Stanniocalcin 2 is positively and negatively controlled by 1,25(OH)2D3 and PTH in renal proximal tubular cells

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Abstract

We have previously identified a second mammalian stanniocalcin (STC2) in humans and demonstrated that STC2 inhibits phosphate uptake in an opossum renal proximal tubular cell line (opossum kidney (OK) cells). However, the regulation of Stc2 gene expression in OK cells is not well understood. In this study, we identified the opossum Stc2 cDNA sequence. The opossum STC2 amino acid sequence had 78.8% homology with human STC2, and has a conserved putative N-linked glycosylation site. Next, we investigated the regulation of Stc2 gene expression by the classical calcium and phosphate-regulating factors 1,25(OH)2D3 and PTH in OK cells. In western blot analysis using affinity-purified anti-STC2 antibody, the secretion of STC2 protein was stimulated by 1,25(OH)2D3 in a dose-dependent manner. By contrast, PTH suppressed the induction of STC2 protein secretion by 1,25(OH)2D3. Real-time PCR analysis revealed that Stc2 mRNA expression was increased by 1,25(OH)2D3 in a dose- and time-dependent manner. In addition, actinomycin D, an RNA synthesis inhibitor, prevented the effects of 1,25(OH)2D3 on Stc2 mRNA expression by PTH. Furthermore, we demonstrated that the renal Stc2 mRNA expression was increased by 1,25(OH)2D3 and decreased by PTH in vivo. These results suggest that STC2 is positively and negatively controlled by 1,25(OH)2D3 and PTH in renal proximal tubular cells.

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Introduction

Inorganic phosphate plays an important role in bone metabolism, energy metabolism, growth, and intracellular signaling (Berner & Shike 1988, Takeda et al. 2004, Rigo et al. 2007). Phosphate homeostasis is controlled by the absorption of phosphate in the intestine and reabsorption in the kidney. The renal proximal tubule is a particularly important tissue in the regulation of serum phosphate levels (Takeda et al. 2004, Nemere 2007).

The serum level of phosphate is regulated by dietary phosphate, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], and PTH, and other factors (Nemere 2007). The type IIa sodium-dependent phosphate cotransporter (NPT2a), which is expressed in the renal proximal tubule, is the major molecule involved in the reabsorption of phosphate (Tenenhouse 2005). 1,25(OH)2D3 has been shown to promote the ability of phosphate uptake with the renal brush border membrane and to directly increase the transcriptional activity of the human and mouse NPT2a genes (Taketani et al. 1998, Yamamoto et al. 2005). By contrast, PTH has been shown to rapidly attenuate NPT2a activity by stimulating apical endocytosis (Murer et al. 1999, Nashiki et al. 2005). In addition, it is well known that 1,25(OH)2D3 suppresses PTH secretion from the parathyroid gland (Beckerman & Silver 1999) and that PTH increases the serum levels of 1,25(OH)2D3 by activating cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1; Brown et al. 1999). Because these phosphate-regulating hormones are regulated by each other through a feedback loop, serum phosphate levels are tightly regulated (Barthel et al. 2007).

Stanniocalcin is a glycoprotein hormone first identified from the corpuscles of Stannius in bony fish (Wagner et al. 1986, Flik et al. 1990). Fish stanniocalcin (STC) acts on gill, gut and kidney as a calcium phosphate-regulating hormone (Lafeber et al. 1988, Sundell et al. 1992, Lu et al. 1994). The related mammalian gene, human STC1, was initially isolated based on its sequence homology to fish STC (Chang et al. 1995). We have previously identified human STC2, a paralogous gene of STC1, and observed its inhibition of the promoter activity of human NPT2a and phosphate uptake in an opossum renal proximal tubular cell line (opossum kidney (OK) cells). We also observed that Stc2 mRNA expression was decreased in the kidney and other
tissues of the hypophosphatemic (Hyp) mouse, which is a model of human X-linked hypophosphatemic (vitamin D-resistant) rickets (XLH; Ishibashi et al. 1998). Yahata et al. (2003) have reported that Stc2 mRNA expression is increased in the kidneys of precocious aging (klotho mutant) mice. Klotho mutant mice exhibit hypervitaminosis D, hypercalcemia, and hyperphosphatemia (Kuro-o et al. 1997, Yoshida et al. 2002). However, Honda et al. (1999) have documented that renal Stc2 mRNA expression is reduced by calcitriol, the active form of vitamin D₃, in rats. Thus, the regulatory mechanisms of renal Stc2 gene expression by 1,25(OH)₂D₃ are still unclear.

In the present study, we identified the cDNA sequence of opossum STC2 and investigated the regulation of Stc2 gene expression by 1,25(OH)₂D₃ and PTH in renal proximal tubular cells.

Materials and methods

Materials

1,25(OH)₂D₃ was obtained from Solvay Pharmaceuticals (Marietta, GA, USA). 22-oxacalcitriol (OCT) and 2β-(3-hydroxypropoxy) calcitriol (ED-71) were kindly provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). Human parathyroid hormone (1–34 fragments) PTH, phorbol 12,13-myristic acetate (PMA), 8-bromo-cyclic AMP (8-bromo-cAMP) and actinomycin D (ActD) were purchased from Sigma-Aldrich. G66976 was purchased from Calbiochem (San Diego, CA, USA).

Cell culture

OK cells were maintained in DMEM (Sigma) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in an incubator with 5% CO₂.

cDNA isolation and DNA sequence analysis

Opossum Stc2 gene information was obtained from a BLAST search on the UCSC Genome Bioinformatics website (http://www.genome.ucsc.edu/) with the amino acid sequence of human STC2. On the basis of this information, oligonucleotide primers specific to the STC2 cDNA sequence were synthesized. The sequences of the upstream and downstream primers were 5'-CCAATGTTGTTGGAGCCGTTCAAGCG-3', and 5'-TCACCTCCGGATATCTGAAATCTCAGC-3' respectively. Opossum STC2 cDNA containing an open reading frame was amplified by RT-PCR using total RNA isolated from OK cells. The amplification products were purified and subcloned into a pGEM-T Easy vector (Promega), and manually sequenced using the dye terminator cycle sequencing method (Applied Biosystems, Foster City, CA, USA). The cDNA sequences of the opossum Stc2 gene have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under the accession number AB462027.

Antibody purification

Using a 12-amino-acid fragment (residues 291–302: EDEQSEYSIDIR) of the human STC2 protein deduced from the corresponding gene sequence, we designed an antigenic peptide. This peptide was added to the cysteine residues to permit coupling of the peptides to keyhole limpet hemocyanin (KLH) as a carrier protein for immunization. Rabbits were immunized by repeated s.c. injection of the KLH-coupled peptides emulsified in complete Freund’s adjuvant on days 1 and 14 and in incomplete Freund’s adjuvant on days 21 and 28. For the affinity purification by a gel column (Cellulofine; Seikagaku-Kogyo, Tokyo, Japan), the antiserum (10 ml) was loaded on the gel coupled with the respective peptide, and the antibodies were eluted with 0.1 mol/l glycine–HCl (pH 2.5), neutralized with 1.0 mol/l Tris, dialyzed into 0.02 mol/l PBS (pH 7.4), and stored at −80 °C.

Immunocytochemical analysis

OK cells grown in plastic cover slips were fixed with 3% (wt/vol) paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 in PBS on ice for 10 min. After blocking with 0.8% BSA in PBS for 1 h, the cells were incubated with anti-STC2 rabbit antibody and a mouse antibody against the cis-Golgi matrix marker protein GM130 (BD Transduction Laboratories, Lexington, KY, USA) for 1 h after which they were washed and labeled with Alexa 488-labeled anti-rabbit IgG (Molecular Probe, Eugene, OR, USA) and Alexa 555-labeled anti-mouse IgG (Molecular Probe). The cells were then washed with Aqua/poly mount and mounted on glass slides (PolyScience, Niles, IL, USA). Slides were analyzed and photographed using a Leica confocal microscope (TCS-SL) (Leica Microsystems GmbH, Mannheim) in the sequential mode.

Western blot analysis

For the analysis of the secreted protein, the conditioned medium was centrifuged at 1500 g for 10 min at 4 °C. The supernatant was concentrated by acetone precipitation. Total kidney protein was prepared by standard procedures (Sambrook et al. 1989). Briefly, the whole kidney was homogenized in lysis buffer containing 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 10 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 mM K⁺.
iodoacetamide. Proteins were electrophoresed on 12% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat dried milk in PBS plus 0.05% Tween 20 (PBS-t) at room temperature for 1 h, membranes were incubated with affinity-purified anti-STC2 antibody in 1% nonfat dried milk in PBS-t. The membranes were washed with PBS-t and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibody at room temperature for 1 h. After washing with PBS-t, the membranes were developed using an ECL plus western blotting system (GE Healthcare, Buckinghamshire, UK).

**RT-PCR and real-time PCR analysis**

Total RNA was extracted with TRIzol reagent (Invitrogen) and then dissolved in RNase-free water. First-strand cDNA was synthesized from 2–5 μg of total RNA using a first-strand cDNA synthesis kit (Invitrogen). Two microliters of the cDNA were then used for quantitative PCR, with 20–30 cycles of amplification, and the PCR products were then separated by electrophoresis using 2% agarose gels. For PCR amplification, the primer sequences were: opossum STC2, 5’-ATGCCCT-GAATGTTAAGCCCATG-3’ and 5’-CACGTAGGTT-CATGCAGTGAGCATGCT-3’; opossum and mouse β-actin, 5’-CTGACCCCTGAGTACCACATGACATC-3’ and 5’-CTGGGTGGTTGAAGGTCTCAGAATGCT-3’; mouse STC2, 5’-AGCCCAAGGAGACCCTCGGTTG-ATTG-3’ and 5’-CAGCAGCAGGTCAAGTCAGTCA-3’; mouse CYP27B1, 5’-ATGGTGAAGAA- TGGCAGAGG-3’ and 5’-TAGTCGCTGGACCACT-3’; rat STC2, 5’-TACCAGTTGACAGCTGGGATG-3’ and 5’-TTAAGGTTCAAGCTGAGG-3’; rat β-actin, 5’-GTCCCATATGCTCTGGTGCTGATC-3’ and 5’-CCAGG-TCTGCTGGACAGATCTG-3’; and rat CYP27B1, 5’-CAAGCAGCAGGTCAAGTCAGTCA-3’ and 5’-TGCAACTTCGTTGCGCCGAC-3’.

**Figure 1** (A) Identification of opossum STC2 and its localization in opossum kidney (OK) cells. Opossum Stc2 cDNA was identified using information obtained from the UCSC genome database website. The opossum STC2 and human STC2 sequences were then aligned. The underlined sequence indicates the predicted signal peptide region. The histidine cluster (HHxxxxHH) is double-underlined. The conserved cysteine residues are indicated by dots. The N-glycosylation site is shown as a closed triangle. The box shows the recognition peptide region of the STC2-specific antibody. (B) OK cells were fixed and double immunolabeled with antibodies specific for STC2 (green) and GM130 (red). Yellow signals are indicative of colocalization. Images were taken by confocal laser scanning microscopy. The data presented are from three independent experiments.
system (ASTEC, Fukuoka, Japan) using GoTaq Master Mix (Promega). Real-time PCR analysis was performed using the LightCycler (Roche Diagnostics). The prepared first-strand cDNA was PCR amplified using SYBR Premix ExTaq (Takara Bio Inc., Shiga, Japan) in a 20 μl reaction volume, with 4 pmol of each primer. β-actin was used as the internal control. The amplification program was 95°C for 10 s followed by 50 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Amplified products were then analyzed using a melting curve, which confirmed the presence of a single PCR product in all reactions (apart from the negative control). The PCR products were quantified by fit point analysis, and results for STC2 and CYP27B1 were normalized to those of β-actin.

**Experimental animals**

Eight-week-old C57BL/6J male mice and 6-week-old Sprague–Dawley (SD) male rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice and rats were maintained with 12h light:12h darkness cycles with free access to regular mouse or rat chow and water under pathogen-free conditions. The breeding and handling of all animals in these experiments was approved by the Animal Experimentation Committee of the University of Tokushima. Mice were administered 1,25(OH)₂D₃ (0.2 μg/mouse) orally. After 7 h, the mice were killed. Rats were fed a low-phosphate diet (0.6% calcium, 0.02% P) for 3 days (Segawa et al. 2002). Then, a bolus of PTH (10 μg/100 g body weight) was injected into the jugular vein. After 0·5 h, the rats were killed.

**Statistical analysis**

Data are expressed as means ± s.d. Statistical significance between the groups was determined using ANOVA followed by post hoc testing using Fisher’s protected least significant difference. P<0·05 was considered to be significant.

**Results**

**Identification of opossum STC2**

We obtained the nucleotide sequence of opossum STC2 from the UCSC genome database website. We then identified the opossum Stc2 cDNA. From the cDNA sequence, we determined both the opossum and human STC2 proteins to be 302 amino acids long. Seventy-eight percent of opossum STC2 was homologous to human STC2 (Fig. 1A). Opossum STC2 had 14 conserved cysteine residues and the same putative N-linked glycosylation site seen in the human gene. However, the C-terminus of the opossum STC2 sequence did not have a cluster of histidines (HHxxxxHH), capable of interacting with divalent metal ions as seen in human STC2 (Moore et al. 1999, Ishibashi & Imai 2002). The specificity of the affinity-purified anti-STC2 antibody was confirmed by western blot analysis and a peptide absorption test (Data not shown). Immunofluorescence analysis was performed to localize the STC2 in OK cells. GM130 has been well characterized as the cis-Golgi matrix maker protein. When cells were stained with anti-STC2 antibody and anti-GM130 antibody, STC2 expression was partially localized to the cis-Golgi matrix, although cells exhibited significant foci of STC2 staining within the cytoplasm (Fig. 1B).

**Regulation of STC2 protein secretion by 1,25(OH)₂D₃ and PTH**

We next investigated the regulation of STC2 protein secretion by 1,25(OH)₂D₃ and PTH in OK cells. As shown in Fig. 2A, the secretion of STC2 protein was...
increased by 1,25(OH)₂D₃ in a dose-dependent manner. In a time course study, secreted STC2 protein was detected after 6 h and beyond (Fig. 2B). Because OCT and ED-71 are known as cell-selective or non-calcemic vitamin D analogs (Murayama et al. 1986, Miyamoto et al. 1993), the possibility that these may uniquely act on the STC2 secretion was examined. In the OK cells, STC2 secretion was also induced by OCT and ED-71, but the magnitude of response was less than that induced by 1,25(OH)₂D₃ (Fig. 2C). Interestingly, PTH markedly suppressed the induction of STC2 protein secretion by 1,25(OH)₂D₃ (Fig. 2D).

**Upregulation of Stc2 mRNA expression by 1,25(OH)₂D₃**

We then determined whether the expression of Stc2 mRNA is affected by 1,25(OH)₂D₃ in OK cells. RT-PCR analysis revealed that Stc2 mRNA levels were increased by 1,25(OH)₂D₃ in a dose-dependent manner (Fig. 3A). In a time course study of 1,25(OH)₂D₃, upregulation of Stc2 mRNA expression was evident at 3 h and reached its peak at 12 h, with a 24-fold increase at that time (Fig. 3B). In addition, OCT and ED-71 were observed to stimulate Stc2 mRNA expression (Fig. 3C). In order to investigate whether upregulation of Stc2 mRNA expression by 1,25(OH)₂D₃ is a result of transcriptional control, we looked at the effects of ActD on STC2 expression. Figure 3D shows that pretreatment with ActD suppressed 1,25(OH)₂D₃-induced Stc2 mRNA expression.

**Downregulation of Stc2 mRNA expression by PTH**

We next investigated the effects of PTH on STC2 expression over multiple doses and times. As shown in Fig. 4A, Stc2 mRNA expression was decreased by PTH in a dose-dependent fashion. In a time course study, PTH reduced Stc2 mRNA expression by 50% at 3 h, and expression returned to basal levels at 12 h (Fig. 4B). To determine whether the inhibitory effect of PTH on Stc2 mRNA expression is mediated by protein kinase A (PKA) and/or protein kinase C (PKC) activation, cells were treated with 8-bromo-cAMP, a specific PKA activator, or PMA, a specific PKC activator. Figure 4C shows that Stc2 mRNA expression was significantly (<40%) decreased by PMA but not affected by 8-bromo-cAMP. In addition, G06976, a specific PKC inhibitor, completely blocked the inhibition of Stc2 mRNA expression by PTH (Fig. 4D).

**Regulation of Stc2 mRNA expression by 1,25(OH)₂D₃ and PTH in vivo**

Finally, we confirmed the effects of 1,25(OH)₂D₃ and PTH on renal Stc2 mRNA expression in vivo. We also
investigated the effects of 1,25(OH)₂D₃ and PTH on renal Cyp27b1 mRNA expression. Figure 5A shows that 1,25(OH)₂D₃ administration in mice significantly increased renal mRNA levels of Stc2 by 160% and decreased renal Cyp27b1 mRNA levels by 5%, compared with control vehicle mice. It has been reported that serum PTH protein and mRNA levels are decreased by a low-phosphate diet (Kilav et al. 1995). Therefore, we fed rats a low-phosphate diet for 3 days, followed by treatment with PTH. After 0·5 h, renal mRNA levels of Stc2 and Cyp27b1 were decreased by 50% and increased by 230%, respectively, in PTH-treated rats compared with PTH-untreated vehicle rats (Fig. 5B). In addition, western blot analysis showed that PTH reduced the protein levels of STC2 in kidney (Fig. 5C).

Discussion

Stanniocalcin is a glycoprotein that maintains the intracellular calcium and phosphate homeostasis (Wagner et al. 1986, Lafeber et al. 1988). In the present study, we have identified opossum STC2 cDNA sequences including the transcriptional start site and coding region. The amino acid sequences of opossum STC2 showed 78% homology to human STC2. The opossum STC2 sequence shows a conserved N-glycosylation site seen in the human sequence. Indeed, secreted opossum STC2 was detected as two bands of 35 and 39 kDa by western blot analysis (Fig. 2). Treatment with N-glycosidase, which removes all N-linked sugar moieties of glycoproteins, showed that these bands were shifted down (data not shown). Moore et al. (1999) have reported that a cluster of four to five histidines in the C-terminal region of STC2 was retained not only in humans but also in the mouse, hamster and macaque genes. Interestingly, the opossum STC2 sequence did not include the cluster of histidines, which might associate with a divalent metal ion. Although the function of the C-terminal region of STC2 has not been clarified, this indicates that some functions of the opossum STC2 might be different from those of other mammalian STC2s. McCudden et al. (2002) have reported that high-affinity mouse STC1-binding sites and/or receptors are present on both the plasma membrane and mitochondria in nephron epithelial cells and liver hepatocytes, and suggested that STC2 interacts with a different receptor. However, high-affinity STC2 receptor and its signaling pathway have not been established. Because we have observed that STC2 inhibits the NPT2a promoter activity and phosphate uptake in OK cells, the great discovery of STC2-specific receptor and its signaling pathway may be accomplished by the luciferase reporter assay using the NPT2a gene promoter or the phosphate uptake assay.
$1,25(OH)_2D_3$ and PTH play a critical role in calcium and phosphate homeostasis in the kidney (Beckerman & Silver 1999, Brown et al. 1999). We first examined the effects of $1,25(OH)_2D_3$ and PTH on the secretion of STC2 protein in OK cells. Western blot analysis indicated that secretion of STC2 protein was increased by $1,25(OH)_2D_3$; however, its secretion was strongly suppressed by PTH. In accordance with our findings regarding STC2 protein secretion, Stc2 mRNA expression was dose- and time-dependently increased by $1,25(OH)_2D_3$. Furthermore, the increase in Stc2 mRNA in response to $1,25(OH)_2D_3$ was completely blocked by actinomycin D. These data suggest that the induction of STC2 protein secretion by $1,25(OH)_2D_3$ is mediated by transcriptional control. Contrary to our findings with $1,25(OH)_2D_3$, PTH significantly suppressed Stc2 mRNA expression. To determine the inhibitory mechanism of PTH on STC2 expression, we examined the signaling pathways of PKA and PKC. Using PMA, 8-bromo-cAMP, and Go¨ 6976, we revealed that activation of the PKC pathway by PTH is an important negative regulator of STC2 expression. These data suggest that PTH suppresses the $1,25(OH)_2D_3$-induced STC2 secretion by PKC-mediated downregulation of its mRNA expression. Finally, we confirmed that Stc2 mRNA expression was regulated by $1,25(OH)_2D_3$ and PTH in vivo. In mice, which were orally administered $1,25(OH)_2D_3$, renal Stc2 mRNA expression was significantly increased. Furthermore, in the kidneys of rats injected with PTH, Stc2 mRNA and protein expression was rapidly reduced (Fig. 5). In addition, the plasma levels of phosphate, not calcium, were increased by $1,25(OH)_2D_3$, but PTH did not change plasma calcium and phosphate levels (data not shown). Because previous studies using klotho mutant mice have suggested that STC2 expression is positively regulated by blood phosphate concentration (Yahata et al. 2003), the renal Stc2 gene expression might be directly and indirectly regulated by $1,25(OH)_2D_3$. Thus, renal Stc2 mRNA expression was strictly controlled by not only $1,25(OH)_2D_3$ but also PTH. Therefore, our data might explain the discrepancy between the previous reports showing that vitamin D enhanced (Yahata et al. 2003) or suppressed renal Stc2 gene expression (Honda et al. 1999).

In conclusion, we have identified a novel STC2 amino acid sequence in opossum and demonstrated that STC2 secretion is positively and negatively regulated by $1,25(OH)_2D_3$ and PTH in OK cells. Furthermore, we have shown that Stc2 mRNA expression is controlled by $1,25(OH)_2D_3$ and PTH in vitro and in vivo.

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.
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