Regulation of CYP24 splicing by 1,25-dihydroxyvitamin D₃ in human colon cancer cells

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Abstract

CYP24 is a well-established vitamin D receptor (VDR) target gene. The active VDR ligand 1,25(OH)₂D₃ regulates its own catabolism by increasing CYP24 expression. It is well known that in the presence of 1,25(OH)₂D₃, VDR binds to VDREs in the promoter region of CYP24 and initiates CYP24 transcription. However, little is known about the role of 1,25(OH)₂D₃ in the posttranscriptional modulation of CYP24. In this study, we investigated the functional significance of 1,25(OH)₂D₃ in CYP24 RNA splicing in colon cancer cells. Using RT-PCR, we found that 1,25(OH)₂D₃ actively induces CYP24 splicing in a time-dependent manner and CYP24 splicing pattern could be cell type or tissue specific. The induction of RNA splicing by 1,25(OH)₂D₃ was mainly CYP24 selective. Treatment of cells with parathyroid hormone inhibited basal CYP24 splicing, but failed to inhibit 1,25(OH)₂D₃-induced CYP24 splicing. Further experiments demonstrated that new RNA synthesis was required for the induction of CYP24 splicing by vitamin D. In addition, alteration of multiple signaling pathways also affected CYP24 splicing and cellular sensitivity in response to vitamin D appeared to correlate with the induction of CYP24 splicing. These results suggest that 1,25(OH)₂D₃ not only regulates CYP24 transcription, but also plays an important role in posttranscriptional modulation of CYP24 by inducing its splicing. Our findings reveal an additional regulatory step that makes the vitamin D mediated action more prompt and efficient.


Introduction

Alternative splicing in relation to cancer has been well investigated (Chen & Manley 2009, Nilsen & Graveley 2010). However, the general RNA splicing (the conversion of precursor-mRNA to functional-mRNA) that makes a gene functional in relation to normal or cancer cells is rarely investigated. It is generally thought that once RNA is transcribed, the immature (or unspliced) RNA is not stable and quickly spliced to mature RNA. Pre-mRNA splicing, the joining of two exons accompanied by the removal of intronic sequence, requires a very large ribonucleoprotein (RNP) complex termed the spliceosome (Wahl et al. 2009). The spliceosome must be guided to the correct sites for splicing. Accumulating evidence suggests that RNA splicing is coupled with transcription activation through a subset of nuclear receptor coregulators (Monsalve et al. 2000, Auboeuf et al. 2002, 2004, 2005, 2007). One of the initial steps in the splicing process is the recognition of the 5' splicing site (SS), which is accompanied in part by the formation of U1 snRNP and the base pairing of the U1 snRNA with the 5' SS sequence. This early U1-5' SS duplex aids the assembly of the 'pre-spliceosome' on the transcript and is then unwound to allow the formation of the U6-5' SS duplex, leading to the creation of the 'active' spliceosome. Interestingly, these two events require proteins that can also act as transcriptional coregulators (Auboeuf et al. 2005). One of these coregulators is NCoA62/Skip, which could interact with vitamin D receptor (VDR) and regulate vitamin D-mediated transcription and splicing (Zhang et al. 2003). It is reported that a dominant negative inhibitor of NCoA62/Skip interferes with appropriate splicing of 1,25(OH)₂D₃-induced transcripts derived from a GH minigene cassette, in which the human GH gene was under the control of the thymidine kinase promoter and four copies of a VDRE from the rat osteocalcin gene (Zhang et al. 2003). These data suggest a potential role for NCoA62/Skip in coupling VDR-mediated transcription to RNA splicing.

Through analysis of CYP24 expression in colon cancer cells using quantitative RT-PCR, we found that different primer sets generated significantly different results in response to vitamin D treatment. This finding prompted us to further carefully investigate whether the active hormone 1,25(OH)₂D₃ regulates endogenous CYP24 RNA splicing in addition to the transcriptional activation in colon cancer cells and whether the splicing pattern could be cell type specific. The spliceosome must be guided to the correct sites for splicing.
specific. CYP24 is the direct target gene of vitamin D; its protein product is 1,25-dihydroxyvitamin D₃ 24-hydroxylase, which is the catabolic enzyme of both 1,25(OH)₂D₃ and 25(OH)D₃. Therefore, the regulation of CYP24 expression is critical for both the protective effects of vitamin D against carcinogenesis and catabolism of vitamin D.

Materials and Methods

Hormones and chemical reagents
1,25(OH)₂D₃ and kinase inhibitors (SB203580 (p38MAPKi) and SP600125 (JNKi)) were purchased from EMD Biosciences (La Jolla, CA, USA). TPA was supplied from the NCI repository (Frederick, MD, USA). Theophylline, actinomycin D, and cycloheximide were purchased from Sigma. 1,25(OH)₂D₃ was dissolved in ethanol and stored at −80°C. Parathyroid hormone (PTH, bovine, 1–34) was purchased from AnaSpec, Inc. (San Jose, CA, USA) and dissolved in PBS. Other reagents were dissolved in DMSO and stored at −20°C.

Cell culture, cell proliferation and biopsy samples
The colon cancer cell lines HT29, Caco-2, and HCT116 were obtained from ATCC (Manassas, VA, USA). The cell lines were maintained and cultured in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine and 1% antibiotic–antimycotic solution (Invitrogen). All cells were treated with hormones or reagents at selected concentrations in 60 mm culture dishes. 1,25(OH)₂D₃ and PTH were used at concentration of 10⁻⁸ and 6×10⁻⁸ mol/l respectively. The concentrations of other reagents were determined by preliminary experiments in our laboratory and from reports in the literature. Cell proliferation was determined by crystal violet assay as described previously (Peng et al. 2010). Biopsy samples were collected from high-risk colon cancer patients during colonoscopy at University of Illinois hospital. Patients’ consents and appropriate approvals for the studies described in this report were obtained in advance. Fresh biopsy samples were quickly frozen in liquid nitrogen and stored at −80°C until RNA isolation.

RNA isolation and RT-PCR
Total RNA extraction, RT and real-time PCRs were performed as described previously (Peng et al. 2004). RNA was further subjected to DNase I (Ambion, Austin, TX, USA) digestion and purification using RNAeasy Mini kit (Qiagen) before reverse transcription. The cycling conditions for PCR were 35 cycles of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C. The cycling conditions for real-time PCR were: 40 cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Real-time PCR product quality was monitored using post-PCR melt curve analysis. GAPDH was amplified for 24 cycles using conventional PCR or using real-time PCR from the same cDNA samples as a housekeeping gene for normalization. All primers were designed and ordered from Integrated DNA Technologies (Coralville, IA, USA). Table 1 lists all the specific primers used in this study. At least two independent experiments of RT-PCR were carried out to confirm the consistency of the data and the representative data are presented.

Immunoblot analysis and immunofluorescent staining
HT29 cell lysates were prepared after 24 h treatment with 10⁻⁸ mol/l 1,25(OH)₂D₃ and subjected to immunoblot analysis (Peng et al. 2004). Procedures for immunostaining and confocal microscopy were described previously (Peng et al. 2006). CYP24 (vitamin D₃ 24-hydroxylase) mouse monoclonal antibody for immunoblotting and immunofluorescent staining was purchased from Abnova Taiwan Corp. (Taipei, Taiwan).

Results

CYP24 splicing in colon cancer cells and human colon tissue samples
To determine the effect of vitamin D on CYP24 splicing in colon cancer cells, we designed specific primers located in exons 1 and 2 of CYP24 gene. Since intron 1 is a small intron (200 bp; Table 1), this pair of primers allowed us to detect both spliced and unspliced CYP24 RNA using RT-PCR. As shown in Fig. 1A, different cell lines exhibited differential CYP24 splicing pattern in the absence of vitamin D treatment. In colon cancer cell lines HT29 and Caco-2, the basal CYP24 RNA remained unspliced or partially spliced, respectively, whereas in HCT116 cells, CYP24 RNA was observed as already spliced; no unspliced CYP24 was observed. 1,25(OH)₂D₃ significantly induced CYP24 splicing in HT29 and Caco-2 cells, suggesting that in addition to transcriptional activation of CYP24, 1,25(OH)₂D₃ also regulates the maturity of CYP24 RNA in these cells. Since basal CYP24 splicing could be cell type specific, we further examined human colon tissue samples directly obtained from 22 patients. As shown in Fig. 1B, five CYP24 splicing patterns were identified, 11 of the 22 patients displayed pattern 1 (in colon aberrant crypt foci (ACF), both unspliced and spliced CYP24 RNA was detectable, however only unspliced CYP24 RNA was detectable in the adjacent normal colon tissue); 2 out of 22 patients displayed pattern 5 (CYP24 remained spliced in both ACF and the adjacent normal tissue samples). Overall, in 15 out of 22 ACF samples (68%), CYP24 remained spliced or partially spliced; whereas in only 8 out of 22 normal colon tissue samples (36%), CYP24 remained spliced or partially spliced; in 64% of normal colon tissue
samples, CYP24 remained unspliced. These results suggest that CYP24 may be a potential oncogene (Horváth et al. 2010) and demonstrate that the regulation of CYP24 splicing is a common phenomenon.

Characterization of vitamin D-induced CYP24 splicing

Since 1,25(OH)2D3 induced CYP24 splicing in colon cancer cells, we further characterized vitamin D-induced CYP24 splicing and expression using HT29 cells. As shown in Fig. 2A, 1,25(OH)2D3 induced CYP24 splicing in a time-dependent manner. CYP24 splicing started to occur after 45 min of 1,25(OH)2D3 treatment; after 120 min of treatment, most of the CYP24 RNA was spliced, suggesting that CYP24 splicing regulation by 1,25(OH)2D3 was very prompt and efficient. To determine that the vitamin D-induced CYP24 splicing was not alternative splicing, we designed another pair of primers located in exons 10 and 11, because intron 10 is also a small intron (98 bp), the splicing at intron 10 can also easily be detected by RT-PCR. Figure 2B shows the CYP24 splicing at different introns after 1,25(OH)2D3 treatment for 2 h. At intron 1, the splicing appeared to be more efficient than the splicing at intron 10 in the presence of 1,25(OH)2D3. These results also suggest that the vitamin D-induced CYP24 splicing is not alternative splicing, but the maturity of CYP24 RNA; which was confirmed by CYP24 protein (24-hydroxylase) expression analyzed by immunoblotting (Fig. 2C). The treatment with 1,25(OH)2D3 induced CYP24 protein (55 kDa) expression, which is associated with the expression of mature (spliced) CYP24 mRNA. In addition, consistent results were obtained by immunofluorescent staining of 24-hydroxylase in cultured HT29 cells, 24-hydroxylase was induced by vitamin D treatment and it was localized in the perinuclear region (data not shown).

Specificity of RNA splicing regulation by vitamin D

Since 1,25(OH)2D3 induced CYP24 splicing and CYP24 is an immediate vitamin D target gene, we examined whether it regulates the splicing of other vitamin D responsive genes. Several vitamin D responsive genes including CYP27B1 (Turunen et al. 2007), VDR (Hussain-Hakimmje & Mehta 2009), and IGFBP5 (Matilainen et al. 2005) were selected for splicing detection keeping CYP24 as a positive control. Figure 3 shows the RT-PCR analysis of the splicing pattern of these genes after 1,25(OH)2D3 treatment of HT29 and HCT116 cells. 1,25(OH)2D3 induced CYP24 splicing as expected, slightly attenuated CYP27B1 splicing and had no significant effect on VDR and IGFBP5 splicing in HT29 cells. In HCT116 cells, all the examined genes remained spliced; 1,25(OH)2D3 increased the expression of the spliced CYP24 and had little effect on CYP27B1 and VDR expression. IGFBP-1 was barely detectable in HCT116 cells. These results suggest that splicing regulation by vitamin D might be specific.

Table 1 Primers used for gene splicing analysis. GAPDH primers were used for both real-time PCR and conventional PCR, all other primers were used for conventional PCR. CYP24Ex1-2 primer pair was the major primer pair used for CYP24 splicing pattern analysis unless indicated.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Locations</th>
<th>Primer sequences (5’-3’)</th>
<th>Spliced (bp)</th>
<th>Unspliced (bp)</th>
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</thead>
<tbody>
<tr>
<td>CYP24 Ex1-2</td>
<td>F (exon 1)</td>
<td>TCA AGA AAC AGC ACA CC</td>
<td>200</td>
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<tr>
<td></td>
<td>R (exon 2)</td>
<td>CGT AGC CTT CTT TGC GGT AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP24 Ex10-11</td>
<td>F (exon 10)</td>
<td>GGT GTT GGG ATC CAG TGA AG</td>
<td>204</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>R (exon 11)</td>
<td>GTG GCC TGG ATG TCG TAT TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP27B1</td>
<td>F (exon 5)</td>
<td>CACCTGAACCACTTTCTGT</td>
<td>132</td>
<td>335</td>
</tr>
<tr>
<td></td>
<td>R (exon 6)</td>
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<td>443</td>
</tr>
<tr>
<td>VDR</td>
<td>F (exon 6)</td>
<td>GAGGAGGAAAGCGGAGATGACCT</td>
<td>192</td>
<td>445</td>
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<tr>
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<td>R (exon 7)</td>
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<tr>
<td>IGFBP5</td>
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<tr>
<td></td>
<td>R (exon 3)</td>
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<tr>
<td>GAPDH</td>
<td>F</td>
<td>ATC ACT GCC ACC CAG AAC AC</td>
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<tr>
<td></td>
<td>R</td>
<td>TTC AGC TCA GGG ATG ACC TT</td>
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</table>

F, Forward; R, reverse.

Figure 1 CYP24 splicing in colon cancer cells and human tissue samples. (A) CYP24 splicing pattern in the absence and presence of vitamin D in colon cancer cells. Three colon cancer cell lines were treated with ethanol (control, C) and 10−8 mol/l 1,25(OH)2D3 (D3) for 4 h and subjected to RT-PCR analysis. Close C from all the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. 1,25(OH)2D3 induced CYP24 splicing in HT29 and Caco-2 cells. (B) CYP24 splicing patterns identified in 22 pairs of human tissue samples. Aberrant crypt foci (A) and adjacent normal colon tissues (N) displayed different CYP24 splicing pattern. The number under each pattern represents the ratio of the selected pattern and in the total number of samples. M, DNA maker. Equal C from both tissue samples were obtained by real-time PCR analysis of 18S expression.
210 X PENG and others · CYP24 splicing regulated by vitamin D

Figure 2 Effect of 1,25(OH)2D3 treatment on CYP24 splicing and protein expression in HT29 cells. (A) HT29 cells were treated with 10−8 mol/l 1,25(OH)2D3 for 30, 45, 60, and 120 min. CYP24 splicing was examined by RT-PCR. 1,25(OH)2D3 (D3) treatment induced CYP24 splicing in a time-dependent manner. GAPDH served as a loading control. (B) CYP24 splicing at different introns after 1,25(OH)2D3 treatment for 2 h. At intron 1, the splicing appeared to be more efficient than the splicing at intron 10 in the presence of 1,25(OH)2D3. (C) Immunoblot analysis of CYP24 protein expression in HT29 cells after 1,25(OH)2D3 treatment for 24 h. 1,25(OH)2D3 also induced CYP24 protein expression, which is associated with the expression of mature (spliced) CYP24 mRNA. M, protein markers showing standard molecular weight; C, control; D3, 1,25(OH)2D3.

Gene or cell specific, although we cannot exclude that vitamin D could also regulate the splicing of other target genes. It is interesting to note that we could not detect spliced IGFBP5 in HT29 cells regardless of vitamin D treatment.

Effect on PTH on 1,25(OH)2D3-induced CYP24 splicing in colon cancer cells

The functional significance of PTH has been closely correlated with vitamin D action (Welsh et al. 1991, Murayama et al. 1999). PTH has been shown to decrease CYP24 expression levels by altering its mRNA stability in AOK-B50 (porcine kidney proximal tubule) cells (Zierold et al. 2001, 2003). Experiments were designed to examine whether PTH inhibits vitamin D-induced CYP24 splicing. As shown in Fig. 4, PTH at 6×10−8 mol/l significantly inhibited the basal CYP24 splicing, especially in HCT116 cells, however, it failed to suppress 1,25(OH)2D3 induced CYP24 splicing. Interestingly, PTH appeared to increase the accumulation of unspliced CYP24 RNA in both HT29 and HCT116 cells in the absence of 1,25(OH)2D3; in the presence of 1,25(OH)2D3, PTH enhanced the expression of spliced CYP24 in HCT116 cells.

Effect of RNA and protein synthesis inhibitors on vitamin D-induced CYP24 splicing

To determine whether new RNA or protein synthesis is required for 1,25(OH)2D3-induced CYP24 splicing, HT29 cells were pretreated with actinomycin D or cycloheximide for 1 h to inhibit RNA or protein synthesis, then the cells were treated with 1,25(OH)2D3 for 1 h to induce CYP24 splicing. As shown in Fig. 5, actinomycin D treatment completely blocked 1,25(OH)2D3-induced CYP24 splicing. When the cells were incubated with cycloheximide and vitamin D together there was a reduction in the spliced CYP24 compared with the splicing induced by vitamin D only in the absence of cycloheximide, indicating that cycloheximide only inhibited 1,25(OH)2D3-induced splicing. These results demonstrate that new RNA synthesis, instead of new protein synthesis, may be required for vitamin D-induced CYP24 splicing. Surprisingly, in the presence of cycloheximide, the unspliced CYP24 appeared to be accumulating in comparison to the control cells (lane 1) in HT29 cell line.

Effect of signaling alterations on 1,25(OH)2D3-induced CYP24 splicing in HT29 cells

It is well established that multiple signaling pathways are regulated by vitamin D treatment or involved in vitamin D action. It is therefore of interest to determine whether these signaling pathways affect vitamin D-induced CYP24 splicing. We selected the signaling pathways that have been reported to be involved in vitamin D action including PKA (Avila et al. 2007, Khanal et al. 2008), PKC (Wang et al. 2010, Buitrago et al. 2011), p38 MAP kinase (Pardo et al. 2006, Brosseau et al. 2010, Buitrago et al. 2011), and c-Jun N-terminal protein kinase (JNK) pathway (Chen et al. 1999, Qi et al. 2002, Brosseau et al. 2010) to examine how activation or inhibition of these signaling pathways would affect vitamin D-induced CYP24 splicing. As shown in Fig. 6, activation of PKA in HT29 cells by theophylline induced CYP24 splicing, but appeared to inhibit CYP24 transcription (Fig. 6A) and had no significant effect on 1,25(OH)2D3-induced CYP24 splicing; whereas PKC activation appeared to attenuate both basal- and vitamin D-induced CYP24 splicing. Inhibition of p38 MAP kinase with SB203580 completely inhibited both transcription and splicing in the absence of 1,25(OH)2D3, but failed to suppress CYP24 splicing in the presence of 1,25(OH)2D3. Inhibition of JNK MAP kinase pathway with SP600125 induced CYP24 splicing in the absence of 1,25(OH)2D3 treatment and also further enhanced 1,25(OH)2D3-induced CYP24 splicing. These results indicate that CYP24 splicing can be affected by multiple signaling pathways and vitamin D could also induce CYP24 splicing by affecting these signaling pathways.
Differential effect of 1,25(OH)2D3 on the splicing of mRNA precursors of vitamin D responsive genes in HT29 and HCT116 cells. HT29 and HCT116 cells were treated for 2 h with 1,25(OH)2D3 and then subject to RT-PCR analysis. Equal Cg from the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. 1,25(OH)2D3 induced CYP24 splicing, slightly attenuated CYP27B1 splicing and had no effect on VDR and IGFBP5 splicing in HT29 cells; in HCT116 cells, most of the RNA remained spliced and vitamin D had little effect on CYP27B1 and VDR, but increased CYP24 mRNA expression.

CYP24 splicing pattern is correlated with cellular sensitivity in response to vitamin D in multiple colon cancer cell lines

We further evaluated CYP24 splicing pattern and cell proliferation in response to vitamin D in multiple colon cancer cell lines side by side. As shown in Fig. 7, 1,25(OH)2D3 treatment significantly inhibited cell proliferation by 42% (P<0.01, n=8) in HT29 cells and by 26% (P<0.01, n=8) in Caco-2 cells. In these vitamin D sensitive cell lines, CYP24 remained unspliced or partially spliced in the absence of 1,25(OH)2D3, inhibition of cell proliferation by vitamin D treatment was accompanied by significant induction of CYP24 splicing. On the other hand, 1,25(OH)2D3 treatment had little or minor inhibitory effect on cell proliferation in HCT116, SW480, and LS174 cells, where CYP24 remained spliced or largely spliced even in the absence of 1,25(OH)2D3, vitamin D treatment had little effect on CYP24 splicing pattern in these cell lines. These results suggest that cellular sensitivity in response to vitamin D might be correlated to CYP24 splicing pattern.

Discussion

In cancer biology, each regulatory point in the control of gene expression (which includes chromatin structure, splicing and polyadenylation of mRNA precursors, translation, and mRNA and protein stability) is subject to profound alterations during the development of most, if not all, cancers (Venables 2006, Mayr & Bartel 2009, Chi et al. 2010, Silvera et al. 2010). Understanding of these alterations is important for both prevention and cancer treatment. Genome-wide approaches have revealed that tumorigenesis often involves large-scale alterations in alternative splicing (Venables et al. 2009); therefore, alternative splicing is well investigated in relation to cancer. The maturation of mRNA precursors (or RNA splicing), although widely reported in the literature, has not been associated with cancer or has not been investigated in response to cancer preventive or therapeutic agents.

We found that in colon cancer cells, CYP24 RNA splicing can be actively regulated by vitamin D treatment and the splicing pattern could be cell type specific. In addition, RNA splicing patterns in the tested cell lines were associated with cellular sensitivity in response to vitamin D. In human tissue samples, CYP24 splicing was also observed: in 68% of the examined ACF samples, CYP24 remained spliced or partially spliced; whereas in only 36% of the examined normal colon tissue samples, CYP24 remained spliced or partially spliced. However, whether the CYP24 splicing pattern is of any diagnostic significance for ACF detection is still not clear. Analysis of a large amount of samples is needed for this correlation. Time-course studies demonstrate that the regulation of CYP24 splicing by vitamin D is very prompt and efficient; however, it appears that there was a reduction in the efficiency of splicing at intron 10 compared with intron 1. It is possible that the reduction in the splicing efficiency between introns 1 and 10 may be dependent on the distance between the two introns. The reduced efficiency of gene splicing based on the position of introns was also reported previously in human neuroblastoma and fibrosarcoma cell lines (Jarrai & Richard 2009). Among the genes tested for splicing regulation by vitamin D, CYP24 seemed to be the major target gene, suggesting that regulation of splicing by vitamin D could be gene selective. However, we cannot rule out that the splicing of other vitamin D target genes might also be regulated by vitamin D. It should be noted that the transcription of VDR and IGFBP5 was not significantly regulated by vitamin D in our experimental setting and whether splicing was coupled with transcription activation or not still needs to be determined, although the experiment
actinomycin D (Act-D, 10^{-5} g/l) or cycloheximide (CHX, 10^{-5} mol/l) for 1 h, then treated with 1,25(OH)_{2}D_{3} for 1 h, CYP24 splicing was analyzed by RT-PCR. GAPDH served as loading control. Actinomycin D treatment blocked 1,25(OH)_{2}D_{3}-induced CYP24 splicing, but cycloheximide did not fully block 1,25(OH)_{2}D_{3}-induced splicing.

Our experiments with signaling alteration indicate that multiple signaling pathways are involved in splicing regulation. Theophylline is a competitive non-selective phosphodiesterase inhibitor (Esayan 2001), which raises intracellular cAMP, activates PKA and inhibits TNF-\( \alpha \) (Marques et al. 1999, Dere et al. 2008). Theophylline induced CYP24 splicing in HT29 cells, however it also inhibited CYP24 transcription; whereas vitamin D stimulated both CYP24 transcription and splicing. Therefore, although vitamin D was reported to activate PKA (Avila et al. 2007, Khan et al. 2008), whether vitamin D induces CYP24 splicing through PKA still needs further investigation. Vitamin D was also reported to activate PKC (Chen et al. 2010, Wang et al. 2010, Centeno et al. 2011), however, vitamin D apparently did not induce CYP24 splicing through activation of PKC, because activation of PKC attenuated CYP24 splicing. Inhibition of p38 MAPK inhibited both CYP24 transcription and splicing, but failed to block vitamin D-induced splicing, suggesting p38 MAPK might not be involved in vitamin D mediated CYP24 splicing.
Interestingly, treatment of HT29 cells with a JNK inhibitor – SP600125 showed similar effect to vitamin D on CYP24 splicing, a question therefore arises: whether vitamin D induces CYP24 splicing by inhibiting JNK signaling. Reports in the literature about the interaction between JNK and vitamin D do not support this concept. Yasunami et al. (2004) demonstrated that SP600125 dose-dependently inhibited the CYP3A4 promoter activity induced by 1,25(OH)2D3; however, SP600125 did not affect 1,25(OH)2D3-induced transactivation of the DR3 via VDR. Buitrago et al. (2011) showed that 1,25(OH)2D3 promotes the phosphorylation of JNK 1/2, the response was fast and maximal phosphorylation of the enzyme was observed at physiological doses of 1,25(OH)2D3. Kondo et al. (2004) showed that 1,25(OH)2D3 treatment of ST2 cells decreased the amount of phosphorylated c-Jun protein (phospho-c-Jun), while the total amount of c-Jun remained constant and the amount of phosphorylated JNK was nearly unchanged by vitamin D treatment. These reports suggest that vitamin D might not inhibit JNK activity, although the interaction between JNK and vitamin D could be cell type or gene specific. Overall, our results suggest that 1,25(OH)2D3-induced CYP24 splicing might involve JNK inhibition.

Previous study showed that vitamin D also induces CYP24 splicing in LNCaP prostate cancer cells (Muindi et al. 2007). However, the mechanism of vitamin D mediated CYP24 splicing remains to be established. In this study, we showed that 1,25(OH)2D3 quickly triggered CYP24 splicing in colon cancer cells, which might not require new protein synthesis, we therefore designed experiments by alteration of signaling transduction to examine whether the non-genomic signaling pathway of vitamin D mediates CYP24 splicing. As discussed above, the results were still inconclusive since alteration of multiple signaling pathways was found to affect CYP24 splicing. Although the genomic pathway of vitamin D in inducing CYP24 splicing was not explored in our study, it is possible that 1,25(OH)2D3, through binding to VDR, interacts with some components of the spliceosome, such as NCoA62/Splicing (Zhang et al. 2003) to trigger the assembling of spliceosome to initiate splicing, however, how CYP24 is specifically selected for vitamin D-induced splicing remains to be a question.

The correlation studies regarding CYP24 splicing induction and cellular sensitivity suggest that the basal level of mature CYP24 might contribute to cellular resistance in response to vitamin D in colon cancer cells. Cells with a high basal level of mature CYP24 could be resistant to vitamin D treatment, since the 24-hydroxylase is immediately available for catabolizing vitamin D. However, sensitive cells such as HT29 and Caco-2 cells express low level of mature CYP24, upon vitamin D treatment, both significant CYP24 induction and inhibition of cell proliferation can be observed in these sensitive cell lines. These data indicate that in sensitive cell lines, while the 24-hydroxylase is not immediately available, genes related to the antiproliferative effect of vitamin D could be activated upon vitamin D treatment.

In summary, this report provides novel information regarding vitamin D-mediated CYP24 splicing and possible association among CYP24 splicing patterns, cell transformation (ACF formation) and cellular sensitivity in response to vitamin D. Our findings shed some light on whether other steroid hormones can regulate the splicing of their selective target genes. Conceptually, similar regulation of immature RNA to its functional states by other steroid hormones is a strong possibility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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