Cellular Signalling 22 (2010) 543–552

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

A role for the phosphatidylinositol 3-kinase – protein kinase C zeta – Sp1 pathway in the 1,25-dihydroxyvitamin D3 induction of the 25-hydroxyvitamin D3 24-hydroxylase gene in human kidney cells

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ARTICLE INFO

Article history:
Received 16 March 2009
Received in revised form 9 October 2009
Accepted 10 November 2009
Available online 14 November 2009

Keywords:
Vitamin D
Gene regulation
CYP24 promoter
GC box
HEK293T cells

ABSTRACT

The molecular mechanisms that underlie non-genomic induction of the 25-hydroxyvitamin D3 24-hydroxylase (*CYP24*) gene promoter by the steroid hormone, 1,25-Dihydroxyvitamin D3 (1,25D), are poorly understood. Although we have previously identified a functional inverted GC-box in the early promoter at −113/−105 bp, it is not known whether this site is important for 1,25D induction of the promoter. Using transfected human embryonic kidney (HEK) 293 T cells, we now report the functional characterisation of the GC-box and that 1,25D induction of the promoter requires PI3-kinase, PKCζ and Sp1 but not Sp3. The data show that 1,25D rapidly stimulates PI3-kinase activity which is required for the activation of PKCζ and the phosphorylation of Sp1. The effects of the PI3-kinase inhibitor, LY294002, and a dominant negative PKCζ mutant on 1,25D induction of wild-type and a GC-box mutated *CYP24* promoter constructs are consistent with the Sp1 site being the target of both kinases. However, these kinases are not required for basal expression of the *CYP24* promoter. The data establish a novel non-genomic mechanism which couples 1,25D to the induction of *CYP24* gene transcription via the PI3-kinase – PKCζ – Sp1 pathway acting through the GC-box.

1. Introduction

1,25-Dihydroxyvitamin D3 (1,25D) is the physiologically active form of vitamin D_3 that classically plays a key role in the regulation of calcium homeostasis and bone metabolism [1–3]. Non-classical roles for 1,25D have now been identified and the hormone possesses anti-proliferative, pro-differentiation and immunosuppressive activities in a variety of cell types [4–6]. The serum level of 1,25D is highly regulated through its synthesis chiefly in the kidney by the mitochondrial cytochrome P450, 25-hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1), and through its inactivation and degradation in the kidney and other tissues by a second mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D 24-hydroxylase (CYP24) [2,3]. 1,25D controls its own level in serum by down-regulation of renal *CYP27B1* gene expression [3] and up-regulation of *CYP24* gene expression in the kidney and various extra-renal tissues. The induction of the *CYP24* gene is one of the most dramatic effects of the hormone and represents an important negative feedback mechanism since excess 1,25D levels are toxic [7].

Our previous studies have established that the genomic activity of the hormone is conferred through two vitamin D responsive elements (VDREs) located about 100 bp apart in the proximal promoter region. There is evidence that in the absence of 1,25D, a VDR/RXR hetero-dimer binds to these VDREs and represses transcription through interactions with a co-repressor complex that includes histone de-acetylase activity [8–10]. After binding of 1,25D to VDR, the co-repressor complex is released permitting the recruitment of co-activator complexes that lead to activation of gene expression [11–14]. We have also identified an Ets-1 binding site (EBS) located near to the proximal VDRE that cooperates with VDR/RXR and is important for maximal transcriptional activation of the *CYP24* gene promoter [15]. Another site, termed vitamin D stimulatory element-1 (VSE), located at about 30 bp upstream of the proximal VDRE of the *CYP24* proximal promoter was found to act in synergy with the Ets-1 site in response to PMA and 1,25D [16]. Neither the EBS nor the VSE regulates basal expression.

Abbreviations: 1,25D, 1,25-Dihydroxyvitamin D3; CYP24, 25-hydroxyvitamin D3 24-hydroxylase; VDRE, vitamin D responsive element; VDR, vitamin D receptor; VSE, vitamin D stimulatory element-1; EBS, Ets-1 binding site.

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doi:10.1016/j.cellsig.2009.11.009

In addition to the genomic activation by 1,25D through VDR/RXR bound to the VDREs, there is evidence that 1,25D in some cell-types can also stimulate rapid non-genomic effects, for example via the ERK1/ERK2/ERK5 and JNK MAP kinase modules [16–19]. There is also evidence that PKC, a branch of the ACG Ser/Thr kinases, is involved in regulating 1,25D induction of *CYP24* expression in rat intestinal epithelial cells [20] and rat costochondral chondrocytes [21]. However, the PKC isozyme involved was not identified in these studies. This is important since there are 10 mammalian PKC isozymes [22]. Using kinase-dead constructs of PKC isozymes in HEK293T cells, we have previously demonstrated that PKCβI has some minor involvement in this response [16]. PKCα, PKCδ and PKCε were not required. However, the roles of other PKC isozymes such as PKC ζ are not known.

Other work from our laboratory has revealed an inverted GC-box at −113/−105 on the *CYP24* promoter [23]. This site is functional and mediates calcitonin induction of *CYP24* gene transcription [23]. However, it is not known whether the GC-box also mediates induction by 1,25D. The aim of the present study was to investigate the role of the GC box in the regulation of *CYP24* expression under basal conditions and induction by 1,25D. Furthermore, we hypothesised that PKCζ and associated signalling molecules which target the GC-box in the promoter of other genes, play a key role in mediating the effects of 1,25D on *CYP24* promoter activation via this transcriptional site.

2. Materials and methods

2.1. Materials

1,25D was purchased from Tetrionics, Inc, Madison, WI. Oligonucleotides were synthesised by Geneworks (Adelaide, South Australia). Cell culture media and associated tissue culture products were from Life Technologies (Grand Island, NY). The dual-luciferase assay kit was supplied by Promega (Madison, WI). VDR monoclonal antibodies 9A7 were purchased from Affinity Bio Reagents, Neshanic Station, New Jersey, USA. Anti-phosphoPKC ζ antibody (Thr410) was a kind gift from Dr Alex Toker (Harvard Medical School, Boston). Anti-Sp1, ERK2, PKCζ, β-actin and PI3-kinase (p85α) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., CA). LY294002 and DOTAP were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and Roche Diagnostics, USA, respectively. Calf intestinal alkaline phosphatase was purchased from Sigma-Aldrich (Sydney, Australia). PKCζ siRNA was obtained from Ambion Inc/Applied Biosystem (Scoresby, Vic, Australia) and negative control siRNA was from Thermo Fisher Scientific (Lafayette, CO). Lipofectamine 2000 and anti-phosphoserine antibody were obtained from Invitrogen Australia Pty Ltd (Mt Waverly, Vic, Australia).

2.2. CYP24 promoter-luciferase constructs and expression plasmids

The wild type construct containing *CYP24* promoter sequence in the pGL3 vector fused with firefly luciferase reporter gene has been used previously [15 and Fig. 1A]. Mutagenesis of the Ets-1 protein binding site (EBS) has been described earlier [15]. Mutations in the GC-box and in the EBS were introduced using the Quik-Change Site Directed Mutagenesis protocol (Stratagene, La Jolla, USA). pRSV-hVDR has been described previously [24]. The kinase dead PKCζ mutant expression clone (PKCζK281M) was a kind gift from Professor I. B. Weinstein [25] and Drosophila specific expression vectors for Sp1 (pPac-Sp1) and Sp3 (pPac-Sp3) were provided by Professor M. Waterman [26].

2.3. Maintenance and transfection of kidney cells

HEK293T cells were obtained from Professor D. Findlay, Hanson Institute, Adelaide, Australia. These cells were maintained in DMEM supplemented with 10% FCS. For transient transfection analysis cells were grown in 175 cm^2 flask to 60–70% confluency, washed once with phosphate buffered saline and removed by trypsinisation. 60,000 cells per well were seeded in 24-well trays containing 400 μl of DMEM with 5% FCS. After the cells had attached to the wells, the medium was removed and cells were washed once with phosphate buffered saline and then 400 μl of serum free RPMI medium was added. Cells were incubated for 1–2 h before transfection. Transfections were carried out with 200 ng of the *CYP24* promoter-luciferase constructs, together with 50 ng of the pRLTK-LUC plasmid (Promega, Corporation, USA) to normalise transfection efficiency using DOTAP as described previously [17]. In some experiments cells were co-transfected with 200 ng of kinase dead PKCζ. Next day, the medium was replaced with 400 μl of RPMI medium and cells were further cultured overnight in the presence of 1,25D (10^{-7} mol/l). In some experiments LY294002, an inhibitor for PI3-kinase activity or vehicle was also added. Next day cells were lysed and luciferase activities in cell lysates were determined by the Dual Luciferase assay kit using a Luminometer model TD 20/20 (Turner Design Instruments, Sunnyvale, CA, USA).

2.4. Generation of stable cell lines with CYP24 promoter luciferase constructs

Stable cell lines were generated in HEK293T cells. Briefly, HEK293T cells (1×10^6) were plated in one 60 mm petri-dish with 5 ml culture medium. Once attached, the cells were transfected with the cocktail of 50μl DNA mixture (10 μg *CYP24* promoter-luciferase construct, 100 ng pEF-IRES-Puro vector) and 30 μg DOTAP. The DNA/DOTAP cocktail was left at room temperature for 20 min before being added to the cells. The transfected cells grew at 37 °C for about 24 h until confluence. The cells were subcultured into two 100 mm petri-dishes with 8 ml culture medium for another 24 h. The medium was replaced with the fresh medium containing 1 μg/ml puromycin to select for transfected cells. This step was repeated after 4–7 days. The cells were pooled into one 25 cm^2 flask and allowed to grow to confluence under the same selection pressure. To conduct the experiments, 60,000 cells per well were seeded in 24 well plates and 1,25D (10^{-7} M) treatments were carried out for 24 h. Next day cells were lysed and firefly luciferase activity in cell lysates was determined by the Luciferase assay kit. The protein content from the cell lysate was also determined using 2 μl of supernatant.

2.5. Transient transfection of Drosophila SL2 cells

Drosophila SL2 cells were maintained with revised Schneider's medium containing 10% FCS at 25 °C without CO_2. Transfections for *Drosophila* SL2 cells were performed using Lipofectamine-2000 (Life Tech. BRL). 2×10^5 cells were seeded into 24 well plates in 400 μl of revised Schneider's medium containing 10% FCS. The cells were allowed to grow for 24 h before transfection. Transfections were performed in triplicate and each transfection was performed using 200 ng of *CYP24* gene promoter luciferase construct and also 10–200 ng of pPac-derived Sp1 and Sp3 expression vectors. Total amount of DNA was adjusted by an addition of pPac vector. All plasmid DNA was diluted to a final volume of 50 μl and mixed with 2 μl of Lipofectamine-2000 diluted in 50 μl of RPMI medium. DNA-Lipofectamine complex formation was achieved by incubating the mixture for 20 min at room temperature and transfected into the cells. Cells were harvested after 48 h and luciferase assays were determined by the DLR assay kit.

2.6. PKCζ siRNA

HEK293T cells were transfected with PKCζ or negative control siRNA (50 nM) using Lipofectamine 2000 according to manufacturer's instructions. Cells were cultured for 96 h before being tested.