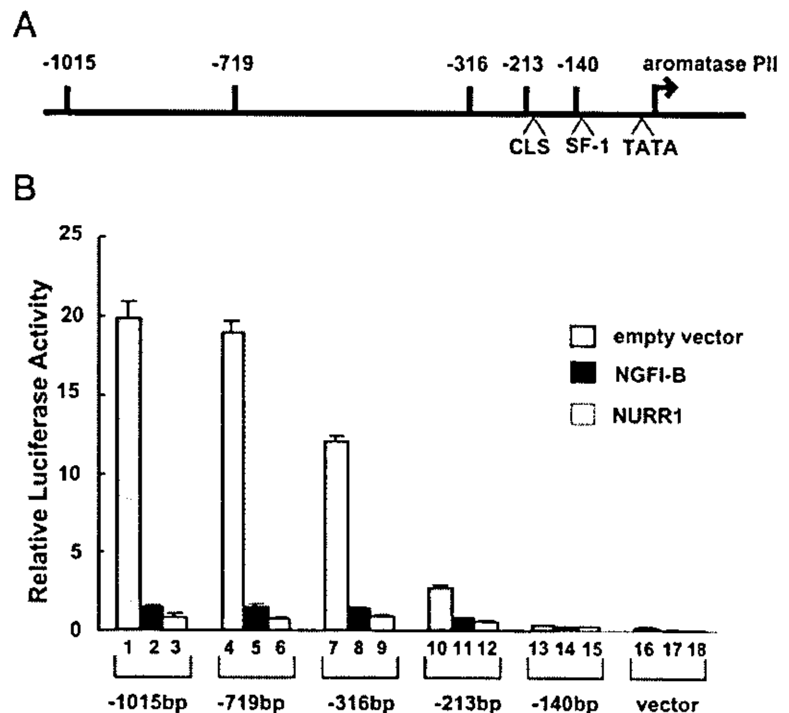

FIG. 6. The promoter-proximal sequence of the ovary-specific aromatase PII promoter contains the *cis*-regulatory element that confers NURR1-mediated repression. A, A diagram indicating the 5′ ends of various deletion constructs in the ovary-specific aromatase PII promoter. Also indicated are the positions of the *cis*-acting elements critical for the promoter activity. CLS, cAMP-responsive element-like sequence. B, The effects of NGFI-B and NURR1 on the transcription activity of various promoter-deletion constructs. The reporter vector alone does not contain any sequence from the aromatase promoter. All samples were treated with forskolin overnight before harvest.

matase gene displays a more delayed response to FSH, thus resembling the pattern observed in the forskolin-treated KGN cells. However, the physiological relevance of the delayed expression of aromatase *in vivo* remains to be explored. Furthermore, the molecular basis for such a delay is unclear. In light of the inhibitory effect of NURR1 and NGFI-B on aromatase gene expression, the transient peak of NR4A expression that precedes the aromatase expression in forskolin-treated KGN cells could contribute to the delay in expression of aromatase and possibly other steroidogenic genes. Conceivably, the resulting delay in aromatase expression could serve to ensure a controlled and coordinated steroidogenic event. In this regard, it would be interesting to determine the effect of FSH on the expression of the NR4A genes in a more physiological setting.

The underlying mechanism of NR4A-mediated transcription repression remains to be elucidated. The NR4A orphan nuclear factors are well known for their ligand-independent transcription activation functions upon binding as a monomer to the NGFI-B response element (5′-AAAGGTCA-3′) (36, 37). In addition, NGFI-B and NURR1 can also form heterodimers with the 9-*cis* retinoic acid X receptor and activate gene expression in a vitamin A-responsive manner (38, 39). Although no transcription repression function has been ascribed to the NR4A subfamily members in the literature, two possible mechanisms of NR4A-mediated repression of aromatase gene expression can be envisioned. In the first scenario, NURR1 and NGFI-B could indirectly inhibit aromatase gene expression by activating the expression of a putative transcription repressor, which in turn, may bind to the ovary-specific PII promoter of the aromatase gene. Interestingly, several known transcription repressors, including TCP8 and ID2, are among the forskolin-activated genes, as revealed by the microarray study. In the second model for NR4A-mediated repression, NR4A may be able to directly bind to the aromatase promoter via a noncanonical site, but such a protein-DNA interaction might be too transient or too weak to be detected by the gel shift assay. In this regard, it is interesting to note that NGFI-B and steroidogenic factor 1 (SF-1) binding sites share the same core sequence (5′-AGGTCA-3′), although the two orphan nuclear receptors require distinct nucleotides 5′ to the core sequence. Nevertheless, it remains possible that a modified form of NR4A or a protein complex between NR4A proteins and their partners may have altered DNA binding specificity such that it would compete with SF-1 for the same DNA binding site at the aromatase promoter, resulting in inhibition of transcription initiation. We do not favor this possibility because *in vitro* translated NR4A proteins were not capable of either binding to the SF-1 site or preventing SF-1 from binding to the same site in a gel shift assay (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site, http://endo.endojournals.org). It is also possible that NURR1 or NGFI-B may be associated with the aromatase promoter via its interaction with another site-specific DNA binding protein. A well-characterized example is glucocorticoid receptor, which can both activate and repress transcription via either a direct interaction with a glucocorticoid response element or interaction with promoter-bound nuclear factor κB or AP1 (40). In the event that NURR1/NGFI-B is associated with the aromatase PII promoter, it is conceivable that the AF1 or AF2 domain of the orphan nuclear receptors may recruit transcription corepressors to achieve transcriptional inhibition.

The NR4A orphan nuclear receptors have been extensively characterized with respect to their physiological roles in the

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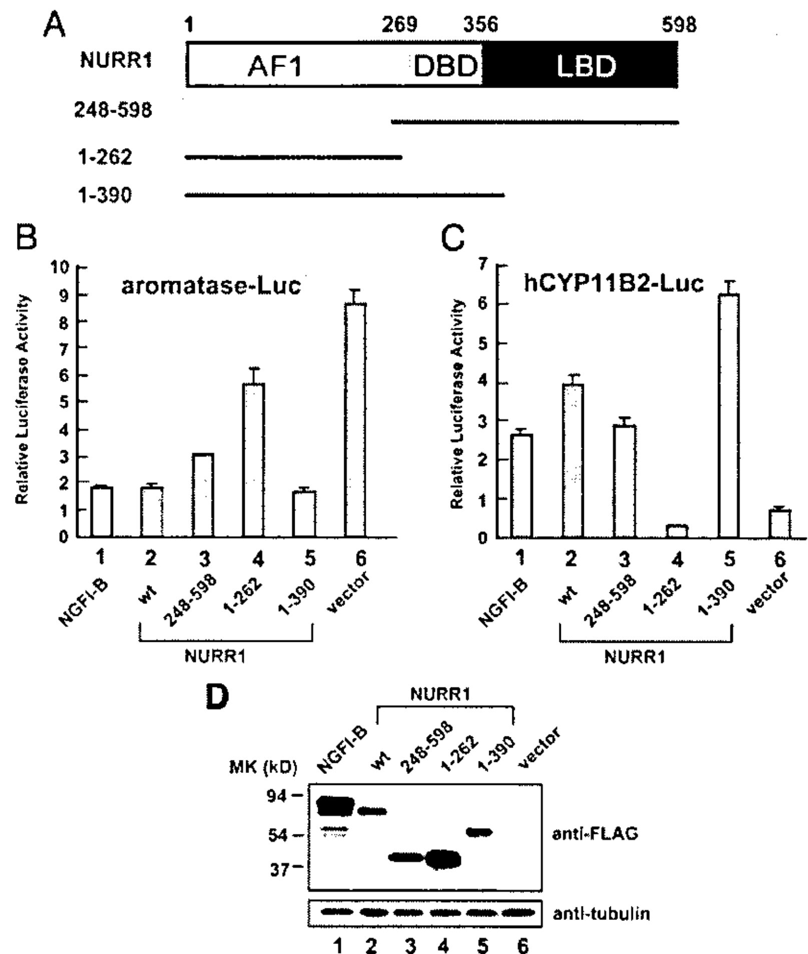

FIG. 7. Domain-mapping study of the NURR1 protein. A, A diagram indicating the multiple functional domains common to nuclear receptor superfamily. Also indicated are the lengths of various NURR1 deletion constructs. The full-length NGFI-B and NURR1, as well as the NURR1 deletion constructs, were compared for their effects on the promoter activity of the aromatase (B) and hCYP11B2 promoters (C). All samples were treated with forskolin at a final concentration of 25 μM. D, An anti-FLAG immunoblot indicating the expression of all tagged NGFI-B and NURR1 proteins in KGN cells. The positions of molecular mass markers (kDa) are indicated on the *left*. An anti-α-tubulin immunoblot is also shown as a loading control.

nervous, immune, and endocrine systems (12, 41). Given the high degree of sequence conservation and similarity in biochemical properties among the NR4A proteins, it is not surprising that their functions *in vivo* appear to be partially redundant. Consistent with this notion, NGFI-B knockout mice have no apparent phenotype (42). On the other hand, Nurr-1 knockout mice die shortly after birth and exhibit severe defects in differentiation of the nigral dopaminergic neurons (43), strongly arguing that the biological functions of the different NR4A genes are not totally exchangeable. In the current study, both ectopically expressed NURR1 and NGFI-B were capable of repressing aromatase promoter activity, yet knockdown of the endogenous NURR1 was sufficient to stimulate aromatase expression. Mutations in NURR1 are associated with familial Parkinson disease (44). It would be interesting to see whether the female Nurr1+/− mice display any phenotypes that could be attributed to elevated expression of aromatase in ovaries. In addition, it is tempting to speculate that the NURR1 mutation carriers in human may also be associated with increased risks of endocrine-related diseases such as breast cancer.

Human aromatase gene is expressed in a broad spectrum of tissues including ovary, bone, adipose tissue, brain, and various fetal tissues (45). Tissue-specific expression of aromatase is conferred largely by tissue-specific promoters and alternative splicing (46). Given the distinct sets of *trans*-acting and *cis*-acting elements involved in regulation of each tissue-specific aromatase promoter, we surmise that NGFI-B/NURR1 may exert differential effects on these aromatase promoters. In light of the important roles of both NURR1 and estrogen in brain function (12, 47), it would be of interest to determine whether NURR1 in the nervous system could influence the local estrogen biosynthesis by regulating aromatase expression from the brain-specific aromatase promoter.

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Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching

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Vitamin D receptor (VDR) is essential for ligand-induced gene repression of 25(OH)D_3 1α-hydroxylase (1α(OH)ase) in mammalian kidney, while this gene expression is activated by protein kinase A (PKA) signaling downstream of the parathyroid hormone action. The mapped negative vitamin D response element (1αnVDRE) in the human 1α(OH)ase gene promoter (around 530 bp) was distinct from those of the reported DR3-like nVDREs, composed of two E-box-like motifs. Unlike the reported nVDREs, no direct binding of VDR/RXR heterodimer to 1αnVDRE was detected. A bHLH-type factor, designated VDIR, was identified as a direct sequence-specific activator of 1αnVDRE. The transactivation function of VDIR was further potentiated by activated-PKA signaling through phosphorylation of serine residues in the transactivation domains, with the recruitment of a p300 histone acetyltransferase co-activator. The ligand-dependent association of VDR/RXR heterodimer with VDIR bound to 1αnVDRE caused the dissociation of p300 co-activators from VDIR, and the association of HDAC co-repressor complex components resulting in ligand-induced transrepression. Thus, the present study deciphers a novel mechanism of ligand-induced transrepression by nuclear receptor via co-regulator switching.

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Introduction

Members of the nuclear receptor (NR) superfamily act as ligand-inducible transcription factors. Fat-soluble NR ligands, such as the steroid/thyroid hormones vitamin A and vitamin D, are believed to exert their biological actions through both positive and negative transcriptional control of specific sets of target genes (Mangelsdorf *et al*, 1995; Chambon, 1996). NR proteins can be divided into several functional domains, with the central highly conserved DNA-binding C domain (DBD) and the less-conserved ligand-binding E domain (LBD) at the C-terminal end present in all members of the NR superfamily. Both the N-terminal A/B and C-terminal E domains are responsible for ligand-inducible NR transactivation functions (Tora *et al*, 1989). While autonomous transactivation function 1 (AF-1) in the A/B domain is constitutively active, it is suppressed by the presence of an unliganded LBD domain. In contrast, AF-2 in the LBD domain is dependent on ligand binding (Tora *et al*, 1989; Beato *et al*, 1995).

In the promoters of target genes transactivated by liganded NRs, homo- or heterodimers of NRs recognize and directly bind to their cognate hormone-responsive elements (HREs) through chromatin remodeling, presumably by ATP-dependent chromatin remodeling complexes (Belandia and Parker, 2003; Kitagawa *et al*, 2003). Liganded NRs bound to their cognate HREs induce the recruitment of a number of histone acetyltransferase (HAT) and non-HAT co-activators to activate transcription (McKenna and O'Malley, 2002). The HAT co-activator complexes CBP/p160 (Onate *et al*, 1995; Kamei *et al*, 1996; Spencer *et al*, 1997) and TRRAP/GCN5 (Yanagisawa *et al*, 2002), and the non-HAT DRIP/TRAP complexes (Fondell *et al*, 1996; Rachez *et al*, 1999) are thought to act as common co-activator complexes for NRs as well as for other classes of DNA-binding activators. In the absence of ligand, NRs bound to HREs appear to be transcriptionally silent due to association with histone deacetylase (HDAC) co-repressor complexes, which are thought to contain NCoR/SMRT, Sin3A and HDACs, along with other components (Chen and Evans, 1995; Heinzel *et al*, 1997; Glass and Rosenfeld, 2000). Thus, ligand binding leads to structural alterations and the switching of NR function from transcriptional inactivation by co-repressors to transcriptional activation via the recruitment of co-activators (Shiau *et al*, 1998).

In sharp contrast to the molecular basis of NR-mediated gene activation, little is known about ligand-induced gene repression at the molecular level. To address this issue, we characterized a negative VDRE (1αnVDRE) in the promoter of the human 25(OH)D_3 1α-hydroxylase (1α(OH)ase) gene (CYP27B1), which is negatively controlled by 1α,25(OH)D_3-bound receptors (VDR) in cultured kidney cells and in the kidneys of intact animals (Murayama *et al*, 1999). 1α(OH)ase is a key enzyme in vitamin D biosynthesis, hydroxylating 25$(OH)_2D_3$ to the active form of vitamin D, 1α,25$(OH)_2D_3$ (Takeyama *et al*, 1997; Panda *et al*, 2001). Expression of the 1α(OH)ase gene is positively and negatively regulated by multiple hormonal factors. 1α,25$(OH)_2D_3$ negatively regulates 1α(OH)ase gene expression through VDR binding to the promoter, while protein kinase A (PKA) signaling downstream of activated parathyroid hormone/parathyroid hormone-related protein (PTH/PTHrP) receptor complexes is thought to be involved in PTH/PTHrP-induced gene induction (Henry, 1985; Brenza *et al*, 1998). 1αnVDRE has been previously mapped to around −500 bp in the human

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1α(OH)ase gene promoter (Murayama *et al*, 1998). However, to our surprise, neither homologous nor related to the previously reported nVDREs in the PTH and PTHrP gene promoters were present in the 1α(OH)ase gene promoter (Demay *et al*, 1992; Falzon, 1996). To our knowledge, the present study was the first to identify the core sequence of 1αnVDRE and to explore the molecular basis of 1α,25(OH)$_2$D$_3$-induced transrepression.

Although the reported nVDREs resemble positive VDREs in that they contain directly repeated AGGTCA motifs spaced by 3 bp (DR3) (Demay *et al*, 1992; Falzon, 1996), the identified 1αnVDRE sequence was composed of two E-box-like motifs and conferred a negative responsiveness to 1α,25(OH)$_2$D$_3$ in a kidney cell line that expressed endogenous 1α(OH)ase gene. Unlike the reported nVDREs, direct DNA binding of VDR/RXR to 1αnVDRE was not detected. The cDNA cloning of a binding factor for 1αnVDRE by yeast expression screening allowed us to identify a bHLH-type transcription factor designated as VDR interacting repressor (VDIR). VDIR acted as an activator on 1αnVDRE by recruiting p300 HAT co-activator complexes in response to activated-PKA signaling. However, 1α,25(OH)$_2$D$_3$-dependent interaction between VDR and VDIR induced p300 dissociation and association of HDAC and Sin3A co-repressors, which resulted in ligand-induced transrepression. Thus, our present findings decipher a novel molecular mechanism of ligand-induced transrepression by a NR.

Results

Mapped core element in 1αnVDRE conferred a positive response to PKA signaling

To identify the core element of the nVDRE in the human 1α-hydroxylase (1α(OH)ase) gene promoter, functional analysis was performed using a series of promoter deletion mutants in a transient expression assay using MCT cells. The MCT cell line is derived from a mouse proximal tubulal cell line that expresses endogenous 1α(OH)ase gene with a negative responsiveness to 1α,25(OH)$_2$D$_3$ (Murayama *et al*, 1998). Using reporter plasmids to supply a thymidine kinase TATA box to potentiate basal transcriptional activity, the core nVDRE region was mapped from −537 to −514 bp upstream of the transcription start site (Figure 1A). 1α,25(OH)$_2$D$_3$-induced repression via the identified 1αnVDRE was confirmed using a synthetic element (data not shown). The mapped sequence, designated as 1αnVDRE, was distinct from the reported DR3-like nVDREs, being composed of two E-box-like motifs (Figure 1B, box). We found that

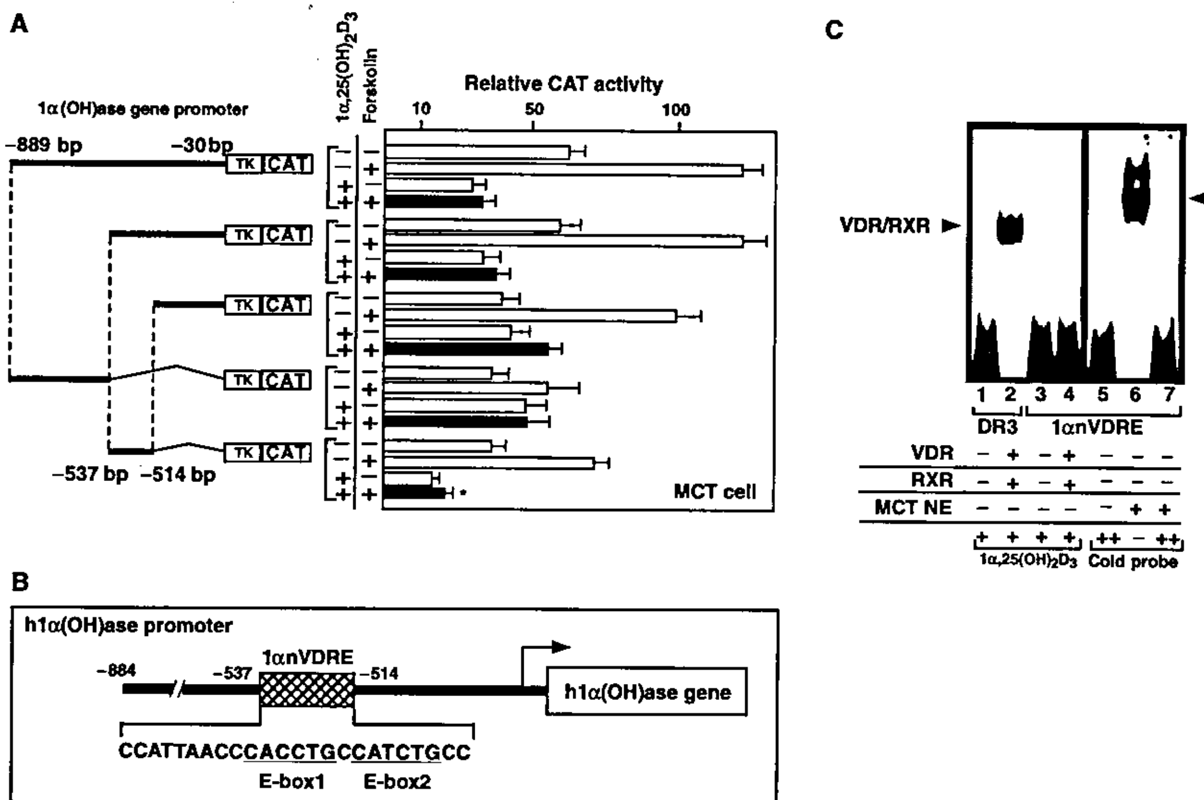

Figure 1 Identification of 1αnVDRE. (**A**) CAT assay using a series of human 1α(OH)ase gene promoter deletion mutants in MCT cells. After 3 h, forskolin (1 × 10^{-8} M), which activates PKA signaling, and 1α,25(OH)$_2$D$_3$ (1 × 10^{-8} M) were added, respectively. 1α(OH)ase gene promoter deletion constructs (−889/−30, −537/−30, −514/−30, −889/−537 and −537/−514) as indicated were transfected in MCT cells. Results shown are representative of five independent experiments. (**B**) Sequence of the 1αnVDRE core element. The 1αnVDRE was composed of two E-box-like motifs in the 1α(OH)ase gene promoter −537 to −514 bp. (**C**) Absence of direct binding between VDR/RXR and 1αnVDRE. A gel mobility shift assay was performed using bacterially expressed recombinant VDR and RXR proteins or MCT cell nuclear extracts together with a radiolabeled probe (10 ng) comprising 1αnVDRE sequence (lanes 3–7). Unlabeled 1αnVDRE oligonucleotides (100 ng) were used as cold competition (lanes 5–7). Radiolabeled probe DR3 (consensus positive VDRE) (10 ng) was used as positive control for DNA binding of liganded VDR/RXR (lanes 1and 2).

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this mapped element also conferred responsiveness to forskolin, an agent used to activate PKA signaling. Interestingly, negative regulation due to 1α,25(OH)$_2$D$_3$ was more pronounced when forskolin was used to potentiate transcription (Figure 1A). As 1α(OH)ase gene expression is induced by PKA signaling downstream of PTH/PTHrP activity (Henry, 1985; Brenza *et al*, 1998), it was possible that the putative core element served as a dual regulatory element for the two oppositely acting hormones. We also found a 1αnVDRE sequence with the identical core motif (−537 to −514 bp) in the mouse 1α(OH)ase promoter, which also exhibited a negative response to 1α,25(OH)$_2$D$_3$ (M Kim, unpublished results).

Previous reports have shown that 1α,25(OH)$_2$D$_3$-induced transrepression through DR3-like nVDREs in the PTH and PTHrP gene promoters requires direct DNA binding of VDR/RXR heterodimers to the nVDREs (Demay *et al*, 1992; Falzon, 1996). Therefore, we examined the DNA binding of VDR/RXR to 1αnVDRE core elements by electrophoresis mobility shift assay (EMSA). Recombinant VDR/RXR heterodimers expressed in *Escherichia coli* effectively bound to a consensus positive VDRE (DR3) containing two AGGTCA core motifs (Ebihara *et al*, 1996; Takeyama *et al*, 1999), while no DNA binding was detected using 1αnVDRE (Figure 1C, left panel). This result confirmed the difference between 1αnVDRE and the reported nVDREs. However, a clear band was observed on 1αnVDRE using MCT nuclear extracts (Figure 1C, right panel), which suggested the presence of an unknown factor that directly bound to 1αnVDRE.

Molecular cloning of a bHLH-type transcription factor, VDIR, as a direct binding factor for 1αnVDRE

To isolate and identify the 1αnVDRE-binding factor, a yeast one-hybrid assay using 1αnVDRE was employed to screen a yeast expression cDNA library derived from MCT cells. Out of 8×10^9 colonies, seven candidates were identified, of which five represented overlapping sequences that encoded a protein designated as VDIR (Figure 2A). VDIR was found to be a bHLH-type factor and appeared to be a mouse homolog of the human E47 (Figure 2B). VDIR also exhibited strong homology, in terms of both motif sequences and genetic organization, to the rat Pan-1 and Pan-2 transcription factors (Vierra and Nelson, 1995) (Figure 2B). The VDIR gene was ubiquitously expressed in many tissues, including the kidney (Figure 2C). To test if VDR controls expressions of VDIR, we examined VDIR transcript levels in VDR-null mouse (Yoshizawa *et al*, 1997). In the mouse kidney, VDIR transcript levels were not altered at all, which suggested that unlike the 1α(OH)ase gene, the VDIR gene was not under the transcriptional control of VDR (Figure 2D).

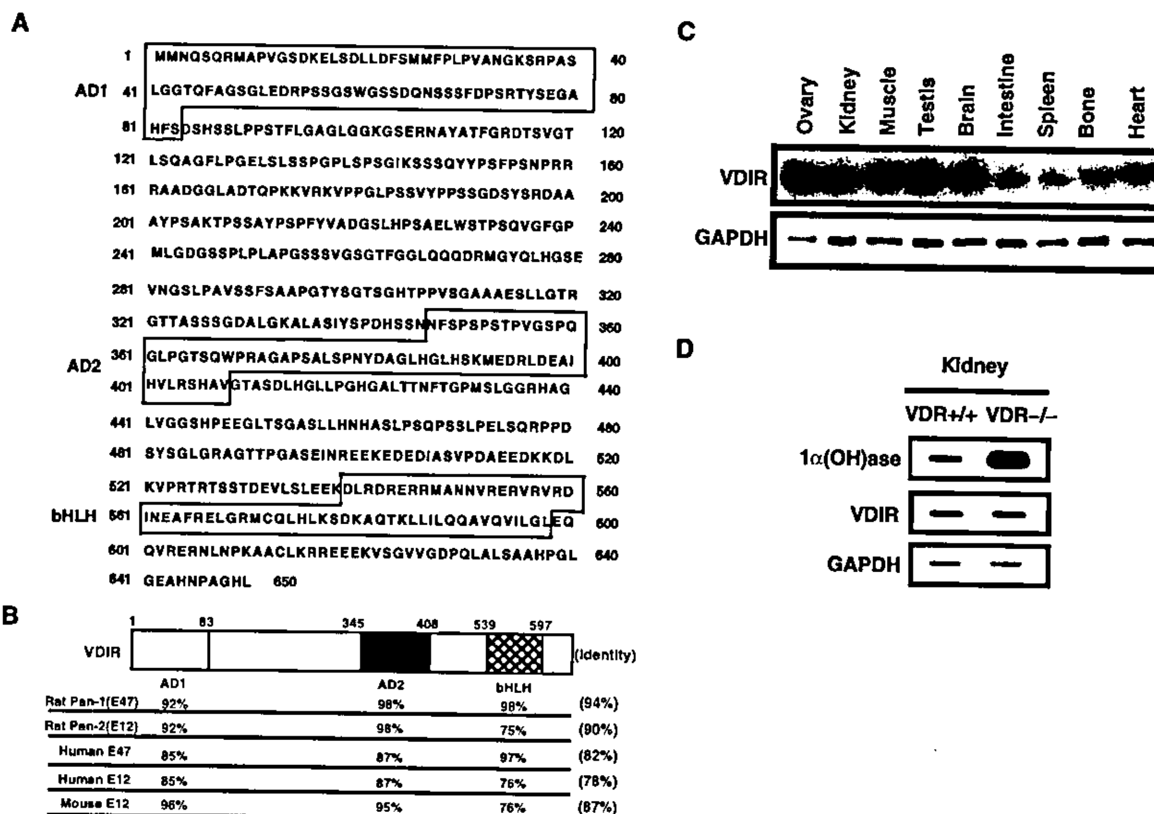

Figure 2 Cloning of the 1αnVDRE-binding factor, VDIR. (**A**) Sequence of VDIR. VDIR has two transactivation domains (AD1 and AD2), and a bHLH motif. (**B**) Functional domain sequence homology between VDIR and members of the bHLH-type activator family (rat Pan-1, E47; rat Pan-2, E12; human E47; human E12; mouse E12). VDIR exhibits a high homology with rat Pan-1 (E47). (**C**) Analysis of VDIR mRNA expression in various tissues. Northern blotting analysis was performed using VDIR open reading frame as a probe. GAPDH was used as an internal control. (**D**) 1α(OH)ase and VDIR gene expression in the kidneys of normal and VDR-deficient mice by Northern blotting. VDR$^{+/+}$: wild-type mice; VDR$^{-/-}$: VDR-deficient mice.

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VDIR is an activator for 1αnVDRE

As VDIR appeared to be a bHLH-type factor and 1αnVDRE was composed of two E-box-like motifs, we tested whether VDIR acted as a DNA sequence-specific regulator on 1αnVDRE using a transient expression assay with MCT cells (Figure 3A). To our surprise, VDIR effectively activated transcription through 1αnVDRE in a plasmid-dose-dependent manner (Figure 3A, left panel). To verify this activator function of VDIR on 1αnVDRE, we also examined other bHLH-type transcription factors, mTFE3 and hE47 (Figure 3A, left panel). hE47 belongs to a family of E2A-type bHLH transcription factors, and is thought to function as an activator, as a homodimer or a heterodimer (Murre *et al*, 1989a, b). mTFE3 is another bHLH-type family factor that binds E-box in functional association with E2A-type bHLH transcription factors (Beckmann *et al*, 1990; Ohkido *et al*, 2003). As expected, hE47 homodimer potently activated transcription of a luciferase reporter gene with 1αnVDRE, while mTFE3 exhibited no activity on 1αnVDRE. Thus, it is likely that VDIR binds, presumably as a homodimer, to 1αnVDRE and activates transcription. Supporting these findings, recombinant VDIR protein effectively bound 1αnVDRE in the absence and presence of VDR/RXR heterodimer. Moreover, while the presence of VDR/RXR heterodimer induced a further band-shift of VDIR, it appeared not to modify VDIR DNA binding (Figure 3B, lanes 6 and 7).

Ligand-induced transrepression of VDIR activation function is mediated by the N-terminal region of VDR

We then tested whether VDR suppressed the VDIR activator function on 1αnVDRE in a ligand-dependent manner (Figure 3C). VDR clearly and potently suppressed VDIR-mediated transcription only in the presence of 1α,25(OH)$_2$D$_3$, while marked ligand-induced transrepression was observed when transcription was activated by VDIR (Figure 3C, lane 6). These findings suggested that liganded VDR-mediated transrepression did not occur in response to basal transcription of the 1α(OH)ase gene, but rather significantly operated only when promoter function was potentiated by active regulators, such as PTH/PTHrP.

The VDR region responsible for ligand-induced VDIR transrepression was mapped using several VDR deletion mutants in a transient expression assay (Figure 3C, middle

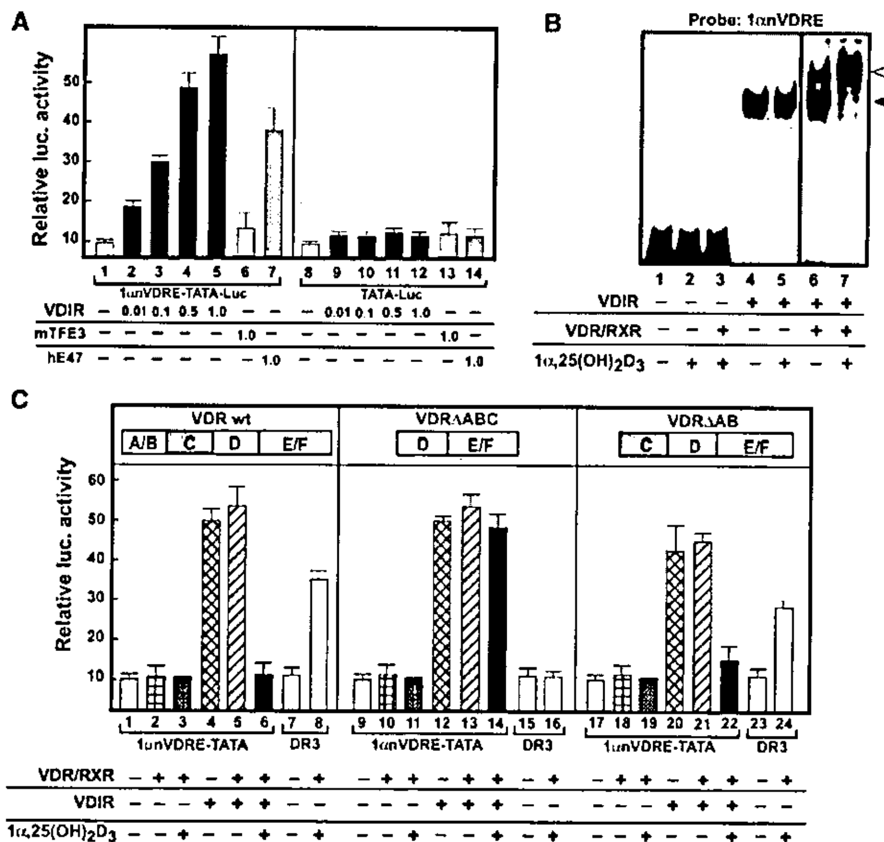

Figure 3 VDIR as an activator for 1αnVDRE. (**A**) Plasmid dose dependency of VDIR activation of nVDRE. Luciferase activity under the control of 1αnVDRE after the transfection of VDIR, mTFE3 or hE47 into MCT cells. MCT cells were cotransfected with LUC reporter plasmid (0.3 μg of nVDRE pGL3 TATA-LUC vector), rat VDR, rat RXR expression vector (0.1 μg of pSG5-rat VDR, pSG5-rat RXR), mTFE3 (1.0 μg of pcDNA3-mTFE3), hE47 (1.0 μg of pcDNA3-hE47) and increasing amounts of pcDNA3-VDIR (0.01–1.0 μg). Empty vector (pcDNA3) was used to keep the total DNA concentration the same. LUC activity is represented as fold induction. Values are mean ± s.d. (**B**) Gel mobility shift assay using bacterially expressed recombinant VDIR, VDR and RXR proteins together with a radiolabeled probe containing 1αnVDRE. The closed arrow indicates VDIR, and the open arrow indicates supershift of the VDR/RXR-VDIR complex. (**C**) Luciferase activity under the control of 1αnVDRE in MCT cells. Wild-type and mutated VDR, RXR, VDIR and 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) were added as indicated. DR3-Luc was used as a positive control for VDR/RXR and 1α,25(OH)$_2$D$_3$. VDR wt: wild-type VDR; VDR ΔABC and VDRΔAB: VDR mutants with deleted N-terminal A–C and AB domains, respectively.

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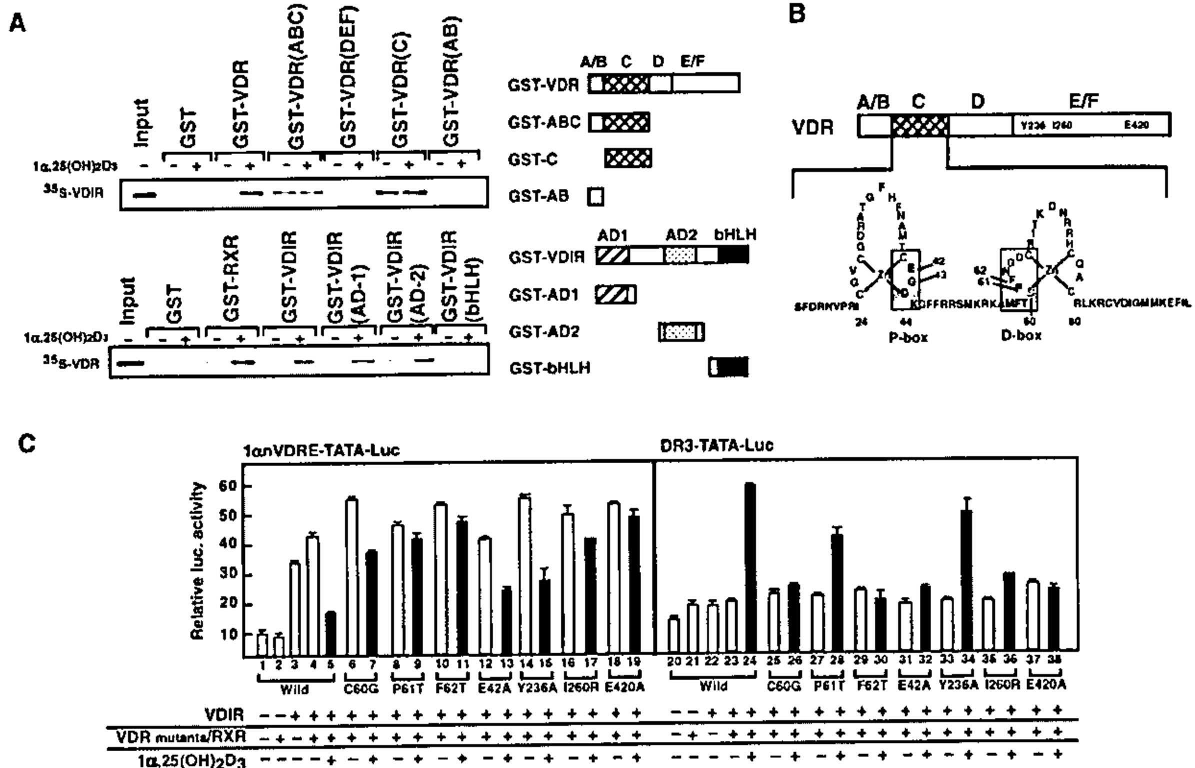

Figure 4 The DNA-binding domain (C-domain) of VDR leeds to the binding of VDIR. (**A**) GST pull-down assay using either GST alone, GST wild-type VDR or GST-fused VDRs deletion mutants together with [^{35}S]-labeled VDIR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (upper panel). GST pull-down assay was observed using either GST alone, GST wild-type VDIR or GST-variant VDIRs together with [^{35}S]-labeled VDR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (lower panel). Right panel: Schematic diagrams of wild-type and variant VDR or VDIR proteins. The specific residues present in each VDR or VDIR variant are indicated. (**B**) Schematic diagram of wild-type VDR and the structure of VDR DNA-binding domain. The P-box is located in the bottom of the first Zn finger, and the D-box is located in the second Zn finger. Amino-acid residues indicating shadow replaced into alanine or threonine residues, which inhibit DNA binding (E42A, P61T and F62T). Y236A and E420A mutants lack co-activator-binding activity. I260R (isoleucine→arginine) mutant lacks heterodimerization of VDR and RXR. (**C**) Transrepression of VDIR via VDR mutants in luc assay. Luciferase activities were tested in either 1αnVDRE or DR3 after co-transfection of either wild-type VDR or point mutant VDRs into MCT cells in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-8} M). This experiment is representative of five independent experiments performed.

and right panels). As expected from the ligand dependency results, ligand-induced transrepression was abolished in mutants that lacked ligand-binding activity (data not shown). A VDR mutant with deleted N-terminal A–C domain was found to be inactive (Figure 3C), although that with a deleted N-terminal A/B domain mutant was active. These data indicate that the C domain of VDR is critically important for ligand-induced VDIR transrepression.

To verify the ligand-induced association between VDR and VDIR, GST pull-down assay with VDR deletion mutants fused to GST protein was performed to detect interactions with full-length VDIR (Figure 4A). The interaction of VDIR with wild-type VDR was dependent on 1α,25(OH)$_2$D$_3$ binding, and only the VDR C domain exhibited clear but ligand-independent interaction with VDIR (Figure 4A, upper panel). Although the VDR DEF domain appeared not to serve as a direct interface for VDR on its own, the DEF domain may contribute to ligand-induced interactions with VDIR through intramolecular associations with the VDR C domain, perhaps altering its structure to make it more accessible for VDIR. In the VDIR molecule, both transactivation domains (AD1 and AD2), which were mapped by generating fusion mutants with GAL4 DNA-binding domain (data not shown), appeared to associate with liganded VDR, while the bHLH domain C-terminal DNA-binding domain showed no interaction with VDR (Figure 4A, lower panel).

To map more precisely the contact site of VDR with VDIR, a series of point mutations were introduced into VDR (Figure 4B). As expected from the ligand-induced interaction between VDIR and VDR, the C-terminal AF-2 core domain appeared to be essential, and its functional state faithfully reflected the level of ligand-induced transactivation or transrepression exhibited by the point mutants (Figure 4C). The E420A mutant, which is lost in co-regulator recruitment but retains its heterodimerization activity for RXR (Kraichely *et al*, 1999), exhibited neither positive nor negative response to 1α,25(OH)$_2$D$_3$ in transcription (Figure 4C, lanes 18, 19, 37 and 38). Another mutant (Y236A), which lacks co-activator-binding activity (Jurutka *et al*, 1997), retained the activity of ligand-induced transrepression, but not transactivation (Figure 4C, lanes 14, 15, 33 and 34). However, the 1α,25(OH)$_2$D$_3$-induced transrepression was undetectable in a mutant (I260R) lacking heterodimerization (Figure 4C, lanes 17 and 36). Thus, these results suggested that heterodimerization with RXR is critical for ligand-induced transrepression.

The replacement of a glutamic acid residue with alanine at amino-acid position 42 (E42A) in the P-box at the base of the

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first Zn finger in the DNA domain abolished ligand-induced transactivation of VDR (Figure 4C, compare lane 31 with 32). This result was in agreement with previous findings that the P-box is critical for the recognition and direct binding of specific DNA elements by cognate nuclear receptors (Schena et al, 1989). Interestingly, ligand-induced transrepression was still retained in this mutant (Figure 4C, lane 13), which suggested that no specific VDRE binding of VDR was required for ligand-induced transrepression. However, both ligand-induced transactivation and transrepression were abolished when an alanine replaced phenylalanine at position 62 residue, part of the D-box of the DNA-binding domain (Jakacka et al, 2001) (Figure 4C, lanes 11 and 30). Thus, together with the observation that VDR does not bind directly to 1αnVDRE (Figure 1C), it is likely that the structure of the VDR DNA-binding domain, particularly the second Zn-finger motif, is critical for ligand-induced interaction and presumably the transrepression of VDIR.

Phosphorylation of VDIR by PKA induced p300 co-activator recruitment

As VDIR acted as an activator on 1αnVDRE, we presumed that VDIR mediated the positive effects of PTH/PTHrP on 1α(OH)ase gene expression through downstream PKA signaling (Henry, 1985; Brenza et al, 1998). Indeed, expression of the PKA catalytic subunit α (PKAα) potentiated VDIR transactivation function (Figure 5A). This potentiation by PKAα was likely to have involved association with the p300 co-activator, initially identified as a PKAα-regulated co-activator (Chrivia et al, 1993), as synergistic potentiation of combined p300 and PKAα was observed (Figure 5A).

Then, to test whether PKAα phosphorylation was linked to p300 recruitment to VDIR, we characterized potential PKAα phosphorylation sites in the VDIR. A series of alanine point mutations that prevented PKAα phosphorylation were introduced into the putative phosphorylation sites (only three representative mutations are displayed). A significant reduction in the

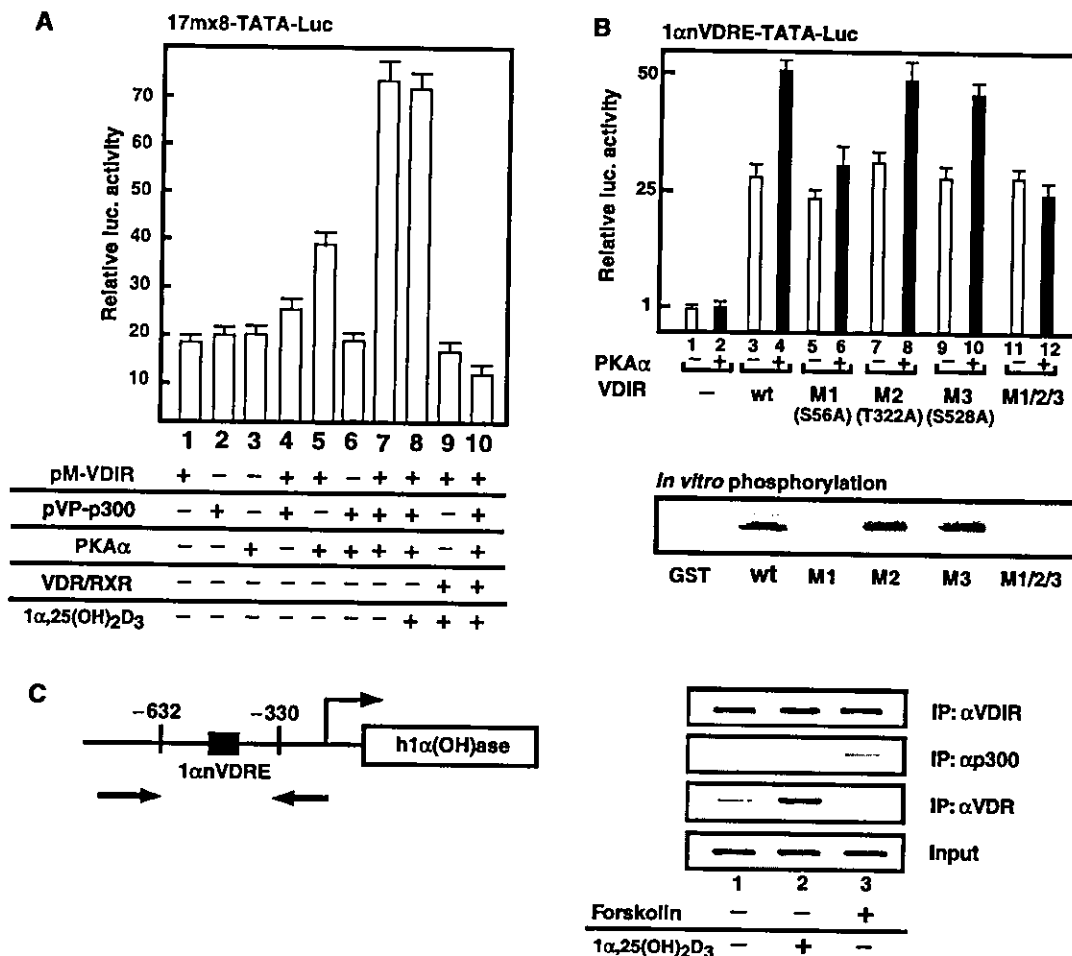

Figure 5 Phosphorylation of VDIR by PKA induced a p300 co-activator recruitment. (A) Association of VDIR and p300 in the mammalian two-hybrid assay. The expression plasmids of fusion proteins with GAL4-DBD (pM) and VP16-AD (pVP) were transiently transfected into MCT cells with a GAL4-DBD-regulated 17mer × 8 TATA luciferase reporter. PKAα or VDR/RXR was co-transfected in the absence or presence of 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) as indicated. (B) Phosphorylation of VDIR by PKAα. Luciferase activity of either wild-type VDIR or its point mutants of potential PKAα phosphorylation residue to alanine was tested on 1αnVDRE with or without PKAα in MCT cells. S56A (M1), T322A (M2) and S528A (M3) were replaced alanine residue, respectively. M1/M2/M3 mutant was indicated to replace alanine residues to all of S56, T322 and S528 amino residues. In the lower panel, the *in vitro* phosphorylation of the VDIR mutants fused with GST by PKAα is shown by *in vitro* phosphorylation assay. (C) ChIP assays demonstrate co-localization of VDIR and p300 in MCF7 cells. In the left schematic diagram, the 1αnVDRE-contained region amplified by PCR in ChIP assays is illustrated. Antibodies used in each assay are indicated on the right panel.

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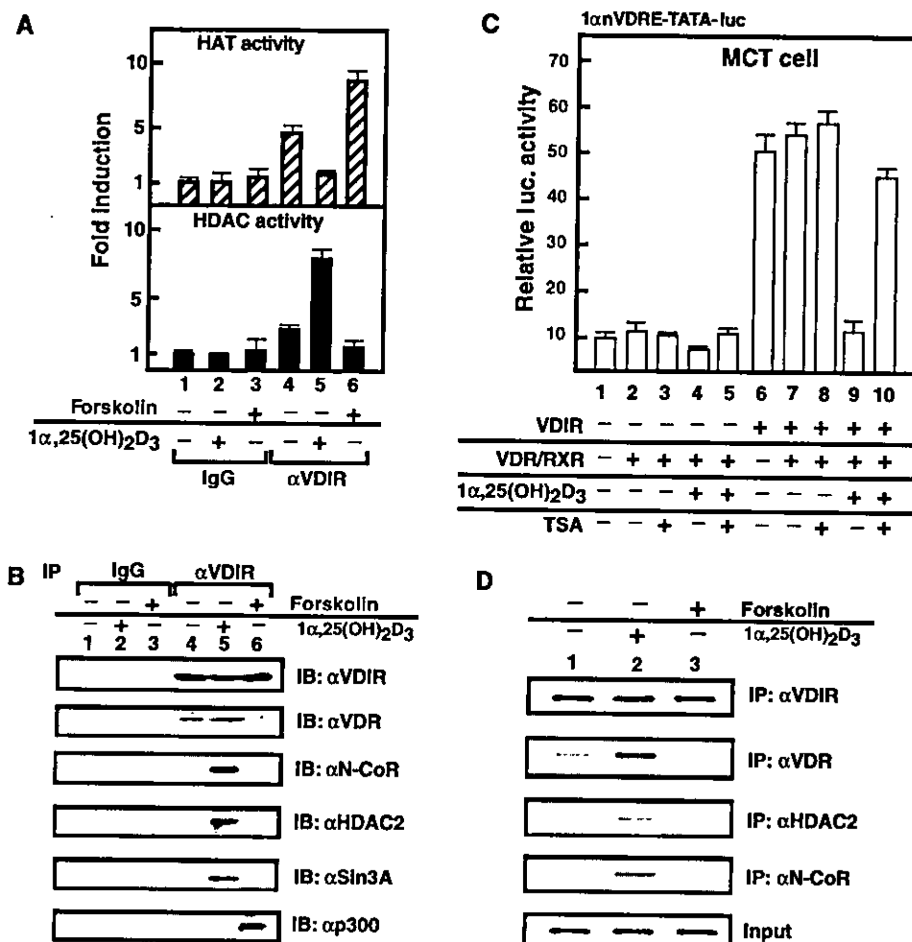

Figure 6 Co-regulator switching upon VDIR for the ligand-induced transrepression by VDR. (A) HAT and HDAC activities of the immunoprecipitated VDIR complexes in the MCT cells. Assays were determined in MCT cells after treatment, in the absence or presence of $1\alpha,25(OH)_2D_3$ and forskolin. Representative graphs corresponding to means ± s.d. for triplicate independent experiments are shown. (B) Forskolin-dependent interaction between p300 and VDIR, and $1\alpha,25(OH)_2D_3$-dependent interaction between HDAC complex and VDIR. Western blotting of the immunoprecipitates with α-VDIR, α-VDR, α-NCoR, α-HDAC2 and α-Sin3A antibodies. (C) Effects of HDAC inhibitor TSA on repression by $1\alpha,25(OH)_2D_3$. Transfections were performed in the presence of TSA (3 mM) in MCT cells. TSA reduced $1\alpha,25(OH)_2D_3$-dependent transrepression. (D) Co-localization of VDIR complex components on 1αnVDRE in ChIP assay. Soluble chromatin was prepared from MCT cells treated with $1\alpha,25(OH)_2D_3$ (1×10^{-8} M) for 45 min and immunoprecipitated with the indicated antibodies.

potentiation of VDIR function by PKAα was found for a mutation at the Ser^{56} residue (Figure 5B, lane 6 in the upper panel), which supported the hypothesis that phosphorylation of serine residues by PKAα enhanced the association of VDIR with p300/CBP, which then potentiated transcription. Reflecting this PKAα-mediated potentiation, PKAα phosphorylation of the VDIR mutant (S56A) *in vitro* was significantly impaired (Figure 5B, lower panel). Furthermore, to test whether PKAα induced p300 recruitment to the VDIR activation region in endogenous gene promoters, ChIP analysis was performed using the human 1α(OH)ase gene promoter region containing 1αnVDRE in MCT cells (Figure 5C). VDIR appeared to be present at 1αnVDRE, while p300 was clearly recruited after forskolin treatment (Figure 5C). The p300 recruitment to VDIR upon the forskolin treatment was also detected in the VDIR immunoprecipitant (Figure 6B).

Ligand-induced transrepression of VDIR by VDR coupled with p300 HAT dissociation and HDAC association

To gain an insight into the ligand-induced VDR transrepression of VDIR function, we examined whether co-repressor complexes associated with VDIR via ligand-induced interaction with VDR (Takeyama *et al*, 1999), thereby suppressing transcription, and whether p300 co-activators disassociated from VDIR upon interaction with liganded VDR. Measurement of HAT and HDAC activities in VDIR immunoprecipitates showed that the highest HAT activity was detected when PKA signaling was induced by forskolin treatment (Figure 6A, upper panel, lane 6). $1\alpha,25(OH)_2D_3$ treatment markedly reduced HAT activity, which was reflected by the dissociation of p300 and the acquisition of HDAC activity (Figure 6A). Treatment with TSA, an HDAC inhibitor (Yoshida *et al*, 1990), abrogated $1\alpha,25(OH)_2D_3$-induced transrepression by VDIR/VDR (Figure 6C), which confirmed the HDAC recruitment. The putative p300/HDAC switching mechanism was further supported by results obtained using VDIR immunoprecipitants (Figure 6B). Moreover, several major HDAC co-repressor components, including N-CoR, HDAC2 and Sin3A, were co-immunoprecipitated with VDIR in a $1\alpha,25(OH)_2D_3$-dependent manner (Figure 6B), and were recruited to the 1α(OH)ase promoter

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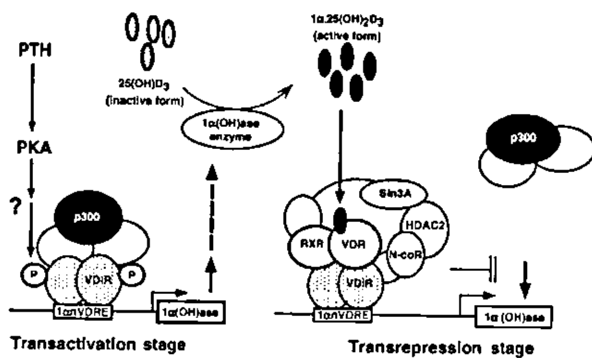

Figure 7 Schematic illustration of the proposed molecular mechanism of 1α,25(OH)$_2$D$_3$-induced transrepression in the 1α-hydroxylase gene promoter. Upon activated-PKA signaling due to PTH, the 1α-hydroxylase gene is transactivated through recruitment of a HAT co-activator complex to VDIR bound to 1αnVDRE, leading to increased serum concentrations of 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ binding to VDR induces association with VDIR, and leads to the dissociation of the HAT co-activator complex, and the recruitment of an HDAC co-repressor complex. This results in ligand-induced transrepression of the 1α(OH)ase gene due to co-regulator switching on VDIR.

as shown by ChIP analysis (Figure 6D). Thus, our findings showed the 1α,25(OH)$_2$D$_3$-dependent switching of co-regulators via VDIR, such that the HDAC co-repressor complex recruited by liganded VDR led to the dissociation of p300 from VDR–VDIR complexes (Figure 7).

Discussion

Identification of a novel nVDRE in the human 1α(OH)ase gene promoter

The 1α(OH)ase gene is one of the best-characterized VDR target genes (Haussler *et al*, 1998). While the VDR target genes are distinguished by being negatively regulated by liganded VDR, regulation of 1α(OH)ase gene expression is more complicated as it is also regulated by PKA signaling activated by liganded PTH/PTHrP receptor (Henry, 1985; Brenza *et al*, 1998; Panda *et al*, 2001). We previously showed that 1α(OH)ase gene expression was highly upregulated in VDR KO mice (Takeyama *et al*, 1997; Murayama *et al*, 1998), similar to hereditary type II rickets patients who suffer from VDR malfunction (Kitanaka *et al*, 1999). Hence, in the present study, we mapped and characterized an nVDRE (1αnVDRE) in the human 1α(OH)ase gene promoter. Our results showed that the identified nVDRE conferred a positive responsiveness to activated-PKA signaling, and that this element appeared to act downstream of PTH/PTHrP. Distinct from the previously reported nVDREs (Demay *et al*, 1992; Falzon 1996), 1αnVDRE contained no AGGTCA-like core motif, present in the binding core elements of many NRs including VDR (Mangelsdorf *et al*, 1995; Ebihara *et al*, 1996; Haussler *et al*, 1998). Instead, 1αnVDRE was composed of two E-box-like motifs. Moreover, no DNA sequences similar to the reported DR3-like nVDREs were present in the entire promoter region, up to 5 kb upstream, in both the human and mouse 1α(OH)ase genes (M Kim, unpublished results). Reflecting the sequence attributes of 1αnVDRE, no direct binding of VDR/RXR heterodimers to the mapped sequence was detected, in contrast to the previously reported nVDREs that readily bind VDR/RXR heterodimers (Demay *et al*, 1992; Falzon 1996). However, EMSA analysis showed that an unknown nuclear factor appeared to bind effectively to 1αnVDRE.

Cloning and characterization of a novel bHLH-type activator as a 1αnVDRE-binding factor

To identify the 1αnVDRE-binding factor, a yeast one-hybrid assay was performed using an MCT cell line cDNA library. This led to the identification of a factor designated VDIR that exhibits motif organization typical of E2A-type activators, including N-terminal transactivation domains (AD) and a C-terminal bHLH-type DNA-binding domain. VDIR appeared to be the mouse homolog of hE47 as the two molecules shared 97% amino-acid sequence identity. Like hE47 (Murre *et al*, 1989a, b; Beckmann *et al*, 1990), VDIR appeared to bind as a homodimer to 1αnVDRE, as determined by EMSA assay using recombinant VDIR. It has been reported that hE47-type transcriptional factors, which are widely expressed, can both homodimerize and heterodimerize with tissue specific-type bHLH proteins, and be responsible for the biological activity of these proteins *in vivo* (Davis *et al*, 1990; Lassar *et al*, 1991). Therefore, we cannot exclude the possibility that an unidentified factor may form a heterodimer with VDIR for more stable DNA binding.

As expected from the VDIR amino-acid sequence and the two E-box-like motifs in 1αnVDRE, VDIR effectively activated transcription via 1αnVDRE binding. 1αnVDRE served as an enhancer, and its function was potentiated through PKA signaling, that is activated by the PTH/PTHrP cell membrane receptors (Henry, 1985). We further found that VDIR was phosphorylated *in vitro* by PKA at several phosphorylation sites in the transactivation domains. A series of point mutations identified the Ser^{58} residue as a significant PKA phosphorylation site, such that phosphorylation of Ser^{58} appeared to be a prerequisite for the PKA-induced transactivation function of VDIR. Thus, VDIR appeared to act as an activator downstream of PKA, and may be responsible, at least in part, for the role of PTH/PTHrP in 1α(OH)ase gene induction.

Ligand-induced transrepression by VDR is mediated via direct binding of VDIR to 1αnVDRE

While ligand-induced transrepression by VDR via 1αnVDRE was detected in the absence of exogenous VDIR expression, it was relatively of low level. However, ligand-induced transrepression by VDR was more evident when transcription was augmented by activated-PKA signaling. Likewise, when higher basal promoter activity was achieved by replacing the intact basal 1α(OH)ase promoter with the much stronger tk promoter, ligand-induced VDR transrepression was much more evident. Supporting these findings, ligand-induced association between VDR and VDIR was detected at the human 1α(OH)ase gene promoter by ChIP analysis (Kitagawa *et al*, 2003). This association was further supported by findings *in vivo* and *in vitro* by nuclear co-immunoprecipitation and GST pull-down assays, respectively.

Modulation of the transactivation function of one activator class by another activator class through their direct association has already been described (McNamara *et al*, 2001; Xu *et al*, 2001). As observed in this study, the ligand-induced association of some nuclear receptors with bHLH-type activators has been shown to either potentiate or suppress the transactivation function of the bHLH activators. Recently, McNamara *et al* reported that nuclear retinoid receptors

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(RARα and RXRγ) suppressed the transactivation function of CLOCK and MOP4, bHLH-type activators, in a ligand-dependent manner, blocking CLOCK/MOP4-mediated gene expression. Further detailed analysis revealed that ligand-induced association of RAR/RXR prevented CLOCK and MOP4 from binding their DNA targets, resulting in suppressed retinoid activity in the CLOCK/MOP4-mediated gene cascade. Like the interaction between VDR and VDIR, the C-terminal AF-2 core motif of RAR/RXR is required for ligand-induced association. However, unlike the VDIR AD domain, the DNA-binding bHLH domains in MOP4 appear to be involved in direct interaction. This discrepancy in the functional domains in terms of interaction with nuclear receptors is hardly surprising due to the completely distinct motif organization between MOP4/CLOCK and VDIR irrespective of the fact that they belong to the same class of bHLH-type activators. This difference may also explain the different modes of nuclear receptor suppressive function on gene expression, as liganded VDR had no inhibitory effect on VDIR DNA binding.

Co-regulator switching in ligand-induced transrepression by VDR

Thus, the present study revealed a novel mechanism of ligand-induced transrepression by nuclear receptors based on co-regulator switching rather than preventing DNA binding of another activator class. The transactivation function of VDIR appeared to require p300 co-activator, presumably as part of a HAT complex (Glass and Rosenfeld, 2000). The functional and physical association of p300 with VDIR was potentiated via the PKA-mediated phosphorylation of several serine residues in the VDIR AD1 domain. This may explain, at least in part, the induction of the 1α(OH)ase gene by the PKA-mediated PTH/PTHrP upregulation, although it is likely from previous reports that there may be other positive regulatory element(s) in the gene promoter (Brenza *et al*, 1998). Interestingly, the association between p300 and VDIR was abrogated by the ligand-induced association of VDR along with major co-repressor complex components. Thus, VDR appeared to be highly effective in switching HAT co-activator complexes to HDAC co-repressor complexes in a ligand-dependent manner upon binding of VDIR to 1αnVDRE, as illustrated in Figure 7. This hypothesis was verified by the finding of both HAT and HDAC activities in immunoprecipitated VDIR complexes. Together, these findings clearly show that co-regulator switching underlies ligand-induced transrepression by VDR.

The molecular mechanism of ligand-induced co-regulator switching involving VDIR remains to be investigated. However, it is evident from its ligand dependency that the VDR LBD plays a crucial role, although this switching is in effect opposite to that of ligand-induced transactivation accompanied by co-activator recruitment. It is presumed from our present findings that ligand-induced association with VDIR allows liganded VDR to retain co-repressor complexes without the recruitment of co-activator complexes. Such ligand-induced switching of co-repressors on VDIR is likely to be accomplished by unique ligand-induced structural alterations in VDR present, thus a unique VDR–VDIR co-repressor complex may be formed. To test this idea, purification and identification of VDR–VDIR complex components is clearly needed to uncover the molecular basis of ligand-induced transrepression by VDR.

Materials and methods

Plasmids

Transfection studies included constructs of a chimeric gene in which the human 1α(OH)ase promoter (−889/−30) and deletion mutants (−537/−30, −514/−30, −889/−537, −537/−514) were inserted into the pGL thymidine kinase (tk)-chloramphenicol acetyltransferase (CAT), and nVDRE (−537/−514) were inserted into the pGL3-Luciferase vector (Promega) driven by TATA promoter. Full-length rat VDR and rat RXR plasmid were described previously (Takeyama *et al*, 1999). Rat VDR point mutants, by PCR mutagenesis, were inserted into pcDNA3 (Invitrogen). Full-length mouse VDIR plasmids were inserted into pcDNA3. Chimeric GST proteins fused with rat VDR and mouse VDIR deletion mutant series were expressed in pGEX-4T (Pharmacia Biotech). pcDNA3-mTFE3 plasmid was kindly provided by Dr K Miyamoto (Tokshima University).

Cell culture and transient transfection assay

MCT cells were maintained in DMEM supplemented with 5% FBS (GIBCO BRL) at 37°C in 5% CO_2. For transfection, cells were plated in DMEM supplemented with 5% charcoal-stripped FBS in 12-well plates 1 day before transfection. Transfections were performed using Lipofectamin Plus (GIBCO BRL) according to the manufacturer's instructions. After 3 h, $1\alpha,25(OH)_2D_3$ (1×10^{-8} M) and/or forskolin (1×10^{-8} M) were added to the culture medium, and the cells were incubated continuously at 37°C for 24 h. CAT and Luciferase assays were performed as described previously (Murayama *et al*, 1998).

Yeast one-hybrid system

The yeast strain YM4271 (CLONETECH), transformed with the yeast expression plasmids pHISi and pLacZi (CLONETECH) containing 3 × 1αnVDRE motifs (CCCACCTGCCATCTGCC), was used to screen a yeast GAL4 activation domain fusion MCT cDNA library (a detailed procedure for the library construction is available upon request). Positive clones were selected on SD medium that lacked Leu and His, but contained 25 mM 3-amino-triazol (3AT). Surviving colonies were assayed for β-galactosidase (X-gal) activity using a colony filter lift assay and incubation in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactosidase according to the manufacturer's instructions (CLONETECH). cDNA from LacZ-positive clones were sequenced across the Gal4/library cDNA and analyzed using the NCBI BLAST search tool.

Gel electrophoresis mobility shift assay

Nuclear extracts were prepared from MCT cells. Recombinant rat VDR, rat RXR proteins fused to GST, were expressed in *E. coli* and bound to glutathione–sepharose 4B beads. GST fusion proteins bound to glutathione–sepharose were cleaved by thrombin protease treatment (25 U/24 h). Double-stranded oligonucleotide DR3 (consensus VDRE, 5′-AGCTTCAGTTCAGGAAGTTCAGT-3′) and human 1αnVDRE (h1αnVDRE 5′-CCATTAACCCACCTGCCATCTGCC-3′) were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Takeyama *et al*, 1999). Reactions were performed using 0.5 μg nuclear extracts in binding buffer (10 mM Tris (pH 7.5), 75 mM KCl, 5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 1 mM DTT, 1 μg poly dI–dC) in a final volume of 20 μl and labeled probes of 10 ng. Samples were incubated for 30 min at room temperature and resolved on 5% polyacrylamide gels run in 0.5 × TAE buffer. Gels were then dried and subjected to autoradiography (Ebihara *et al*, 1996).

Northern blotting

Northern blot analysis was performed as previously described (Takeyama *et al*, 1997). cDNA fragments of N-terminal mouse 1α hydroxylase and VDIR full-length were used as probes.

GST pull-down assay

VDIR and VDR deletion mutant proteins fused to GST were expressed in *E. coli* and bound to glutathione–sepharose 4B beads (Pharmacia Biotech). [^{35}S]methionine labeling of proteins was carried out by *in vitro* translation using a TNT-coupled transcription–translation system (Promega). GST-VDR (or GST-VDIR) was preincubated with $1\alpha,25(OH)_2D_3$ (10^{-6} M) for 15 min at room temperature. GST fusion proteins and [^{35}S]methionine-labeled proteins were then incubated in Net-N+ buffer for 2 h. After

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successive washes in Net-N+ buffer, proteins were resolved by SDS-PAGE and visualized by autoradiography (Kitagawa *et al*, 2003).

HAT/HDAC assay

Whole MCT cell lysates were immunoprecipitated with a-VDIR antibody and then incubated with or without 10 μg calf thymus histones (Sigma) and [^{3}H]-labeled acetyl CoA (4.7 Ci/mmol, Amersham) for 30 min at 30°C, spotted onto Whatman P-81 filters, and washed extensively with sodium carbonate buffer (pH 9.1). Radioactivity remaining on the filter was then quantitated by liquid scintillation counting (Yanagisawa *et al*, 2002). HDAC assays were carried out using the HDAC fluorescent activity assay kit according to the manufacturer's instructions (BIOMOL, Inc.).

Mammalian two-hybrid assay

MCT cells were co-transfected with 17mer × 8-Luc reporter plasmid, pM-VDIR and pVP-p300 with pSG5-rat VDR and pSG5-rat RXR in the presence of PKAα. After 3 h, 1α,25(OH)$_2$D$_3$ was added to the culture medium, and the cells were incubated for 24 h at 37°C. Luciferase assays were performed as described above.

In vitro kinase assay

MCT cells transfected with pcDNA3-Flag-PKAα were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl$_2$, 2 mM EDTA, 12.5 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium vanadate, 1 mM PMSF, 1% Triton-X) with protease inhibitors (Kato *et al*, 1995). Whole cell lysate supernatants were immunoprecipitated with Anti-FLAG M2-Agarose Affinity Gel (Sigma), and washed three times in TBS buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM PMSF, 2 mM DTT, 1 mM sodium vanadate) with protease inhibitors and twice in Tris-HCl (pH 7.5) buffer. Reactions consisted of 4 μl 5 × kinase buffer (100 mM Tris-HCl (pH 7.5), 50 mM MgCl$_2$, 0.5 mM ATP), 2 μl immunoprecipitate, [γ-^{32}P]ATP and GST-VDIR in a final volume of 20 μl and were incubated for 20 min at 30°C. Reaction products were resolved by SDS-PAGE and visualized by autoradiography (Watanabe *et al*, 2001).

Immunoprecipitation

Whole cell lysate supernatants in TNE buffer (10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.15 M NaCl, 0.1% NP-40) containing protease inhibitors were immunoprecipitated with α-VDIR antibody and then added to G-sepharose beads. After successive washes in TNE buffer, proteins were resolved by SDS-PAGE and Western blotted using α-VDR antibody (Neo Markers), α-HDAC2 antibody (ABR), α-p300 antibody (Santa Cruz Biotechnology) or α-Sin3A antibody (Santa Cruz Biotechnology) (Yanagisawa *et al*, 1999).

ChIP assay

ChIP analyses were performed using the ChIP assay kit (Upstate Biotechnology), as described previously (Kitagawa *et al*, 2003). Whole cell lysates of MCF7 cells were immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designed (h1αp5'(632) 5'-ATTCCCATGTCTGGAAGGAG-3' and h1αp3'(−330) 5'-CAGTGAGCCCAGCCCCTTTA-3') and PCR conditions optimized to allow semiquantitative measurement. Conditions used were 25 cycles of 30 s at 90°C, 15 s at 58°C and 1 min at 72°C. PCR products were visualized on 2% agarose/TAE gels.

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Regulation of transforming growth factor-β and bone morphogenetic protein signalling by transcriptional coactivator GCN5

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Smad proteins are intracellular signalling mediators of transforming growth factor-β (TGF-β) superfamily. In the nucleus, activated Smad complexes regulate transcriptional responses of the target genes in cooperation with transcriptional coactivators and corepressors. To identify new components of transcriptional complexes containing Smad proteins, we purified DNA-binding proteins from human breast cancer MCF-7 cell nuclear extract using a Smad-binding DNA element as bait, and identified a coactivator GCN5 as a direct partner of activated Smad complexes. GCN5 is structurally similar to PCAF, which was previously identified as a coactivator for receptor-regulated Smads (R-Smads) for TGF-β signalling pathways. GCN5 functions like PCAF, in that it binds to TGF-β-specific R-Smads, and enhances transcriptional activity induced by TGF-β. In addition, GCN5, but not PCAF, interacts with R-Smads for bone morphogenetic protein (BMP) signalling pathways, and enhances BMP-induced transcriptional activity, suggesting that GCN5 and PCAF have distinct physiological functions *in vivo*. Moreover, silencing of the GCN5 gene by RNA interference results in repression of transcriptional activities induced by TGF-β. In conclusion we identified GCN5 as a Smad-binding transcriptional coactivator which positively regulates both TGF-β and BMP signalling pathways.

Introduction

Members of the transforming growth factor-β (TGF-β) superfamily are multifunctional proteins that regulate various cellular responses, including cell proliferation, differentiation, migration, and apoptosis (Roberts & Sporn 1990). The TGF-β superfamily includes TGF-βs, activins and inhibins, bone morphogenetic proteins (BMPs), and Müllerian inhibiting substance. Members of the TGF-β superfamily bind to type II and I serine/threonine kinase receptors and transduce intracellular signals by Smad proteins (Heldin *et al.* 1997; Shi & Massagué 2003). Type II receptor kinases are constitutively active; upon ligand binding and complex formation with type I receptors, type II receptor kinases transphosphorylate type I receptors, resulting in activation of Smads by type I receptor kinases.

Smads are classified into three groups depending on their roles in signalling; receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads are direct substrates of the type I receptors. R-Smads are phosphorylated at the C-terminal SSXS motif by serine/threonine kinase receptors and form heteromeric complexes with Co-Smads.

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The Smad complexes translocate into the nucleus, where they regulate the transcription of various target genes. Smad3 and Smad4, but not Smad2, directly bind to DNA through their N-terminal MH1 domains (Yagi *et al.* 1999). The Smad binding element (SBE), containing a CAGACA sequence, is known as a consensus DNA sequence for Smads 3 and 4 binding, and is present in promoter regions of many TGF-β response genes, including the plasminogen activator inhibitor-1 (PAI-1) gene (Dennler *et al.* 1998).

Transcriptional coactivators p300 and CREB binding protein (CBP) have been shown to interact with R-Smads in a ligand-dependent manner (Feng *et al.* 1998; Janknecht *et al.* 1998; Nishihara *et al.* 1998; Shen *et al.* 1998). p300 and CBP have intrinsic histone acetyltransferase (HAT) activity, which facilitates transcription by decreasing chromosome condensation through histone acetylation and by increasing the accessibility of transcription factors with the basal transcription machinery (Bannister & Kouzarides 1996). Thus, p300/CBP positively regulate Smad-mediated transcriptional activation. In addition to p300/CBP, another transcriptional coactivator, p300 CBP associated factor (PCAF), which belongs to the GCN5-related N-acetyltransferase (GNAT) superfamily, has been reported to interact with Smads 2 and 3, to facilitate transcription induced by TGF-β (Itoh *et al.* 2000). Other proteins involved in Smad regulation include c-Ski, SnoN and TGIF. These proteins interact with Smads 2, 3 and/or 4 in the nucleus. They compete with p300/CBP and recruit histone deacetylases to Smad complexes, resulting in transcriptional repression (Akiyoshi *et al.* 1999; Stroschein *et al.* 1999; Wotton *et al.* 1999).

GCN5 is a transcriptional coactivator that functions as a HAT to promote transcriptional activation of various genes (Candau *et al.* 1996; Wang *et al.* 1997). GCN5 is structurally related to PCAF; therefore, it is also termed PCAF-B (Yamauchi *et al.* 2000). However, the function of GCN5 in TGF-β superfamily signalling was previously unknown. In the present study, we identified GCN5 as a Smad-binding transcriptional coactivator. In contrast to PCAF, which preferentially enhances transcriptional activity induced by TGF-β, GCN5 enhances transcriptional activities for both TGF-β and BMP.

Results

GCN5 interacts with activated Smad complexes

To identify new components of transcriptional complexes containing Smad proteins, we performed DNA affinity purification (DNAP) using human breast cancer MCF-7 cell nuclear extract. Biotinylated 3xCAGA oligonucleotide, to which activated Smads 3 and 4 binds directly (Dennler *et al.* 1998), was used to purify DNA-binding proteins. The CAGA-binding proteins from the nuclear extract were separated by SDS-PAGE followed by silver nitrate staining (Fig. 1A). The proteins that bound to wild-type 3xCAGA, but not to mutated 3xCAGA, were subjected to in-gel trypsin digestion, followed by mass spectrometry analysis using Autoflex (Bruker). Peptide mass fingerprinting, and database searching with the peptide mass spectra obtained, revealed that one of the proteins was human GCN5 (Fig. 1A, arrow).

To further characterize the association of GCN5 with the CAGA sequence, we performed the DNAP assay using lysates of transfected COS7 cells (Fig. 1B). GCN5 bound to CAGA in the presence of both Smad3 and a constitutively active form of TGF-β type I receptor, TβR-I(TD), whereas GCN5 alone did not bind to CAGA. Moreover, GCN5 was not detected when mutant 3xCAGA was used. These findings suggest that GCN5 is incorporated in the activated Smad complexes. It is important to note that the MCF-7 nuclear extract used for purification in the present study was derived from cells that were not stimulated with TGF-β. We therefore investigated whether the extract contained activated Smad complexes by immunoblotting. Phospho-Smad2, phospho-Smad3, and Smad4 were detected in the nuclear extract (data not shown), suggesting that MCF-7 is activated by endogenous TGF-β to some extent, and that the nuclear extract contains activated Smad complexes.

To investigate whether GCN5 is indeed incorporated in the activated Smad complex on DNA *in vivo*, we performed a chromatin immunoprecipitation (ChIP) assay using the promoter of a TGF-β-responsive endogenous target gene, human PAI-1 (Fig. 2A). Since an anti-GCN5 antibody that could be used for immunoprecipitation experiments was not available, we generated a stable HaCaT human keratinocyte cell line expressing FLAG-GCN5. Expression of FLAG-GCN5 in the selected clone (HG15) was detected by immunoblotting analysis (Fig. 2B). One hour after treatment with TGF-β, binding of endogenous Smad2/3 to one of the CAGA regions in the PAI-1 promoter (nucleotides −280 to −272) was observed (Fig. 2C, left panel). Moreover, immunoprecipitation of FLAG-GCN5 revealed that the recruitment of FLAG-GCN5 was greatly increased when Smad2/3 binding occurred (Fig. 2C, right panel). The binding of FLAG-GCN5 in the absence of TGF-β was at background levels for protein G (data not shown). The recruitment of Smad2/3 and GCN5 was also detected in other CAGA regions in the human PAI-1 promoter (nucleotides −730 to −722 and nucleotides

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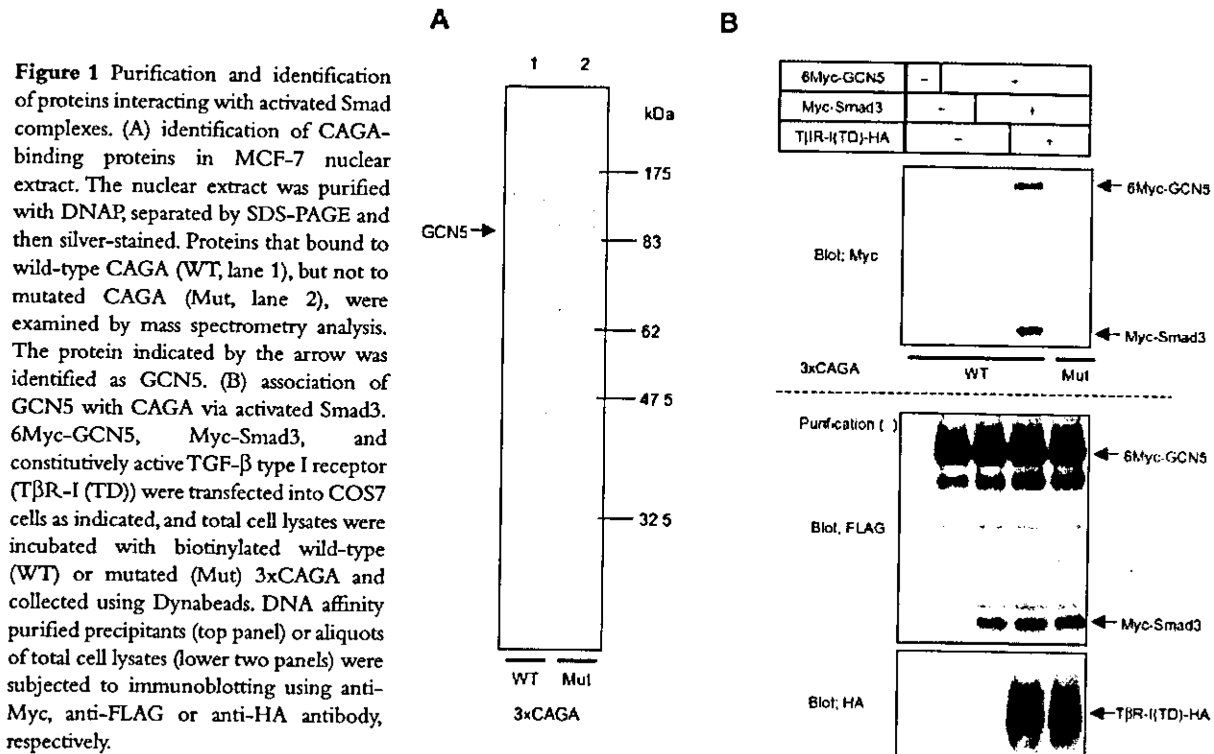

Figure 1 Purification and identification of proteins interacting with activated Smad complexes. (A) identification of CAGA-binding proteins in MCF-7 nuclear extract. The nuclear extract was purified with DNAP, separated by SDS-PAGE and then silver-stained. Proteins that bound to wild-type CAGA (WT, lane 1), but not to mutated CAGA (Mut, lane 2), were examined by mass spectrometry analysis. The protein indicated by the arrow was identified as GCN5. (B) association of GCN5 with CAGA via activated Smad3. 6Myc-GCN5, Myc-Smad3, and constitutively active TGF-β type I receptor (TβR-I (TD)) were transfected into COS7 cells as indicated, and total cell lysates were incubated with biotinylated wild-type (WT) or mutated (Mut) 3xCAGA and collected using Dynabeads. DNA affinity purified precipitants (top panel) or aliquots of total cell lysates (lower two panels) were subjected to immunoblotting using anti-Myc, anti-FLAG or anti-HA antibody, respectively.

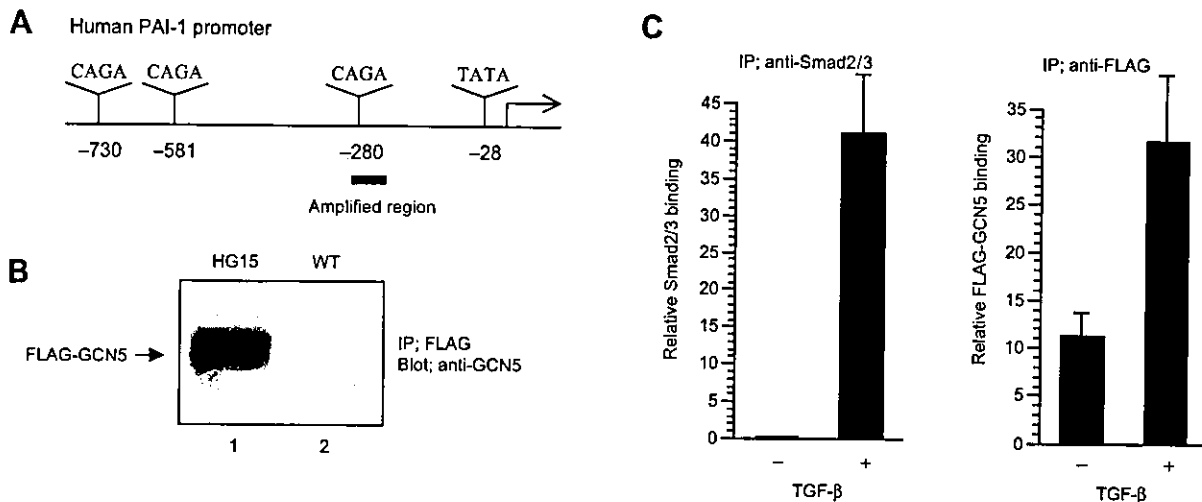

Figure 2 Binding of GCN5 to the human PAI-1 promoter. (A) a schematic diagram of the human PAI-1 promoter. A specific pair of primers covering nucleotides −280 to 205 was designed to amplify the CAGA region located at −280 to 272. (B) expression of FLAG-GCN5 in HaCaT stable transfectants. Exogenous expression of FLAG-GCN5 was detected in a HaCaT cell line constitutively expressing FLAG-GCN5 (HG15, lane 1) compared with wild-type HaCaT (WT, lane 2) by FLAG immunoprecipitation followed by immunoblotting using anti-GCN5 antibody. (C) TGF-β-dependent recruitment of Smad2/3 and FLAG-GCN5 to the PAI-1 promoter. Soluble chromatin was prepared from HG15 cells untreated or treated with TGF-β3 (40 pM) for 1 h, and immunoprecipitated with Smad2/3 antiserum (left panel) or anti-FLAG M2 antibody (right panel). Final DNA extracts were amplified using the primer pair described above (A). The data shown are the means of quantitative real-time PCR experiments, performed in triplicate and normalized to human GAPDH gene expression levels.

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−580 to −572, data not shown). These data suggested that GCN5 is recruited to the promoter region of the PAI-1 gene together with Smad2/3 in a TGF-β-dependent manner.

GCN5 associates with R-Smads for TGF-β and BMP in a ligand-dependent fashion

We next determined the physical interaction of GCN5 with all R-Smads and with Co-Smad in transfected COS7 cells. GCN5 associated with Smads 2 and 3 in the presence of TβR-I(TD), and with Smads 1 and 5 (but not Smad4 or Smad8) in the presence of constitutively active BMP type IB receptor (BMPR-IB(QD)) (Fig. 3A).

PCAF, which is structurally similar to GCN5, also potentiates TGF-β signalling (Itoh *et al.* 2000). To determine the differences between GCN5 and PCAF, we examined physical interactions of PCAF with Smads using transfected COS7 cells (Fig. 3B). PCAF associated with Smad2 and Smad3 in a ligand-dependent fashion, as previously reported (Itoh *et al.* 2000), whereas it failed to associate with Smad1, Smad5, Smad8 and Smad4. From observations of the differences in association with Smad1 and Smad5, it was thought that GCN5 and PCAF might play different roles in BMP signalling.

We next determined the GCN5-interacting domain of R-Smads using deletion mutants of Smad3 (Fig. 3C). GCN5 binds to Smad3C (MH2 domain +Linker region) and Smad3D (MH2 domain), but not to Smad3A (MH1 domain) or Smad3B (MH1 domain +Linker region), suggesting that GCN5 associates with R-Smads through their MH2 domains.

To determine the Smad interaction region in GCN5, we prepared two deletion mutants of GCN5, i.e. GCN5N and GCN5C (Fig. 3D, bottom). GCN5N lacks the HAT and bromo domains in the C-terminal region, whereas GCN5C possesses them, but lacks the N-terminal region. We expressed full-length GCN5, or either of its two deletion mutants, together with Smad3 and TβR-I(TD) in COS7 cells, and determined their interaction (Fig. 3D, top). Both GCN5N and GCN5C interacted with Smad3 in the presence of TβR-I(TD), suggesting that GCN5 binds to Smad3 through at least two regions in GCN5.

GCN5 enhances TGF-β and BMP signalling

Smads have intrinsic transcriptional activity when fused to the Gal4 DNA-binding domain (Liu *et al.* 1996, 1997). In order to determine whether GCN5 potentiates the intrinsic transcriptional activity of TGF-β, R mutant mink lung epithelial (Mv1Lu) cells lacking TβR-I were transfected with a luciferase reporter gene containing multiple Gal4 binding sites upstream of a minimal promoter, Gal4-Smad3 constructs, TβR-I(TD), and GCN5 (Fig. 4A). Gal4-Smad3 facilitated TGF-β dependent transcription, which was further enhanced in the presence of GCN5. Furthermore, we examined the transcriptional activity of GCN5 in TGF-β signalling using $(CAGA)_9$-MLP-lux in three different types of cells. Transcriptional activation of $(CAGA)_9$-MLP-lux by TGF-β was enhanced by expression of GCN5 in C2C12 cells (Fig. 4B), 293T cells (Fig. 4C), and R mutant Mv1Lu cells (data not shown). These results demonstrate that GCN5 positively regulates TGF-β signalling.

We next investigated the effect of the deletion mutant GCN5N on the transcriptional activity induced by TGF-β. GCN5N lacks a catalytic domain, but contains a Smad-interacting region. Transfection of GCN5N suppressed transcriptional activation of $(CAGA)_9$-MLP-lux induced by TβR-I(TD) in a dominant-negative fashion (Fig. 4D).

Because GCN5, but not PCAF, associates with BMP-specific R-Smads (see Fig. 3A,B), we next tested the effects of GCN5 and PCAF on BMP signalling with a BMP-responsive reporter assay using Id1-MLP-Lux. As shown in Fig. 4E, while GCN5 potentiated the transcriptional activation induced by BMPR-IB(QD), PCAF had no effect. These findings suggest that GCN5 is involved in BMP signalling as well as in TGF-β signalling, whereas PCAF is likely to play a less important role in BMP signalling.

Endogenous GCN5 is required for TGF-β signalling

To confirm the function of GCN5 in TGF-β signalling, we employed RNA interference (RNAi) to inhibit endogenous expression of GCN5. The efficiency of the specific RNAi oligonucleotide for GCN5 was confirmed by the reduction in mRNA for endogenous GCN5 (Fig. 5A) and expression of co-transfected FLAG-GCN5 protein (Fig. 5B) in 293T cells. The TGF-β-induced transcriptional activity was significantly reduced in the presence of RNAi specific for GCN5 in transfected 293T cells (Fig. 5C). Similar results were obtained by using HaCaT cells (data not shown). These data support the conclusion that GCN5 functions as a coactivator in the Smad signalling pathway.

Discussion

In the present study, we identified GCN5 as a Smad-binding transcriptional coactivator. GCN5 is structurally similar to PCAF which was previously identified as a coactivator for TGF-β-specific R-Smads (Itoh *et al.* 2000). Similar to PCAF, GCN5 binds to TGF-β-specific

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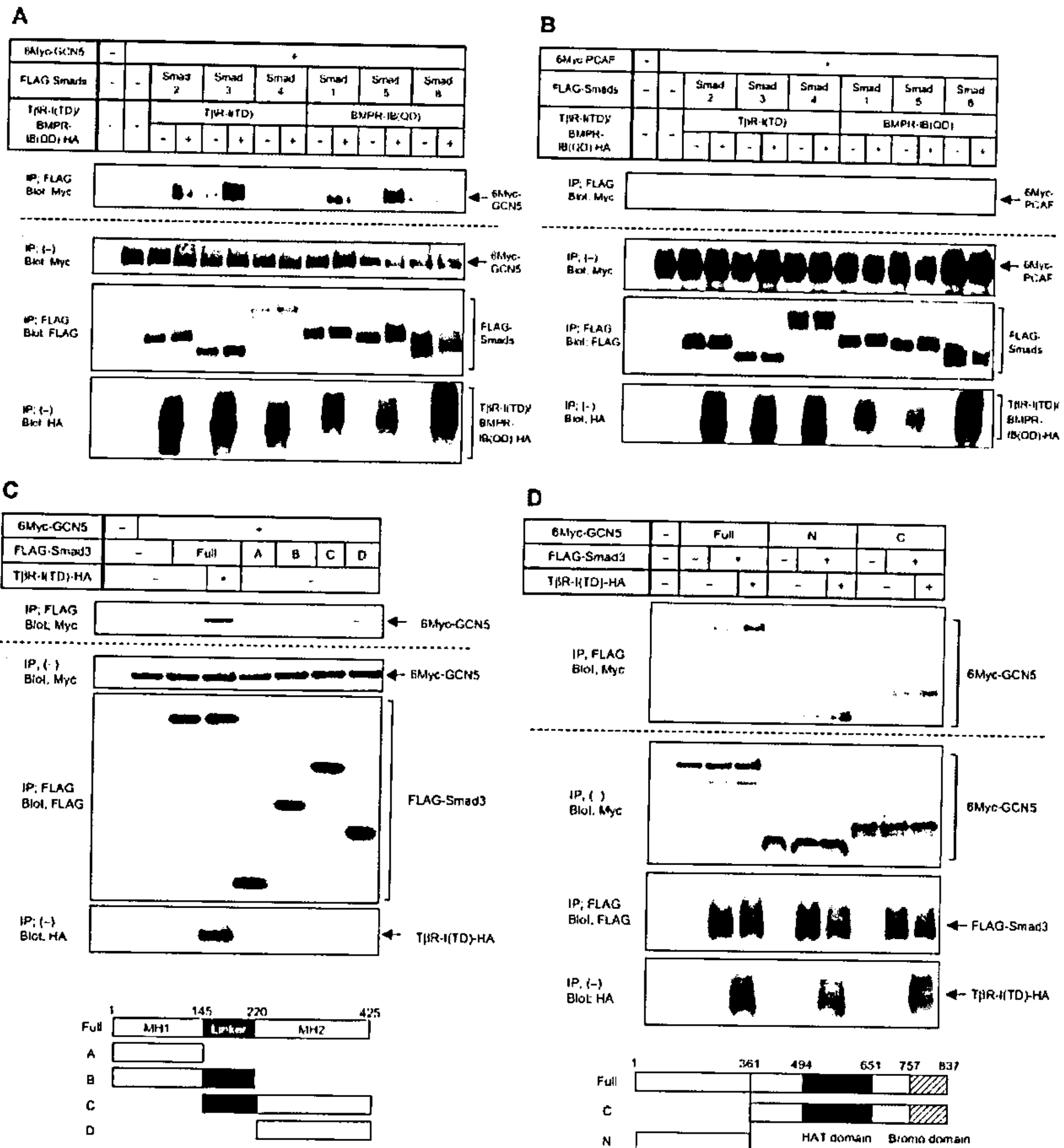

Figure 3 Physical interaction of GCN5 or PCAF with Smads. Physical interaction of Smads with (A) GCN5 or (B) PCAF was investigated in mammalian cells. COS7 cells were transfected with indicated expression plasmids, and interaction of Smads with GCN5 or PCAF was examined by FLAG-immunoprecipitation of Smads followed by Myc-immunoblotting of GCN5 (A, top panel) or PCAF (B, top panel). Expression levels of each protein were determined by immunoblotting using FLAG, Myc or HA antibodies (A, B, lower three panels). (C) GCN5 interaction domain in Smad3. A schematic representation of Smad3 deletion mutants is shown (bottom scheme). The GCN5 interaction domain of Smad3 was determined by immunoprecipitation and immunoblotting, as described in (A, top). The lower three panels show expression levels of transfected proteins. (D) Smad3 interaction regions in GCN5. Structures of GCN5 deletion mutants used are shown (bottom scheme). Smad3 interaction regions were examined by immunoprecipitation and immunoblotting as described in (A, top). Expression levels of transfected proteins are shown in the lower three panels.

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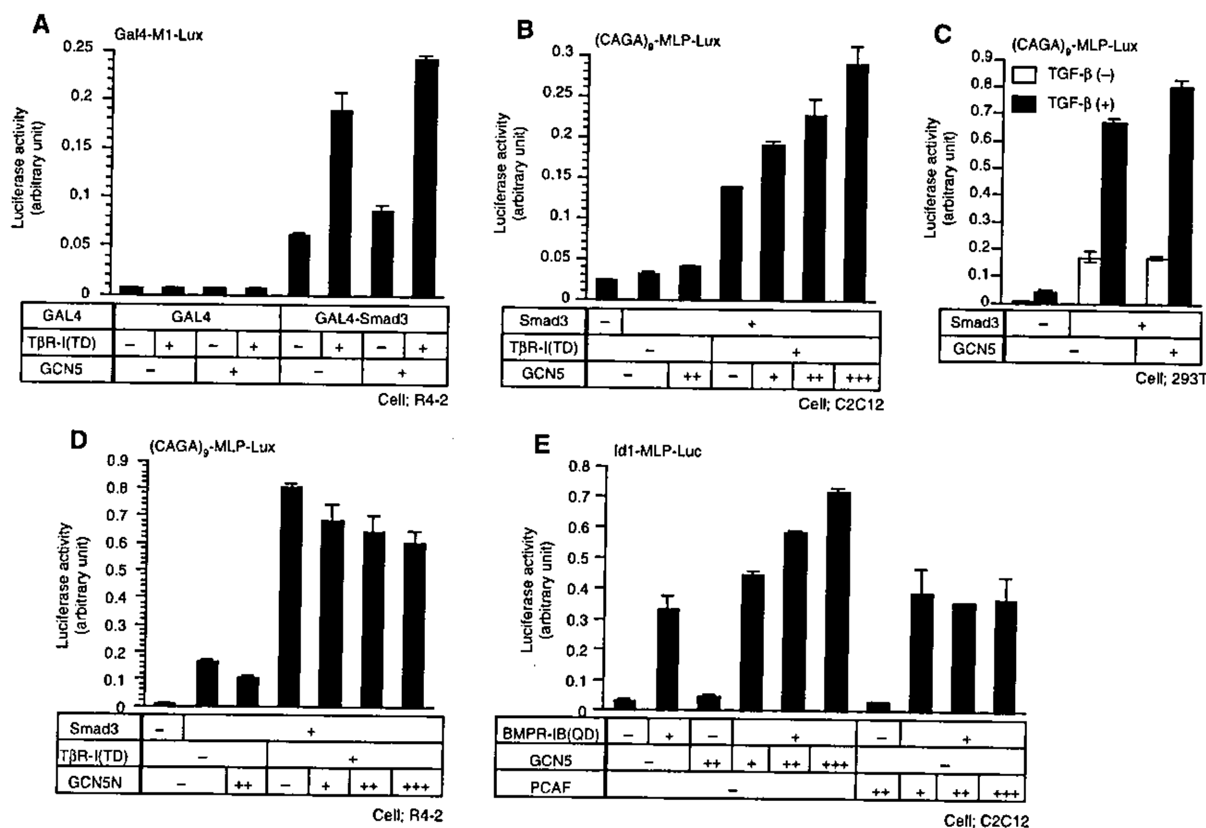

Figure 4 Enhancement of TGF-β and BMP signalling by GCN5. (A) potentiation of transcriptional activity of Gal4-Smad3 by GCN5. R mutant Mv1Lu (R4-2) cells were co-transfected with Gal4-M1-lux and the indicated expression plasmids, and luciferase activities were determined. (B, C) transcriptional activity induced by (B) TβR-I(TD) or (C) TGF-β3 (40 pM) using $(CAGA)_9$-MLP-lux. (B) C2C12 cells or (C) 293T cells were transfected with the indicated plasmids and luciferase activities were determined. +, ++ and +++ are 0.05, 0.1 and 0.2 μg of DNA, respectively, transfected into cells. (D) dominant-negative effect of amino-terminal region of GCN5. The effects of GCN5 amino-terminal deletion mutant (N) on $(CAGA)_9$-MLP-lux transcription induced by TβR-I(TD) were examined. The GCN5 deletion mutant shown in Fig. 3D was used. +, ++ and +++ are 0.05, 0.1 and 0.2 μg of DNA, respectively, transfected into R4-2 cells. E, transcriptional effects of GCN5 and PCAF on Id1-MLP-lux transcription induced by BMPR-IB(QD). C2C12 cells were transfected with the plasmids indicated, and luciferase activities were determined. +, ++ and +++ are 0.05, 0.1 and 0.2 μg of DNA, respectively, transfected into C2C12 cells.

R-Smads in a ligand-dependent manner in transfected COS7 cells. The binding region in Smad3 is its MH2 domain, which has an intrinsic transcriptional activity. Many transcriptional coactivators, such as p300/CBP, PCAF and ARC105, have been reported to interact with Smads via MH2 domain (Feng *et al.* 1998; Janknecht *et al.* 1998; Nishihara *et al.* 1998; Shen *et al.* 1998; Itoh *et al.* 2000; Kato *et al.* 2002). However, it is currently unknown whether these transcriptional coactivators play similar or distinct roles under physiological conditions. It should also be determined in the future whether multiple transcriptional coactivators interact with Smads at the same time, and regulate transcription of target genes in cooperative fashions. Thus, a further fine mapping of the GCN5-binding in Smads should be carried out in the future.

Human GCN5 consists of 837 amino acid residues, with the HAT domain located at amino acid residues 494–651. GCN5 also contains a bromo domain, between amino acids 757–837, which binds to acetylated lysine residues (Smith *et al.* 1998). In the present study, we showed that both N-terminal region (GCN5N) and C-terminal region (GCN5C) of GCN5 interact with Smad3 in a TGF-β-dependent fashion, suggesting that GCN5 binds to Smad3 through at least two regions in GCN5. This is in contrast to PCAF, which binds to Smad3 through its N-terminal region (Itoh *et al.* 2000). However, this finding is not surprising because p300 also

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