−104 bp) containing E-box motifs was mapped as nVDRE, presumably as well as the more upstream region (−850 to −600 bp) (Fig. 2B). The E-box-type elements in promoters of negative target genes for VDR are illustrated in Table 1. The two mapped E-box motifs in the hPTHrP gene promoters are identical in sequence to those of the 1αnVDRE in the 1α(OH)ase promoter, although the space between the two E-box motifs is different (Table 1).

An E-Box-Type Element in the hPTH Promoter Functions as a Negative VDRE through the VDR/RXR Heterodimer

We previously identified direct repeats of E-box motifs (CANNTG) as nVDRE (1αnVDRE) in the 1α(OH)ase promoter (9, 24). We examined whether the mapped E-box-type element (−87 to −60 bp) in the hPTH promoter alone serves as an nVDRE using a reporter assay in MCT cells. A clear negative response was only observed with the intact promoter (hPTH460) when compared with a positive control hPTH promoter (hPTH460) (Fig. 3B, lanes 3 and 4). An hPTH promoter mutant lacking the E-box-type element (hPTHΔE-box) was unable to confer the negative response to 1α,25(OH)$_2$D$_3$ (Fig. 3B, lanes 5–8), suggesting this motif functions as an nVDRE. Indeed, synthetic short elements (hPTHnVDRE) alone were sufficient to confer an expected negative response to 1α,25(OH)$_2$D$_3$ (Fig. 3B, lanes 11 and 12). Therefore, we concluded that these elements are the nVDRE for the hPTH gene promoter.

To directly test DNA binding and the requirement for heterodimerization of VDR with RXR on ligand-induced transrepression of the E-box-like motif in the hPTH promoter, two classes of VDR mutants were applied. One class of the two VDR mutants lacked dimerization ability (VDR I260R and I384R) (27). These mutants lost their transrepressive function upon the hPTHnVDRE (Fig. 3C, lanes 4 and 6). The second class of VDR mutant lacked DNA binding ability for DR3-type-positive VDRE (VDR E42A) (24), and indeed impaired ligand-induced transactivation was observed (Fig. 3C, lanes 7 and 8). However, this mutant was still potent to transrepress the hPTH gene promoter (Fig.

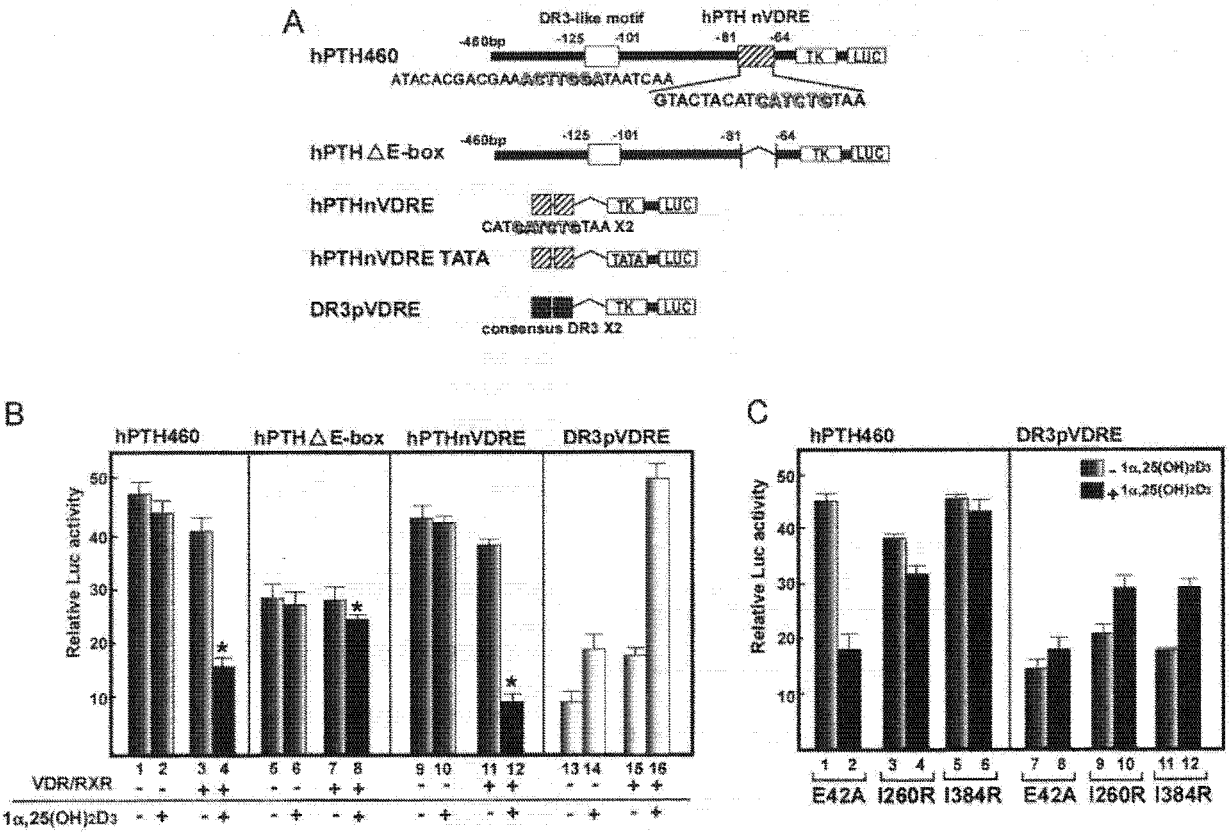

Fig. 3. 1α,25(OH)$_2$D$_3$-Induced Transrepression of hPTH Gene by VDR Mediates an E-Box-Type nVDRE in the Promoter

A, Schematic representation of an hPTH promoter and a mutant deleted E-box. The previously reported DR3-core motif and an E-box motif are located in the −460-bp region. B, A mutant promoter deleted E-box motif in hPTH promoter (hPTHΔE-box) lost the negative response to 1α,25(OH)$_2$D$_3$. The luciferase (LUC) activity was measured as described in Fig. 2A. C, Transrepression of VDIR via VDR mutants measured by luciferase assay. E42A mutant inhibits DNA binding affinity, and I260R and I384R mutants lack heterodimerization of VDR and RXR. Luciferase assay was performed with either hPTH460 or DR3 reporter after cotransfection of wild-type VDR or mutant VDRs into MCT cells.

3C, lanes 1 and 2). Thus, these findings suggest that transrepression of the hPTH gene promoter by VDR requires heterodimerization, but not necessarily direct DNA binding of VDR/RXR heterodimer.

VDIR Is Indispensable for the Transrepression Function by VDR/RXR through the E-Box-Type Element in the hPTH Promoter

Because we have previously shown that VDIR transactivates through physical interaction with 1αnVDRE (24), we then assessed whether VDIR directly binds to the identified hPTHnVDRE by EMSA. No direct DNA binding of recombinant VDR/RXR heterodimer to the hPTHnVDRE as well as the reported DR3-like nVDRE was observed (Fig. 4A, lanes 5–8). However, clear DNA binding was detected for positive DR3 VDRE as a positive control (Fig. 4A, lanes 1 and 2). A recombinant VDIR bound to the hPTHnVDRE, similar to 1αnVDRE (Fig. 4B, lanes 4 and 8). The specificity of DNA binding of VDIR to the hPTHnVDRE was further confirmed by competition with a large excess of cold 1αnVDRE oligomers or *vice versa* (Fig. 4B, lanes 5, 6, 9, and 10). In fact, a ligand-induced transrepression by VDR/RXR on hPTH promoter (hPTH460) was enhanced by overexpression of VDIR (Fig. 5A, lanes 1 and 2) when compared with conditions without VDIR overexpression (Fig. 3B, lanes 3 and 4). Conversely, when an endogenous VDIR protein was knocked down using RNA interference (RNAi) (see Fig. 5C, *right panel*), no transrepression by VDR/RXR through both of the hPTH460 and hPTHrP850 was observed (Fig. 5B), and indeed VDIR RNAi abrogated the transrepression of the endogenous mouse (m) PTHrP gene expression by activated VDR in MCT cells (*left panel* in Fig. 5C). Moreover, in our preliminary experiment using primary cultured cells of parathyroid glands, we also observed that VDIR ablation by RNAi derepressed the endogenous mPTH expression by activated VDR (data not shown). These results suggest that ligand-induced transrepression by VDR/RXR heterodimer requires VDIR upon the hPTHnVDRE. This idea is supported by the result that a VDR-point mutation (P61T) failed to transrepress through hPTHnVDRE but still retained ligand-induced transactivation through the positive VDRE (DR3 pVDRE) (Fig. 5A, lanes 4 and 8). This mutant where proline is replaced with threonine at position 62 in the DNA binding domain was unable to interact with VDIR *in vitro* (Fig. 5D). Thus, this functional abrogation of VDR by the P61T mutation verified a significant role of VDIR in the transrepression.

Previously, we demonstrated that VDIR acted as an activator on the 1αnVDRE by recruiting p300 HAT coactivator complexes in response to activated-protein kinase A signaling. Also, activation of VDR-induced recruitment of HDAC corepressor complex to the VDIR for transrepression (24). We therefore examined whether the transactivation function of VDIR bound on the hPTHnVDRE was transrepressed by liganded VDR/RXR through recruitment of an HDAC corepressor. As expected, VDIR clearly activated transcription through hPTH nVDRE (Fig. 6A, lane 6), but activated VDR attenuated this transactivation by VDIR (Fig. 6A, lane 8). An HDAC inhibitor, trichostatin A (TSA) abrogated ligand-induced transrepression through VDIR and VDR/RXR (Fig. 6A, lanes 9 and 10), confirming that HDAC activity is required for transrepression. We then tested coregulator recruitment to the nVDREs by an avidin-biotin complex DNA binding assay. As previously reported (24), treatment with forskolin, a protein kinase A activator to stimulate VDIR function, induced p300 HAT recruitment to nVDREs (see *upper panel* of Fig. 6B). In contrast, when the cells were treated with 1α,25(OH)$_2$D$_3$, HDAC2, but neither HDAC1 nor 3 (data not shown), was recruited to VDR/VDIR bound upon hPTHnVDRE as well as 1αnVDRE (Fig. 6B, *lower panel*). Altogether, these findings demon-

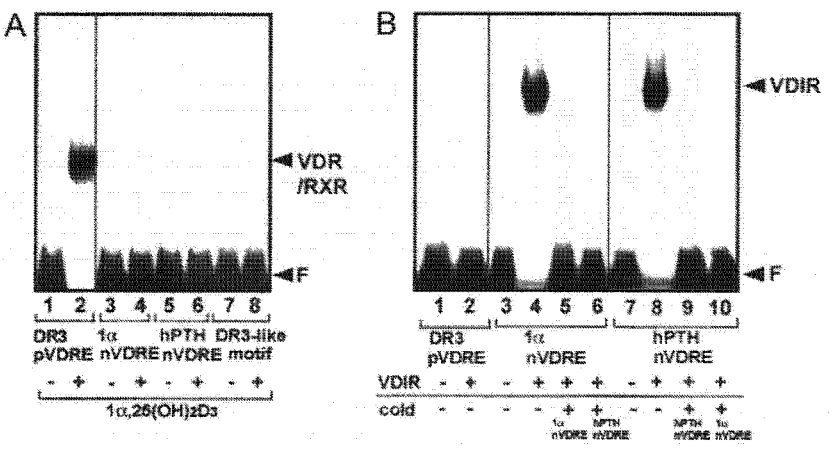

Fig. 4. E-Box-Type nVDREs Physically Interact with VDIR, But Not VDR/RXR

A and B, EMSA was performed with 1αnVDRE, hPTHnVDRE as a designed probe, and direct repeat (DR) 3 oligomers as a positive VDRE. VDR and RXR recombinant proteins were expressed as GST fusions. Specific representative cold-hPTHnVDRE or cold-1αnVDRE oligomers were used at a 50-fold molar excess. F, Free probe.

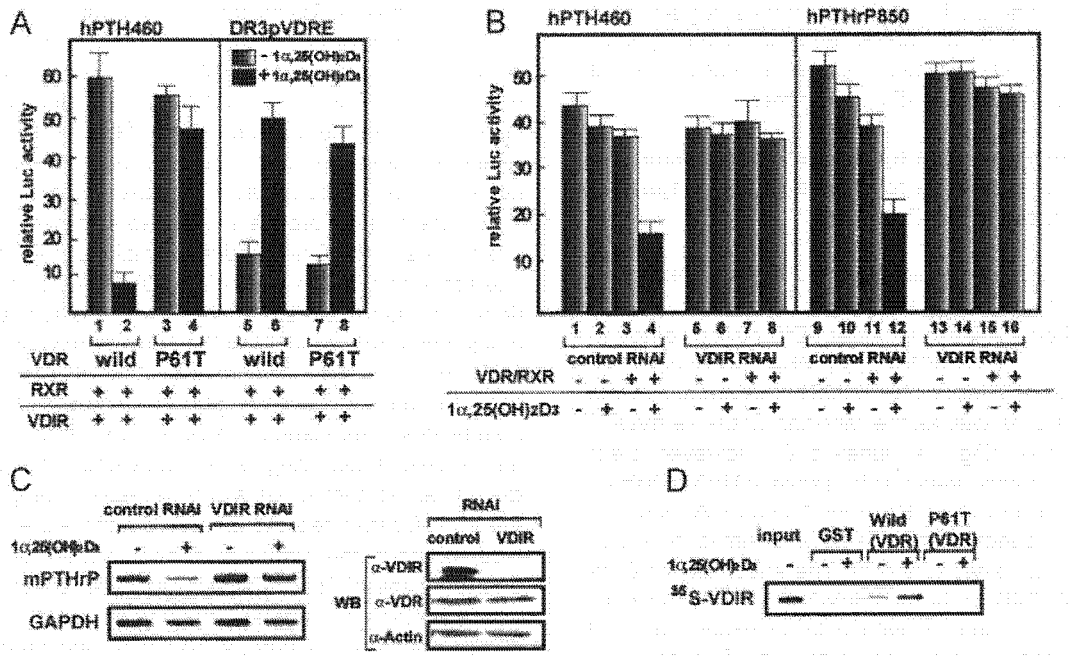

Fig. 5. Direct DNA Binding of VDIR to the hPTH nVDRE Is Essential for Ligand-Dependent Transrepression by VDR

A, A P61T mutant abolished 1α,25(OH)$_2$D$_3$-induced transrepression activity using a luciferase assay. Luciferase activity was assessed as shown in Fig. 2A. B, VDIR is required for 1α,25(OH)$_2$D$_3$-induced transrepression by VDR in the hPTH460 and hPTHrP850. For VDIR-specific knockdown, whole-cell extracts were prepared from MCT cells transfected with 100 nM siRNA. C, Endogenous PTHrP gene was analyzed by RT-PCR. To assess gene-knockdown effects by siRNA of VDIR, Western blots were performed using α-VDIR, α-VDR and α-actin (as a control). D, P61T mutant loses binding activity with VDR. GST-alone, GST-VDR wild-type, or GST-P61T mutant was incubated with ^{35}S-labeled VDIR, which was translated *in vitro*, in the presence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) or not. DR, Direct repeat; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot.

strated that ligand-induced transrepression by VDR/RXR with VDIR upon hPTHnVDRE also mediates HDAC2 recruitment, presumably coupled to HAT coactivator dissociation. Such ligand-induced coregulator switching through VDR/RXR and VDIR upon the hPTHnVDRE appears similar to that reported for 1αnVDRE (24).

E-Box-Type Elements Act as a Class of nVDRE

Considering that vitamin D plays a number of roles in various target tissues, it is likely that there are a number of as-yet-unidentified target genes whose expression is under negative control by VDR. The

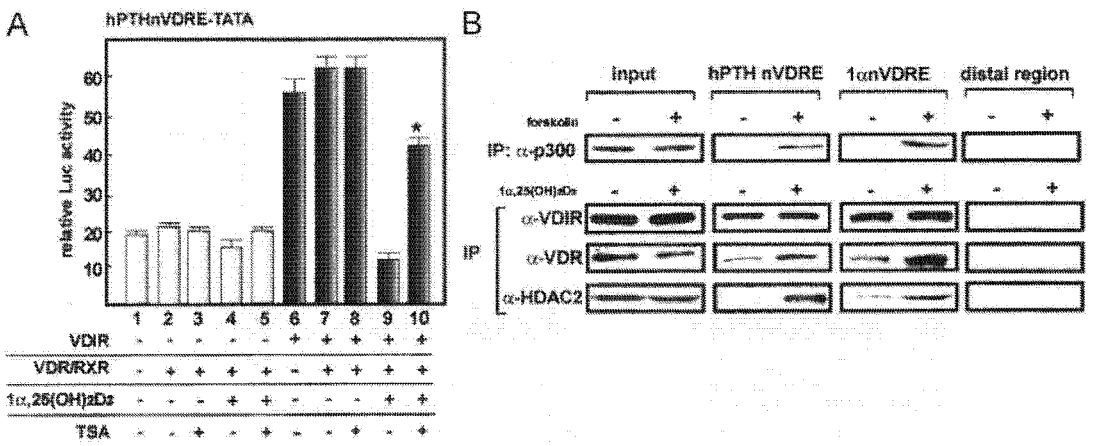

Fig. 6. The 1α,25(OH)$_2$D$_3$-Induced Transrepression by VDR upon the hPTHnVDRE Requires HDAC2

A, The transfected cells were treated with TSA (10^{-7} M) for 24 h before harvest. B, A ligand-induced HDAC2 recruitment to VDR/RXR and VDIR bound upon the hPTHnVDRE. Two copies of hPTHnVDRE and h1αnVDRE oligomers (as a control) were conjugated to the beads and incubated with whole-cell extracts from MCT cells treated in the presence and absence of 1α,25(OH)$_2$D$_3$ and forskolin. The interactants with oligo-beads were detected by Western blots using individual antibody. IP, Immunoprecipitation.

present model might explain at the molecular level the negative response to 1α,25(OH)$_2$D$_3$ in the expression of at least some VDR-negative target genes. E-box type elements in the h1α(OH)ase and hPTH gene promoters may be representative of those in other gene promoters. In this respect, it would be intriguing to identify the genes negatively regulated by VDR and analyze the negative VDREs in those gene promoters. There may be other classes of nVDRE because modes of ligand-induced transrepression generally appear diverse among nuclear receptors (18, 28).

A Cell Type-Specific Coregulator Complex Is Mediated by Ligand-Induced Transrepression by VDR

Although the enzyme is expressed in many nonrenal tissues besides kidney, including skin, the regulation of 1α(OH)ase gene expression is different between kidney and skin cells (29). In the previous report, we revealed that ligand-induced transrepression by VDR through E-box type nVDRE in the h1α(OH)ase gene promoter is reproducible only in the renal tubule cell line (MCT) that endogenously expresses 1α(OH)ase, VDIR and VDR genes (24). Such ligand-induced transrepression was undetectable in other cell lines, even other kidney-derived cell lines (data not shown). These results suggest transrepression by liganded VDR may require a cell type-specific coregulator (16, 17). Similarly, because the PTH gene is expressed only in the parathyroid and thymus, a tissue-specific factor may also be indispensable there for transrepression of the PTH gene by liganded VDR. It remains unclear whether such a tissue cell type-specific coregulator is identical or not in both parathyroid and renal tubule cells. Even though HDAC appears principally responsible for transrepression, it was unexpected that TSA, even at much higher concentrations than the normally applied range, could not fully abrogate 1α,25(OH)$_2$D$_3$-induced transrepression by VDR. This may imply that other histone modifiers such as histone methylase/demethylase are involved in this transrepression. In this sense, biochemical identification of complex(es) containing both VDR/RXR and VDIR clearly requires identification of tissue-specific factor(s) involved in 1α,25(OH)$_2$D$_3$-induced transrepression.

MATERIALS AND METHODS

RT-PCR

The male VDR KO mice have been described previously (26). 1α,25(OH)$_2$D$_3$ (10 μg/kg) or vehicle (medium chain triglyceride) were administered orally three times a week. After the final 24 h, total RNA was extracted from several mice (12-wk-old mice) using an ISOGEN Kit (Nippon Gene). One microgram of total RNA was reverse-transcribed by Manuscript (Invitrogen, Carlsbad, CA). The PCR was performed for 35 cycles consisting of 40 sec of denaturing at 95 C, 40 sec annealing at 59 C (mPTH/mPTHrP) or 57 C [m1α(OH)ase], and then 40 sec extension at 72 C. For RT-PCR, the following primers were used: mPTH 5′-ATGATGTCTGCAAACACCGTGG-3′ and 5′-ATCAGCTTTGTCTCCCTCACCAG-3′; mPTHrP 5′-CTGGTTCAGCAGTGGAGCTC-3′ and 5′-GTTAGGGGACACCTCCGAGGT-3′; mouse 1α(OH)ase 5′-AAACACAGACATGACCCAGG-3′ and 5′-GCAGCGCCATGCACCTGCAG-3′; mouse glyceraldehyde-3-phosphate dehydrogenase 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (30).

Plasmid and RNAi

Deletion mutants of the hPTH and hPTHrP promoters were generated by PCR and cloned into the pGL3-TK Luciferase vector (Promega, Madison, WI). The two hPTH VDRE sequences (5′-CATCATCTGTAA-3′) were inserted into the pGL3-Luciferase vector driven by a thymidine kinase (TK) and TATA promoter, respectively. The small interfering RNA (siRNA) sequences of VDIR were designed by Dharmacon (Lafayette, CO) (23).

Cell Culture and Transient Transfection

MCT cells (derived from proximal tubules of mouse kidney) were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) (Invitrogen) at 37 C, 5% CO_2. For transfection, cells were cultured in DMEM supplemented with 5% charcoal-stripped FBS. Cells were transfected with expression vector using Lipofectamin Plus (Invitrogen) according to the manufacturer's instructions. After treatment with vehicle or ligand (1×10^{-8} M), cells were further cultured for 24 h. Luciferase assays were performed as described (31). siRNA transfection were as described (23).

EMSA

Recombinant VDR, RXRβ, and VDIR were expressed in *E. coli* as glutathione-*S*-transferase (GST)-fusion proteins and purified by digestion with thrombin after affinity column chromatography. Double-stranded oligonucleotide probes were end-labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase. For reaction, 10 ng of recombinant proteins were preincubated in binding buffer [10 mM Tris-HCl (pH 7.5), 75 mM KCl, 5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 1 mM dithiothreitol] with/without 10^{-8} M 1α,25(OH)$_2$D$_3$ for 20 min on ice. Ten microliters of labeled probes were added in each sample. After incubation for 30 min at room temperature, the samples were resolved on 5% polyacrylamide gels and run in 0.5× Tris-EDTA buffer. The gel was dried and subjected to autoradiography. The following sequences of double-stranded oligonucleotides were used as probes: [1αnVDRE; 5′-CCATTAACCCACCTGCCATCTGCC × 2–3′, hPTHnVDRE: 5′-CATCATCTGTAAC × 2–3′, DR3-like motif; 5′-GTGTGTATGTGCTGCTTT × 2–3′]. Assays were performed as described (24).

Template-Pull-Down Assay (Avidin-Biotin Complex DNA Binding Assay)

3′-End biotin conjugated sense and antisense DNA were incubated at 100 C to anneal and cooled slowly to room temperature. The following sequences of double-stranded oligonucleotides were used as probes: [1αnVDRE; 5′-CCATTAACCCACCTGCCATCTGCC × 2–3′, hPTHnVDRE: 5′-CATCATCTGTAAC × 2–3′, distal regin (negative control); 5′-GGCATGCATC × 2–3′]. To prepare bead-DNA complex, biotin-conjugated double-strand DNA was mixed with 50% slurry avidin-beads. Cells were cultured in DMEM supplemented with 5% charcoal-stripped FBS for 24 h, after treatment with vehicle or ligand (1×10^{-8} M). Cells were lysed in lysis buffer [10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.15 M

NaCl, 0.1% Nonidet P40]. Whole cell lysates were immunoprecipitated by bead-DNA complex containing poly (deoxyinosine-deoxycytosine) (32). Proteins were resolved on SDS-PAGE and Western blots were visualized with α-E47 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), α-VDR antibody (NeoMarkers; Lab Vision, Fremont, CA), α-HDAC2 antibody (Affinity BioReagents, Golden, CO), and α-p300 antibody (Santa Cruz Biotechnology) (24).

Acknowledgments

We thank I. Takada for helpful discussions and Chugai Pharmaceutical Co. Ltd. for the $1\alpha,25(OH)_2D_3$ relative compound. We also thank H. Higuchi for preparation of the manuscript.

Received May 26, 2006. Accepted November 1, 2006.

Address all correspondence and requests for reprints to: Shigeaki Kato, The Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. E-mail: uskato@mail.ecc.u-tokyo.ac.jp.

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to S.K.).

Disclosure Statement: The authors have nothing to disclose.

REFERENCES

1. DeLuca HF 2004 Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 80: 1689S–1696S
2. Salle BL, Delvin EE, Lapillonne A, Bishop NJ, Glorieux FH 2000 Perinatal metabolism of vitamin D. Am J Clin Nutr 71:1317S–1324S
3. Silver J, Naveh-Many T 2005 Vitamin D and the parathyroids. In: Feldman D, Pike JW, Glorieux FH, eds. Vitamin D. 2nd ed. San Diego: Elsevier Academic Press; 537–549
4. Bouillon R, Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257
5. Brenza HL, DeLuca HF 2000 Regulation of 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression by parathyroid hormone and 1,25-dihydroxyvitamin D3. Arch Biochem Biophys 381:143–152
6. Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S 1997 25-Hydroxyvitamin D3 1α-hydroxylase and vitamin D synthesis. Science 277:1827–1830
7. Demay MB, Kiernan MS, DeLuca HF, Kronenberg HM 1992 Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 89: 8097–8101
8. Liu SM, Koszewski N, Lupez M, Malluche HH, Olivera A, Russell J 1996 Characterization of a response element in the 5′-flanking region of the avian (chicken) PTH gene that mediates negative regulation of gene transcription by 1,25-dihydroxyvitamin D3 and binds the vitamin D3 receptor. Mol Endocrinol 10:206–215
9. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T, Kato S 1998 The promoter of the human 25-hydroxyvitamin D3 1 α-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1 α,25(OH)2D3. Biochem Biophys Res Commun 249:11–16
10. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S 1999 Positive and negative regulations of the renal 25-hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1α,25(OH)2D3 in intact animals. Endocrinology 140:2224–2231
11. Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13:325–349
12. Rachez C, Freedman LP 2001 Mediator complexes and transcription. Curr Opin Cell Biol 13:274–280
13. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
14. Whitfield GK, Jurutka PW, Haussler CA, Hsieh JC, Barthel TK, Jacobs ET, Dominguez CE, Thatcher ML, Haussler MR 2005 Nuclear vitamin D receptor: structure-function, molecular control of gene transcription, and novel bioactions. In: Feldman D, Pike JW, Glorieux FH, eds. Vitamin D. 2nd ed. San Diego: Elsevier Academic Press; 219–261
15. Umesono K, Murakami KK, Thompson CC, Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell 65:1255–1266
16. Glass CK, Rosenfeld MG 2000 The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14:121–141
17. McKenna NJ, O'Malley BW 2002 Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465–474
18. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, Kadowaki T, Takeuchi Y, Shibuya H, Gotoh Y, Matsumoto K, Kato S 2003 Cytokines suppress adipogenesis and PPAR-γ function through the TAK1/TAB1/NIK cascade. Nat Cell Biol 5:224–230
19. McNamara P, Seo SP, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA 2001 Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell 105: 877–889
20. Nissen RM, Yamamoto KR 2000 The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev 14:2314–2329
21. Falzon M 1996 DNA sequences in the rat parathyroid hormone-related peptide gene responsible for 1,25-dihydroxyvitamin D3-mediated transcriptional repression. Mol Endocrinol 10:672–681
22. Russell J, Ashok S, Koszewski NJ 1999 Vitamin D receptor interactions with the rat parathyroid hormone gene: synergistic effects between two negative vitamin D response elements. J Bone Miner Res 14:1828–1837
23. Fujiki R, Kim MS, Sasaki Y, Yoshimura K, Kitagawa H, Kato S 2005 Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. EMBO J 24:3881–3894
24. Murayama A, Kim MS, Yanagisawa J, Takeyama K, Kato S 2004 Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. EMBO J 23:1598–1608
25. Sela-Brown A, Russell J, Koszewski NJ, Michalak M, Naveh-Many T, Silver J 1998 Calreticulin inhibits vitamin D's action on the PTH gene in vitro and may prevent vitamin D's effect *in vivo* in hypocalcemic rats. Mol Endocrinol 12:1193–1200

26. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16:391–396
27. Kraichely DM, Collins 3rd JJ, DeLisle RK, MacDonald PN 1999 The autonomous transactivation domain in helix H3 of the vitamin D receptor is required for transactivation and coactivator interaction. J Biol Chem 274: 14352–14358
28. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK 2005 A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-γ. Nature 437:759–763
29. Flanagan JN, Wang L, Tangpricha V, Reichrath J, Chen TC, Holick MF 2003 Regulation of the 25-hydroxyvitamin D-1α-hydroxylase gene and its splice variant. Recent Results Cancer Res 164:157–167
30. Gunther T, Chen ZF, Kim J, Priemel M, Rueger JM, Amling M, Moseley JM, Martin TJ, Anderson DJ, Karsenty G 2000 Genetic ablation of parathyroid glands reveals another source of parathyroid hormone. Nature 406:199–203
31. Kitagawa H, Fujiki R, Yoshimura K, Mezaki Y, Uematsu Y, Matsui D, Ogawa S, Unno K, Okubo M, Tokita A, Nakagawa T, Ito T, Ishimi Y, Nagasawa H, Matsumoto T, Yanagisawa J, Kato S 2003 The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. Cell 113:905–917
32. Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y, Kato S 2003 Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423:545–550

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Oncogene (2007), 1–8

www.nature.com/onc

ORIGINAL ARTICLE

Id2 gene-targeted crosstalk between Wnt and retinoid signaling regulates proliferation in human keratinocytes

A Memezawa[1,2], I Takada[1], K Takeyama[1], M Igarashi[1], S Ito[1], S Aiba[2], S Kato[1,3] and AP Kouzmenko[1,3]

[1]*Department of Nuclear Signaling, Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan;* [2]*Department of Dermatology, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan and* [3]*ERATO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan*

We investigated the effect of all-*trans*-retinoic acid (atRA) on proliferation in several human skin cell lines and found that antiproliferative potency of atRA correlated with the endogenous activity of canonical Wnt signaling. In HaCaT keratinocytes, we found that atRA significantly suppressed the expression of Id2, a member of the inhibitor of differentiation family of transcription factors that regulate cell growth and differentiation. However, no apparent change in the expression of other Wnt targets, like c-Myc or cyclin D1, was observed. Retinoid-induced Id2 gene suppression was associated with decreased levels of histone H3 and H4 acetylation and histone H3 Lys-4 methylation, and with recruitment of the LSD1 demethylase at the Wnt-response element (WRE) (TCF/LEF-binding site), in the Id2 gene promoter. None of such changes was detected at the WRE of c-Myc and cyclin D1 gene promoters. Inhibition of Id2 by short interfering RNA (siRNA) had a similar effect on the proliferation of HaCaT cells as exposure to atRA, whereas anti-β-catenin siRNA significantly inhibited its antiproliferative effect. These data suggest that down-regulation of Id2 gene expression through transcriptional convergence between Wnt and retinoid signaling pathways underlies the antiproliferative effect of retinoids in keratinocytes, and provide evidence of gene-targeted crosstalk between signaling pathways.
Oncogene advance online publication, 19 February 2007; doi:10.1038/sj.onc.1210320

Keywords: keratinocytes; retinoids; Wnt; proliferation; Id2

Introduction

Retinoids, structural and functional analogues of vitamin A, play fundamental role in the regulation of growth, differentiation, homeostasis and apoptosis in many cells and tissues. The physiological action of retinoids is primarily mediated by the activities of two classes of nuclear receptors, retinoic acid receptors (RARα, RARβ and RARγ) and retinoid X receptors (RXRα, RXRβ and RXRγ). Upon ligand binding, these receptors form RXR-RAR heterodimers or RXR-RXR homodimers, which then bind to DNA response elements, RAREs or RXREs, leading to recruitment of activator or repressor complexes to modulate the expression of target genes. Retinoid receptors interact with intracellular mediators of other signaling pathways and modulate their regulatory action on their corresponding target genes, thereby increasing complexity of the pleiotropic effects of retinoids owing to crosstalk with different signaling pathways (Mark *et al.*, 2006).

Vitamin A deficiency has been implicated in the etiology of various epithelial tumors. The chemopreventive and chemotherapeutic effects of some natural and synthetic retinoids, such as all-*trans*-retinoic acid (atRA), for various cancers, including head and neck, cervical, neuroblastoma, promyelocytic leukemia and cutaneous squamous cell carcinoma, have been well documented in clinical trials and experiments on animal models (Niles, 2004; Okuno *et al.*, 2004 and references therein). However, the molecular mechanisms of the antiproliferative action of retinoids in skin cells remain generally unclear.

Canonical Wnt signaling is activated by Wnt peptide ligands through interaction with frizzled (Fzd) plasma membrane receptors. This leads to stabilization and nuclear translocation of the intracellular transducer β-catenin. In the nucleus, β-catenin associates with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptional factors. TCF/LEF–β-catenin complexes bind to TCF/LEF-binding sites, or Wnt-response elements (WRE), in the promoters of target genes, leading to their transcriptional activation. The crucial role of Wnt signaling in skin physiology and disease has been well established (Alonso and Fuchs, 2003; Weeraratna, 2005). Canonical

Correspondence: Professor S Kato and Dr AP Kouzmenko, Department of Nuclear Signaling, Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo, 113-0032, Japan.
E-mails: uskato@mail.ecc.u-tokyo.ac.jp and alexk@iam.u-tokyo.ac.jp
Received 26 September 2006; revised 21 December 2006; accepted 8 January 2007

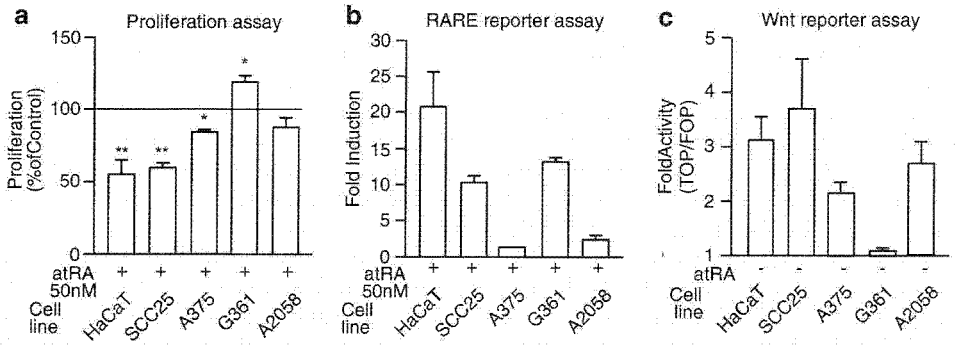

Figure 1 Retinoid-induced reduction in the rate of proliferation correlates with the activity of endogenous Wnt signaling in skin cells. (**a**) Cells were treated with vehicle or 50 nM atRA and proliferation was estimated after 48 h of treatment. Data presented as the mean ± s.d. percentage of BrdU incorporation in the presence of atRA relatively to incorporation of the vehicle control taken as 100% for each cell line from four independent experiments each in quadruplicate. $*P<0.01$ and $**P<0.001$ (**b**) Activities of endogenous retinoid signaling in skin cell lines. Cells were transfected with RARE-dependent luciferase reporter plasmid and treated overnight with vehicle or 50 nM atRA. Data presented as the ratio of luciferase activity in the presence of atRA to its activity in the presence of vehicle. (**c**) Activities of endogenous Wnt signaling in skin cell lines. Cells were transfected with either Wnt-responsive TOPFLASH or non-responsive FOPFLASH reporter plasmid and luciferase activity in cell lysates was analysed 24 h post-transfection. Data presented as the ratio of TOPFLASH to FOPFLASH reporter activities, with ratio 1 indicating no Wnt/β-catenin-specific reporter activation. The data in (**b**) and (**c**) represent the mean ± s.d. of three independent experiments, each in triplicate.

Wnt signaling converges with various hormone-signaling pathways through interaction with corresponding nuclear receptors. This crosstalk has been shown to play an important role in the regulation of many vital biological processes, including cell proliferation and carcinogenesis (Moon *et al.*, 2004; Mulholland *et al.*, 2005 and references therein).

Initially, RA was shown to suppress the expression of the Wnt/β-catenin-dependent reporter through direct interaction between RAR and β-catenin (Easwaran *et al.*, 1999). However, in different systems RA was found to increase levels and stability of the nuclear β-catenin, thereby enhancing the Wnt/β-catenin signaling (Liu *et al.*, 2002; Otero *et al.*, 2004). Conversely, Wnt/β-catenin enhances RAR transactivation (Easwaran *et al.*, 1999) and synergizes with RAR in activation of RA-inducible genes (Szeto *et al.*, 2001; Tice *et al.*, 2002).

We have studied the effect of atRA on proliferation in several human skin cell lines and found that the antiproliferative potency of atRA correlated with the endogenous activity of canonical Wnt signaling, but not retinoid signaling. Interaction between Wnt and retinoid signaling in regulation of proliferation has been further investigated in detail in HaCaT cells (Boukamp *et al.*, 1988), a widely accepted human keratinocyte model system. Here, we show that transcriptional downregulation of Id2 gene expression through crosstalk between Wnt and retinoid signaling pathways underlies the antiproliferative effect of retinoids in keratinocytes. Our data indicate that Id2 may represent a beneficial therapeutic target in treatment of epithelial tumors.

Results

We studied the effects of retinoids on cell proliferation in several human keratinocyte (HaCaT and SCC25) and melanocyte (A2058, A375 and G361) lines. After 48 h of exposure to 50 nM atRA, the rate of proliferation significantly decreased in HaCaT keratinocytes and squamous cell carcinoma SCC25 cells, slightly decreased in A375 and A2058 melanoma cells, but somewhat increased in G361 melanoma cells (Figure 1a).

To investigate the factors responsible for selective effects of atRA on proliferation, activities of endogenous signaling pathways in these cell lines were analysed by expression of pathway-specific reporter genes. No apparent association was observed between the effects of atRA on cell proliferation and retinoid signaling, judged by the induction of RARE-dependent reporter expression (Figure 1b). A375 cells showed the lowest activation of the reporter, but exhibited a small yet statistically significant reduction of BrdU incorporation in response to atRA. In comparison, G361 and SCC25 cells with high and seemingly comparable levels of endogenous retinoid signaling contrasted sharply in their response to atRA.

β-catenin-mediated Wnt signaling has been shown to activate the expression of many key regulators of proliferation, such as cyclin D1 and c-Myc. Therefore, we analysed endogenous Wnt signaling in these cells by using TOPFLASH (with a functional WRE) and FOPFLASH (as a normalizing control with mutated WRE that does not bind TCF/LEF factors) reporters. There appeared to be a correlation between the activity of endogenous Wnt signaling and the retinoid-induced decrease in the rate of proliferation (Figure 1c). Growth of G361 cells with almost silent Wnt and high levels of endogenous retinoid signaling was rather stimulated by atRA. In contrast, proliferation of A375 cells with low levels of endogenous retinoid signaling decreased when treated with atRA.

We analysed the expression of genes encoding Wnt ligands and mediators of the retinoid and Wnt signaling pathways in two cell lines that contrasted most in their

proliferative response to atRA: HaCaT keratinocytes and G361 melanocytes. Expression of mRNA for three RAR receptors, Wnt ligands, Fzd receptors and β-catenin in these cells in the absence and presence of atRA was estimated by reverse transcription-polymerase chain reaction (RT-PCR). Consistent with the reporter expression data, cells of both lines express RAR receptors, albeit at different levels. Induction of RARβ expression in response to atRA was more readily apparent in HaCaT cells, probably owing to the higher basal levels of expression of RARα and RARγ in comparison with G361 cells. Both cell lines also express various Frz plasma membrane receptors for Wnt ligands. However, expression of Wnt ligands was only detectable in HaCaT, but not in G361 cells (Figure 2a).

As β-catenin has a dual role as a structural protein and intracellular Wnt signal transducer, we compared levels of nuclear β-catenin in these cell lines by immunocytochemical staining. Although levels of β-catenin mRNA appeared to be higher in G361 cells, it was only reflected in stronger plasma membrane staining, whereas the cyto- or nucleoplasmic fluorescent staining of β-catenin protein in these cells was not visible (Figure 2b). Lack or below-detectable levels of Wnt ligand expression is consistent with the apparent silence of the Wnt reporter and absence of nuclear staining of β-catenin in G361 cells.

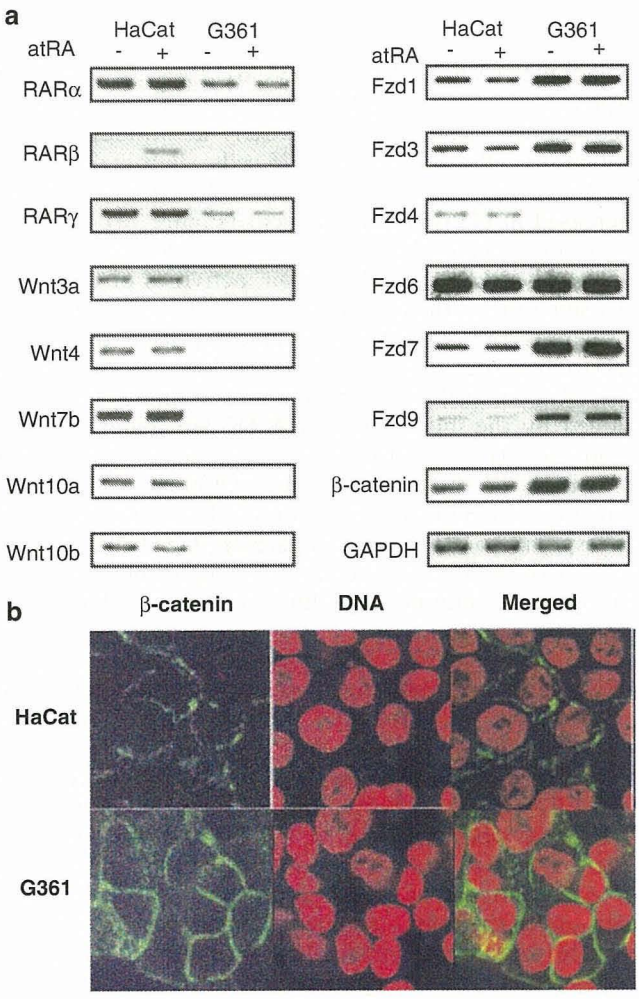

Figure 2 Expression of Wnt ligands and mediators of Wnt and retinoid signaling pathways in HaCaT keratinocytes and G361 melanocytes. (**a**) Levels of mRNA for indicated proteins from intact cells and cells treated with 50 nM atRA were analysed by RT-PCR. Shown data are the most representative experiment of several independent experiments. (**b**) Immunocytochemical staining of β-catenin in HaCaT and G361 cells.

To investigate further correlation between Wnt activity and action of retinoids, we analysed effects of activation and suppression of Wnt signaling in HaCaT cells on their proliferation in response to atRA. In these cells, additional activation of Wnt by treatment with Wnt3a resulted in a stimulation of cell growth in the presence of vehicle. However, there was a more prominent decrease in the rate of proliferation in response to atRA. In contrast, inhibition of Wnt with anti-β-catenin short interfering RNA (siRNA) markedly reduced both the basal rate of proliferation and antiproliferative effect of atRA in comparison to control cells transfected with nonspecific anti-GFP siRNA (Figure 3a). As total cellular levels of β-catenin do not reflect levels of the nuclear β-catenin that mediates Wnt, as it can be clearly seen above when comparing levels of β-catenin mRNA and Wnt activity in HaCaT and G361 cells (Figures 1c and 2), for control of Wnt activation by the Wnt3a ligand and suppression by anti-β-catenin siRNA, we used the activity of Wnt/β-catenin-dependent reporter as a functionally more relevant marker (Figure 3b).

Overall, the above data show that the antiproliferative action of atRA involves crosstalk between Wnt and retinoid signaling pathways.

Next, we investigated potential target genes of this crosstalk in the HaCaT keratinocytes. Expression of several well-characterized Wnt targets (see www.stanford.edu/~rnusse/pathways/targets.html) and key regulators of cell-cycle progression were analysed by RT-PCR. Treatment with atRA produced no discernable differences in the levels of mRNA for cyclin D1, c-Myc, or EGFR and there was a slight decrease for CD44. However, exposure to atRA caused a significant reduction in Id2 gene expression (Figure 4a and b).

Next, we investigated whether this suppression of the Id2 expression was direct and mediated by transcriptional interaction between RARs and β-catenin. Chromatin immunoprecipitation (ChIP) assay experiments showed that atRA induced recruitment of the RARs to the WRE of the Id2 (Figure 5a), c-Myc (Figure 5b) and cyclin D1 (not shown) gene promoters. However, only in the Id2 gene atRA triggered a marked reduction in the levels of histone H4 and H3 (not shown) acetylation and histone H3 K-4 methylation, a clear mark of transcriptional repression. This was associated with retinoid-dependent recruitment of the LSD1 demethylase to the WRE in the promoter of the Id2 gene (Figure 5a). At the same time, expression of the LSD1 itself was not affected by atRA. Despite a slight increase in the β-catenin binding, combined treatment with Wnt3a and atRA had no readily detectable effect on LSD1 recruitment to the WRE, probably owing to sensitivity

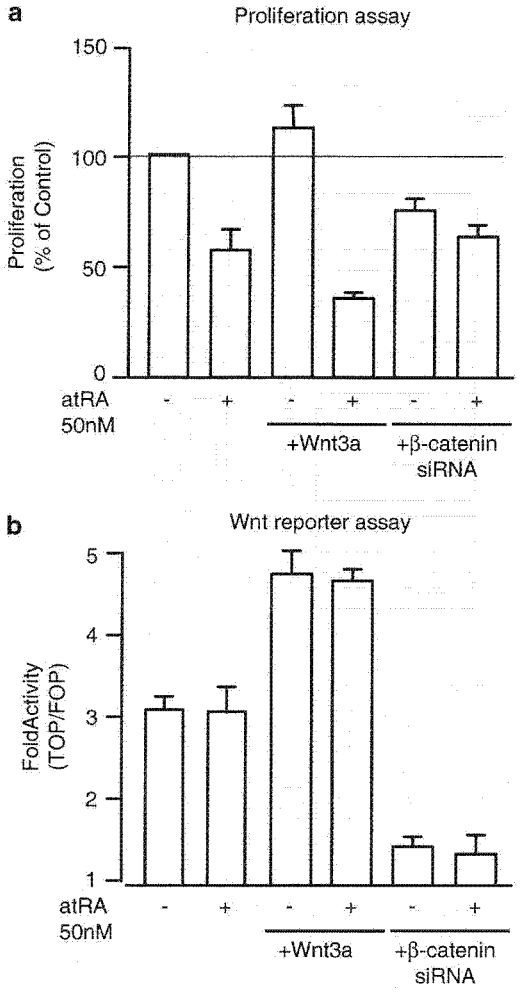

Figure 3 Effect of atRA on HaCaT cell proliferation depends on Wnt activation. (**a** and **b**) For Wnt activation cells were grown in the presence of 50 ng/ml Wnt3a, and for Wnt suppression cells were transfected with β-catenin siRNA. Cells were treated with vehicle or atRA for 48 h. (**a**) Proliferation analysed as described in the legends for Figure 1a. Proliferation of cells transfected with β-catenin siRNA presented as percentage of proliferation rates under the same conditions of cells transfected with control non-targeting siRNA, (**b**) Wnt/β-catenin-dependent reporter activity was used as an indicator of Wnt signaling after treatment with Wnt3a or transfection with siRNAs.

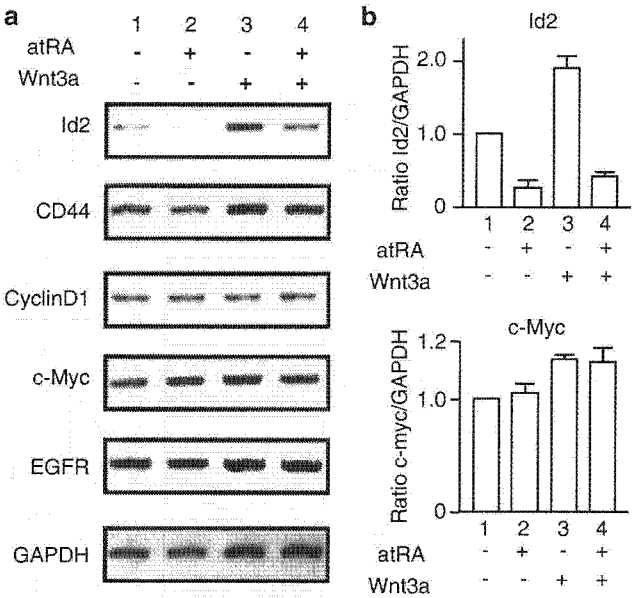

Figure 4 Effect of atRA on Wnt target genes in HaCaT keratinocytes. (**a**) Wnt target gene expression in HaCaT cells treated with vehicle or 50 nM atRA in the presence or absence of 50 ng/ml Wnt3a was analysed by RT-PCR. (**b**) Id2 and c-Myc mRNA expression was further analysed by semi-quantitative real-time RT-PCR experiments. Data presented as the ratio of Id2 and c-Myc expression to GAPDH (normalizing control) expression based on real time PCR efficiency of the primers. The ratio from the control experiment was taken as 1.

limits of the ChIP assay (data not shown). Consistent with the levels of c-Myc and cyclin D1 mRNA (Figure 4a and b), no such change was apparent in the promoters of c-Myc (Figure 5b) and cyclin D1 (not shown) genes.

We were not able to identify in the Id2 gene promoter a DNA sequence resembling known RARE or RXRE, and no functional RARE was identified in the cloned Id2 promoter in earlier studies (Neuman *et al.*, 1995). Nonetheless, we investigated whether RAR recruitment to the WRE at the Id2 promoter was entirely dependent on the binding of β-catenin. Transfection with β-catenin targeting siRNA significantly reduced the amounts of β-catenin bound to the Id2 gene WRE and it was associated with seemingly reciprocal reduction in the atRA-induced binding of RARs (Figure 5c). This suggests that RAR binding to the WRE in the Id2 gene promoter is indirect and mediated by β-catenin.

It has been reported that retinoic acid does not affect levels of cyclin D1 mRNA, but promotes the ubiquitination and consequent degradation of cyclin D1 protein, and that proteasomal inhibitors block retinoid-induced decline in cyclin D1 (Ma *et al.*, 2005). In a different experimental system, glucocorticoid-induced decline in proliferation of neural stem cells was abrogated in the presence of 0.5 μM of a potent proteasomal inhibitor MG132, reflecting an involvement of the ubiquitin proteasomal pathway (Sundberg *et al.*, 2006). Furthermore, Id2 has been identified as a potential substrate for some E3 ubiquitin ligase complexes and, thus, can be targeted for proteasomal degradation (Lasorella *et al.*, 2006). Therefore, we investigated whether MG132 would be able to counteract the antiproliferative action of atRA in HaCaT cells.

Although without detectable effect on the total cellular β-catenin (data not shown), treatment with MG132 mildly stimulated proliferation of HaCaT cells (Figure 6a), presumably owing to stabilization of signaling β-catenin (Aberle *et al.*, 1997). Nevertheless, inhibition of proteasomal proteolytic activity did not abrogate reduction in the rate of cell proliferation in response to atRA (Figure 6a). This indicates that ubiquitin-targeted proteasomal degradation of cyclin D1, Id2 or any other proliferation-promoting factor is unlikely to play a major role in mechanisms of the antiproliferative action of retinoids in human keratinocytes. It should be noted, however, that besides the use

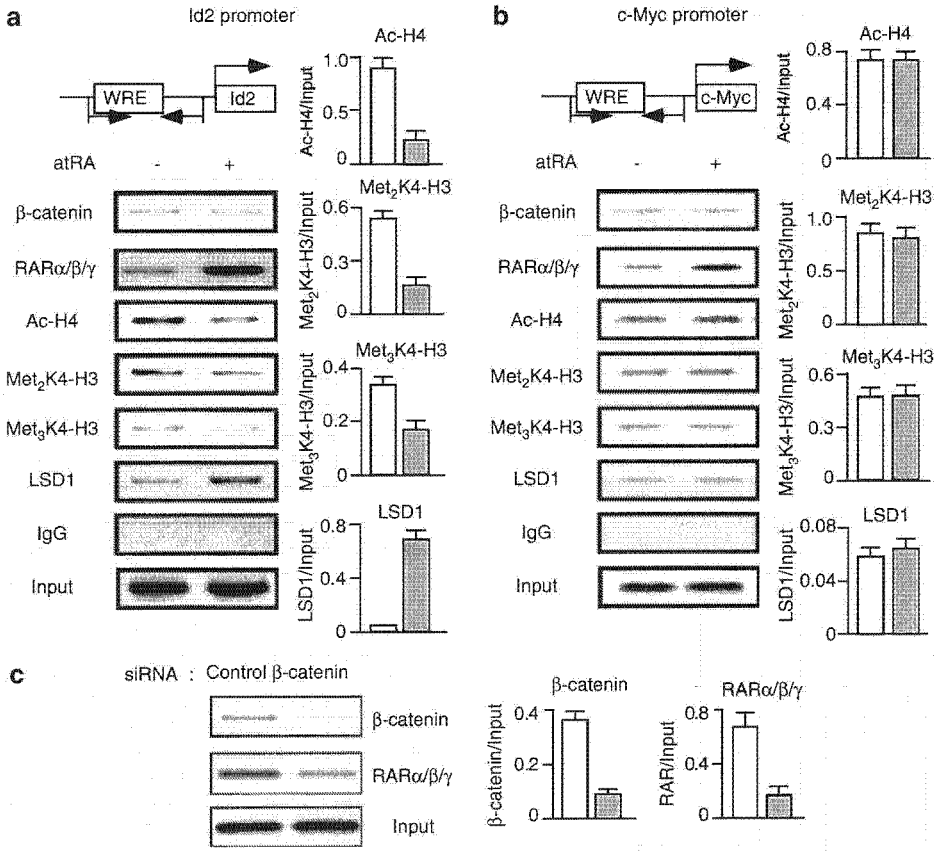

Figure 5 Downregulation of Id2 expression in HaCaT keratinocytes through transcriptional convergence between Wnt and retinoid signaling pathways. (**a**–**c**) ChIP assay of WRE in the Id2, (**a** and **c**), and c-Myc, (**b**) gene promoters in HaCaT cells using antibodies against the indicated proteins. (**a** and **b**) Levels of LSD1, acetylated histone H4 (Ac-H4), dimethyl- and trimethyl-Lys4 histone H3 (Met_2K4-H3 and Met_3K4-H3, respectively) at the WRE of these promoters from control cells (light bars) and cells treated with atRA (dark bars) were further analysed by semiquantitative real-time PCR; (**c**) Knockdown of β-catenin decreased atRA-dependent recruitment of RAR to the Id2 gene WRE. HaCaT cells were transfected with non-targeting (control, light bars) or β-catenin (dark bars) siRNA and treated with 50 nM atRA. RT-PCR, semiquantitative real time PCR and ChIP assay experiments were performed several times, and the most representative data are presented.

of different cell lines, retinoic acid concentrations used in experiments reported by Ma *et al.* (2005) were about two orders of magnitude higher than those used in our study. It is conceivable that higher doses are required to activate the proteasomal regulatory pathway.

The significance of Id2 gene suppression in the mechanism of the retinoid-induced decrease in rate of proliferation in HaCaT cells was further tested by inhibition of Id2 expression with siRNA. We found that transfection with siRNA targeting Id2 mRNA resulted in a significant reduction in the rate of cell proliferation. Exposure to atRA did not markedly decrease the proliferation rate of these cells in comparison to control cells transfected with anti-GFP siRNA (Figure 6b). Thus, transcriptional suppression of Id2 is a pivotal mechanism in the control of keratinocyte proliferation by retinoids.

Discussion

The Id (inhibitor of differentiation or DNA binding) helix–loop–helix transcription factors represent an important family of regulators of cellular growth and differentiation. Ectopic expression of Id proteins abrogates differentiation and can lead to cellular immortalization. In clinical studies, expression of members of the Id family significantly correlated both with cancer progression and overall prognosis, implying an important role of these regulatory proteins in carcinogenesis (Sikder *et al*, 2003). Among other members, Id2 was found to play a crucial role in the regulation of differentiation, cell cycle and proliferation in epithelial cells. It acts as a dominant negative antagonist of Rb and abrogates the tumor suppressor function of Rb, thereby promoting entry into S phase and cell proliferation (Lasorella *et al.*, 2001, 2005).

In different cell systems, retinoids have been reported to downregulate (Neuman *et al*, 1995; Wagsater *et al.*, 2003) and upregulate (Buzzard *et al.*, 2003; Nigten *et al*, 2005) the Id2 gene expression. Overexpression of another helix–loop–helix transcription factor NSCL was shown to abrogate RA-induced downregulation of Id2 in F9 cells (Neuman *et al.*, 1995). The Id2 gene promoter harbors no DNA sequence, resembling known RAREs or RXREs (Neuman *et al.*, 1995), and RAR

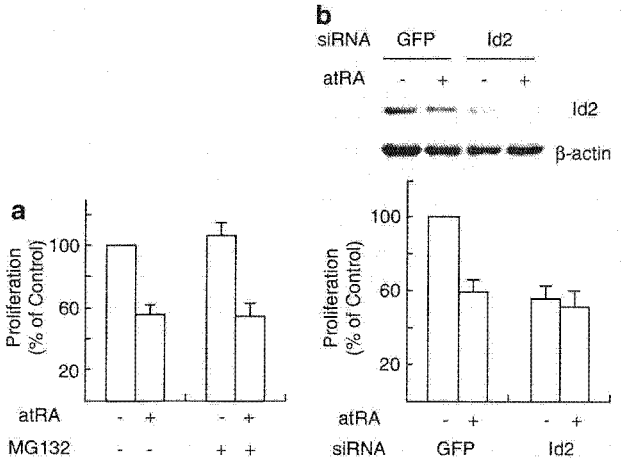

Figure 6 Transcriptional repression of the Id2 gene underlies the antiproliferative effect of retinoids in HaCaT keratinocytes. (**a**) Inhibition of proteasomal protease activity does not abrogate the antiproliferative action of atRA in HaCaT cells. Cells were treated with 50 nM atRA or vehicle in the presence or absence of 1 μM MG132, and the rates of proliferation were analysed after 48 h of treatment. (**b**) Id2 extinction decreases rate of HaCaT cell proliferation. Cells were transfected with either anti-GFP or anti-Id2 siRNA and treated with vehicle or 50 nM atRA for 48 h. Levels of Id2 protein were analysed by Western immunoblotting with anti-Id2 antibody. (**a** and **b**) Proliferation rates were estimated and the data presented as described in Figure 1a. The data represent the mean$\pm$s.d. of three independent experiments.

binding to the Id2 WRE is β-catenin-dependent (Figure 5c) Thus, effects of atRA on the Id2 gene expression appear to be cell context-dependent and unlikely to be mediated by direct binding of the RARs to the gene promoter DNA.

We have shown that Id2 suppression through transcriptional interaction between Wnt and retinoid signaling transducers plays a key role in the control of keratinocyte proliferation by retinoids. Our data are in agreement with recent reports that Id2 abrogates γ-irradiation-induced cell-cycle arrest in human keratinocytes (Baghdoyan *et al.*, 2005), and that upregulation of Id2 by UVB stimulates proliferation and predisposes keratinocytes to carcinogenesis by preventing their normal differentiation program (Simbulan-Rosenthal *et al.*, 2005). Significantly, among potential target genes, crosstalk affects expression of only some (such as Id2 or, to a lesser degree, CD44), but not other (such as cyclinD1, c-Myc or EGFR) genes. This suggests the existence of mechanisms for gene-specific convergence of signaling pathways.

Nuclear receptors interact and recruit various regulatory complexes to target gene promoters. The protein composition of these complexes determines the overall transcriptional effect exerted by the receptor (Yanagisawa *et al.*, 2002; Kitagawa *et al.*, 2003; Ohtake *et al.*, 2003). Interaction of retinoid receptors with β-catenin at the Id2 gene WRE presumably leads to recruitment of repressor complexes containing histone deacetylase and demethylase activities. Indeed, we were able to detect a retinoid-dependent recruitment to WRE in the Id2 gene promoter of LSD1 demethylase (Figure 5a), shown to be essential for histone H3 Lys-4 demethylation (Shi *et al.*, 2004; Lee *et al.*, 2005). As histone demethylases and deacetylases are believed to be present in non-related regulatory complexes, the observed pattern of histone modification at the Id2 gene implies sequential or independent recruitment to the WRE of different chromatin remodeling complexes. For prospective studies, the data presented here raise an important question about the factors and mechanisms that specify which potential target genes are affected by crosstalk of signaling pathways. Determination of the composition of retinoid-dependent regulatory complexes modulating Wnt target genes in keratinocytes may lead to understanding of such mechanisms and identification of novel therapeutic targets for skin cancer treatment.

Materials and methods

Cell lines

A375 and SCC25 lines were purchased from the ATCC. A2058 and G361 lines were purchased from the Health Science Research Resources Bank (Osaka, Japan). The immortalized keratinocyte cell line HaCaT was generously provided by Dr N Fusenig (German Cancer Research Center, Heidelberg, Germany).

Proliferation assay

Cells were plated in 96-well plates in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% charcoal-stripped fetal calf serum and treated with vehicle or 50 nM atRA (Sigma, St Louis, MO, USA). When indicated, cells were treated with 50 ng/ml Wnt3a (R&D Systems, Minneapolis, MN, USA) or 1 μM MG132 (Calbiochem, San Diego, CA, USA). Proliferation was assessed using Cell Proliferation ELISA, BrdU (Chemiluminescent) Kit (Roche Diagnostics, Mannheim, Germany) with TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA, USA).

Transfection and reporter assay

Transfection and luciferase expression assays were performed as described previously (Kouzmenko *et al.*, 2004). Cells transfected with RARE-tk-Luc reporter were treated with vehicle or 50 nM atRA.

siRNA experiments

Cells were transfected with siRNA duplexes targeting β-catenin mRNA (Ambion, siRNA ID#42816, Austin, TX, USA), or with a mixture of siRNA duplexes targeting CCTGTGGACGACCCGATGAGC and GATCGCCCTGG ACTCGCATCC sequences in the Id2 gene (Baghdoyan *et al.*, 2005). siRNA targeting AAGATGAACTTCAGGGTCAGC sequence in the GFP gene or non-targeting Silencer Negative Control#1 siRNA (Ambion) were used in control experiments. Cells were transfected twice with given siRNA at 48 h interval, plated 12 h after the last round of transfection into 96-well plates and treated with vehicle or 50 nM atRA for the next 48 h before the proliferation assay.

RT-PCR

Total RNA was prepared using Isogen (Nippon Gene, Toyama, Japan) and reverse transcription and PCR reagents were from Takara (Otsu, Japan). Real time PCR was performed with SYBR Premix Ex Taq (Takara) and

monitored using Smart Cycler II apparatus and software (Cepheid, Sunnyvale, CA, USA).

ChIP assay

The ChIP assay was performed as described earlier (Kouzmenko *et al*., 2004). ChIP grade or ChIP assay tested antibodies were purchased from Upstate (Lake Placid, NY, USA), Abcam (Cambridge, UK) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). For RAR ChIP, we used anti-RAR M-454 antibody (Santa Cruz Biotechnology) that recognized all three RAR isoforms. At least two independent pairs of primers were used for PCR at the WRE of the each Id2, c-myc and cyclin D1 gene promoter.

Acknowledgements

We thank Professor NE Fusenig (German Cancer Research Center, Heidelberg, Germany) for a kind gift of HaCaT cells, and members of the Department of Nuclear Signaling for discussions and advice. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology (SK), and by the Kato Nuclear Complex Project grant from the Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency (JST).

References

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. (1997). β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* **16**: 3797–3804.

Alonso L, Fuchs E. (2003). Stem cells in the skin: waste not, Wnt not. *Genes Dev* **17**: 1189–1200.

Baghdoyan S, Lamartine J, Castel D, Pitaval A, Roupioz Y, Franco N *et al*. (2005). Id2 reverses cell cycle arrest induced by γ-irradiation in human HaCaT keratinocytes. *J Biol Chem* **280**: 15836–15841.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* **106**: 761–771.

Buzzard JJ, Wreford NG, Morrison JR. (2003). Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat sertoli cells. *Endocrinology* **144**: 3719–3721.

Easwaran V, Pishvaian M, Salimuddin, Byers S. (1999). Cross-regulation of β-catenin-LEF/TCF and retinoid signaling pathways. *Curr Biol* **9**: 1415–1418.

Kitagawa H, Fujiki R, Yoshimura K, Mezaki Y, Uematsu Y, Matsui D *et al*. (2003). The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in William's syndrome. *Cell* **113**: 905–917.

Kouzmenko AP, Takeyama K, Ito S, Furutani T, Sawatsubashi S, Maki A *et al*. (2004). Wnt/β-catenin and estrogen signaling converge *in vivo*. *J Biol Chem* **279**: 40255–40258.

Lasorella A, Rothschild G, Yokota Y, Russell RG, Iavarone A. (2005). Id2 mediates tumor initiation, proliferation, and angiogenesis in Rb mutant mice. *Mol Cell Biol* **25**: 3563–3574.

Lasorella A, Stegmuller J, Guardavaccaro D, Liu G, Carro MS, Rothschild G *et al*. (2006). Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. *Nature* **442**: 371–374.

Lasorella A, Uo T, Iavarone A. (2001). Id proteins at the cross-road of development and cancer. *Oncogene* **20**: 8326–8333.

Lee MG, Wynder C, Cooch N, Shiekhattar R. (2005). An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature* **437**: 432–435.

Liu T, Lee YN, Malbon CC, Wang HY. (2002). Activation of the β-catenin/Lef-Tcf pathway is obligate for formation of primitive endoderm by mouse F9 totipotent teratocarcinoma cells in response to retinoic acid. *J Biol Chem* **277**: 30887–30891.

Ma Y, Feng Q, Sekula D, Diehl JA, Freemantle SJ, Dmitrovsky E. (2005). Retinoid targeting of different D-type cyclins through distinct chemopreventive mechanisms. *Cancer Res* **65**: 6476–6483.

Mark M, Ghyselinck NB, Chambon P. (2006). Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol* **46**: 451–480.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. (2004). WNT and β-catenin signalling: diseases and therapies. *Nat Rev Genet* **5**: 691–701.

Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC. (2005). Interaction of nuclear receptors with the Wnt/β-catenin/Tcf signaling axis: Wnt you like to know? *Endocr Rev* **26**: 898–915.

Neuman K, Nornes HO, Neuman T. (1995). Helix–loop–helix transcription factors regulate Id2 gene promoter activity. *FEBS Lett* **374**: 279–283.

Nigten J, Breems-de Ridder MC, Erpelinck-Verschueren CA, Nikoloski G, van der Reijden BA, van Wageningen S *et al*. (2005). ID1 and ID2 are retinoic acid responsive genes and induce a G0/G1 accumulation in acute promyelocytic leukemia cells. *Leukemia* **19**: 799–805.

Niles RM. (2004). Signaling pathways in retinoid chemoprevention and treatment of cancer. *Mutat Res* **555**: 81–96.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K *et al*. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* **423**: 545–550.

Okuno M, Kojima S, Matsushima-Nishiwaki R, Tsurumi H, Muto Y, Friedman SL *et al*. (2004). Retinoids in cancer chemoprevention. *Curr Cancer Drug Targets* **4**: 285–298.

Otero JJ, Fu W, Kan L, Cuadra AE, Kessler JA. (2004). β-catenin signaling is required for neural differentiation of embryonic stem cells. *Development* **131**: 3545–3557.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA *et al*. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**: 941–953.

Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. (2003). Id proteins in cell growth and tumorigenesis. *Cancer Cell* **3**: 525–530.

Simbulan-Rosenthal CM, Trabosh V, Velarde A, Chou FP, Daher A, Tenzin F *et al*. (2005). Id2 protein is selectively upregulated by UVB in primary, but not in immortalized human keratinocytes and inhibits differentiation. *Oncogene* **24**: 5443–5458.

Sundberg M, Savola S, Hienola A, Korhonen L, Lindholm D. (2006). Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of cyclin D1. *J Neurosci* **26**: 5402–5410.

Szeto W, Jiang W, Tice DA, Rubinfeld B, Hollingshead PG, Fong SE *et al*. (2001). Overexpression of the retinoic acid-responsive gene Stra6 in human cancers and its synergistic induction by Wnt-1 and retinoic acid. *Cancer Res* **61**: 4197–4205.

Tice DA, Szeto W, Soloviev I, Rubinfeld B, Fong SE, Dugger DL *et al.* (2002). Synergistic induction of tumor antigens by Wnt-1 signaling and retinoic acid revealed by gene expression profiling. *J Biol Chem* **277**: 14329–14335.

Wagsater D, Sirsjo A, Dimberg J. (2003). Down-regulation of ID2 by all-*trans* retinoic acid in monocytic leukemia cells (THP-1). *J Exp Clin Cancer Res* **22**: 471–475.

Weeraratna AT. (2005). A Wnt-er wonderland – the complexity of Wnt signaling in melanoma. *Cancer Metastasis Rev* **24**: 237–250.

Yanagisawa J, Kitagawa H, Yanagida M, Wada O, Ogawa S, Nakagomi M *et al.* (2002). Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. *Mol Cell* **9**: 553–562.

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Archives of Biochemistry and Biophysics xxx (2006) xxx–xxx
ABB
www.elsevier.com/locate/yabbi

Multiple co-activator complexes support ligand-induced transactivation function of VDR

Kazuyoshi Yamaoka [b], Masayo Shindo [a], Kei Iwasaki [b], Ikuko Yamaoka [b], Yoko Yamamoto [a], Hirochika Kitagawa [a], Shigeaki Kato [a,b,*]

[a] *Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[b] *ERATO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchisi, Saitama 332-0012, Japan*

Received 20 June 2006, and in revised form 26 July 2006

Abstract

Vitamin D receptor (VDR) mediates a wide variety of vitamin D actions through transcriptional controls of target genes as a ligand-dependent transcription factor. The transactivation by VDR is known to associate with two co-activator complexes, DRIP/TRAP and p160/CBP, through physical interaction with DRIP205 and p160 members (TIF2) components, respectively. However, functional difference between the two co-activator complexes for VDR co-activation remains unclear. In the present study, to address this issue, a series of point mutants in VDR helix 12 were generated to test the functional association. Alanine replacement of VDR valine 418 resulted in loss of DRIP205 interaction, but it was still transcriptionally potent with ability to interact with TIF2. Surprisingly, the V421A mutant was only partially impaired in transactivation without co-activator interaction, implying presence of a putative co-activator/complex. Thus, these findings suggest that ligand-induced transcriptional controls by VDR require a number of known and unknown co-regulator complexes, that may support the tissue-specific function of VDR.

Keywords: Vitamin D; Vitamin D receptor; Co-activator; Co-regulator complex; Ligand-induced transactivation; Helix 12; Vitamin D 24-hydroxylase

Calciotropic hormone 1, 25$(OH)_2D_3$, the active form of vitamin D_3, regulates calcium homeostasis as well as cellular proliferation and differentiation [1]. Most biological actions of 1, 25$(OH)_2D_3$ are believed to be mediated through transcriptional control of a particular set of target genes by the vitamin D receptor (VDR)[1] [2,3]. The VDR is a member of the nuclear receptor (NR) gene superfamily, and acts as a ligand-inducible transcription factor by heterodimerizing with another NR member, RXR [4]. Like other members of the NR superfamily, VDR structure is divided into several functional domains. The most highly conserved DNA binding domain (C) is located centrally whilst the less highly conserved ligand binding domain (E) is located at the C-terminal end [5]. Most nuclear receptors harbor both N-terminal activation function 1 (AF-1) and C-terminal AF-2 domains [6]. However, the VDR appears to lack the significant N-terminal AF-1 function due to its relatively short A/B domain.

In the promoters of target genes, VDR/RXR heterodimers recognize and directly bind to cognate vitamin D responsive elements (VDREs) [6], following recruitment of a number of co-regulators and co-regulatory complexes. Such co-regulators and complexes appear to transiently associate with VDR, and their recruitment is considered to be highly regulated and cyclic among the complexes [7]. A group of co-regulator complexes that normally form large complexes with multiple components and support ligand-dependent transcriptional control of NRs have been identified, and have been classified into three categories according to their function [8]. The major function of the first class of com-

* Corresponding author. Fax: +81 3 5841 8477.
E-mail address: uskato@mail.ecc.u-tokyo.ac.jp (S. Kato).

[1] *Abbreviations used:* VDR, vitamin D receptor; NR, nuclear receptor; AF-1, activation function 1; VDREs, vitamin D responsive elements; HAT, histone acetyltransferase.

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.07.015

plexes is chromatin remodeling, which involves the ATP-dependent dynamic remodeling of chromatin structure [9–13]. Chromatin remodeling complexes utilize energy from ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. For VDR, a specific ATP-dependent chromatin remodeling complex, WINAC, has been biochemically identified [11]. A second co-regulator complex class regulates transcriptional control directly, through physical interaction with general transcription factors and RNA polymerase II [14,15]. The DRIP/TRAP co-activator complex has been well-described as a representative of this complex class to co-activate liganded NRs in vivo and in vitro.

Members of a third co-regulator complex class are classified to modify histone tails covalently, for example, by acetylation, methylation and phosphorylation, in promoter nucleosomal arrays [16–19]. p160 family members/p300/CBP histone acetylase (HAT) co-activator complex has been well characterized as a typical member of this class. This type of HAT co-activator complex harbors one of three p160 HAT family members (SRC-1 [18], TIF2 [20,21], AIB1 [22–26]) and one of CBP/p300 HAT [27] together with other components. In addition of such co-regulator complexes to define histone acetylation state, the other histone modifying enzyme complexes to methylate histones appear to support the ligand-dependent transcriptional activation by NRs [28,29]. Though the DRIP/TRAP non-HAT and the p160/CBP HAT complexes have been described to co-activate VDR [7,30], it remains to be addressed if the other histone modifying enzyme complexes are required for VDR transactivation.

Ligand-dependent interaction of NRs with co-activator complexes are mediated through physical interaction of consensus LXXLL and related motifs present on specific complex components and the C-terminal helix 12 (H12) of the NR LBD domain [31]. The ligand-induced association of NRs with complexes is stabilized by ligand binding-induced shifting of NR H12. The molecular basis of this ligand-induced association has been deduced by X-ray analysis [32,33].

Considering the tissue-specific gene regulation by VDR from a variety of phenotypic abnormalities seen in VDR KO mice [34], the present study was undertaken to examine whether VDR requires multiple types of transcriptional co-regulator complexes. To address this issue, we generated a series of VDR H12 mutants and tested co-activation of the VDR mutants by two classes of different co-activator complexes (DRIP/TRAP and p160/CBP complexes). A VDR H12-point mutant, that selectively lost its ability to physically interact with the TRAP/DRIP complex, was still potent in ligand-induced transactivation. Another VDR H12-point mutant, that was unable to associate with either co-activator in vitro, still retained ligand-induced transactivation. Thus, taken together, our results suggest that multiple known and unknown types of co-activator complex support ligand-induced transactivation by VDR.

Material and methods

Materials

Rat VDR cDNA in pSG5 [30] was applied to generate a series of alanine point replaced mutants in the VDR H12. Mutagenesis was performed through PCR with specific sets of oligonucleotide primers. For GST fusion proteins of the VDR point mutants, the cDNAs of VDR LBD point mutants were inserted into pGEX-4T-1. For one-hybrid analysis of the VDR mutants with TIF2, TIF2 cDNA was fused with a cDNA of VP16 activation domain to generate a TIF2 chimeric protein with VP16.

Cell culture and transient transfection

COS-1 cells (derived from monkey kidney) and 293T cells (derived from human kidney) were maintained in DMEM supplemented with 5% FBS (Gibco) at 37 °C, 5% CO_2. For transfection, cells were cultured in DMEM (phenol red-free) supplemented with 5% charcoal-stripped FBS. Cells were transfected with expression vectors of either VDR, RXR, co-activators, or the combined and were harvested for 24 h, after treatment with vehicle or 1, 25$(OH)_2D_3$ (1×10^{-8} M). For one-hybrid analysis, the expression vectors of VDR mutants and VP16-TIF2 chimeric protein were transfected and incubated for 24 h in the presence of 1, 25$(OH)_2D_3$ (1×10^{-8} M). The total cell extracts prepared from the transfectants were applied for a luciferase assays as described [11].

Western blotting

The transfected COS-1 cells used for the luciferase assay were also utilized for Western blotting analysis to measure the expression levels of the VDR mutants. Briefly the transfectants were lysed in TNE (10 mM Tris–HCl, pH 7.8, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotini) buffer. Cell lysates were separated by 8% SDS–polyacrylamide gel eletrophoresis, transferred onto polyvinylidene difluoride membranes (Milliopore) and detected with rat anti-VDR monoclonal antibody (NeoMarkers) and anti-rat IgG antibody conjugated with horseradish peroxidase (Dako).

GST pull-down assay

For the GST pull-down assay, TIF2 and DRIP205 were in vitro-translated and incubated with GST-fused a series of VDR point mutants deleting the A/B and C domain as illustrated in Fig. 3, immobilized on glutathione–Sepharose beads, in the absence or presence of 1, 25$(OH)_2D_3$ (1×10^{-6} M) [35].

Chromatin immunoprecipitation

Soluble chromatin from 293T cells derived from human kidney, prepared using the chromatin immunoprecipitation assay kit (Upstate), was immunoprecipitated with antibodies against the indicated proteins [11,36]. Specific primer pairs were designed to amplify the promoter region of human vitamin D 24 hydroxylase (5′-GGGAGGCGCGTTCGAA-3′ and 5′-TCCTATGCCCAGGGAC-3′), from genomic DNA. PCR conditions were optimized to allow semi-quantitative measurement and PCR products were visualized on 2% agarose/TAE gels.

Results

Several, but not all, point mutations in VDR helix 12 impair ligand-induced transactivation function

As illustrated in Fig. 1, a series of alanine replacements were introduced into the transactivation core domain (AD core) in the VDR H12 domain. The amino acid

Please cite this article as: Kazuyoshi Yamaoka et al., Multiple co-activator complexes support ligand-induced transactivation function of VDR, Archives of Biochemistry and Biophysics (2006), doi:10.1016/j.abb.2006.07.015.

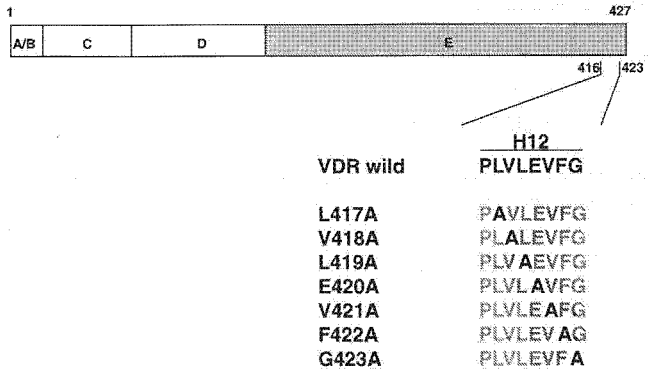

Fig. 1. Schematic representation of VDR point mutants. Rat VDR protein is schematically displayed, and alanine point replacement in H12 domains are indicated in the lower panel.

residue number of rat VDR H12 is adjusted to those of human VDR, since the length and amino acid residues of H12 is identical between rat and human. We then examined the ligand-induced transactivation function of the VDR mutants by a transient expression assay with their expression vectors and a luciferase reporter plasmid containing a consensus VDRE (DR3) in its promoter. The plasmids were transfected with a renal cell line, COS-1 cells as indicated in Fig. 2.

Ligand-induced transactivation of VDR in the presence of RXR was severely impaired by alanine replacement at 417Leu, 420Glu, 422Phe, while only partial reductions in transactivation were observed in V418A, L419A and V421A VDR H12 mutants. Unexpectedly, the 423 glycine replacement (G423A) rather potentiated ligand-induced transactivation of VDR. Thus, the loss of ligand-induced transactivation of 417, 420 and 422 VDR point mutations was expected to be caused by abrogation of co-activator interaction through H12. In contrast, the retained though diminished activities of the other mutants suggest there was still a retained ability to interact with co-activators in a ligand-dependent manner in some mutants. The transcriptional activities of the VDR mutants appeared unlikely due to the mutant expression levels, when the expression levels were estimated by Western blot analysis (see lower panel in Fig. 2). Note that we could confirm the same observations in human kidney cell line 293T cells (data not shown).

Different usage of co-activators for VDR H12-point mutants

To verify whether transcriptionally active VDR H12 mutants are capable of interacting with the best characterized co-activators, ligand-induced interaction of VDR mutants with DRIP205/TRAP220 and TIF2 co-activators was tested. DRIP205/TRAP220 was originally identified to physically interact in vitro with liganded VDR as a non-HAT DRIP/TRAP co-activator complex component. This complex then co-activated VDR as observed by an in vitro transcription assay [15]. TIF2, one of three p160 HAT co-activator family members, has been well described to associate with VDR in a ligand-dependent manner. By a GST pull-down assay, the inactive VDR mutants (L417A, E420A and F422A) were expectedly seen to lose their co-activator interactions (Fig. 3). Surprisingly, a transcriptionally active VDR mutant (V421A) was also unable to associate with either co-activator, and the V418A mutant interacted with only TIF2, but not DRIP205. To confirm these observations, a one-hybrid system with the VDR mutants and a TIF2 chimeric protein fused with a VP16 activation domain for a sensitive detection, was used to detect ligand-induced interaction of the VDR mutants with TIF2. Reflecting the observation in vitro, TIF2 interactions were observed in the V418A, L419A, and G423A mutants, but not in the V421A mutant in COS-1 cells (Fig. 4) and 293T cells (data not shown).

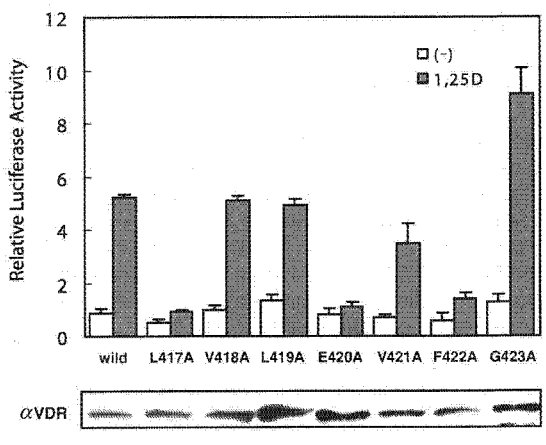

Fig. 2. H12 point mutations of VDR caused altered transactivation function of liganded VDR. The expression vectors of VDR mutants were transfected with COS-1 cells in the presence of 1, $25(OH)_2D_3$ (1×10^{-8} M). The transfected cells were applied for a luciferase analysis. The averages of the results from three independent experiments are shown with ±SD (upper panel). The expression levels of the VDR mutants were verified by Western blotting with a specific antibody for VDR (lower panel).

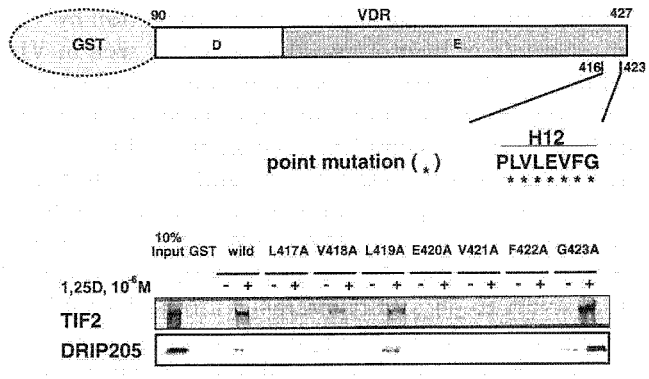

Fig. 3. Differential associations of VDR mutants with co-activators. Singular print mutations (*) introduced into H12 in the GST-VDR were the same as shown in Fig. 1. Two best known co-activators (TIF2 and DRIP205) were in vitro translated and incubated with the bacterially expressed VDR chimeric mutants fused with GST in the absence (−) or presence (+) of 1, $25(OH)_2D_3$ (1×10^{-6} M). The association was visualized on SDS–PAGE.

Please cite this article as: Kazuyoshi Yamaoka et al., Multiple co-activator complexes support ligand-induced transactivation function of VDR, Archives of Biochemistry and Biophysics (2006), doi:10.1016/j.abb.2006.07.015.

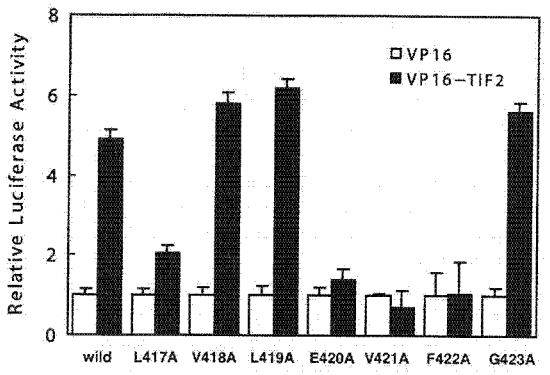

Fig. 4. The V421A VDR mutant failed to functionally associate with TIF2. As described in Fig. 2, a luciferase assay was performed for one-hybrid assay to detect ligand-dependent association of the VDR mutants with TIF2. Although V421A mutant was only partially impaired in the ligand-induced transactivation, this mutant failed to interact with TIF2, in accordance with in vitro GST-pull down assay (see Fig. 3).

VDR H12 mutants are recruited to the target promoter in vivo

We could not still exclude the possibility that several H12 point-mutations disable VDR from associating with the target promoter, leading to impaired transactivation in the transient expression assay, even though the expression levels of the VDR mutants were unaltered by the point mutations. To address this issue, we tested if these mutants are recruited to the endogenous target promoter of human vitamin D 24 hydroxylase [24(OH)ase] gene, that is well known to contain typical VDRE [37] by ChIP analysis. As shown in Fig. 5, V418A and V421A were recruited to the promoters, and the transcriptionally inactive VDR mutants were also recruited (data not shown). These findings clearly suggest that promoter targeting of VDR/RXR heterodimer to the target promoter does not directly couple with transactivation function of liganded VDR. As the HAT co-activators recruited to ligand VDR are presumed to acetylate histones for histone modification, histone modification may be dispensable for promoter targeting of VDR.

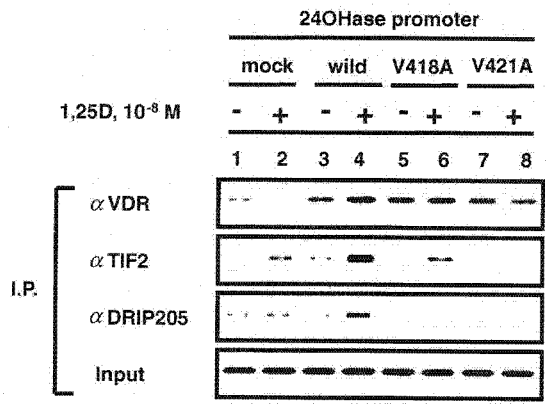

Fig. 5. The VDR point mutants were recruited to the human vitamin D 24-hydroxylase gene promoter. ChIP analysis was performed in the human vitamin D 24-hydroxylase gene promoter in 293T cells transfected with the indicated plasmids shown in the figure. The immobilized chromatin immunoprecipitated by a specific antibody was used for PCR to detect the factor bindings as described in Materials and methods.

Discussion

Is chromatin remodeling and histone modification independent from VDR-mediated gene regulation?

In our study, we have already reported that ligand-induced transactivation and transrepression mediated VDR requires chromatin remodeling through one class of ATP-dependent chromatin remodeling complex, WINAC [11,38]. Ligand-independent association of VDR with WSTF protein in the WINAC complex as well as non-specific but significant interaction of the WSTF bromo domain with acetylated histone appear to assist targeting of VDR to specific DNA binding regions in the VDR target gene promoters [38]. At the present time it is unclear whether chromatin remodeling is coupled with histone modification in VDR-mediated gene regulations. For sex steroid hormone receptors, histone modification appears indispensable prior to chromatin remodeling since steroid receptor recruitment to the target promoter is dependent on ligand binding [39]. This is presumably accompanied by recruitment of histone modifying enzyme co-regulator complexes that undergo histone modification. However, unlike steroid receptors, we observed ligand-independent recruitment of VDR to the target promoters, implying a possibility that promoter targeting of VDR does not require histone modification. This idea was further supported by the observations that the V421A VDR H12 mutant is unable to associate with the p160 HAT co-activators, but was still recruited to the promoter (Fig. 5). Thus, it is more likely that WINAC associating with VDR remodels the target chromosomal areas to expose VDREs, leading to stable DNA binding of VDR/RXR.

The two classes of co-activator have distinct sites of direct contact with VDR AF-2

By generating a series of VDR mutants, we have shown in this study that point-mutations in VDR H12 regions result in loss of ligand binding-dependent ability to associate with the tested co-activators, DRIP205 and TIF2. This would agree with the recent view that H12 serves as a direct interface for co-activator interaction through physical interaction with LXXLL motifs of the co-activators [40,41]. By detailed analysis of the interaction of VDR H12 mutants with co-activators in vitro, we found that a VDR H12 mutant (V418A) lacking ligand-induced ability to associate with DRIP205 is still capable of interacting with the other class of co-activator TIF2 (Figs. 3 and 4). These findings were unexpected, since the H12 domain is well established to serve as the direct interface to recruit DRIP205 in the DRIP/TRAP co-activator complex [42]. Indeed, loss of the interaction with DRIP205 was also confirmed to impair ligand-induced transactivation of VDR in vitro. As this V418A mutant was still transcriptionally ligand-inducible, this mutation appears unlikely to abrogate the indispensable H12 property of ligand-induced H12

Please cite this article as: Kazuyoshi Yamaoka et al., Multiple co-activator complexes support ligand-induced transactivation function of VDR, Archives of Biochemistry and Biophysics (2006), doi:10.1016/j.abb.2006.07.015.

shifting. Moreover, ligand-induced recruitment to VDR was seen in the VDR target gene promoter by ChIP analysis (Fig. 5). Thus, together with these findings, it is most likely that ligand-induced transactivation of VDR is supported by multiple classes of co-activator complex.

A third co-activator complex for VDR?

Accumulating evidence of co-regulator/co-regulator complex identification suggests that ligand-induced transactivation of nuclear receptors is supported by a number of co-regulators/co-regulator complexes [43,44]. Each co-regulators complex is believed to govern a distinct process in gene regulation.

However, at least among co-activators, functional redundancy is observed in our in vitro transactivation assays. In the present study, the V421A VDR H12 mutant was unexpectedly transcriptionally active even though this mutant lost its ability to interact with either class of co-activator complexes, both of which have been shown indispensable for ligand-induced transactivation of VDR in vitro [12,14,15]. These findings indicate the possible existence of other co-activator complexes. Although histone acetylation is well described to trigger chromatin activation for following gene expression, methylation of specific residues of histone tails has recently proposed to also enable chromatin active for transcription [28,29]. In this respect, like the other NR members, histone methylase complex(es) recruited to liganded VDR may be potent enough to activate chromatin through functional association with HAT complexes, leading co-activation of liganded VDR. To verify this idea, identification of the third critical co-activator/co-activator complex using several distinct approaches is required. Such studies would enhance our understanding of the spatial and temporal function of VDR in various target tissues.

Acknowledgments

We thank Teijin Pharma Ltd. for the 1α, 25$(OH)_2D_3$. We also thank H. Higuchi for manuscript preparation. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to S.K.).

References

[1] H.F. DeLuca, Adv. Exp. Med. Biol. 196 (1986) 361–375.
[2] R. Bouillon, W.H. Okamura, A.W. Norman, Endocr. Rev. 16 (1995) 200–257.
[3] M.R. Walters, Endocr. Rev. 13 (1992) 719–764.
[4] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, Cell 83 (1995) 835–839.
[5] M.R. Hughes, P.J. Malloy, D.G. Kieback, R.A. Kesterson, J.W. Pike, D. Feldman, B.W. O'Malley, Science 242 (1988) 1702–1705.
[6] M.R. Haussler, G.K. Whitfield, C.A. Haussler, J.C. Hsieh, P.D. Thompson, S.H. Selznick, C.E. Dominguez, P.W. Jurutka, J. Bone Miner. Res. 13 (1998) 325–349.
[7] S. Kim, N.K. Shevde, J.W. Pike, J. Bone Miner. Res. 20 (2005) 305–317.
[8] C.K. Glass, M.G. Rosenfeld, Genes Dev. 14 (2000) 121–141.
[9] D.V. Fyodorov, J.T. Kadonaga, Cell 106 (2001) 523–525.
[10] T. Ito, M. Bulger, M.J. Pazin, R. Kobayashi, J.T. Kadonaga, Cell 90 (1997) 145–155.
[11] H. Kitagawa, R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, S. Kato, Cell 113 (2003) 905–917.
[12] B. Lemon, C. Inouye, D.S. King, R. Tjian, Nature 414 (2001) 924–928.
[13] G.J. Narlikar, H.Y. Fan, R.E. Kingston, Cell 108 (2002) 475–487.
[14] W. Gu, S. Malik, M. Ito, C.X. Yuan, J.D. Fondell, X. Zhang, E. Martinez, J. Qin, R.G. Roeder, Mol. Cell 3 (1999) 97–108.
[15] C. Rachez, Z. Suldan, J. Ward, C.P. Chang, D. Burakov, H. Erdjument-Bromage, P. Tempst, L.P. Freedman, Genes Dev. 12 (1998) 1787–1800.
[16] T. Heinzel, R.M. Lavinsky, T.M. Mullen, M. Soderstrom, C.D. Laherty, J. Torchia, W.M. Yang, G. Brard, S.D. Ngo, J.R. Davie, E. Seto, R.N. Eisenman, D.W. Rose, C.K. Glass, M.G. Rosenfeld, Nature 387 (1997) 43–48.
[17] Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass, M.G. Rosenfeld, Cell 85 (1996) 403–414.
[18] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Science 270 (1995) 1354–1357.
[19] J. Yanagisawa, H. Kitagawa, M. Yanagida, O. Wada, S. Ogawa, M. Nakagomi, H. Oishi, Y. Yamamoto, H. Nagasawa, S.B. McMahon, M.D. Cole, L. Tora, N. Takahashi, S. Kato, Mol. Cell 9 (2002) 553–562.
[20] H. Hong, K. Kohli, A. Trivedi, D.L. Johnson, M.R. Stallcup, Proc. Natl. Acad. Sci. USA 93 (1996) 4948–4952.
[21] J.J. Voegel, M.J. Heine, C. Zechel, P. Chambon, H. Gronemeyer, EMBO J. 15 (1996) 3667–3675.
[22] S.L. Anzick, J. Kononen, R.L. Walker, D.O. Azorsa, M.M. Tanner, X.Y. Guan, G. Sauter, O.P. Kallioniemi, J.M. Trent, P.S. Meltzer, Science 277 (1997) 965–968.
[23] H. Chen, R.J. Lin, R.L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M.L. Privalsky, Y. Nakatani, R.M. Evans, Cell 90 (1997) 569–580.
[24] H. Li, P.J. Gomes, J.D. Chen, Proc. Natl. Acad. Sci. USA 94 (1997) 8479–8484.
[25] A. Takeshita, G.R. Cardona, N. Koibuchi, C.S. Suen, W.W. Chin, J. Biol. Chem. 272 (1997) 27629–27634.
[26] J. Torchia, D.W. Rose, J. Inostroza, Y. Kamei, S. Westin, C.K. Glass, M.G. Rosenfeld, Nature 387 (1997) 677–684.
[27] V.V. Ogryzko, R.L. Schiltz, V. Russanova, B.H. Howard, Y. Nakatani, Cell 87 (1996) 953–959.
[28] E. Metzger, M. Wissmann, N. Yin, J.M. Muller, R. Schneider, A.H. Peters, T. Gunther, R. Buettner, R. Schule, Nature 437 (2005) 436–439.
[29] Y. Sedkov, E. Cho, S. Petruk, L. Cherbas, S.T. Smith, R.S. Jones, P. Cherbas, E. Canaani, J.B. Jaynes, A. Mazo, Nature 426 (2003) 78–83.
[30] K. Takeyama, Y. Masuhiro, H. Fuse, H. Endoh, A. Murayama, S. Kitanaka, M. Suzawa, J. Yanagisawa, S. Kato, Mol. Cell. Biol. 19 (1999) 1049–1055.
[31] D.M. Heery, E. Kalkhoven, S. Hoare, M.G. Parker, Nature 387 (1997) 733–736.
[32] A.K. Shiau, D. Barstad, P.M. Loria, L. Cheng, P.J. Kushner, D.A. Agard, G.L. Greene, Cell 95 (1998) 927–937.
[33] A.K. Shiau, D. Barstad, J.T. Radek, M.J. Meyers, K.W. Nettles, B.S. Katzenellenbogen, J.A. Katzenellenbogen, D.A. Agard, G.L. Greene, Nat. Struct. Biol. 9 (2002) 359–364.
[34] T. Yoshizawa, Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto, S. Kato, Nat. Genet. 16 (1997) 391–396.
[35] T. Imai, K. Matsuda, T. Shimojima, T. Hashimoto, Y. Masuhiro, T. Kitamoto, A. Sugita, K. Suzuki, H. Matsumoto, S. Masushige, Y. Nogi, M. Muramatsu, H. Handa, S. Kato, Biochem. Biophys. Res. Commun. 233 (1997) 765–769.

Please cite this article as: Kazuyoshi Yamaoka et al., Multiple co-activator complexes support ligand-induced transactivation function of VDR, Archives of Biochemistry and Biophysics (2006), doi:10.1016/j.abb.2006.07.015.

[36] Y. Shang, X. Hu, J. DiRenzo, M.A. Lazar, M. Brown, Cell 103 (2000) 843–852.
[37] K.S. Chen, H.F. DeLuca, Biochim. Biophys. Acta 1263 (1995) 1–9.
[38] R. Fujiki, M.S. Kim, Y. Sasaki, K. Yoshimura, H. Kitagawa, S. Kato, EMBO J. 24 (2005) 3881–3894.
[39] V. Kumar, P. Chambon, Cell 55 (1988) 145–156.
[40] J.J. Voegel, M.J. Heine, M. Tini, V. Vivat, P. Chambon, H. Gronemeyer, EMBO J. 17 (1998) 507–519.
[41] C.X. Yuan, M. Ito, J.D. Fondell, Z.Y. Fu, R.G. Roeder, Proc. Natl. Acad. Sci. USA 95 (1998) 7939–7944.
[42] C. Rachez, B.D. Lemon, Z. Suldan, V. Bromleigh, M. Gamble, A.M. Naar, H. Erdjument-Bromage, P. Tempst, L.P. Freedman, Nature 398 (1999) 824–828.
[43] B. Belandia, R.L. Orford, H.C. Hurst, M.G. Parker, EMBO J. 21 (2002) 4094–4103.
[44] L.P. Freedman, Cell 97 (1999) 5–8.

Please cite this article as: Kazuyoshi Yamaoka et al., Multiple co-activator complexes support ligand-induced transactivation function of VDR, Archives of Biochemistry and Biophysics (2006), doi:10.1016/j.abb.2006.07.015.