Biochemical and Biophysical Research Communications 436 (2013) 632–637

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Insulin-like growth factor binding protein-3 modulates osteoblast differentiation via interaction with vitamin D receptor

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ARTICLE INFO

Article history:
Received 6 April 2013
Available online 11 June 2013

Keywords:
IGFBP-3
VDR
1,25-$(OH)_2D_3$
Osteoblast differentiation

ABSTRACT

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) is a secreted glycoprotein that reduces the bioavailability of IGFs. This glycoprotein has both IGF-dependent and -independent effects on cell growth. However, the mechanisms responsible for the IGF-independent actions of IGFBP-3 are not fully understood. In the present study, we used multiple methodologies including glutathione S-transferase pull-down assay and co-immunoprecipitation to demonstrate that IGFBP-3 can directly interact with vitamin D receptor (VDR) *in vitro* and *in vivo*. Furthermore, immunofluorescence co-localization studies showed that IGFBP-3 and VDR could co-localize in the cell nucleus. Reporter gene experiment showed that IGFBP-3 negatively regulates the growth hormone promoter activity induced by ligand-activated VDR. Moreover, real-time RT-PCR demonstrated that IGFBP-3 can inhibit the osteocalcin and CYP24a1 mRNA transcription induced by 1,25-$(OH)_2D_3$ in osteoblastic cells. Finally, alkaline phosphatase activity significantly decreased in osteoblastic cells when the cells were transfected with IGFBP-3 in the presence of 1,25-$(OH)_2D_3$. In conclusion, these studies provide evidence that overexpression of IGFBP-3 suppresses osteoblastic differentiation regulated by VDR in the presence of 1,25-$(OH)_2D_3$. These findings reveal a novel mechanism by which IGFBP-3 functions.

1. Introduction

Insulin-like growth factor (IGF) binding proteins (IGFBPs) play an integral role in modifying the effects of IGFs on a wide variety of cell types [1,2]. IGFBP-3, a major IGFBP species in circulation, binds 75 – 90% of circulating IGF-I in a large ternary complex consisting of IGFBP-3, acid-labile subunit (ALS), and IGF [3,4]. The IGFBPs are known to modulate the actions of IGFs in circulation as well as in the immediate extracellular environment. Interestingly, apart from the ability of IGFBPs to inhibit or enhance IGF actions, IGFBPs also exhibit very clear, distinct biological effects independent of the IGF-I [5]. These IGF-independent actions contribute to the diversity of biological outcomes caused by IGFBPs. In the case of IGFBP-3 and -5, these IGF-independent effects can be either growth inhibitory or stimulatory, depending on the cellular context [6,7]. IGFBP-3 is a well-documented inhibitor of cell growth and/or promoter of apoptosis. IGFBP-3 is closely associated with the chondrocyte nucleus in human articular cartilage [8,9]. This glycoprotein located in both the cytoplasm and nuclei may regulate transcription of hormone receptors, which is important in cartilage differentiation [8]. IGFBP-3 concentration is also increased in patients with reduced bone mineral density [10]. These studies suggest that IGFBP-3 may regulate osteoblast differentiation through unknown mechanisms.

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. This receptor regulates the expression of target genes by binding to 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) [11,12]. The classic role of VDR is to stimulate calcium absorption in the intestines, maintain normocalcemia, and indirectly regulate bone mineralization [13]. 1,25-$(OH)_2D_3$ induces the differentiation of osteoblasts through the sequential induction of cell cycle arrest, the maturation of extracellular matrix, and ultimately, bone mineralization [14–16]. Treating osteoblasts with 1,25-$(OH)_2D_3$ inhibits proliferation, upregulates osteoblast-associated genes, such as osteocalcin and osteopontin, and stimulates calcium accumulation [17]. The MG-63 and U2-OS cell lines are considered to represent a population of undifferentiated human osteoblast-like cells. These cell lines can respond to 1,25-$(OH)_2D_3$ by expressing alkaline phosphatase (ALP) activity and osteocalcin [18,19].

These studies shows that IGFBP-3 interacts with VDR both *in vitro* and in living cells. Immunofluorescence colocalization and nuclear cytoplasmic fractionation results indicated that IGFBP-3 and the VDR co-localized predominantly to the nucleus.

Abbreviations: IGFBP-3, insulin-like growth factor binding protein-3; VDR, vitamin-D receptor; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D3; Oste, osteocalcin; ALP, alkaline phosphatase; VDRE, vitamin D response element.

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http://dx.doi.org/10.1016/j.bbrc.2013.04.111

Further exploration revealed that IGFBP-3 inhibited VDR transcriptional regulation induced by 1,25-$(OH)_2D_3$. Moreover, IGFBP-3 repressed ALP activity in osteoblast differentiation. When VDR was knocked down, IGFBP-3 had no effect on ALP induced by 1,25-$(OH)_2D_3$. Thus, our results suggest that IGFBP-3 may alter the 1,25-$(OH)_2D_3$-induced expression of osteocalcin and ALP activity during osteoblast differentiation.

2. Materials and methods

2.1. Reagents

Antibodies against IGFBP-3, VDR, RXR, Histone1, GST were from Santa Cruz Biotechnology (Santa Cruz, CA). 1,25$(OH)_2D_3$ and antibodies against Flag- and HA-tag were purchased from Sigma (Deisenhofen, Germany). Antibodies against LAMP-1 were purchased from Abcam (Cambridge, UK). Peroxidase-conjugated secondary antibodies, rhodamine (TRITC) and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Zhongshan Goldenbridge Biotechnology Corporation (Beijing, China). 1,25$(OH)_2D_3$ was dissolved in 95% ethanol by volume. 1,25$(OH)_2D_3$ was dissolved in 95% ethanol by volume. Whenever 1,25$(OH)_2D_3$ was used, 95% ethanol was also used so that the concentration of ethanol (<0.1%) was equal in all wells.

2.2. Cell culture

All human cell lines were maintained in our laboratory. 293T, HeLa and MG-63 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. DMEM and the antibiotics (penicillin/streptomycin) were purchased from Gibco Life Technologies (Karlsruhe, Germany). For each cell line, a cell stock was prepared 5 to 7 d after resuscitation.

2.3. Immunoblotting (IB) analysis

Adherent cells were harvested with a cell scraper (Costars, Cambridge, MA) in ice-cold lysis buffer ((0.5% NP-40, 20 mM Tris–HCl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 10 mM PNPP, and 10 μg/ml aprotinin). Cell lysates were resolved by SDS–PAGE before transferring to nitrocellulose membranes (Pall Corporation, Pensacola, FL). Nitrocellulose membranes were then incubated with 5% (w/v) nonfat dry milk in TBST washing buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 60 min at room temperature to block nonspecific protein binding. Primary antibodies were applied to the membranes overnight at 4 °C. After washing thrice with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies for 60 min at room temperature and then washed again. Bands were visualized using Super Signal chemiluminescence (Pierce Chemical, Rockford, IL).

2.4. Glutathione S-transferase (GST) pulldown assays

The GST-VDR fusion vector encoded the full-length IGFBP-3/IGFBP-5/RXR/VDR molecule. These fusion proteins were produced in *Escherichia coli* BL21 (DE3) after isopropyl-β-dthiogalactoside induction for 10 h at room temperature. The bacteria were disrupted by sonication, and recombinant GST fusion proteins were captured from cell lysates using glutathione-Sepharose beads (Sigma, Deisenhofen, Germany). The purified GST-IGFBP-3 that was bound to the beads was incubated with 293T cell lysates, which were transiently transfected with Flag-VDR. After incubation, they were separated by centrifugation. The bound proteins were analyzed by SDS–PAGE followed by IB using an anti-VDR antibody. Experiments were repeated three times.

2.5. Co-immunoprecipitation

Cells (293T) were transiently transfected with pCMV-Flag-VDR and pCMV-HA-IGFBP-3 or a control vector. After transfection for 24 h, cells were stimulated with 1,25$(OH)_2D_3$ (10^{-8} M) for 24 h. The cells were then suspended in 50 mM Tris–HCl buffer (pH 7.5) with 0.5% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 10 μg/ml aprotinin; Roche, Mannheim, Germany). Anti-Flag antibodies (1 μg; Sigma, Deisenhofen, Germany) were added to each sample and placed on a rotating stirrer at 4 °C for 12 h. Then, protein-G Sepharose 4 Fast Flow beads (30 μl, Sigma, Deisenhofen, Germany) were added to each sample according to the manufacturer's protocol. The solutions were rotated overnight at 4 °C, and the beads were then washed three times with Tris–HCl buffer (50 mM; pH 7.5) with 0.5% Triton X-100.

2.6. Immunofluorescence

MG-63. cells were transfected with plasmids expressing pCMV-Flag-VDR, pCMV-HA-IGFBP-3, or a pCMV vector. After transfection for 24 h, cells were stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) for 12 h. The cells were then fixed with 4% (w/v) paraformaldehyde in PBS for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature. Cells were incubated overnight at 4 °C with a VDR antibody (rabbit anti-human, 1:50) and an IGFBP-3 antibody (goat anti-human, 1:50). Then, they were incubated with TRITC or FITC-conjugated secondary antibodies (1:100) for 60 min at room temperature. The cells were incubated with 4,6-diamidine-2-phenylindole (DAPI; 1 μg/ml) and sealed with coverslips. Images were obtained using a laser scanning confocal microscope (Leica, Deerfield, IL).

2.7. Nuclear and cytoplasmic fractionations

Cell pellets were lysed with buffer A (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) and swirled for 10 s. The cell lysates were incubated for 5 min on ice and centrifuged for 6 min at 1500 rpm and 4 °C. The supernatants were considered to be cytoplasmic fractions. The cellular pellets were resuspended in buffer A and treated again as described above to eliminate cytoplasmic remainders. Finally, the cellular pellets were extracted with SDS–PAGE sample buffer for 5 min at 95 °C and centrifuged (12,000 rpm, 5 min at 20 °C), and the resulting supernatants were considered to be nuclear fractions.

2.8. Reporter gene luciferase assay

Two kinds of luciferase reporter genes were used in our experiments. The pGL3-basic-VDRE luciferase reporter is a luciferase gene that is driven by four copies of VDRE (5-AGCTTCAGGT CAAGGAGGTCAGAG-3). The pGL3-basic-Oste was constructed as previously reported [19]. Cells were transiently co-transfected with one of the three luciferase reporter plasmids (0.5 μg; expressing firefly luciferase) containing the VDREs and an internal control reporter plasmid (0.005 μg; pRL-TK) per well in a 24-well plate. The pRL-TK vector expressing the Renilla luciferase gene was used to control for transfection efficiency. The cell lysates were prepared and assayed for firefly and Renilla luciferase activity using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Luminescence was quantified with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

2.9. ALP assay

Cells were seeded in 24-well plates at a density of 10^4 cells per well and transfected with plasmids expressing HA-IGFBP-3, Flag-VDR, vector control, or siRNA against the VDR (siRNA-VDR). After transfection for 24 h, the cells were treated with 1,25-$(OH)_2D_3$ (10^{-8} M) or vehicle control for 24 h. Cells were treated according to methods reported previously [6].

2.10. RNA extraction and quantitative real-time RT-PCR analysis

RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Then, the RNA was reverse-transcribed into first-strand cDNA using the Reverse Transcriptase Kit (Promega, Madison, WI, USA). For real-time quantitative RT-PCR analysis, SYBR Premix Ex Tag™ TaKaRa, Japan) analysis of the cDNA were performed using the Bio-Rad iCycler with a MyiQ Real-time PCR Detection System. Expressing of IGFBP-3 or the VDR gene was normalized to beta-actin expression.

2.11. Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. Student's *t*-test was used for statistical comparisons. Statistical significance was set to $P < 0.01$.

3. Results

3.1. Characterization of the interaction between IGFBP-3 and the VDR

To investigate the specificity of the VDR and IGFBP-3 bond, we performed GST pulldown experiments using recombinant fused IGFBP-3 linked to GST. Compared with GST alone, the GST-IGFBP-3 protein bound VDR, and no GST band was detected. We used GST-RXR or GST-IGFBP-5 as positive control (Fig. 1A) and found that both RXR and IGFBP-5 bound with the VDR. When equal amounts of Flag-IGFBP-3 were incubated with decreasing amounts of the GST-VDR protein (200, 100, 50, and 25 μg), the level of IGFBP-3 appeared to decrease with decreasing amounts of the GST-VDR protein in a dose-dependent manner (Fig. 1B).

To investigate the specificity of VDR binding to IGFBP-3 *in vivo*, 293T cell lysates were transfected with HA-IGFBP-3 and Flag-VDR. Using anti-HA antibodies, Flag-VDR and its interacting molecules were immuneprecipitated (Fig. 1C). VDR was found in the immunoprecipitates of cells that were co-transfected with HA-IGFBP-3 and Flag-VDR, and no IGFBP-3 was detected in control immunoprecipitates. Another immunoprecipitation experiment was performed with anti-Flag antibodies (Fig. 1D). A substantial amount of IGFBP-3 was detected, indicating an association between HA-IGFBP-3 and Flag-VDR proteins.

To further confirm the association between IGFBP-3 and VDR, a semi-endogenous co-immunoprecipitation experiment was performed with exogenous IGFBP-3 and endogenous VDR in MG-63 cells. VDR was immunoprecipitated with an anti-Flag antibody that could capture Flag-IGFBP-3 in cell lysates, and no VDR was detected by IB when the cells were transfected with a Flag-vector control (Fig. 1E). These results indicate that binding of IGFBP-3 and VDR is specific.

3.2. Intracellular co-localization of IGFBP-3 and the VDR

To investigate whether biochemical interaction between IGFBP-3 and VDR is also present in living cells, we compared their co-localizations in MG-63 cells. Flag-VDR (stained in green fluorescence) and HA-IGFBP-3 (stained in red fluorescence) were predominantly found in the nucleus (Fig. 2A). Also, Flag-VDR and HA-IGFBP-3 both co-localized in the MG-63 cells nucleus (merged panel in Fig. 2A). To confirm these observations, nuclear cytoplasmic fractionation was applied to HeLa and MG-63 cells that were transiently transfected with HA-IGFBP-3 and Flag-VDR. As expected, the nuclear protein Histone 1 was retrieved in the nuclear fraction, and the lysosomal protein LAMP-1 was found exclusively in the cytoplasmic fraction. This results suggests that a clean separation between nuclear and cytoplasmic proteins was achieved (Fig. 2B). A small proportion of IGFBP-3 and VDR was retained in the cytoplasm, but both localized predominantly in the cell nucleus. These results are in agreement with those obtained using immunofluorescence. Taken together, these data suggest that IGFBP-3 colocalizes with VDR in the nucleus where they interact with each other.

3.3. IGFBP-3 negatively regulates the ligand-induced transcriptional activity of the vitamin D response element (VDRE)

VDR binds vitamin D response elements (VDREs) within target gene promoters with high affinity. Canonical VDREs consists of a direct repeat of the 5′-AGG/TTCA-3′ motif or a minor variation of this motif separated by three nucleotides [20]. We investigated the ability of IGFBP-3 to modulate the 1,25-$(OH)_2D_3$-induced transcription of promoters including VDREs. Using pGL3-basic-VDREs and pRL-TK for luciferase assays, IGFBP-3 was found to significantly decrease VDR-mediated addition of reporter activity in the presence of 1,25-$(OH)_2D_3$ (Fig. 3A, HeLa; (Fig. 3B), 293T). The ratios of firefly to Renilla luciferase activity (Fluc/Rluc) were 4.35 and 2.34 when the VDR was transfected singly, but the ratios were reduced to 3.09 and 1.62 when IGFBP-3 was co-transfected with VDR in the presence of 1,25-$(OH)_2D_3$ ($P < 0.01$) in HeLa and 293T cells, respectively. Although the ratios changed from 1.15 to 0.53 and from 1.21 to 0.49 in the 293T or HeLa cells, respectively, no statistical differences were noted in the absence of 1,25-$(OH)_2D_3$.

To confirm that IGFBP-3 decreases VDR transduction activity, we repeated the dual-luciferase assay using pGL3-basic-Oste (Fig. 3C and D) and pRL-TK. We observed similar decreases in luciferase activity when cells were co-transfected with IGFBP-3 and VDR compared with cells transfected with VDR alone in the presence of 1,25-$(OH)_2D_3$. With pGL3-basic-Oste, the ratios decreased from 28.18 to 19.34 in HeLa cells ($P < 0.01$, Fig. 3C) and from 19.07 to 11.35 in 293T cells ($P < 0.01$, Fig. 3D). In the absence of 1,25-$(OH)_2D_3$, no statistical differences were observed between cells co-transfected with IGFBP-3 and VDR and those transfected with VDR alone. Together, our results suggest that IGFBP-3 reduces the transcriptional activity of VDR in the presence of 1,25-$(OH)_2D_3$.

3.4. Effect of IGFBP-3 on VDR function

To further investigate the role of IGFBP-3 in modulating bone cell differentiation, we examined its effect on a bone differentiation marker, ALP, which could be induced by VDR and 1,25$(OH)_2D_3$. We investigated the role of IGFBP-3 in modulating ALP activity in MG-63 cells, which is considered to represent a population of undifferentiated human osteoblast-like cells [6,19]. ALP activity decreased from 1.12 to 1.05 when transfected with IGFBP-3 in the absence of 1,25$(OH)_2D_3$ after 30 min (Fig. 4A). However, in the presence of 1,25$(OH)_2D_3$, the ALP activity of IGFBP-3 decreased from 1.68 to 1.17 after 30 min ($P < 0.01$). These results suggest that IGFBP-3 down-regulates ALP activity in the presence of 1,25$(OH)_2D_3$. Moreover, we used siRNA against VDR (siRNA-VDR) to determine whether decreased ALP activity was associated with interactions between IGFBP-3 and VDR. Significant decrease in ALP activity was observed when cells were treated with IGFBP-3 compared with the vector control in the presence of

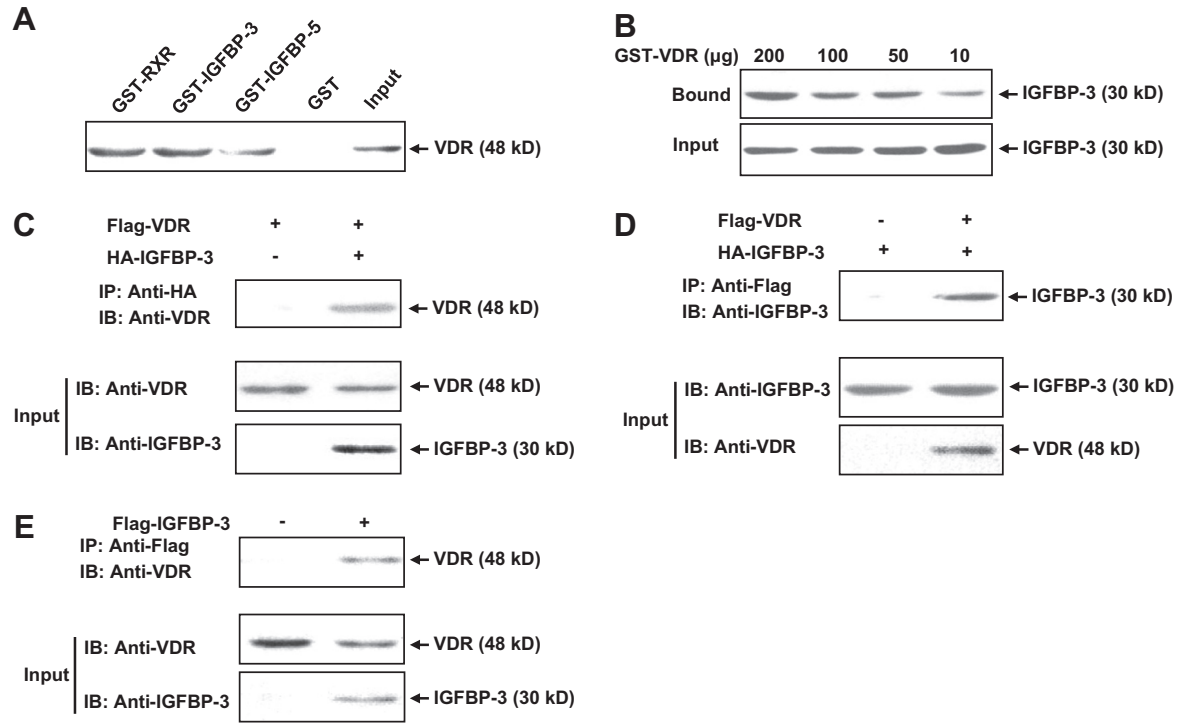

Fig. 1. Characterization of the interaction between IGFBP-3 and VDR. (A) Equal volumes of 293T cell lysates that were transiently transfected with pCMV-Flag-VDR along with GST, GST-RXR, GST-IGFBP-3 or GST-IGFBP-5 immobilized on glutathione-Sepharose beads. (B) Equal amounts of IGFBP-3 were incubated with decreasing amounts of GST-VDR. Bound IGFBP-3 and an equal volume of cell lysates (input) were detected by IB with an anti-IGFBP-3 antibody. (C) Cells (293T) were transfected with Flag-VDR with either pCMV-HA vector control or HA-IGFBP-3 and stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) for 24 h after transfection. Immunoprecipitations (IP) were performed with anti-HA antibodies, and immune complexes were analyzed by IB using anti-VDR antibodies. (D) IP was performed with anti-Flag antibodies, and immune complexes were analyzed by IB using anti-IGFBP-3 antibodies. E Semi-endogenous IP was conducted with HeLa cell lysates that were transiently transfected with HA-IGFBP-3 or the HA-vector. Cell lysates were incubated with anti-VDR antibodies, and immune complexes were analyzed by IB using anti-VDR antibodies.

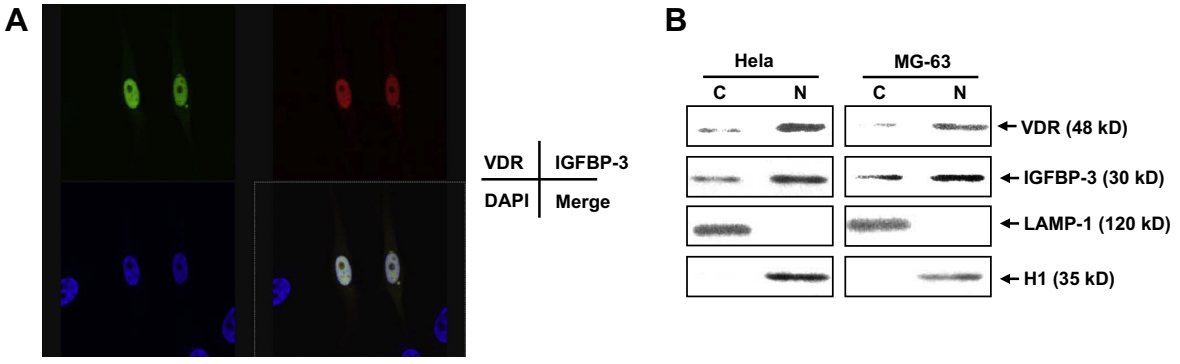

Fig. 2. Cellular co-localization of VDR and IGFBP-3. (A) Cells (MG-63) were transiently co-transfected with plasmids expressing HA-IGFBP-3 and Flag-VDR. After transfection for 24 h, cells were stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) for 24 h. Control experiments used 293T and HeLa cells transfected with an HA-vector and a Flag-vector (data not shown). (B) Cells (HeLa and MG-63) co-transfected with constructs expressing IGFBP-3 and VDR were treated with 1,25-$(OH)_2D_3$ (10^{-8} M) for 24 h before extracting the nuclear and cytoplasmic fractions. N, nucleus; C, cytoplasm.

1,25$(OH)_2D_3$ ($P < 0.01$; Fig. 4B). When the cells were transfected with siRNA-VDR, no obvious differences in ALP activities were observed between cells treated with IGFBP-3 or a vector control in the presence of 1,25$(OH)_2D_3$. These data suggest that the influence of IGFBP-3 on ALP activity is due to its interaction with VDR and inhibition of VDR function.

To determine whether endogenously expressed IGFBP-3 is also inhibitory, we studied the effects of knocking down IGFBP-3 expression on the 1,25-$(OH)_2D_3$ response. MG-63 cells were used to examine the effects of endogenous IGFBP-3 on CYP24A1 gene expression. The effect of IGFBP-3 siRNA-mediated knockdown is shown in Fig. 4C. Endogenous IGFBP-3 levels were found to be significantly decreased. As expected from the previous finding that IGFBP-3 overexpression inhibits 1,25$(OH)_2D_3$-induced CYP24A1 expression, IGFBP-3 knockdown was found to increase CYP24A1 expression induced by 1,25$(OH)_2D_3$ (Fig. 4D). Moreover, osteocalcin expression was also increased in IGFBP-3 knockdown MG-63 cells, which was consistent with the effect of IGFBP-3 overexpression (Fig. 4E). These results are thus in agreement with previous data showing that IGFBP-3 inhibits osteoblast differentiation induced by VDR and 1,25-$(OH)_2D_3$.

4. Discussion

The discovery of IGF-independent mechanisms of cell growth, differentiation and apoptosis by IGFBPs provides indirect evidence

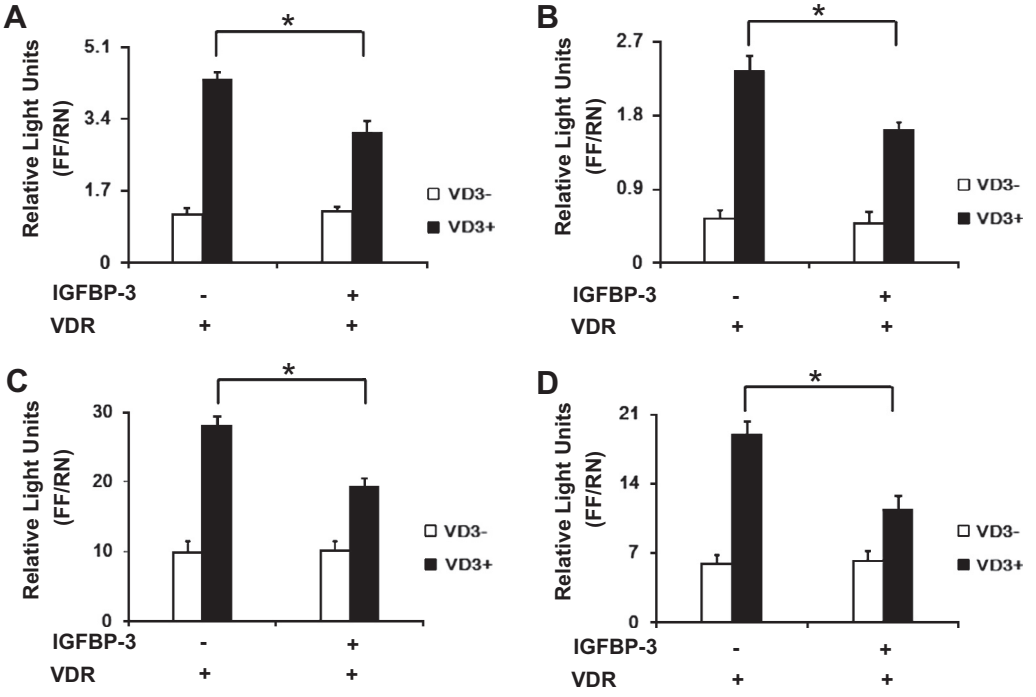

Fig. 3. IGFBP-3-inhibited VDR transcriptional activity in the presence of $1,25(OH)_2D_3$. 293T (left) and HeLa (right) cells were transiently co-transfected with plasmids (pCMV-Flag-IGFBP-3 with pCMV-Myc-VDR or the pCMV vector control). One kind of luciferase reporter plasmid (expressing the firefly luciferase gene), and the pRL-TK vector (expressing the Renilla luciferase gene). After a 24 h of transfection, the cells were treated with $1,25\text{-}(OH)_2D_3$ (10^{-8} M) (black bars) or vehicle control (white bars) for 24 h. (A and B) The luciferase reporter plasmid pGL3-basic-VDRE was used. (C and D) The luciferase reporter plasmid pGL3-basic-Oste was used. Each experimental condition was performed in triplicate and applied in three independent experiments. Values are expressed as mean ± SD. $^*P < 0.01$ (Student's *t*-test).

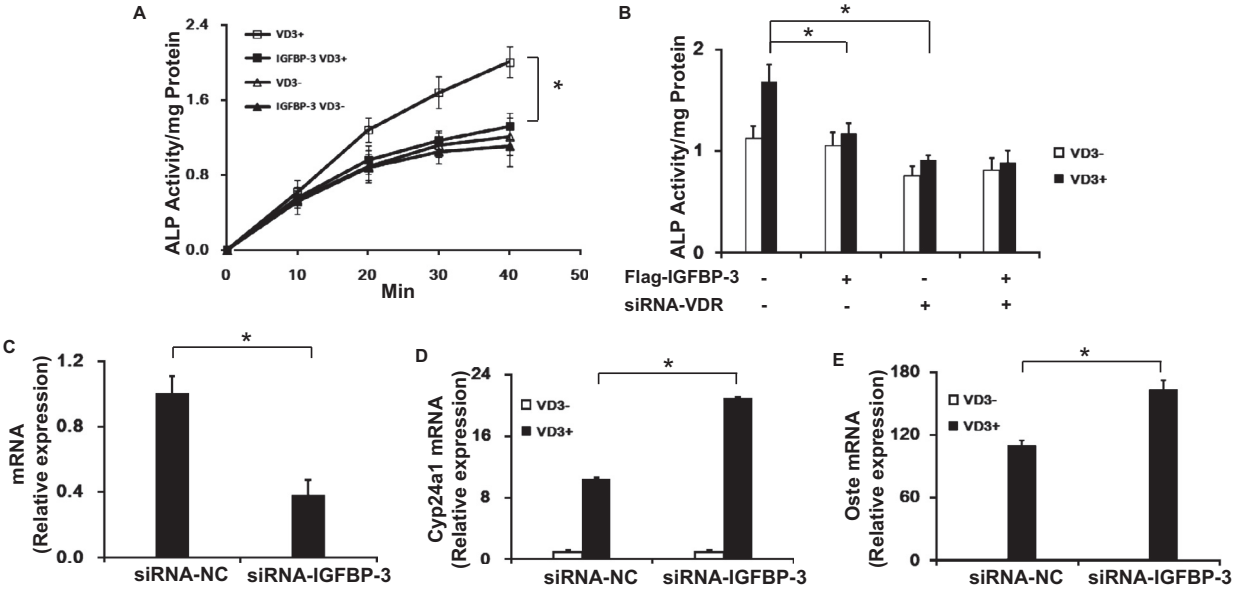

Fig. 4. Effect of IGFBP-3 on the function of VDR. (A) Cell lysates were prepared from MG-63 cells transiently transfected with constructs expressing IGFBP-3 [treated with $1,25(OH)_2D_3$ (10^{-8} M) (open triangle) or vehicle control (filled triangle) for 24 h after transfection] or vector control [treated with $1,25(OH)_2D_3$ (10^{-8} M) (open squares) or vehicle control (filled squares) for 24 h after transfection]. Lysates were assayed for ALP activity at 10, 20, 30, and 40 min. (B) MG-63 cells were washed, scraped and suspended in TXM buffer, and the lysates were assayed for ALP activity. Each sample was expressed as the rate of ALP activity/mg protein. (C) siRNA against IGFBP-3 (siRNA-IGFBP-3) suppresses IGFBP-3 mRNA expression in MG-63 cells. Effects of IGFBP-3 knockdown on ligand-induced expression of VDR targets, CYP24A1 (D) and osteocalcin. (E) Cells were transfected with the control negative control siRNA (siRNA-NC) or siRNA-IGFBP-3 and treated with or without $1,25(OH)_2D_3$ (10^{-8} M). Values for normalized mRNA expression were relative to those of cells treated with siRNA-IGFBP-3 and $1,25(OH)_2D_3$ (10^{-8} M). Values are expressed as mean ± SD. $^*P < 0.01$ (Student's *t*-test).

for the presence of IGFBP binding proteins [21]. Previous work has shown that IGFBP-5 interacts directly with VDR and modulates $1,25(OH)_2D_3$ response [6]. These results suggest that the nuclear receptor may be an important part of the IGF-independent functions of IGFBPs. In this study, we first demonstrated an interaction between IGFBP-3 and VDR. IGFBP-3 and VDR co-localized mainly in the nucleus. Furthermore, we presented evidence that IGFBP-3 inhibits VDRE-mediated gene expression, osteocalcin expression and ALP activity in osteoblast cells mediated by $1,25(OH)_2D_3$. Knockdown of VDR reduced the effect of IGFBP-3. Moreover,

knockdown of IGFBP-3 enhanced the expression of osteocalcin and Cyp24a1, which was induced by VDR and $1,25(OH)_2D_3$.

We examined the impact of IGFBP-3 on VDRE transcription activities mediated by VDR and $1,25(OH)_2D_3$. Both activities were induced by $1,25(OH)_2D_3$, but they were significantly reduced when the cells were co-transfected with both IGFBP-3 and VDR compared with cells transfected only with VDR (Fig. 3A and B). In the presence of $1,25(OH)_2D_3$, these results were confirmed by the repression of the transcriptional activity of the osteocalcin promoter (Fig. 3C and D) when IGFBP-3 was co-transfected with VDR compared with transfection with VDR only. These results clearly demonstrate that IGFBP-3 is able to restrict the transcriptional activation of VDR and exerts an inhibitory effects on the cellular responsiveness to 1,25(OH)2D3. Interestingly, IGFBP-5 and IGFBP-6 blocked $1,25(OH)_2D_3$-induced transcriptional activity.

We examined the effects of IGFBP-3 on the activity of a bone differentiation marker, ALP. A significant decrease in ALP activity was observed when the plasmids expressing IGFBP-3 were transiently transfected into MG-63 cells in the presence of $1,25(OH)_2D_3$ (Fig. 4). When the endogenous VDR was knocked down by siRNA, no obvious changes in ALP activities were noted between cells transfected with an IGFBP-3 expression vector or a vector control. These results suggest that IGFBP-3 influences ALP activity because of its interaction with VDR. Furthermore, when endogenous IGFBP-3 was knocked down by siRNA, ALP activity induced by VDR and $1,25(OH)_2D_3$ significantly increased compared with negative siRNA (Fig. 4).

In summary, we have demonstrated that IGFBP-3 participates in the VDR-$1,25(OH)_2D_3$ signaling pathway, several related results suggest that IGFBP-3 acts as a VDR corepressor when it interacts with TR. Our study revealed a novel link between IGFBP-3 and $1,25(OH)_2D_3$-VDR signaling, providing a potential mechanism underlying the nuclear role of IGFBP-3. These findings may advance the understanding of the molecular mechanisms responsible for VDR signaling in osteoblasts.

Acknowledgments

This work was supported by the National Natural Science of Foundation of China (81171723, 30872638) and the Foundation for Distinguished Young Talents in Higher Education of Guangdong, China (LYM11041).

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