Table 1 List of increased and decreased genes related to the cytoskeleton in vaginas of mice exposed neonatally to DES

Accession no.	Gene name	Fold changes
AF053235	Cytokeratin 16 (CK16)	2352
Y09227	Small proline-rich protein (SPR) 3	1552
X91825	SPR 1b	326
X99251	Repetin	111
J03458	Filaggrin	104
AJ005566	SPR 2H	87·4
AJ005564	SPR 2F	64·0
AJ005561	SPR 2C	26·9
V00830	CK10	24·3
AB012042	CK6β	24·3
K02108	Keratin complex 2, gene 6A	24·3
AJ005562	SPR 2D	23·4
X03491	CK4	19·0
U09189	Loricrin	17·8
AF057156	SPR 1A	16·0
AJ005559	SPR 2A	16·0
AJ005560	SPR 2B	12·6
M10937	CK1	12·6
AJ005568	SPR 2J	10·6
AJ005569	SPR 2K	8·9
U13921	CK13	8·6
AB012033	CK6α	8·6
AJ005559	SPR 2A	7·7
AB017202	Nidogen-2	7·5
AJ005567	SPR 2I	6·7
AJ005565	SPR 2G	3·6
AJ005563	SPR 2E	3·5
M22832	CK18	−23·4
X15662	CK 8	−8·6
M12481	Actin beta	−6·1

quantify the expression of putative targets of neonatal DES exposure. Table 3 shows a list of genes whose expression was changed more than 3-fold as measured by Q-PCR. The list includes growth factors and cytokines, membrane receptors/proteins, intracellular/signaling factors, transcription factors and their regulators, nuclear proteins, protease inhibitors and miscellaneous genes.

We have analyzed the factors involved in vaginal cell proliferation and differentiation focusing on growth factors and cytokines (Miyagawa *et al.* 2004). The novel finding in the present study is that genes related to the IL-1 system (IL-1L1 and IL-1 RII) were increased in expression in the neoDES vagina. Thus, the IL-1 system is also possibly involved in the persistent vaginal epithelial cell proliferation and differentiation. We also found that several growth factor-binding proteins were increased (IGF-binding protein (IGFBP)-2 and fibroblast growth factor (FGF)-binding protein (FGFBP)-1) and decreased (IGFBP-3 and latent transforming growth factor-β binding protein (LTBP)-2) in the neoDES vagina as compared with controls. As to IGF-I signaling, its receptor (IGF-IR) was decreased in the neoDES vagina. It is a common property of receptor tyrosine kinase that ligand binding and phosphorylation of receptor ultimately result in downregulation (Pastan & Willingham 1983). Although their ligands (IGFs) did not show 3-fold change on DNA array, these growth factors systems are possibly involved in persistent proliferation of epithelial cells in the neoDES vagina. To confirm this hypothesis, we further analyzed two signaling pathways, the IL-1 and IGF-I cascades, in the neoDES vagina.

Table 2 List of increased and decreased genes related to the immune system in vaginas of mice exposed neonatally to DES

Accession no.	Gene name	Fold changes
AF109905	MHC class III regions Hsc70t	7·5
L38444	T cell-specific guanine nucleotide triphosphate-binding protein (TGTP)	−8·0
AV092014	Peptidoglycan recognition protein (Pglyrp)	−6·7
AF039663	AC133 antigen homolog	−5·9
X13333	CD14 antigen	−5·7
V00746	MHC class I antigen H-2K	−5·5
U69488	Viral envelope like protein (G7e)	−5·3
AB001489	Phosphatidylinositol glycan class R	−4·9
AF076482	Peptidoglycan recognition protein precursor (Pgrp)	−4·9
M58156	MHC class I antigen H-2K-b	−4·4
X00246	MHC class I antigen with a Set 1 repetitive element	−4·4
X16202	MHC class I antigen Q4	−4·3
AF109906	MHC class III region RD	−4·1
X58609	MHC class I antigen Q2-k	−4·0
AJ132098	Vanin-1	−3·9
AF029215	MRC OX-2	−3·7
X58861	Complement component 1 q subcomponent α	−3·6
M18837	MHC class I Q4 beta-2-microglobulin	−3·5

IL-1 signaling in the neoDES vagina

Cytokine networks regulate epidermal tissue homeostasis and keratinocyte proliferation and differentiation (Schroder 1995). Using an *in vitro* skin equivalent model, IL-1 induction regulates epidermal proliferation and differentiation through the induction of KGF/FGF7 and GM-CSF in fibroblasts (Szabowski *et al.* 2000). Therefore, it seemed to be convincing that the IL-1 system also has functions in vaginal epithelial cell proliferation and differentiation.

The time-course of gene expression (IL-1α, IL-1β, IL-1RI, IL-1RII, IL-1ra and IL-1L1) was examined in vaginas from 60-day-old OVX control mice after a single injection of E_2 (Fig. 2A). E_2 administration rapidly induced IL-1RI expression within 1 h (5·1-fold compared with 0 h); IL-1RI expression subsequently declined slowly. Expression of the other genes was not changed but IL-1L1 expression increased from 24 h, peaking at 36 h (43·3-fold). In neoDES vaginas, expression of IL-1α (4·2-fold) and IL-1 RII (30·1-fold) was increased together with high-level expression of IL-1L1 (Fig. 2B). To study the tissue distribution of these factors, epithelium and stroma from neoDES vaginas were separated by incubation in trypsin, followed by dissection and RT-PCR analysis. IL-1α and IL-1L1 were found to be primarily expressed in the epithelium whereas IL-1RI and IL-1RII were expressed in both epithelium and stroma (Fig. 2C).

To investigate the participation of IL-1 signaling in the neoDES vagina, we tested the activation status of SAPK/JNK, which is one of the downstream factors of IL-1 (Karin *et al.* 1997). As shown in Fig. 2D, SAPK/JNK (particularly JNK1) was phosphorylated in the neoDES vagina but only slightly phosphorylated in controls. Then, we tested the mRNA expression of KGF and GM-CSF in stromal tissue in the control and neoDES vaginas. In both control and neoDES vaginas, KGF and GM-CSF mRNA were expressed; however, unexpectedly, their expression levels showed no change (Fig. 2E).

IGF-I signaling in the neoDES vagina

In mouse uterus, locally produced uterine IGF-I mediates the effects of E_2 on growth and cell proliferation (Klotz *et al.* 2002). In the neoDES vagina, IGFBP-2 levels were increased, but IGFBP-3 and IGF-IR levels were decreased (Table 3). These data raised the possibility that the IGF-I pathway is modulated in the neoDES vagina, therefore, we further analyzed this signaling pathway in the vagina.

Table 3 List of increased genes related to signalling factors in the vagina of mice exposed neonatally to DES

	Increased		
	Accession No.	Gene name	Fold changes microarray/Q-PCR
Growth factors and cytokines	AJ250429	Interleukin-1 like protein 1 (IL-IL1)	7·7/124
Membrane receptors/proteins	X59769	IL-1 receptor type II (IL-IRII)	4·6/30·1
	M84683	Mucin 1 (episialin)	8·0/3·5
Intracellular/signaling factors	U42443	MECA39/branched chain aminotransferase 1 (BCAT)	4·4/42·5
	81580	IGF-binding protein 2 (IGFBP2)	7·2/23·9
	AF065441	FGF-binding protein 1 (FGFBP1)	6·7/8·0
	AF058798	14-3-3 protein sigma	4·1/4·1
Transcription factors and their regulators	X83106	Mad	6·1/14·1
	AF049702	E74-like factor 5 (Elf5)	4·9/6·3
	L36435	Basic domain/leucine zipper transcription factor	4·0/4·1
Proteases and protease inhibitors	AA615849	Single WAP motif protein 2	68·6/2688
	AF063937	Squamous cell carcinoma antigen 2 (Scca2)	90·5/832
	M95545	T cell-specific protein (Tcl-30)	7·2/258
	AF002719	Secretory leukoprotease inhibitor (SLPI)	27·9/146
	X68680	Bikunin	5·9/30·9
	D30785	Neuropsin	6·3/16·8
	X16490	Plasminogen activator inhibitor type II	5·1/7·4
	U54705	Maspin	4·9/6·5
Miscellaneous genes	AW045533	Farnesyl diphosphate synthetase	4·1/6·4
	AB013137	Glutaredoxin	11·7/4·5
	AJ223066	Fatty acid binding protein (Fabpe)	7·2/4·3
	AW124836	Ceroid-lipofuscinosis, neuronal 8 (Cln8)	6·5/4·0
	D16215	Flavin-containing monooxygenase	4·4/3·5
	Y14384	Arachidonate 12-lipoxygenase pseudogene 2	14·4/3·4
	X74154	Cellular retinoic acid binding protein II	7·0/3·1

Table 3 *(continued)* List of decreased genes related to signalling factors in the vagina of mice exposed neonatally to DES

	Decreased		
	Accession No.	Gene name	Fold change microarray/Q-PCR
Growth factors and cytokines	U27267	LPS-induced C-X-C chemokine (LIX)	−8·3/−11·4
	M29464	Platelet-derived growth factor a (PDGF-a)	−5·5/−5·3
Membrane receptors/proteins	AF004874	Latent TGF-β binding protein-2 (LTBP2)	−7·7/−33·0
	D86563	Rab4	−6·7/−8·2
	X94998	Fibromodulin	−6·3/−7·3
	M38337	Milk fat globule-EGF factor 8 protein (MFG-E8)	−5·5/−6·6
	AF056187	IGF-1 receptor (IGF-1R)	−3·7/−4·2
Intracellular/signaling factors	AI840868	Nedd4-like	−3·9/−6·0
	M93422	Adenlyl cyclase type VI	−4·6/−5·6
	U50631	Heat-responsive protein (HRP12)	−4·1/−4·6
	AI851250	Sprouty protein with EVH-1 domain 2	−3·2/−4·5
	X81581	IGFBP3	−9·6/−3·9
	L07918	GDP-dissociation inhibitor (D4/LyGDI)	−3·9/−3·2
	AI845584	Dual specificity phosphatase 6 (Dusp6)	−4·3/−3·0
	U88327	Suppressor of cytokine signalling-2 (SOCS-2)	−3·5/−3·0
Transcription factors and their regulators	AJ223069	TCF-3	−6·5/−17·4
	X80339	Six1	−7·5/−7·2
	D26532	Core binding factor alpha 2 (PEBP2aB2)	−4·0/−5·9
	U33005	TBC1	−4·9/−5·9
	AI447619	Zinc finger protein 161	−4·4/−5·4
	V00727	C-fos	−3·9/−5·2
	M94623	Aryl-hydrocarbon receptor (AhR)	−4·6/−4·3
	AF074600	LIM-only family of nuclear LIM protein 4	−4·1/−3·7
	U32395	Mad4	−3·7/−3·7
	AF017085	BAP-135 homolog (Diwslt)	−4·4/−3·2
	AI854510	Similar to KIAA0130 gene product	−4·0/−3·1
DNA/RNA and nuclear proteins	AI849928	Cyclin D	−8·0/−6·3
	AB028921	NAKAP95	−6·3/−4·3
	X67209	Npdc-1	−3·9/−3·8
Proteases and protease inhibitors	AW121336	WAP domain protein HE4 (He4)	−17·1/−28·3
Miscellaneous genes	X57437	L-histidine decarboxylase	−6·6/−13·0
	M22679	Alcohol dehydrogenase class I	−9·8/−10·1
	M77497	Cytochrome P450 2f2	−8·4/−6·1
	D00466	Apolipoprotein	−4·1/−3·5
	M83218	Intracellular calcium-binding protein (MRP8)	−6·6/−3·2

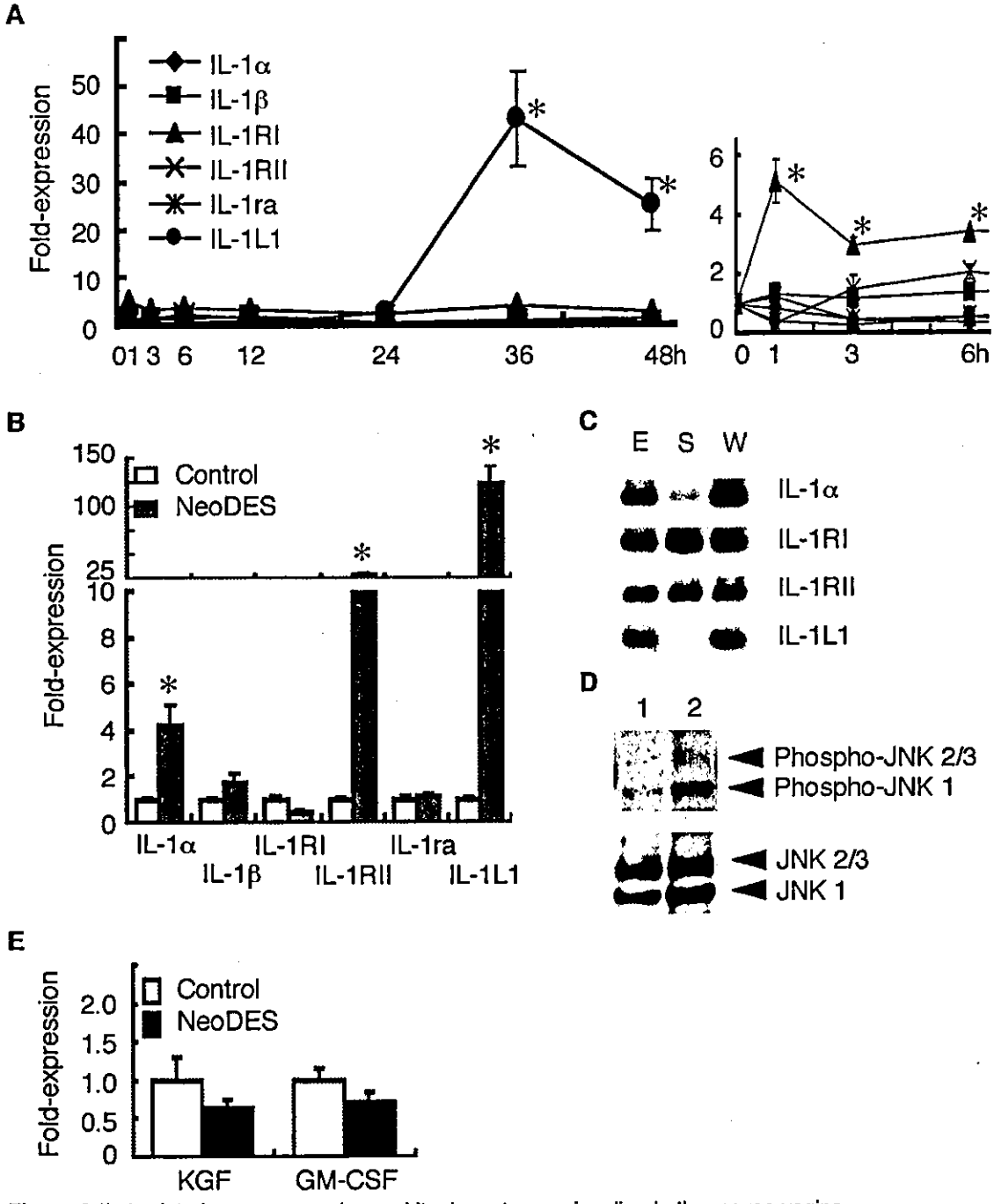

Figure 2 IL-1-related gene expression and its downstream signaling in the mouse vagina. (A) Time-course analysis of IL-1-related gene expression. Samples were from vaginas of mice 0 (control), 1, 3, 6, 12, 24, 36 and 48 h after a single injection of 50 ng E_2/g BW. The right panel shows an enlarged view of the 0–6 h range of the left panel. More than three mice were used per each group. Error bars represent the standard error. (B) Expression profiles of IL-1-related genes in vaginas of 60-day-old control and neoDES mice. (C) Tissue distribution of IL-1α, IL-RI, IL-1RII and IL-1L1 mRNA expression in the neoDES vagina. E, epithelium; S, stroma; W, whole tissue. (D) Activation status of JNK was detected by anti-phospho-JNK antibody. The samples are from control (lane 1) and neoDES mice (lane 2). (E) Expression of KGF and GM-CSF mRNA in vaginal stromal tissue in control and neoDES vaginas.

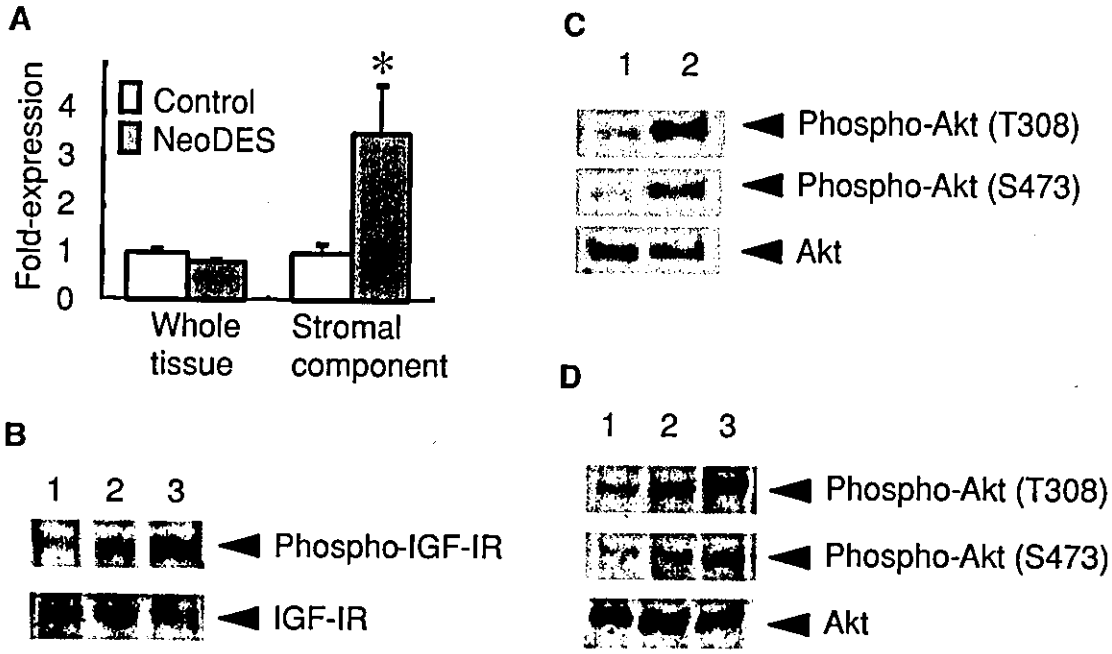

Figure 3 Activation status of the IGF signaling pathway in the neoDES vagina. (A) Expression of IGF-I mRNA in vaginal tissue in control and neoDES vaginas. More than three mice were used in each group. Error bars represent the standard error. (B) Phosphorylation of IGF-IR was detected by anti-phospho-IGF-IR antibody. The samples were from vaginas of OVX mice treated with oil (lane 1), DES (lane 2) and neoDES mice (lane 3). (C) IGF-I-induced phosphorylation of Akt in the adult OVX mouse vagina. The samples were from vaginas of OVX mice treated with saline (lane 1) and long R3 IGF-I (lane 2). (D) Activation status of Akt was detected by anti-phospho-Akt antibodies. The samples were from vaginas of OVX mice treated with oil (lane 1), DES (lane 2) and neoDES mice (lane 3).

IGF-I mRNA level in the whole tissue was not altered between control and neoDES vaginas (Fig. 3A and Miyagawa *et al.* 2004). However, when compared in the stromal component, IGF-I mRNA level was increased in the neoDES vagina (Fig. 3A). As shown in Fig. 3B, distinct phosphorylation of IGF-IR was detected in the neoDES vagina and it is similar to vaginas of mice treated with DES successively as a positive control. IGF-IR in vaginas of oil-treated control mice showed a low level of phosphorylation, although IGF-IR protein was expressed. To further characterize the factors downstream of IGF-I signaling in the neoDES mouse, we examined the activation status of Akt, which is known as one of the major regulatory factors in IGF-I signaling (Martin *et al.* 2000). As in the mouse uterus (Klotz *et al.* 2002), IGF-I administration induced phosphorylation of Akt in vaginas of adult OVX mice (Fig. 3C). Furthermore, we detected Akt activation in the vaginas of neoDES and DES-treated mice but not in oil-treated controls (Fig. 3D).

Discussion

We examined the global expressions of mRNAs, focusing on factors involved in cell signaling in the neoDES vagina, which shows persistent vaginal hyperplasia and keratinization (Takasugi *et al.* 1962, McLachlan *et al.* 1980, Iguchi 1992). First, we intended to clarify the overall expression patterns of genes in the neoDES vagina which seemed to be useful for further analysis. Using DNA microarray, we found altered expression of several genes in the neoDES vagina compared with controls and detected genes showing altered expression which are candidates for mediating the persistent proliferation and differentiation. Nonetheless, each gene needs to be elucidated in detail; in this report, we

focused on the two signaling pathways, the IL-1 and IGF-I systems, which are possibly involved in cellular signaling in the vagina.

IL-1 signaling

The cytokine IL-1 elicits pleiotropic biological effects in various tissues, including the regulation of epidermal differentiation and inflammation in skin (Dinarello 1998). Although the function of IL-1L1 remains undescribed (Barton *et al.* 2000), its abundant expression in skin and vaginal epithelium suggests that it may be involved in epithelial cell differentiation (Mulero *et al.* 1999). IL-1L1 expression was elevated 24 h after E_2 administration, similar to other differentiation markers such as CK1 (data not shown). Intriguingly, expression of IL-1α, which showed no change in expression after estrogen stimulation in adult OVX mice, was increased in the neoDES vagina. Although IL-1α and IL-1L1 may not be estrogen-primed genes, these data suggested that IL-1 signaling has a functional role in the maintenance of epithelial cell differentiation, rather than estrogen-primed cell proliferation in the vagina. The negative regulator of IL-1 signaling, IL-1RII, was also highly expressed in the neoDES vagina. Stimuli that induce IL-1 gene expression in keratinocytes *in vitro*, also strongly stimulate IL-1RII expression (Groves *et al.* 1995), and a high level of IL-1RII is expressed in keratinocytes in psoriasis (Groves *et al.* 1994). Thus, the increased expression of IL-1RII in the neoDES vagina showing ovary-independent stratification and keratinization is not surprising.

IL-1 stimulates the activity of the transcription factors AP-1 and nuclear factor κB (NF-κB). These effects are mediated through activation of SAPK/JNK and inhibitor of NF-κB kinases respectively. In the present study, we found that JNK1 was activated in the neoDES vagina. IL-1 induced in the epidermis is known to regulate KGF and GM-CSF in the dermis through AP-1 (Szabowski *et al.* 2000). We supposed that there is the presence of similar regulation by IL-1 signaling in both epidermis and vaginal epithelium. KGF and GM-CSF were expressed in both control and neoDES vaginas and their expression levels were not different. Therefore, it is unlikely that KGF and GM-CSF have direct positive roles in epithelial proliferation and differentiation in the vagina. Actually, estrogen treatment did not induce mRNA expression of KGF and GM-CSF in the vagina (Masui *et al.* 2003, Miyagawa *et al.* 2004), and KGF-null mice are fertile and morphologically normal (Guo *et al.* 1996). This is in contrast to skin, where KGF effects are crucial. Defects in the skin and hair follicles were observed in KGF-null mice (Guo *et al.* 1996). Overexpression of KGF in transgenic mice induced altered epidermal growth and differentiation, including wrinkled skin and increased epidermal thickness (Guo *et al.* 1993). Thus, KGF contributed less to vaginal epithelial differentiation than to that of skin, however, non-physiological amounts of KGF treatment may affect vaginal keratinocyte differentiation. It was reported that neonatally KGF-treated mouse vaginas showed persistent epithelial differentiation (Hom *et al.* 1998). Although we indicated differences between skin and vagina, both showed stratification and keratinization in the epithelium, and the roles of the IL-1 system, KGF, GM-CSF and also JNK1 phosphorylation in the vagina remain unknown.

IGF-I signaling

In the mouse uterus and vagina, epithelial proliferation appears to be a paracrine event mediated by stromal soluble factors, but these factors have not been identified (Cooke *et al.* 1997, Buchanan *et al.* 1998). Although the stromal contribution to persistent activation of vaginal epithelium is less than that of normal E_2-induced action in the vagina (Cunha *et al.* 1977, Miyagawa *et al.* 2004), some stromal factors must be contributing to the persistent activation in the neoDES vagina. Such candidates were not directly detected on DNA array data, which is probably due to the conservative adoption of 3-fold differences in the present microarray analysis. In the present study, IGF-I mRNA derived from vaginal stroma was increased by estrogen treatment as compared with controls but was not changed at whole tissue levels. This IGF-I signaling may be enhanced by its growth factor-binding factors, which influence growth factor availability and activity, because IGFBP-3 levels were decreased. As a result, IGF-IR is phosphorylated, leading to Akt phosphorylation, which suggests that IGF-I signaling is active in the neoDES vagina, where it may regulate several biological processes. Of course, other growth factors have a potential involvement

in persistent signaling in the neoDES vagina. A large amount of epithelial cells makes it difficult to detect genes derived from stroma. We need to further analyze the gene expression from the stromal component. Still, IGF-I is one of the candidates of stromal factors which mediate the vaginal epithelial cell proliferation.

We found that IGFBP-3 was downregulated whereas IGFBP-2 was highly expressed in the neoDES vagina. IGFBP-2 generally regulates IGF-induced action by binding to IGFs. Although IGFBP-2 inhibits IGF action, IGFBP-2 is overexpressed in various malignant tissues, often correlating with tumorigenesis (Zumkeller 2001). It should be noted that some of the effects of IGFBP-2 overexpression on cell proliferation are proposed to be independent of IGFs (Slootweg *et al.* 1995, Badinga *et al.* 1999). Therefore, the elevated levels of IGFBP-2 we detected could be directly involved in the inappropriate epithelial proliferation in neoDES vaginas.

Akt phosphorylation

In the present study, we found that Akt was phosphorylated by estrogen in the mouse vagina, which was similar to the uterus, as described in the demonstration that Akt was a functional mediator of estrogen-induced cell proliferation (Klotz *et al.* 2002). In addition, expression of active Akt in MCF-7 cells results in estrogen-independent tumor growth (Faridi *et al.* 2003). Akt promotes cell survival through phosphorylation and inactivation of several pro-apoptotic targets, such as Bad and forkhead transcription factors which indirectly affect NF-κB and p53 (Brunet *et al.* 1999, Downward 1999, Vivanco & Sawyers 2002). These observations indicate that Akt is involved in estrogen-dependent and/or -independent cell proliferation and even carcinogenesis. The present study showed that Akt phosphorylation occurred in the neoDES vagina even in the absence of estrogen stimulation by OVX, and it may be involved not only in the persistent cell proliferation but also in carcinogenesis in the neoDES vagina later in life.

Other cellular events in the neoDES vagina

We detected additional genes that are expected to be involved in vaginal epithelial cell differentiation. For example, various proteases and protease inhibitors were highly expressed in neoDES vaginas. The protease inhibitors are involved in keratinocyte differentiation or epidermal wound repair (Tamechika *et al.* 1996, Cui *et al.* 1999, Katz & Taichman 1999, Li *et al.* 2000, Zhu *et al.* 2002). The serine protease, neuropsin, is expressed in superficial layers of keratinized epithelium in the mouse vagina (Katsu *et al.* 2002). These genes are thought to be involved in fibrinolysis, inflammation, cell migration and growth factor proteolysis by regulating the activity of other proteases (Travis & Salvesen 1983, Carrell *et al.* 1989, Potempa *et al.* 1994). Thus, proteases and protease inhibitors identified in this study may be involved in the differentiation of mouse vaginal epithelium.

Application to DES syndrome in the human

Although estimates vary, about four million women have been exposed to DES during pregnancy (Newbold 1993). Epidemiological studies indicate an increased incidence of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma in women exposed to DES *in utero* (Herbst 2000, Hatch *et al.* 2001, Palmer *et al.* 2002). In addition to the aberrant cellular signal transduction, a hypothesis for the association between DES and high-grade disease is that DES *in utero* possibly induced permanent alteration in the immune system. Profound alterations in the immune system, especially of natural killer cells, were found in mice treated neonatally with DES (Kalland 1982). In the present study, we demonstrated that expression of genes related to the immune system was decreased, which could lead to a locally reduced immune function in neoDES mice. In humans, permanent alterations in the immune system may lead to a reduced ability to fight off a genital infection, such as human papilloma virus, thereby increasing the susceptibility to secondary risk factors for carcinogenesis. Further characterization of the perinatally estrogen-exposed mouse model is needed to fully understand the potential risk of the carcinogenic effects of estrogens.

In conclusion, we characterized the gene expression pattern of the neoDES vagina. In such vaginas, the IGF-I signaling cascade was activated, resulting in Akt phosphorylation. Aberrant Akt phosphorylation is one of the key regulators of the vagina showing persistent proliferation. We also

found JNK-1 phosphorylation status and expression of various genes were changed. These complicated expressions of genes and protein phosphorylation status lead to vaginal changes and the present findings give insight into the persistent activation in vaginas from neonatally DES-exposed mice.

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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 tubular 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

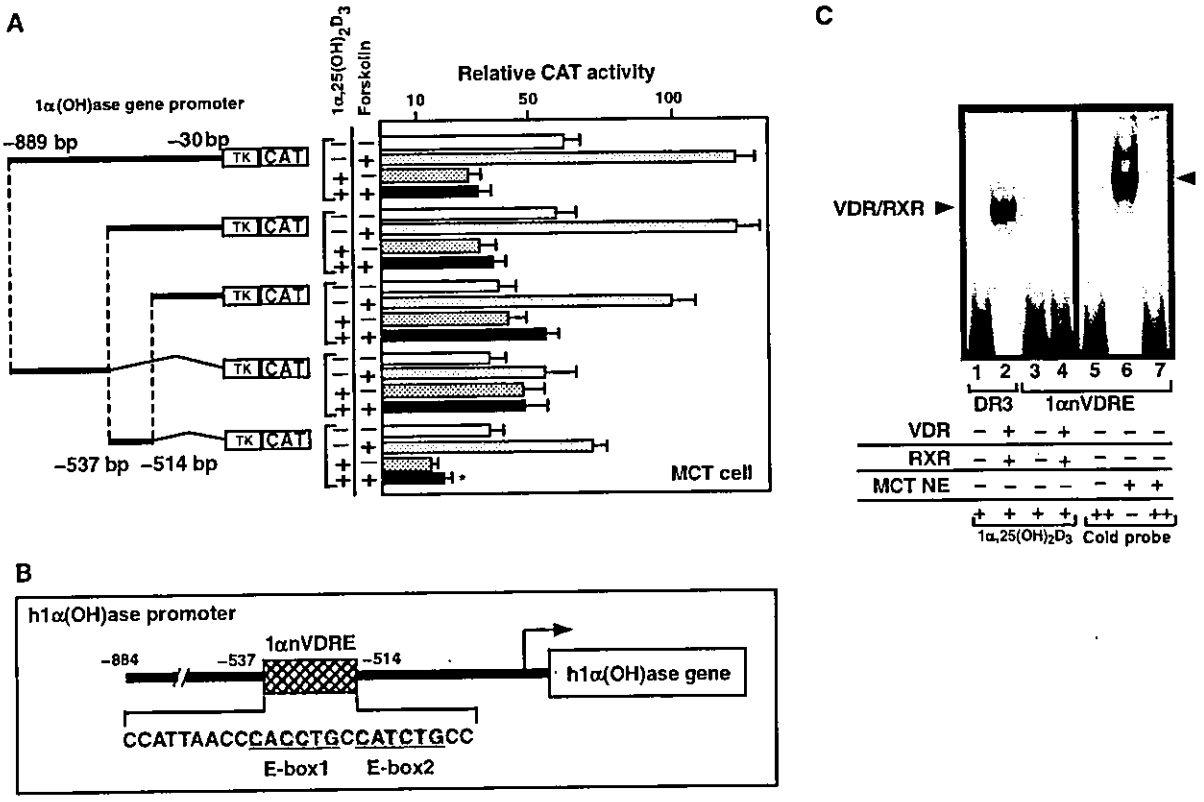

Figure 1 Identification of 1αnVDRE. (A) CAT assay using a series of human 1α(OH)ase gene promoter deletion mutants in MCT cells. After 3 h, forskolin (1×10^{-6} M), which activates PKA signaling, and 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) were added, respectively. 1α(OH)ase gene promoter deletion constructs (−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 1 and 2).

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).

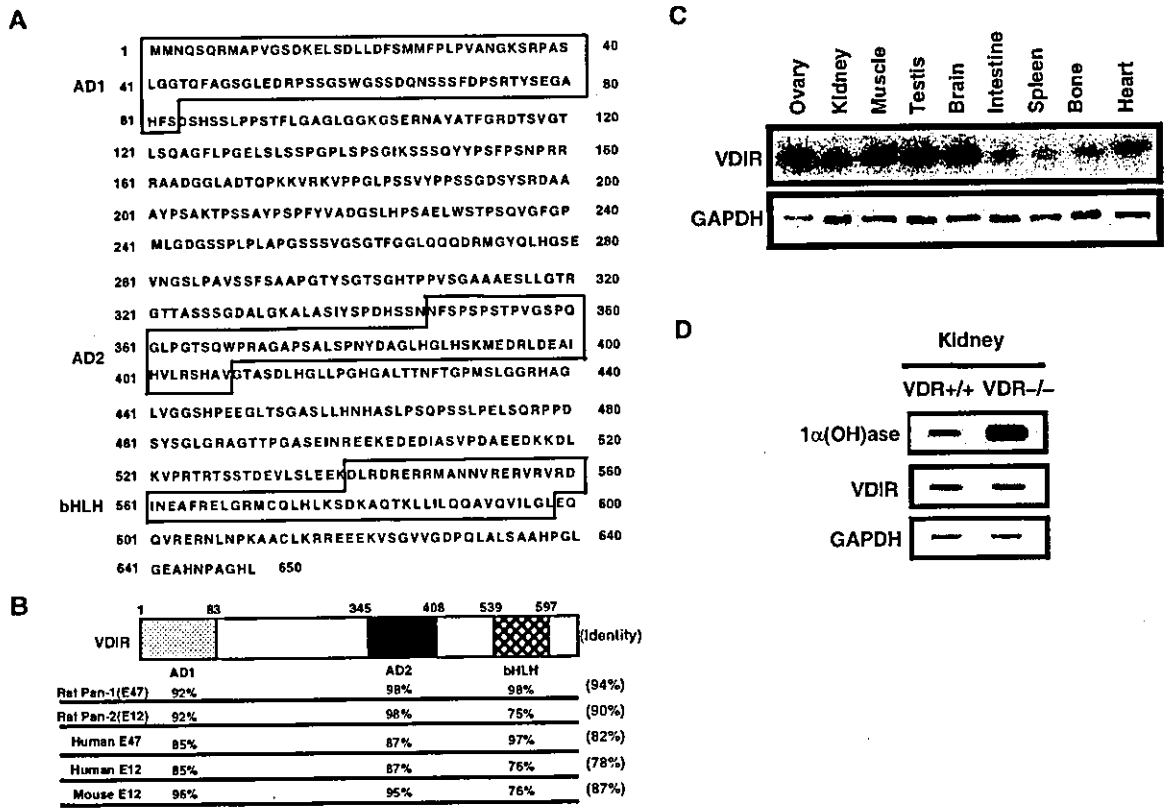

Figure 2 Cloning of the 1αnVDRE-binding factor, VDIR. (**A**) Sequence of VDIR. VDIR has two transactivation domains (AD1 and AD2), and a bHLH motif. (**B**) Functional domain sequence homology between VDIR and members of the bHLH-type activator family (rat Pan-1, E47; rat Pan-2, E12; human E47; human 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.

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 $1\alpha,25(OH)_2D_3$, 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

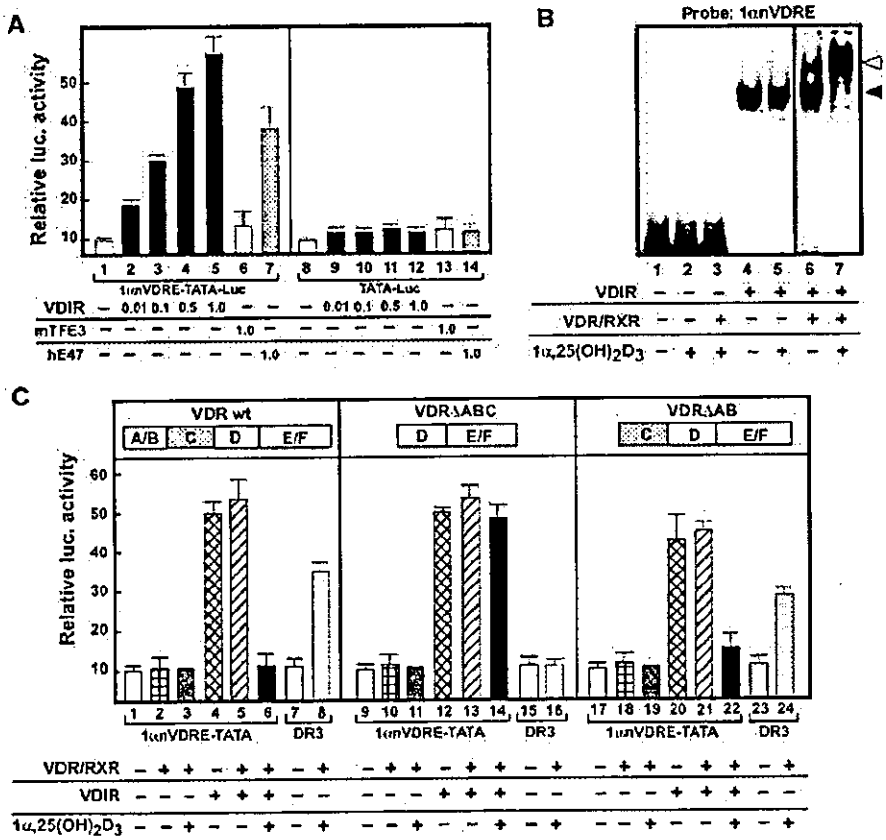

Figure 3 VDIR as an activator for 1αnVDRE. (**A**) Plasmid dose dependency of VDIR activation of nVDRE. Luciferase activity under the control of 1αnVDRE after the transfection of VDIR, mTFE3 or hE47 into MCT cells. MCT cells were cotransfected with LUC reporter plasmid (0.3 μg of nVDRE pGL3 TATA-LUC 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 $1\alpha,25(OH)_2D_3$ (1×10^{-8} M) were added as indicated. DR3-Luc was used as a positive control for VDR/RXR and $1\alpha,25(OH)_2D_3$. VDR wt: wild-type VDR; VDR ΔABC and VDRΔAB: VDR mutants with deleted N-terminal A–C and AB domains, respectively.

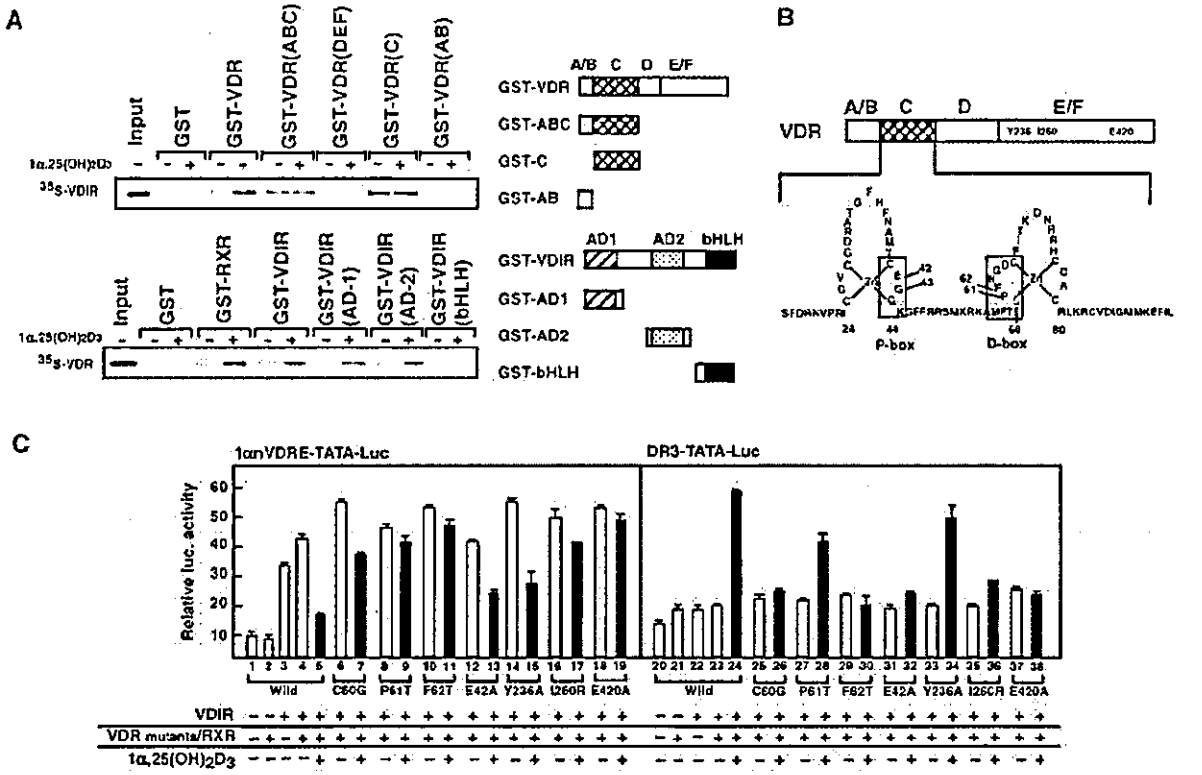

Figure 4 The DNA-binding domain (C-domain) of VDR leeds to the binding of VDIR. (**A**) GST pull-down assay using either GST alone, GST wild-type VDR or GST-fused VDRs deletion mutants together with [^{35}S]-labeled VDIR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (upper panel). GST 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

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

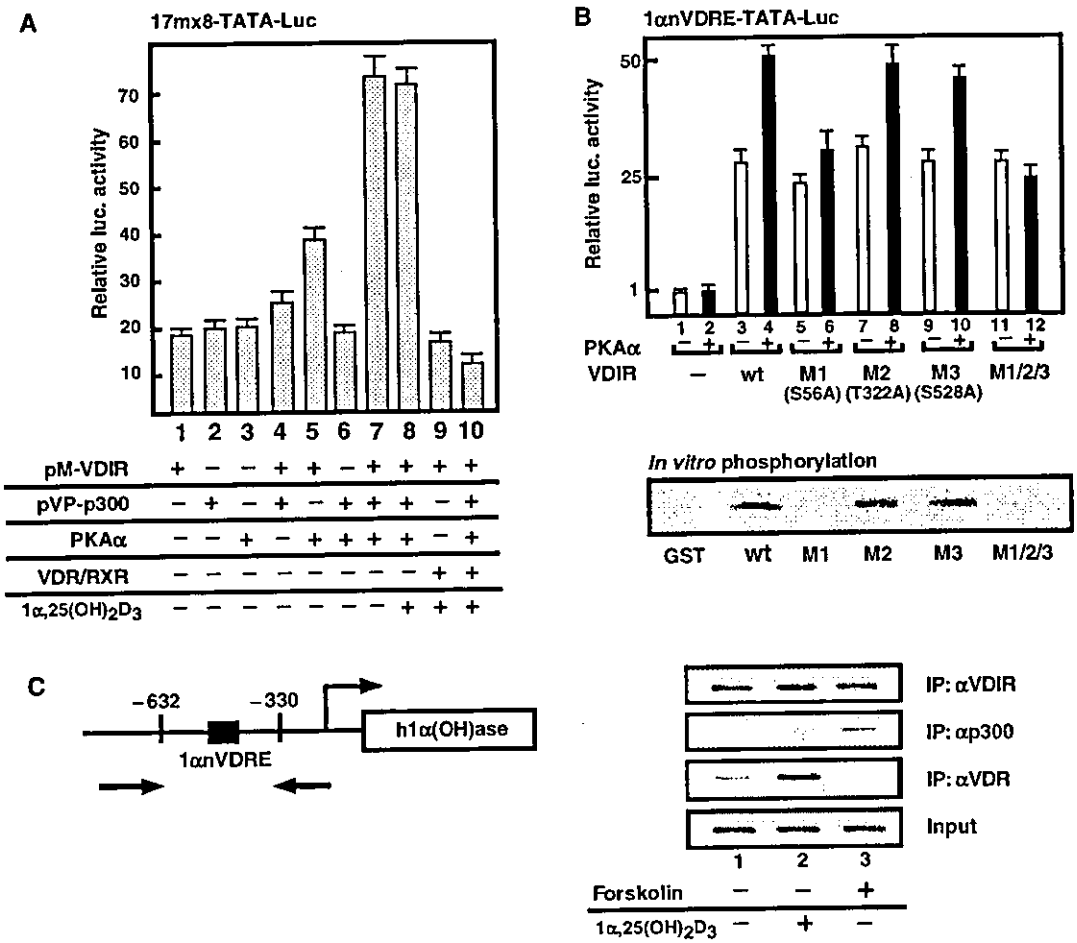

Figure 5 Phosphorylation of VDIR by PKA induced a p300 co-activator recruitment. (**A**) Association of VDIR and p300 in the mammalian two-hybrid assay. The expression plasmids of fusion proteins with GAL4-DBD (pM) and VP16-AD (pVP) were transiently transfected into MCT cells with a GAL4-DBD-regulated 17mer × 8 TATA luciferase reporter. PKAα or VDR/RXR was co-transfected in the absence or presence of $1\alpha,25(OH)_2D_3$ (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.

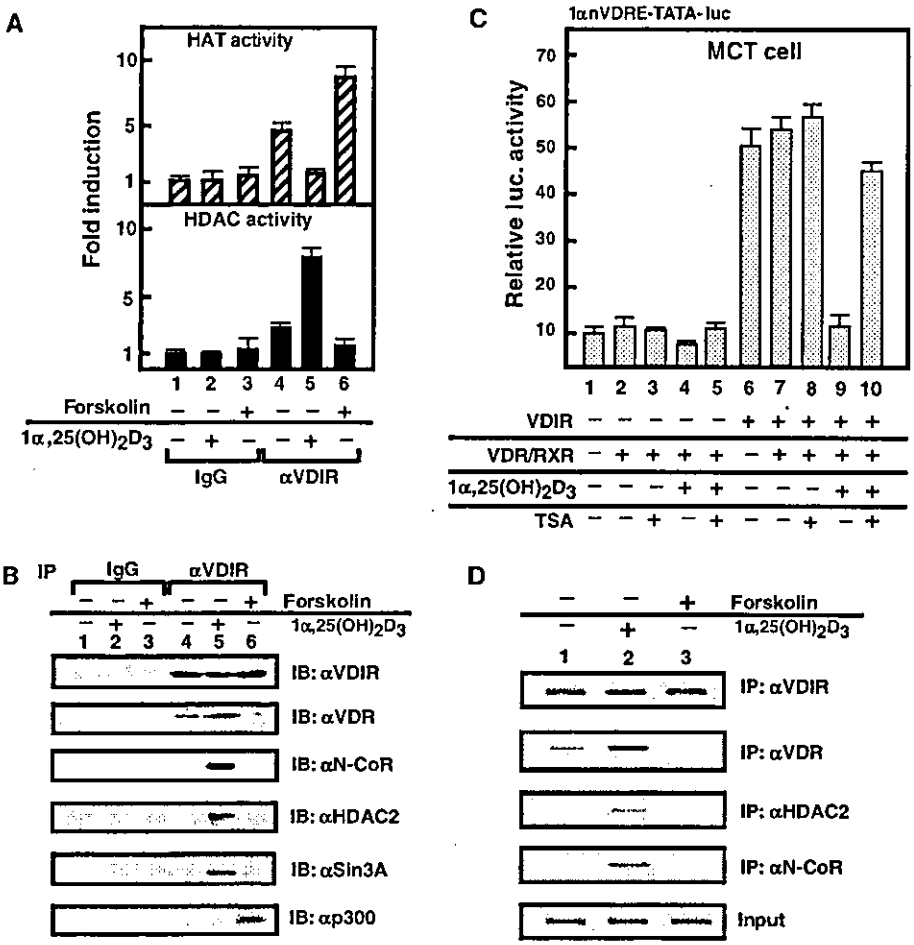

Figure 6 Co-regulator switching upon VDIR for the ligand-induced transrepression by VDR. (**A**) HAT and HDAC activities of the immunoprecipitated VDIR complexes in the MCT cells. Assays were determined in MCT cells after treatment, in the absence or presence of $1\alpha,25(OH)_2D_3$ and forskolin. Representative 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

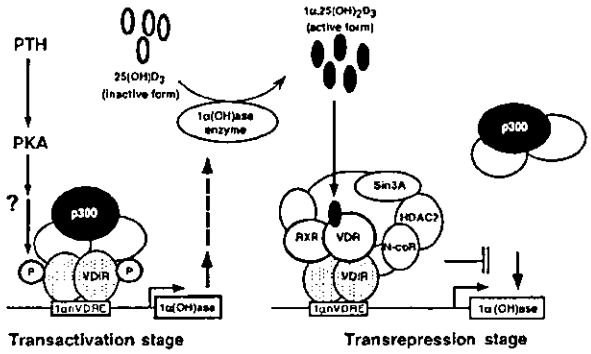

Figure 7 Schematic illustration of the proposed molecular mechanism of $1\alpha,25(OH)_2D_3$-induced transrepression in the 1α-hydroxylase gene promoter. Upon activated-PKA signaling due to PTH, the 1α-hydroxylase gene is transactivated through recruitment of a HAT co-activator complex to VDIR bound to 1αnVDRE, leading to increased serum concentrations of $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$-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

(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, 1α,25$(OH)_2D_3$ (1×10^{-8} M) and/or forskolin (1×10^{-6} 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 1α,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

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′-ATTCCCATCTCTGGAAGGAG-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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