-actin-06 probe. We calculated the ratio of CYP27B1 to β-actin from each sample.

Statistical Analyses

Data are presented as means ± SEM. Statistical analyses were performed by one-way ANOVA with a post hoc Fisher protected least significant difference test. P values < 0.05 were considered significant.

Results

Cloning of Porcine CYP27B1 cDNA in LLC-PK1 Cells

We and other investigators have recently succeeded in the cloning of rat, mouse, and human CYP27B1 cDNA (4–8,11), and these clones showed high homology with one another. We performed RT-PCR with oligonucleotide primers in which the sequences were relatively conserved in these species to obtain partial sequences of porcine CYP27B1 cDNA. RT-PCR of total RNA from LLC-PK1 cells yielded an 889-bp and a 621-bp sequence (RT-PCR-1 and RT-PCR-2, respectively) (Figure 1), and they showed significant homology with human, rat, and mouse CYP27B1 cDNA. We then performed 3'9-RACE and 5'9-RACE to clone the full length of its cDNA. As shown in Figure 2, the nucleotide sequence of porcine CYP27B1 cDNA was 2316 bp in length and contained an open-reading frame of 1512 bp, starting at base 36 and ending at base 1547. The predicted protein was composed of 504 amino acids, and showed 88, 81, and 80% sequence identities to the human, rat, and mouse enzyme, respectively (Figure 3A). It was particularly noteworthy that the ferredoxin binding site at Lys-367, Lys-371, and Arg-375, and the heme-binding domain recognized from residues 446 to 464 were almost completely conserved among these species. On the contrary, the predicted protein had only 43% amino acid identity to vitamin D3 25-hydroxylase, and had less than 35% identity to other proteins including 25-hydroxyvitamin D3 24-hydroxylase (CYP24) (26). We therefore concluded that this is the porcine homologue of CYP27B1.
Effect of cAMP on CYP27B1 mRNA Expression
LLC-PK₁ cells were incubated with humoral factors, and expression of CYP27B1 mRNA was measured by the quantitative RT-PCR/Southern blot procedure. We first examined the time course of the effect of 8-bromo-cAMP on CYP27B1 mRNA expression. When cells were incubated with 500 μmol/L 8-bromo-cAMP, CYP27B1 mRNA expression was significantly increased at 3-, 6-, 12-, and 24-h incubations (294 ± 42, 342 ± 47, 314 ± 53, and 261 ± 76%, respectively, P < 0.05) (Figure 4, A and C). The maximal induction of CYP27B1 mRNA level by 8-bromo-cAMP was seen at 6 h. To confirm the effect of cAMP on CYP27B1 gene expression, we examined whether the effect of 8-bromo-cAMP was reversible. LLC-PK₁ cells were incubated with 500 μmol/L 8-bromo-cAMP for 6 h, and then medium was changed to the medium without 8-bromo-cAMP (●) or unchanged (○). An arrow indicates the time the medium was changed. An arbitrary value of 100 was assigned to the sample before treatment (control). Values represent means ± SEM of four independent experiments. *P < 0.05 compared with control. P < 0.05) (Figure 4, A and C). The maximal induction of CYP27B1 mRNA level by 8-bromo-cAMP was seen at 6 h. To confirm the effect of cAMP on CYP27B1 gene expression, we examined whether the effect of 8-bromo-cAMP was reversible. LLC-PK₁ cells were incubated with 500 μmol/L 8-bromo-cAMP for 6 h, and then medium was changed into that without 8-bromo-cAMP (●) or unchanged (○). An arrow indicates the time the medium was changed. An arbitrary value of 100 was assigned to the sample before treatment (control). Values represent means ± SEM of four independent experiments. *P < 0.05 compared with control. P < 0.05) (Figure 4, A and C). The maximal induction of CYP27B1 mRNA level by 8-bromo-cAMP was seen at 6 h. To confirm the effect of cAMP on CYP27B1 gene expression, we examined whether the effect of 8-bromo-cAMP was reversible. LLC-PK₁ cells were incubated with 500 μmol/L 8-bromo-cAMP for 6 h, and then medium was changed into that without 8-bromo-cAMP (●) or unchanged (○). An arrow indicates the time the medium was changed. An arbitrary value of 100 was assigned to the sample before treatment (control). Values represent means ± SEM of four independent experiments. *P < 0.05 compared with control. P < 0.05) (Figure 4, A and C). The maximal induction of CYP27B1 mRNA level by 8-bromo-cAMP was seen at 6 h. To confirm the effect of cAMP on CYP27B1 gene expression, we examined whether the effect of 8-bromo-cAMP was reversible. LLC-PK₁ cells were incubated with 500 μmol/L 8-bromo-cAMP for 6 h, and then medium was changed into that without 8-bromo-cAMP (●) or unchanged (○). An arrow indicates the time the medium was changed. An arbitrary value of 100 was assigned to the sample before treatment (control). Values represent means ± SEM of four independent experiments. *P < 0.05 compared with control.
cAMP generally occurs through the activation of PKA. We therefore examined the effect of the PKA inhibitor H-89 on cAMP-induced CYP27B1 mRNA expression. Combined treatment with 10 \( \mu \text{mol/L} \) H-89 and 500 \( \mu \text{mol/L} \) 8-bromo-cAMP significantly reduced CYP27B1 mRNA levels (202 ± 10\%) compared to treatment with 500 \( \mu \text{mol/L} \) 8-bromo-cAMP only (Figure 6C).

**Effect of Vitamin D\(_3\) on CYP27B1 mRNA Expression**

We next examined the effects of vitamin D\(_3\) metabolites on CYP27B1 mRNA expression in LLC-PK\(_1\) cells. As shown in Figure 4B, 10 nmol/L 1\(\alpha\),25(OH)\(_2\)D\(_3\), the active form of vitamin D\(_3\), had no effect on CYP27B1 mRNA expression after 3-, 6-, 12-, and 24-h incubations. Treatment with other vitamin D\(_3\) metabolites, 100 nmol/L 25(OH)D\(_3\) and 100 nmol/L 24,25(OH)\(_2\)D\(_3\), did not alter the CYP27B1 mRNA levels, nor did 10 and 100 nmol/L 1\(\alpha\),25(OH)\(_2\)D\(_3\) (Figure 7). We also measured the level of CYP24 mRNA in LLC-PK\(_1\) cells. Partial sequence of porcine CYP24 cDNA was cloned, and deduced amino acid sequence was shown in Figure 3B. The quantitative RT-PCR was performed using porcine CYP24-specific 24-H-03 and 24-H-04 primers, followed by Southern blotting with 24-H-05 oligonucleotide probe (Table 1). Treatment with 10 and 100 nmol/L 1\(\alpha\),25(OH)\(_2\)D\(_3\) markedly stimulated CYP24 mRNA expression, and 100 nmol/L 25(OH)D\(_3\) had a weak stimulatory effect on CYP24 mRNA expression, but 100 nmol/L 24,25(OH)\(_2\)D\(_3\) failed to stimulate expression at all (Figure 7).

**Combined Effect of cAMP and Vitamin D\(_3\) on CYP27B1 mRNA Expression**

The effect of coincubation with 8-bromo-cAMP and 1\(\alpha\),25(OH)\(_2\)D\(_3\) was examined. Although CYP27B1 mRNA expression was significantly increased by 500 \( \mu \text{mol/L} \) 8-bromo-cAMP treatment, combined treatment with 500 \( \mu \text{mol/L} \) 8-bromo-cAMP and 10 nmol/L 1\(\alpha\),25(OH)\(_2\)D\(_3\) did not reduce its expression (Figure 8). In physiologic conditions in which elevated levels of 1\(\alpha\),25(OH)\(_2\)D\(_3\) have been generated, the cAMP levels would tend to be low due to the suppression of PTH and due to the elevation of serum calcium. Hence, the effect of a combination of lower cAMP concentrations (5 and 50 \( \mu \text{mol/L} \) 8-bromo-cAMP) and 10 nmol/L 1\(\alpha\),25(OH)\(_2\)D\(_3\) was also examined. As shown in Figure 8, any combined treatment had no difference with cAMP treatment alone.

**Discussion**

PTH and 1\(\alpha\),25(OH)\(_2\)D\(_3\) are the two major physiologic factors involved in the regulation of 1\(\alpha\)-hydroxylase and 24-hydroxylase activities of 25(OH)D\(_3\) in the kidney (13–15,17,29). The mechanism of regulation of CYP27B1 has...
been studied only in terms of its enzyme activity, whereas the molecular mechanism of regulation of CYP24 gene has been clarified (17,29). Recent cloning of rat (4,5), mouse (6), and human (7,8,11) CYP27B1 cDNA has made it possible to investigate the molecular mechanism of regulation of the CYP27B1 gene. In this study, we have cloned porcine CYP27B1 cDNA, and demonstrated that the CYP27B1 mRNA level was significantly increased by cAMP, but unaffected by 1α,25(OH)₂D₃ in LLC-PK₁ cells.

LLC-PK₁ cells, originally isolated from an unknown site in the renal cortex of the Hampshire pig, retain many functional characteristics of renal proximal tubular cells (23). In a previous study, Condamine et al. demonstrated that LLC-PK₁ cells, but not opossum kidney cells, are able to produce 1α,25(OH)₂D₃ (18). They found that the production of 1α,25(OH)₂D₃ was stimulated by phosphate deprivation or insulin-like growth factor-I treatment of these cells. These findings are in agreement with the results of several studies on regulation of the enzymatic activity of CYP27B1 in vivo (19). On the basis of their report, we attempted to clone porcine CYP27B1 cDNA in LLC-PK₁ cells. The porcine CYP27B1 cDNA was approximately 2.3 kb long, and consistent with the size of the transcript from pig kidney tissue by Northern blot analysis (4). Moreover, porcine cDNA has extremely high homology to human CYP27B1 cDNA, and their ferredoxin binding site and heme-binding domain matched perfectly. Thus, we confirmed that this clone is the porcine homologue of CYP27B1 cDNA.

PTH has been shown to stimulate CYP27B1 enzyme activity in cultured avian and mammalian kidney cells (30,31). PTH increases intracellular cAMP levels by stimulating adenylate cyclase activity. The effect of PTH on CYP27B1 enzyme activity is mediated mainly by the cAMP signaling pathway (14). In this study, we have shown that the elevation of intracellular cAMP has a stimulatory effect on CYP27B1 mRNA expression. Both 8-bromo-cAMP and forskolin increased the CYP27B1 mRNA level in LLC-PK₁ cells. This effect of 8-bromo-cAMP was reversible and was blocked by the PKA inhibitor. Our observation is in agreement with the enzymatic data described previously (14), and shows that the elevation of CYP27B1 enzyme activity by cAMP was due to elevation of its mRNA level. In a promoter analysis of the mouse cyp27b1 gene, PTH markedly increased its promoter activity in AOK-B50 cells, a modified LLC-PK₁ cell line that expresses stably transfected PTH/PTHrP receptors (21,32). They also observed that stimulation of promoter activity by PTH could be mimicked by forskolin. Accordingly, cAMP-induced expression of CYP27B1 mRNA would be mostly a result of transcriptional activation rather than the reduction of degradation in this gene.

Although 1α,25(OH)₂D₃ is known to be a downregulator of the enzyme activity of CYP27B1 (15), our data failed to demonstrate that 1α,25(OH)₂D₃ had an inhibitory effect on the expression of CYP27B1 mRNA in LLC-PK₁ cells. The addition of 1α,25(OH)₂D₃ to 8-bromo-cAMP-treated cells did not reduce its mRNA level either. By contrast, 1α,25(OH)₂D₃
markedly increased CYP24 mRNA expression in LLC-PK₁ cells. This is in agreement with the previous data that 1α,25(OH)₂D₃ induces CYP24 mRNA levels in vivo (17). Our findings concerning CYP24 mRNA expression mean that the transactivation system by 1α,25(OH)₂D₃ was well conserved in these cells, whereas no transrepression system by 1α,25(OH)₂D₃ was demonstrated. In the transfection analysis by Brenza et al. (21), 1α,25(OH)₂D₃ also failed to suppress basal promoter activity of mouse cyp27b1 gene in AOK-B50 cells. Reduction of CYP27B1 enzyme activity by 1α,25(OH)₂D₃ itself may be a different mechanism from the direct suppression of CYP27B1 mRNA expression. Another possibility is that loss of transrepression in the CYP27B1 gene in response to 1α,25(OH)₂D₃ was specific to LLC-PK₁ cells. Several genes are negatively controlled at the transcription level by 1α,25(OH)₂D₃, and this transrepression apparently occurs by mechanisms different from the well known transactivation mechanism (33). An unknown cofactor required for negative regulation by 1α,25(OH)₂D₃ may be deficient in these cells. Additional studies will be needed to clarify this issue.

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References


