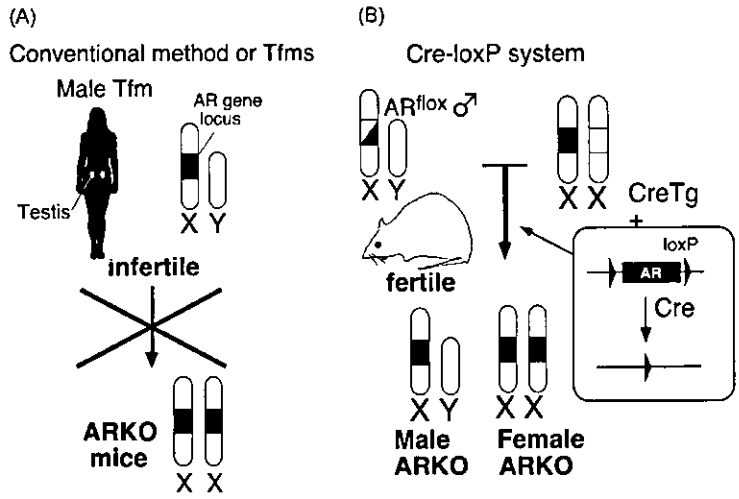

Fig. 1. Strategy for generating the ARKO mouse line. (A) The *AR* gene is located on the X chromosome. As male Tfm animals are infertile, the mutated *AR* gene cannot be transmitted to the next generation. (B) In the first step, floxed AR mice that carry a functional *AR* gene flanked by loxP sites are generated by the introduction of loxP sites into the first exon of the *AR* gene by homologous recombination in ES cells. By mating subsequent mice with CMV-Cre transgenic mice, the *AR* gene is disrupted during embryogenesis.

genetic models of loss of AR function in mice [15,16] and gain of AR function in *Drosophila* [17].

2. Androgen receptor inactivation by gene targeting using the Cre-loxP system in mice

As shown in Fig. 1A, there were both basic and technical difficulties in generating AR knockout (ARKO) mice. The *AR* gene is located on the X chromosome [18], and therefore exists as a single copy in 46, XY males, in which androgen exerts its most profound effects. As male mice lacking a functional *AR* gene would be expected to show Tfm abnormalities with complete infertility [10,13,14], successful targeted disruption of the *AR* gene, essential for reproduction, necessarily prohibits its transmission to the next generation. Thus, it was impossible to generate an ARKO mouse line by either breeding or conventional gene targeting methods. Furthermore, as all Tfm model animals are genetically male, it was impractical to generate genetically female animals homozygous for *AR* gene mutations.

To avoid this problem, we applied the Cre-loxP system [19] to establish an ARKO mice line (Fig. 1B). We first generated floxed AR mice, in which the *AR* gene locus was flanked by loxP sites. Floxed AR mice were fully fertile and showed normal expression of AR protein. We then crossed these mice with mice that expressed the Cre recombinase ubiquitously under the control of a CMV promoter, and obtained male and female ARKO mice at theoretical Mendellian frequencies.

3. Female-typical appearance of male ARKO mice

The appearance of male ARKO mice is shown in Fig. 2. ARKO males exhibited female-typical external appearance, such as a vagina with a blind end, and a clitoris-like phallus, instead of a penis and scrotum. Male reproductive organs, including seminal vesicles, vas deferens, epididymis and prostate were absent in ARKO males. However, no ovaries or uteri were observed, although small inguinal testes were present. Histological examination of the testes showed that spermatogenesis was severely arrested. From these results, it was clear that while AR was not required for the formation of testis, it was essential for the development of male reproductive organs and spermatogenesis.

Estimation of plasma hormone levels in ARKO males revealed markedly lowered androgens, but similar estradiol levels to that of wild-type males. This suggested that it was possible to investigate the effect of androgens independently in ARKO mice as only AR was disrupted, leaving estrogen receptors intact.

4. Late onset of obesity in male ARKO mice

A characteristic change was seen in the growth of ARKO males (Fig. 3). Until 10 weeks of age, ARKO males exhibited growth retardation with growth curves indistinguishable from that of wild-type female littermates. However, thereafter, the growth of ARKO males rapidly increased, such that at 12 weeks of age, male ARKO mouse body weights exceeded that of wild-type male littermates (Fig. 3A). This late onset of drastically increased ARKO male growth curve led to the clear development of obesity, with 30-week-old ARKO males, showing significantly increased wet tissue weights in subcutaneous, infrarenal and intraperitorial white adipose tissues (WATS) (Fig. 3B). Such clear increases were not detected in WATs of 8-week-old ARKO males. As no significant alterations in serum lipid parameters or food intake were observed, our results suggested that AR may serve

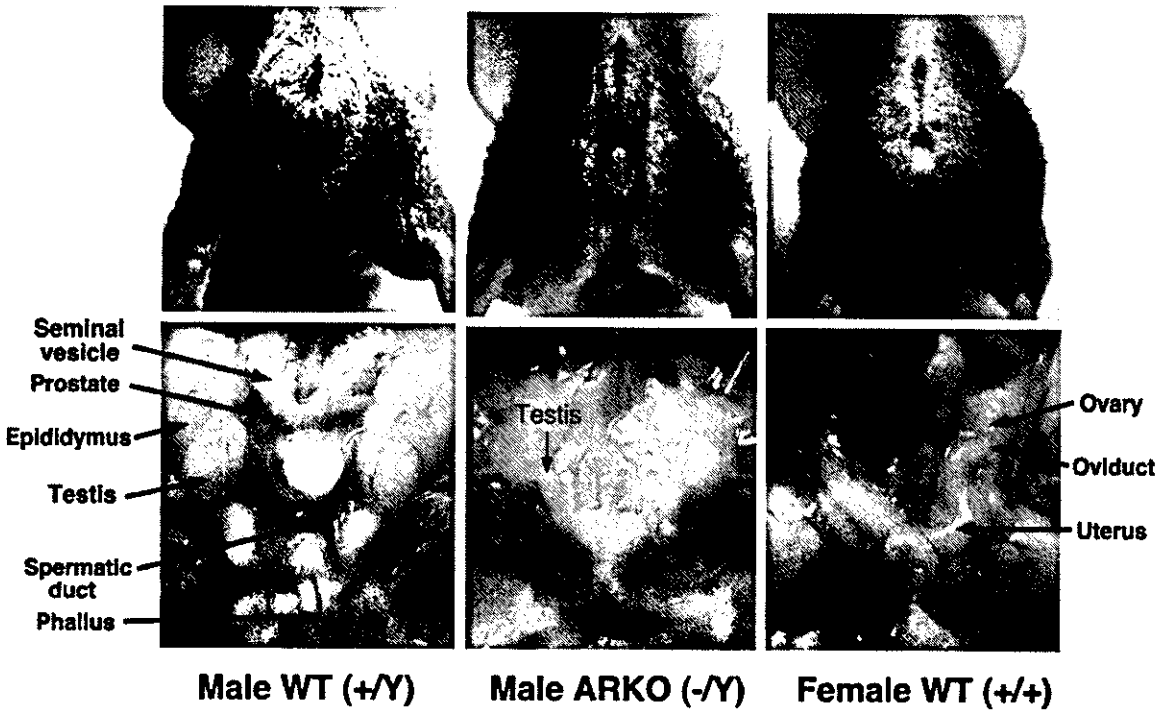

Fig. 2. Male ARKO mice are characterized by female-typical appearance, including a clitoris-like phallus and, a vagina with a blind end as well as the absence of internal male and female reproductive organs, except for the presence of atrophic testes.

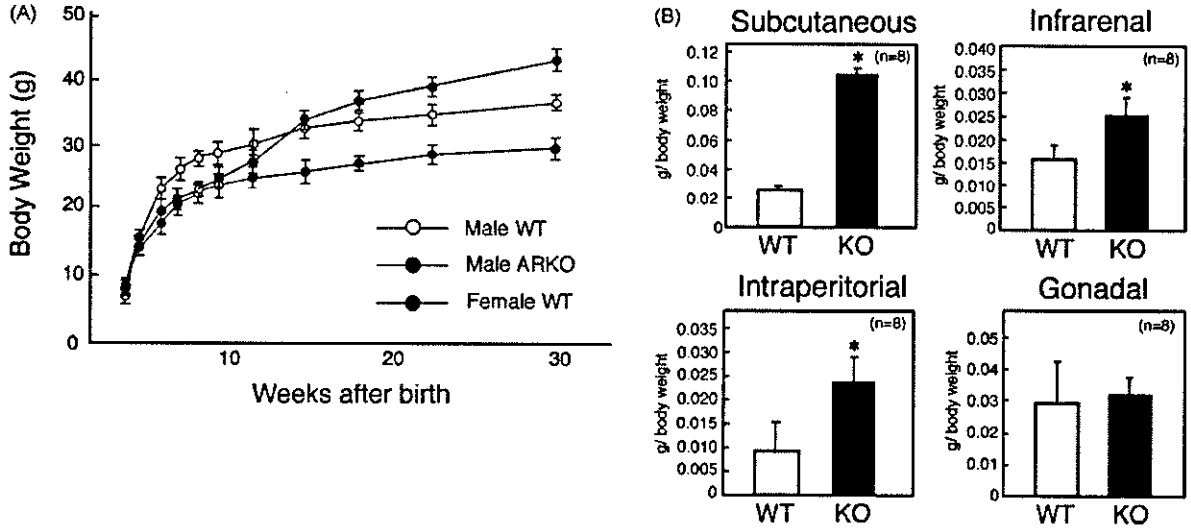

Fig. 3. Late onset of obesity in male ARKO mice. (A) Growth curves of wild-type male and female mice, and ARKO male mice. (B) Increased wet-weights of white adipose tissues in male ARKO mice.

as a negative regulator of adipocyte development in adult males.

5. Androgen-dependent neurodegeneration by polyQ-expanded human AR in *Drosophila*

A unique example of the tissue specific effects of an AR defect is Kennedy's disease. Kennedy's disease, or spinal and bulbar muscular atrophy (SBMA), is a rare degenerative disease of the motor neurons characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30–50 years of age [11]. Previous analyses of Kennedy's disease revealed expansions in the number of trinucleotide CAG repeats in the first exon of the *AR* gene, that generated expanded polyQ stretches in the A/B domain of the AR protein [9,12,20]. It was found that disease onset occurred when these repeat stretches encoded more than

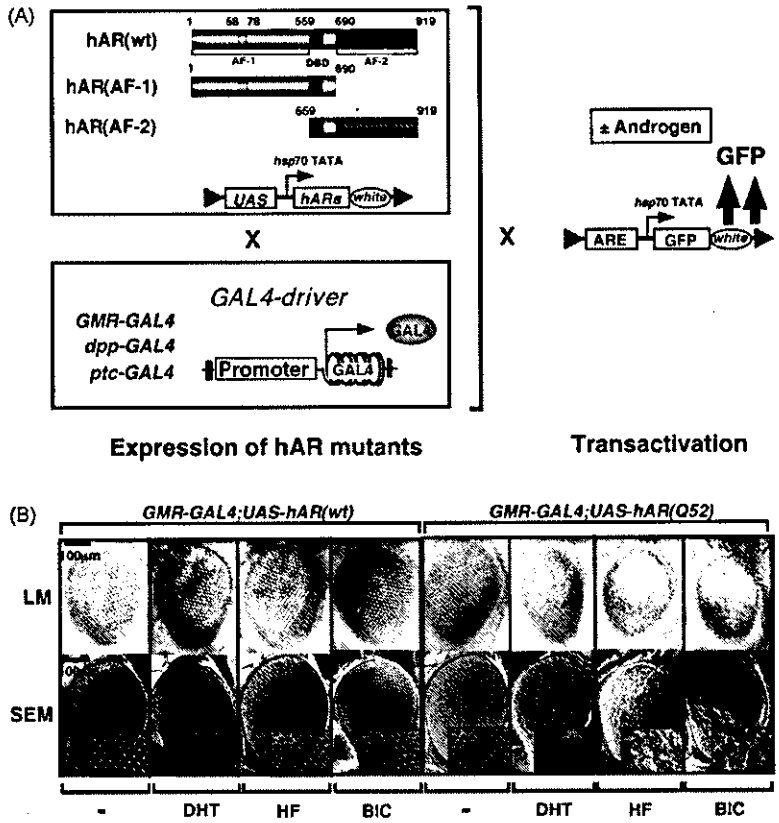

Fig. 4. Ligand-induced degeneration in photoreceptor neurons due to mutant hAR containing expanded polyQ stretches. (A) Expression of human AR constructs in *Drosophila* eyes using the GAL4-UAS system. To monitor the ligand-induced transactivation of hAR, hAR-expressing flies are further crossed to flies carrying a *GFP* reporter gene. Thus, GFP expression was induced by ligand-bound AR that recognized the consensus androgen response element (*ARE*) in the GFP promoter. Location of the polyglutamine (polyQ) region in relation to the DNA binding domain (DBD). Transactivation function-1 (AF-1) region is localized within the N-terminal A/B domain, and transactivation function-2 (AF-2) region is localized within the C-terminal E/F domain containing the ligand binding domain (LBD). (B) Rough-eye phenotype induced in hAR (Q52) lines by DHT or AR antagonist. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult fly-eye tissue.

40 glutamine residues, compared to a range of 15–35 polyQ residues in normal individuals.

As the onset of Kennedy's disease occurred in adult men rather than women, even those women carrying AR mutations in the A/B domains, we reasoned that the binding of significant amounts of androgen and the subsequent structural alteration of mutant AR was likely to be a critical step in the onset of Kennedy's disease. To test this hypothesis, we investigated the role of human AR (hAR) mutants with expanded polyQ stretches in neurodegeneration. To this end, we established a *Drosophila* model that ectopically overexpressed a mutated AR in photoreceptor neurons (Fig. 4). We first expressed wild-type and mutated hAR in photoreceptor neurons in developing eye discs under the glass multimer reporter (*GMR*) gene promoter [21] using the *Drosophila melanogaster* GAL4-UAS system [22]. To monitor the ligand-induced transactivation function of hAR, hAR-expressing flies were further crossed to flies carrying a *GFP* reporter gene, such that GFP expression was induced by the binding of ligand-bound AR to the consensus *ARE* in the GFP promoter [23]. Expressed hAR proteins were then detected as red fluorescence in situ using an immunofluorescent antibody.

Although, eyes that expressed a mutant hAR containing an expanded 52-stretch polyQ (Q52) appeared normal, dietary ingestion of dihydroxytestosterone (DHT) or androgen antagonists induced marked degeneration and apoptosis of the photoreceptor neurons, despite the mutant hAR retaining only reduced transactivation function (Fig. 4). Ligand-independent toxicity was detected in fly eyes expressing truncated polyQ-expanded A/B domains alone, but this was abrogated by the co-expression of unliganded LBD domains. Thus, our results suggested that hormone binding and subsequent structural alteration of hAR mutants with nuclear localization appeared to be critical for Kennedy's disease onset, and that the fly-eye model may be useful for the development of novel therapeutic approaches to Kennedy's disease.

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AC, Santa Cruz) for immunoprecipitation; a monoclonal antibody against haemagglutinin A (HA; 1867423, Roche), a polyclonal antibody against Myc (SC789, Santa Cruz), and antibodies against phosphylated (Ser 473) or total Akt (9270, New England Biolabs).

Statistical analysis

Results shown are the mean ± s.d. We analysed data by one-way analysis of variance (ANOVA). Individual statistical differences were determined by Scheffe's multiple range comparison test.

Accession numbers

The sequences of mouse and human ERas can be retrieved from DDBJ/GenBank/EMBL with accession numbers AB093573 and AB093575.

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Modulation of oestrogen receptor signalling by association with the activated dioxin receptor

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Environmental contaminants affect a wide variety of biological events in many species. Dioxins are typical environmental contaminants that exert adverse oestrogen-related effects[1]. Although their anti-oestrogenic actions[2,3] are well described, dioxins can also induce endometriosis[4–7] and oestrogen-dependent tumours[8,9], implying possible oestrogenic effects. However, the molecular mechanism underlying oestrogen-related actions of dioxins remains largely unknown. A heterodimer of the dioxin receptor (AhR) and Arnt, which are basic helix–loop–helix/PAS-family transcription factors, mediates most of the toxic effects of dioxins[10,11]. Here we show that the agonist-activated AhR/Arnt heterodimer directly associates with oestrogen receptors ER-α and ER-β. This association results in the recruitment of unliganded ER and the co-activator p300 to oestrogen-responsive gene promoters, leading to activation of transcription and oestrogenic effects. The function of liganded ER is attenuated. Oestrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in AhR$^{-/-}$ or ER-α$^{-/-}$ ovariectomized mice. Our findings suggest a novel mechanism by which ER-mediated oestrogen signalling is modulated by a co-regulatory-like function of activated AhR/Arnt, giving rise to adverse oestrogen-related actions of dioxin-type environmental contaminants.

ERs, which are members of the nuclear receptor (NR) family[12,13], and AhR/Arnt are both ligand-dependent transcription factors. Ligand-activated AhR heterodimerizes with Arnt and activates the transcription of dioxin target genes such as *CYP1A1* (refs 10,11) through xenobiotic response elements (XREs). ERs bind to oestrogen response elements (EREs) and activate transcription in an oestrogen-dependent manner. This transcriptional activation

requires the recruitment of co-activator complexes[13–18], including histone acetyltransferase (HAT) complexes containing p300 and CREB binding protein (CBP). In view of previous reports that AhR ligands exhibit oestrogen-related adverse effects, it is possible that ER-mediated oestrogen signalling might cross-talk with AhR-mediated signalling through an unknown mechanism that regulates transcription. We therefore decided to examine whether AhR/Arnt heterodimer could transcriptionally affect ER transactivation functions, thereby modulating oestrogen signalling.

To monitor the transactivation function of endogenous receptors, luciferase reporter plasmids bearing consensus binding elements—ERE for ERs, and XRE for AhR/Arnt—were transfected into MCF-7 cells, a breast cancer cell line known to express both receptors endogenously[2]. Although the synthetic AhR ligand 3-methylcholanthrene (3MC) effectively activated transcription through XRE[10], 17β-estradiol (E2) did not, as expected (Fig. 1a). However, to our surprise, 3MC alone activated ERE-mediated transcription in the absence of E2 (Fig. 1a). In the presence of E2, ERE-mediated transcription was decreased by the addition of 3MC. Western blotting showed that the amount of ligand-induced transactivation did not simply reflect variations in receptor numbers (Fig. 1a). 3MC alone decreased AhR and ER-α protein levels, in agreement with previous reports[19].

We then examined the effect of AhR/Arnt on ER-mediated transcription by using exogenous receptors in Ishikawa cells, a uterine tumour cell line. Again, 3MC potently stimulated ERE-mediated transcription in the absence of E2 when both ER (either ER-α or ER-β) and AhR/Arnt were expressed, whereas it lowered the E2-induced transactivation function of ERs (Fig. 1b) without binding directly to ERs (Supplementary Fig. 1a) or affecting expression levels of ERs (data not shown). This activation effect of 3MC requires ERE (Fig. 1b, lanes 1–4), ER-α (lanes 7 and 8), AhR (lanes 9 and 10) and Arnt (lanes 11 and 12). To verify that an AhR ligand does indeed exert oestrogenic action through direct binding to AhR, other AhR ligands were further tested. More stable ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]-pyrene and β-naphthoflavone acted as agonists, like 3MC (Fig. 1c), whereas the oestrogenic action of 3MC was blocked by either a known AhR antagonist, α-naphthoflavone or a pure oestrogen antagonist, ICI182,780 (Fig. 1d). The modulation of transcription activity by AhR/Arnt observed with ERs was not detected on other NRs including glucocorticoid receptor, progesterone receptor, vitamin D receptor (VDR), retinoic acid receptor and peroxisome proliferator activated receptor-γ (PPAR-γ) (data not shown).

Because ERs possess two transactivation functions, AF-1 and AF-2, in the amino-terminal A/B and carboxy-terminal E/F regions, respectively[16,20], we examined the functional association of AhR/Arnt with these two regions using ER deletion mutants (HE15 for AF-1 domain, and HE19 for AF-2 domain) (Supplementary Fig. 1b) in Ishikawa cells. The N-terminal A/B regions of ER-α and ER-β were required for stimulation of ERE-mediated transcription by AhR/Arnt, whereas we detected no modulation of AF-2 functions (Fig. 1e)[20]. Thus, 3MC-bound AhR/Arnt might modulate the functions of ERs through association with the N-terminal A/B regions. This possibility was supported by the observation that

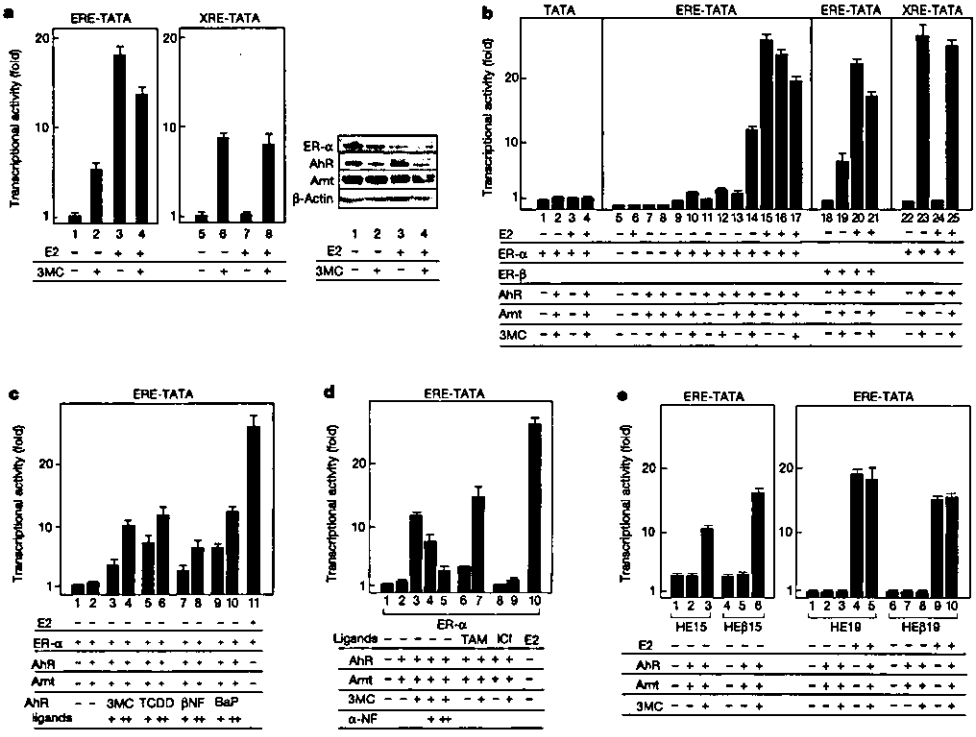

Figure 1 Activation of unliganded ER function by liganded dioxin receptor heterodimer. **a**, A dioxin receptor ligand activates transcription mediated through an ERE. MCF-7 cells were transfected with the reporter plasmids ERE-luciferase or XRE-luciferase in the presence or absence of E2 (10 nM) and 3MC (1 μM). Luciferase assays were performed with the cell extracts. All values are means ± s.d. for at least three independent experiments. **b**, Liganded AhR/Arnt induces the transactivation function of ERE-bound unliganded ER. Ishikawa cells transfected with the indicated plasmids were subjected to luciferase assays. **c**, Transactivation of unliganded ER by the other AhR agonists. **d**, Potentiation of ERE-mediated transcription by liganded AhR/Arnt is blocked by an antagonist for either ER-α or AhR. Cells treated with tamoxifen (TAM; 100 nM), ICI182,780 (ICI; 100 nM), 3MC (+, 100 nM; ++, 1 μM), TCDD (+, 10 nM; ++, 100 nM), β-naphthoflavone (β-NF; +, 100 nM; ++, 1 μM), benzo[*a*]pyrene (BaP; +, 10 nM; ++, 100 nM), α-naphthoflavone (α-NF; +, 100 nM; ++, 1 μM). **e**, Potentiation of ERE-mediated transcription by AhR/Arnt is mediated by the ERs A/B regions.

3MC-bound AhR/Arnt potentiates the transactivation function of ER-α in the presence of the ER-α AF-1 agonist/AF-2 antagonist tamoxifen (Fig. 1d)[16].

We then tested whether a 3MC-dependent physical interaction occurred between AhR/Arnt and ERs. Irrespective of E2 binding, endogenous ER-α in MCF-7 cells, and tagged ER-α overexpressed in COS-1 cells, were found to co-immunoprecipitate with 3MC-bound AhR, but not with unliganded AhR, only when Arnt was co-expressed (Fig. 2a and b). In agreement with the functional interaction between AhR/Arnt and the A/B region of ER-α (Fig. 1e), a 3MC-dependent interaction between AhR/Arnt and HE15 was observed, but not between AhR/Arnt and HE19 (ref. 12). Although ER-β, like ER-α, also associated with AhR in a 3MC-dependent fashion, no other receptors tested showed such an association (Fig. 2b).

Moreover, a direct interaction between AhR, but not Arnt, and A/B regions from both ER-α and ER-β could be mapped by an *in vitro* glutathione *S*-transferase (GST) pull-down assay (Fig. 2c). It therefore seems that, upon ligand binding and nuclear translocation[10], AhR heterodimerizes with nuclear Arnt and then associates with unliganded ER-α or ER-β, which are constitutively in the nucleus[16], through direct interaction with their A/B regions. Further analyses by GST pull-down assay mapped the small regions of the A/B region of ER-α (residues 40–120), the A/B region of ER-β (residues 33–55)[21], and the helix–loop–helix/PAS domain of AhR[22], which are indispensable for direct interaction *in vitro* (Fig. 2d and Supplementary Fig. 2a). An ER-α mutant lacking the AhR-interacting region (ER-α ΔAhR) failed to be activated by AhR/Arnt but responsiveness to E2 was still retained, supporting the idea that the interaction is required for AhR ligand-induced activation of the ER function (Fig. 2e).

To explore the molecular mechanisms of the 3MC-dependent transactivation function of AhR and ERs, we used co-immunoprecipitation to examine whether p300 was recruited to the complex, because both AhR and ERs have been independently reported to require p300/CRB as a co-activator[10,16,18,23]. p300 was recruited to ER-α in the presence but not the absence of E2 (Fig. 2f, lanes 2 and 4). However, even in the absence of E2, p300 associated with 3MC-bound AhR/Arnt and unliganded ER-α to form a complex (Fig. 2f, lane 3). Recruitment of the p160 family co-activator SRC-1 (ref. 13; Fig. 2f, lane 3), TIF2 or AIB1 (data not shown) to AhR/Arnt-associated ERs were not detected. Thus, the co-activator complex required to activate transcription by the unliganded ERs associated with liganded AhR/Arnt might be distinct from both co-activator

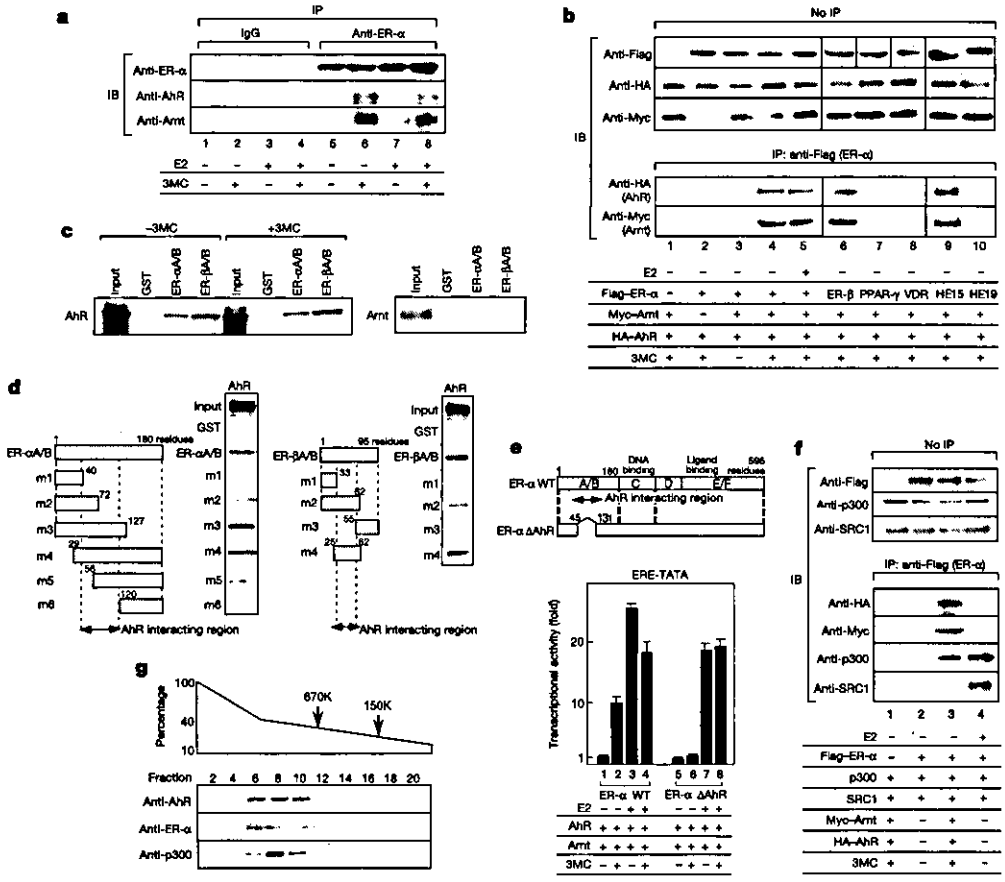

Figure 2 3MC-dependent interaction of ERs with AhR/Arnt. **a**, 3MC-dependent but E2-independent interaction of endogenous ER-α with AhR/Arnt in MCF-7 cells. Cells were subjected to immunoprecipitation (IP) with mouse anti-ER-α or normal mouse immunoglobulin as a control. The immunoprecipitates were western blotted (IB) with specific antibodies as indicated. **b**, E2-independent, 3MC-dependent interaction of exogenous ERs with AhR/Arnt in COS-1 cells. The transfected cells were subjected to immunoprecipitation and then western blotting. PPAR, peroxisomeproliferatoractivated receptor; VDR, vitamin D receptor. **c**, Direct but 3MC-independent interaction of AhR with ER-α and ER-β in an *in vitro* GST pull-down assay. **d**, Mapping the interaction domains of ER-α and ER-β with AhR. **e**, The AhR-interacting core region in the ER-α A/B domain is required for ER-α activation by AhR/Arnt. Luciferase assays with the indicated ER derivative. **f**, Recruitment of p300 co-activator to a complex containing unliganded ER-α and 3MC-bound AhR/Arnt. **g**, AhR/ER–α/p300 form a complex on glycerol gradient analysis. The Flag–AhR associated proteins in stable transformant HeLa cells were fractionated by molecular mass by a glycerol gradient assay.

complexes for the unassociated receptors. Indeed, ER-α and p300 were detected in the same fractions as Flag ($[EYKEEEK]_2$)-tagged AhR fractionated by a glycerol gradient, suggesting that they form a complex with a relative molecular mass (M_r) larger than 670,000 (670K) (Fig. 2g).

To investigate whether the observed association between AhR and ERs occurred on EREs in endogenous target gene promoters of MCF-7 cells, we performed a chromatin immunoprecipitation (ChIP) analysis with *pS2* and c-*fos* gene promoters[17]. Interestingly, 3MC induced binding of ER-α to ERE, as did E2, with AhR/Arnt recruitment. As expected, 3MC induced the recruitment of AhR/Arnt, but not ER-α, to the *CYP1A1* promoter XRE (Fig. 3a). Reflecting the recruitment of the receptors, acetylation of histone H4 was observed in the promoters (Fig. 3a), indicating the possible recruitment of a HAT co-activator complex to the receptors. The expression of these genes was accordingly induced by 3MC or E2 (Fig. 3d). Thus, the 3MC-dependent association between AhR/Arnt and ER-α seems to promote the binding of unliganded ER-α to EREs.

A ChIP assay involving sequential immunoprecipitation confirmed the 3MC-dependent association of AhR/Arnt with ER-α on

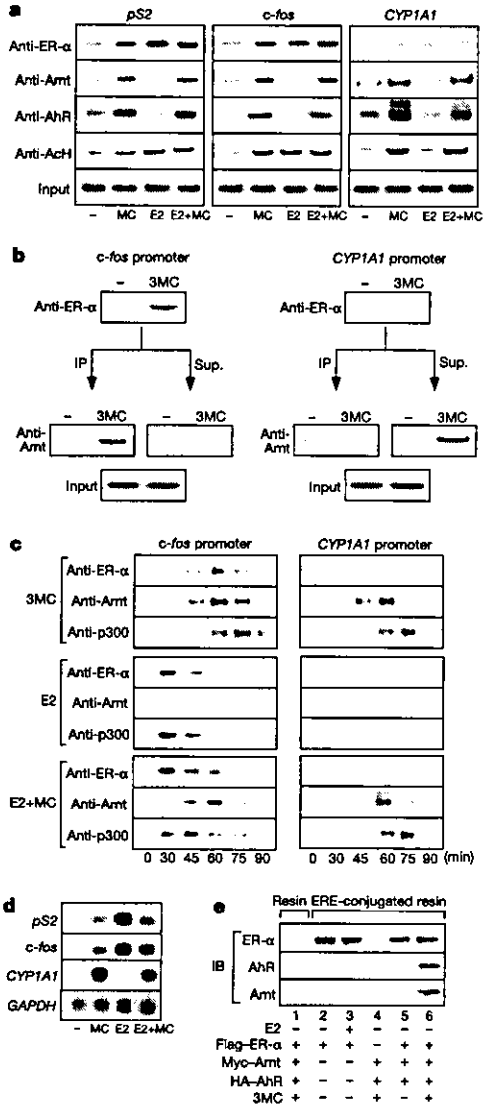

Figure 3 3MC-dependent recruitment of AhR/Arnt to ER-α bound on oestrogen-responsive gene promoters. **a**, 3MC-dependent interaction with AhR/Arnt induces ERE binding of unliganded ER-α to E2 responsive gene promoters in MCF-7 cells. For ChIP analyses, soluble chromatin prepared from MCF-7 cells treated with ligands for 45 min was immunoprecipitated with the indicated antibodies. The final DNA extracts were amplified using specific sets of primer pairs to detect the c-*fos*, *pS2* and *CYP1A1* gene promoters as indicated. **b**, 3MC-dependent association of AhR/Arnt with ER-α bound to E2-responsive gene promoters. The immunoprecipitates and their supernatants were sequentially applied for ChIP analysis as indicated. **c**, Dynamics of ER-α–Arnt–p300 assembly on ligand-responsive gene promoters. Occupancy of the c-*fos* and *CYP1A1* promoters by ER-α, Arnt and p300 at different times after ligand treatments. **d**, Induction of target genes examined by northern blot analysis. **e**, Complex formation of AhR–Arnt–ER-α on ERE through ER-α as revealed by ABCD assay.

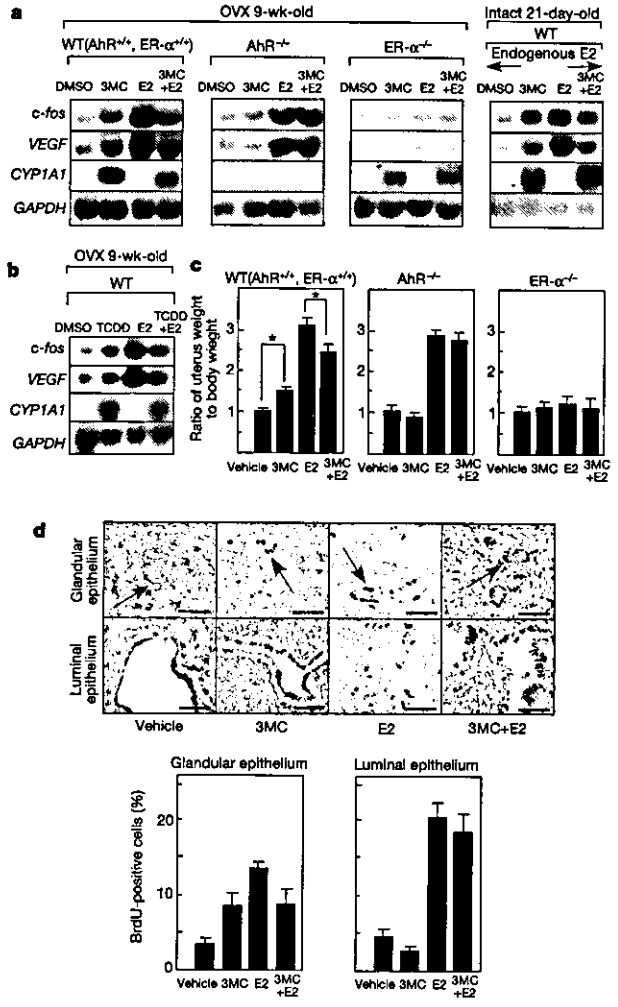

Figure 4 Oestrogenic actions of 3MC in mouse uterus are mediated by AhR and ER-α. **a**, **b**, Induction of E2-responsive genes by AhR agonists is mediated by both AhR and ER-α. Nine-week-old ovariectomized (OVX) mice and intact 21-day-old female mice of the indicated genotypes were injected with the ligands. Three hours later, total RNA was extracted from the uterus, then subjected to northern blot analysis with cDNAs for the target genes for E2 (c-*fos*, *VEGF*) and for 3MC (*CYP1A1*); *GAPDH* cDNA was used as an internal control. WT, wild type. **c**, The 3MC-induced increase in uterine wet weight (measured as the ratio of uterine wet weight in milligrams to body weight in grams) in ovariectomized mice was abolished by inactivation by either AhR or ER-α. The *t*-test shows a significant difference ($P < 0.01$) between 3MC-treated ($n = 9$) and olive-oil-treated ($n = 9$) wild-type mice. There is no significant difference ($P > 0.2$) between 3MC-treated ($n = 4$) and olive-oil-treated ($n = 4$) animals in either $AhR^{-/-}$ and $ER\text{-}\alpha^{-/-}$ mice. All values are means ± s.e.m. **d**, Induction of endometrial cell proliferation by 3MC and E2. BrdU-positive cells (brown) are indicated by arrows.

ERE (Fig. 3b). A time-course ChIP assay showed that ER-α, AhR and p300 HAT were simultaneously recruited to the *c-fos* promoter, presumably upon the binding of 3MC to AhR (Fig. 3c).

To verify the interaction of AhR/Arnt with ER-α bound to ERE in the promoters, the formation of a complex with ERE was tested by avidin–biotin-conjugated DNA(ERE) (ABCD) precipitation[24] (Fig. 3e). ER-α bound to consensus ERE (Fig. 3e, lanes 2, 3, 5 and 6), whereas AhR/Arnt alone did not (Fig. 3e, lane 4). However, in the presence of ER-α, AhR/Arnt was recruited to ERE in a 3MC-dependent manner (Fig. 3e, lanes 5 and 6). In the transient luciferase assay, the binding of ER-α to ERE and the activation function of both AhR and Arnt were required for the activation of ER-α through ERE by AhR/Arnt (Supplementary Fig. 3a, lanes 3, 7, and 8), whereas the AF-1 and AF-2 activities of ER-α and the DNA-binding capacity of the AhR/Arnt heterodimer were dispensable (Supplementary Fig. 3a, lanes 4–6).

Finally, we tested whether AhR-ligand-dependent AhR–ER interaction was responsible for the oestrogenic actions of AhR agonists in the absence of oestrogens on gene expression in intact animals. In addition to the induction of the *CYP1A1* gene, treatments with 3MC (Fig. 4a) and TCDD (Fig. 4b) for 3 hours stimulated the expression of the oestrogen-responsive genes *c-fos*[25] and *vascular endothelial growth factor* (*VEGF*)[26] in the uteri of ovariectomized wild-type mice (Fig. 4a, b). This oestrogenic action of 3MC in the uterus was also detected in intact 21-day-old female mice, whereas the AhR agonists exhibited anti-oestrogenic activities in the presence of high doses of oestrogen (Fig. 4a). There have been conflicting reports on the induction of *c-fos* by AhR ligands: one is that AhR ligands repress the E2-induced expression of *c-fos*[3]; the other is that AhR ligands themselves induce the expression of *c-fos*[27]. The 3MC-mediated activation of oestrogen target genes was completely abolished in both $AhR^{-/-}$ (ref. 28) and ER-$\alpha^{-/-}$ (ref. 29) ovariectomized mice, although each receptor knockout mouse strain retained ligand responsiveness (Fig. 4a) and the expression (Supplementary Fig. 4a) of the other intact receptor. The injection of 3MC led to increases in uterine wet weight, as did that of E2 (Fig. 4c). This action of 3MC was again abolished in both $AhR^{-/-}$ and ER-$\alpha^{-/-}$ mice (Fig. 4c).

To examine whether the increased uterine wet weight was due to the proliferation of endometrial cells, DNA synthesis in uterine epithelial cells was examined by labelling with bromodeoxyuridine (BrdU). Ovariectomized mice treated with 3MC exhibited enhanced cell proliferation in the glandular epithelium, as did E2-treated mice (Fig. 4d). Proliferation of the luminal epithelium was enhanced by E2 but not by 3MC.

The present findings indicate that the oestrogenic action of AhR agonists might be exerted through a direct interaction between AhR/Arnt and unliganded ER and by the formation of functional units bound to EREs that activate transcription, at least in uterine gene induction and cellular proliferation. The most marked manifestation of the possible oestrogenicity of dioxins could be seen as their linking to endometriosis[4–7], because oestrogen is the major factor in the stimulation of proliferation of these cells. Thus, AhR expressed in the uterine glandular epithelium[30] might respond to dioxins by associating with unliganded ERs, which then stimulates oestrogen-dependent cell proliferation. In contrast, AhR agonists exhibit anti-oestrogenic activities in the presence of high doses of E2 in animals[3] and cultured cell lines[2]. We also found that AhR/Arnt repressed E2-bound ER function, which is consistent with these previous reports. However, whereas most previous studies have not examined or mentioned the effects of AhR ligands in the absence of E2, we addressed this issue carefully in the present study. Thus, oestrogen concentrations, which vary with age, oestrous cycle, tissues and other factors, might define the oestrogenic/anti-oestrogenic actions of the AhR ligands in intact animals. Our present model, in which AhR potentiates unliganded ERs but represses liganded ER, might be an explanation of these previous findings, and it will be of interest to identify the other components of the liganded AhR–ER-α complex involved in the oestrogenic/anti-oestrogenic actions of dioxins. Our proposal is that one of the molecular mechanisms for the oestrogen-related adverse effects of dioxin-type environmental contaminants is the modulation of oestrogen receptor signalling by dioxin-dependent association with dioxin receptor. □

Methods

Plasmids

Full-length complementary DNAs of AhR and Arnt were inserted into pcDNA3 vectors (Invitrogen). Three consensus EREs[16] and XREs[22] were inserted into the promoter of luciferase pGL3-basic vector to generate ERE-TATA–luciferase and XRE-TATA–luciferase, respectively. ER-α ΔAhR was generated by the deletion of 45–131 residues from ER-α. The other mutants of ER-α and ER-β were as described previously[21].

Transfection and luciferase assay

Human endometrium cancer-derived Ishikawa cells, human breast cancer-derived MCF-7 cells, green monkey COS-1 cells and human 293T cells maintained in DMEM supplemented with 10% FBS were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS before assays. Cells at 40–50% confluence were transfected with the indicated plasmids (0.25 μg ERE-Luc, 0.1 μg XRE-Luc, 0.025 μg ER-α, AhR and Arnt were transfected) using Lipofectamine reagent (Gibco BRL) in 12-well Petri dishes. Total amounts of cDNA were adjusted by supplementing with empty vector up to 1.0 μg. Cells were treated with E2 (100 nM) and 3MC (1 μM). Luciferase activity was determined with the Luciferase Assay System (Promega)[16]. As a reference plasmid to normalize transfection efficiency, 25 ng pRL-CMV plasmid (Promega) was co-transfected in all experiments. Results are given as means ± s.d. for at least three independent experiments.

Immunoprecipitation and GST pull-down assay

Whole cell extracts[17] were used for immunoprecipitation with either anti-ER-α or anti-Flag antibody (anti-ER-α Ab-4 from Neo Markers; anti-Flag from Santa Cruz Biotechnology) after western blotting with anti-ER-α (Chemicon), anti-Arnt (Santa Cruz Biotechnology), anti-AhR (Santa Cruz Biotechnology), anti-p300 (Upstate Biotechnology), anti-SRC-1 (Santa Cruz Biotechnology), anti-Flag, anti-haemagglutinin and anti-Myc (Invitrogen) antibodies. Normal mouse immunoglobulin was used as a control. For immunoprecipitation of overexpressed proteins, cells were transfected as indicated with Flag-tagged ERs (5 μg), haemagglutinin-tagged AhR (3 μg), Myc-Arnt (5 μg), SRC-1 (0.7 μg) and p300 (0.7 μg) in the presence or absence of 3MC and E2. For the GST pull-down assay, AhR and Arnt were translated *in vitro* and incubated with either GST, GST–ER-α(A/B) or GST–ER-β(A/B) immobilized on glutathione–Sepharose beads[17].

Purification and separation of AhR-interacting complexes

HeLa nuclear extracts were loaded on an M2 anti-Flag agarose gel (Kodak). After being washed with binding buffer, the bound proteins were eluted from the agarose by incubation overnight with 2.5–5.0 ml of the Flag peptide (Kodak) in binding buffer (0.2 mg ml^{-1}). For fractionation on a glycerol gradient, eluents were layered on the top of a 13-ml linear 100–10% glycerol gradient and centrifuged for 16 h at 40,000 r.p.m. in an SW40 rotor (Beckman). Each fraction was western blotted with anti-AhR, anti-ER-α and anti-p300 antibodies. The protein standards used were β-globulin (M_r 158K) and thyroglobulin (667K)[17].

Chromatin immunoprecipitation

Soluble chromatins of MCF-7 cells prepared with the acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) were immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designated to amplify the promoter regions of the *c-fos* (5′-GAAGAGTGGAGAAGGG-3′ and 5′-GAAGCTGTGCTTACGG-3′), *pS2* (5′-AAAGAATTAGCTTAGGCC-3′ and 5′-ACCTTAATCCAGGTCC-3′) or *CYP1A1* (5′-CTTCGCCATCCATTCC-3′ and 5′-GGGACTCCTCTTCGAC-3′) genes from the extracted DNA. Optimal PCR conditions to allow semiquantitative measurement were used on 2% agarose/Tris-acetate-EDTA gels[17]. As a usual condition, cells were treated with ligands for 45 min. The inductions of the target genes were examined by northern blot analysis in MCF-7 cells treated with the ligands for 3 h.

ABCD precipitation

Avidin resin (Promega) was incubated with biotin-conjugated consensus ERE oligonucleotides, followed by incubation with cell lysates in lysis buffer (20 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100 and 1 mM dithiothreitol) for 30 min. The subsequent ERE–protein complexes trapped on the resin were then eluted and western blotted[24].

Oestrogen responses in uterus

Nine-week-old female C57BL/6 mice with the indicated genotypes were ovariectomized. After 2 weeks the mice were treated with 3MC (4 mg kg^{-1}), TCDD (40 μg kg^{-1}), and/or E2 (20 μg kg^{-1}) in olive oil for 3 h. Total RNA was extracted from the uteri by Isogen (Wako Co.) and then subjected to northern blot analysis with cDNAs for the target genes for E2 (*c-fos*, *VEGF*) and for 3MC (*CYP1A1*), with *GAPDH* cDNA (encoding glyceraldehydes-3-

phosphate dehydrogenase) as an internal control[16]. For experiments with intact mice, 21-day-old female mice were used.

For uterine weight analysis, mice were treated with ligands for 3 days, and the ratio of uterine wet weight to body weight was calculated, followed by *t*-test analysis. Results are given as means ± s.e.m.

For the BrdU labelling experiment, ovariectomized mice were treated with ligands for 3 days, then injected with BrdU (30 mg kg^{-1}). Paraffin sections from the uteri 8 h after BrdU injection were immunostained with anti-BrdU monoclonal antibody by using the BrdU Labeling and Detection Kit 1 (Roche), and the percentage of BrdU-positive epithelial cells in the sections was calculated.

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Insulin-regulated hepatic gluconeogenesis through FOXO1–PGC-1α interaction

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Hepatic gluconeogenesis is absolutely required for survival during prolonged fasting or starvation, but is inappropriately activated in diabetes mellitus. Glucocorticoids and glucagon have strong gluconeogenic actions on the liver. In contrast, insulin suppresses hepatic gluconeogenesis[1–3]. Two components known to have important physiological roles in this process are the forkhead transcription factor FOXO1 (also known as FKHR) and peroxisome proliferative activated receptor-γ co-activator 1 (PGC-1α; also known as PPARGC1), a transcriptional co-activator; whether and how these factors collaborate has not been clear. Using wild-type and mutant alleles of FOXO1, here we show that PGC-1α binds and co-activates FOXO1 in a manner inhibited by Akt-mediated phosphorylation. Furthermore, FOXO1 function is required for the robust activation of gluconeogenic gene expression in hepatic cells and in mouse liver by PGC-1α. Insulin suppresses gluconeogenesis stimulated by PGC-1α but co-expression of a mutant allele of FOXO1 insensitive to insulin completely reverses this suppression in hepatocytes or transgenic mice. We conclude that FOXO1 and PGC-1α interact in the execution of a programme of powerful, insulin-regulated gluconeogenesis.

Two transcriptional components that are targets of insulin signalling, and that can activate the process of gluconeogenesis in liver, are FOXO1 and PGC-1α. FOXO1 has been shown to bind directly to the promoters of gluconeogenic genes and activate the process of glucose production[4–6]. FOXO1 is directly phosphorylated by Akt, a key protein kinase downstream of the insulin receptor[7,8]. This phosphorylation results in exclusion of FOXO1 from the nucleus. A second transcriptional component controlled by insulin and having a role in gluconeogenesis is the co-activator PGC-1α. PGC-1α is induced in liver on fasting, and is elevated in several models of diabetes or deficiency in insulin signalling. Notably, expression of PGC-1α at physiological levels turns on the entire programme of gluconeogenesis[9,10].

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

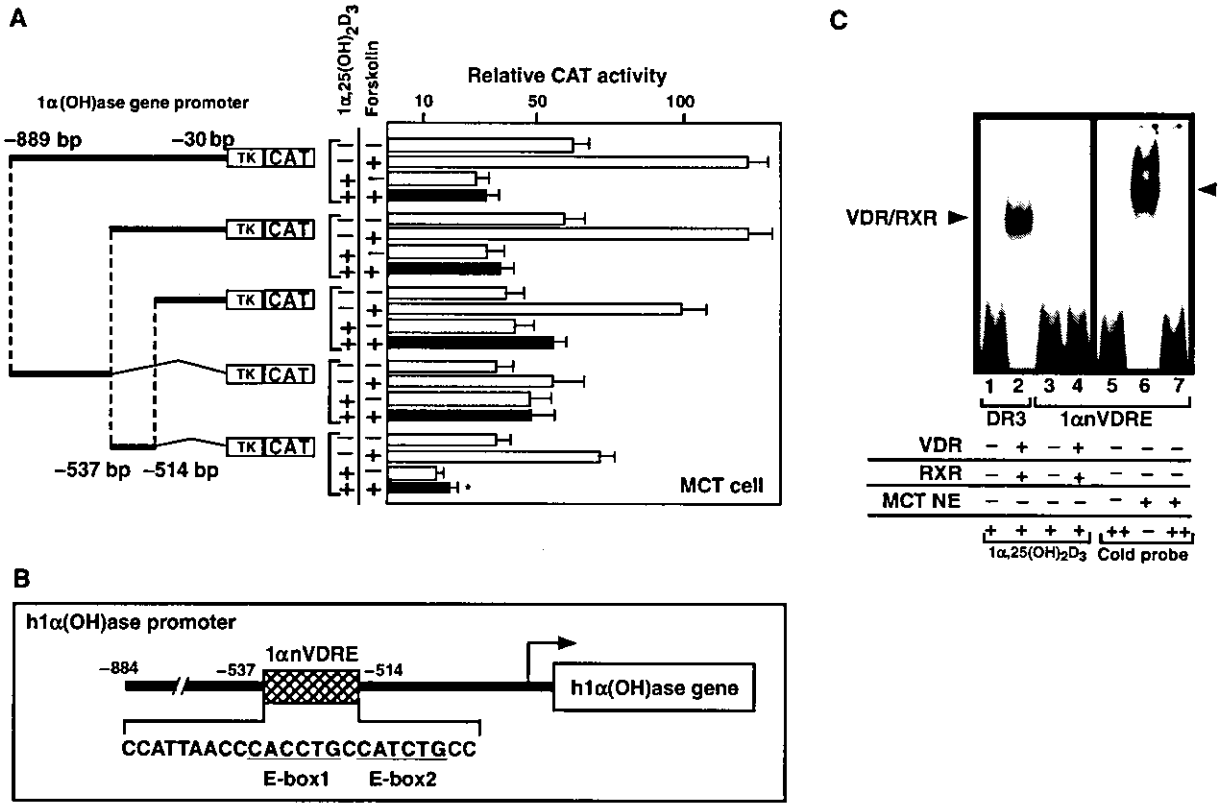

Figure 1 Identification of 1αnVDRE. (**A**) CAT assay using a series of human 1α(OH)ase gene promoter deletion mutants in MCT cells. After 3 h, forskolin (1×10^{-8} M), which activates PKA signaling, and $1\alpha,25(OH)_2D_3$ (1×10^{-8} M) were added, respectively. 1α(OH)ase gene promoter deletion constructs (−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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ (M Kim, unpublished results).

Previous reports have shown that $1\alpha,25(OH)_2D_3$-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).

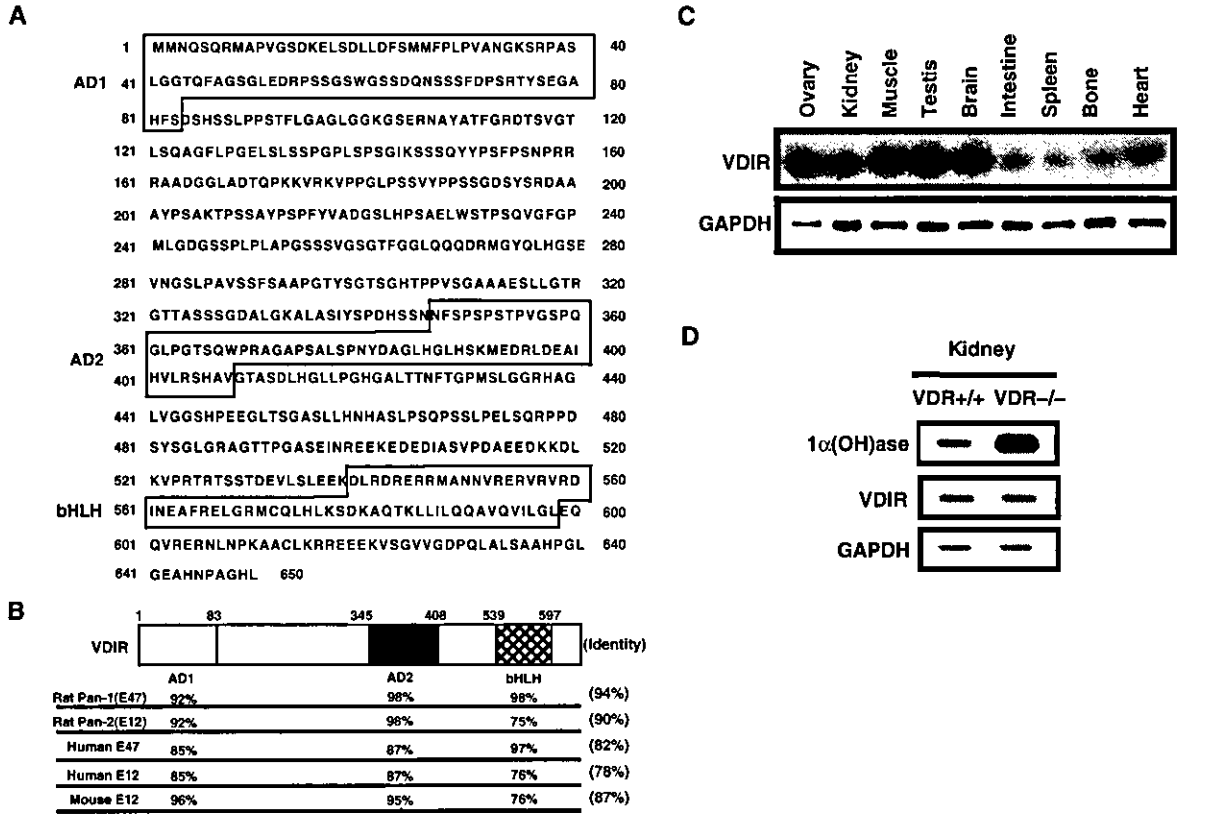

Figure 2 Cloning of the 1αnVDRE-binding factor, VDIR. (**A**) Sequence of VDIR. VDIR has two transactivation domains (AD1 and AD2), and a bHLH motif. (**B**) Functional domain sequence homology between VDIR and members of the bHLH-type activator family (rat Pan-1, E47; rat Pan-2, E12; human E47; human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

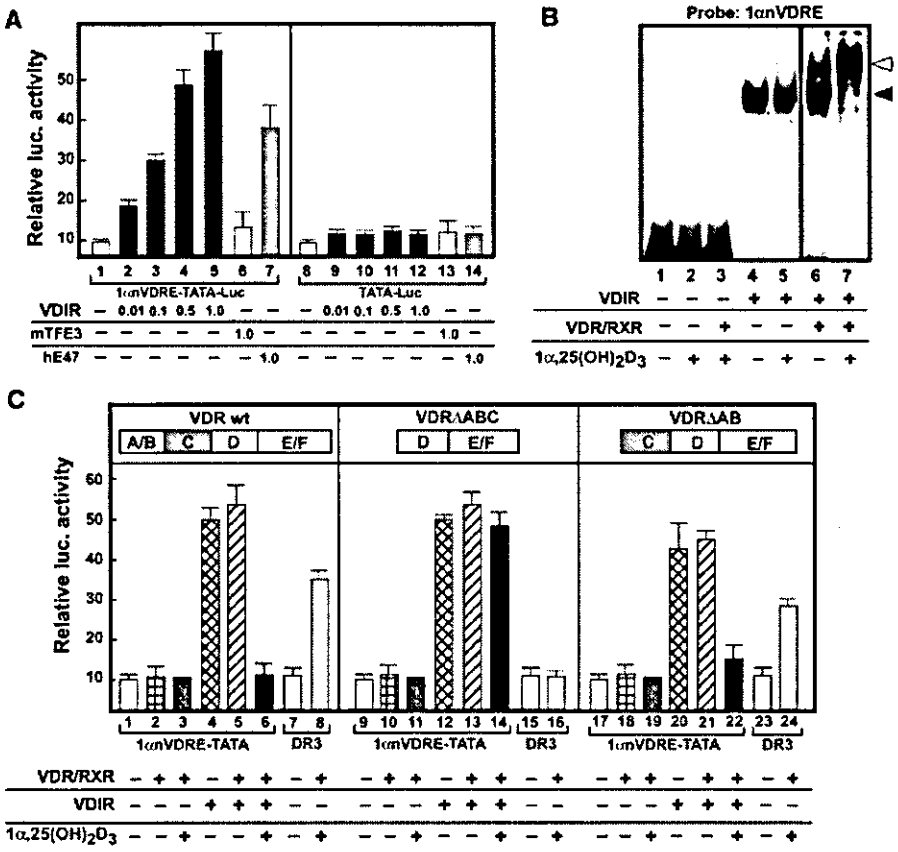

Figure 3 VDIR as an activator for 1αnVDRE. (**A**) Plasmid dose dependency of VDIR activation of nVDRE. Luciferase activity under the control of 1αnVDRE after the transfection of VDIR, mTFE3 or hE47 into MCT cells. MCT cells were cotransfected with LUC reporter plasmid (0.3 μg of nVDRE pGL3 TATA-LUC 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.

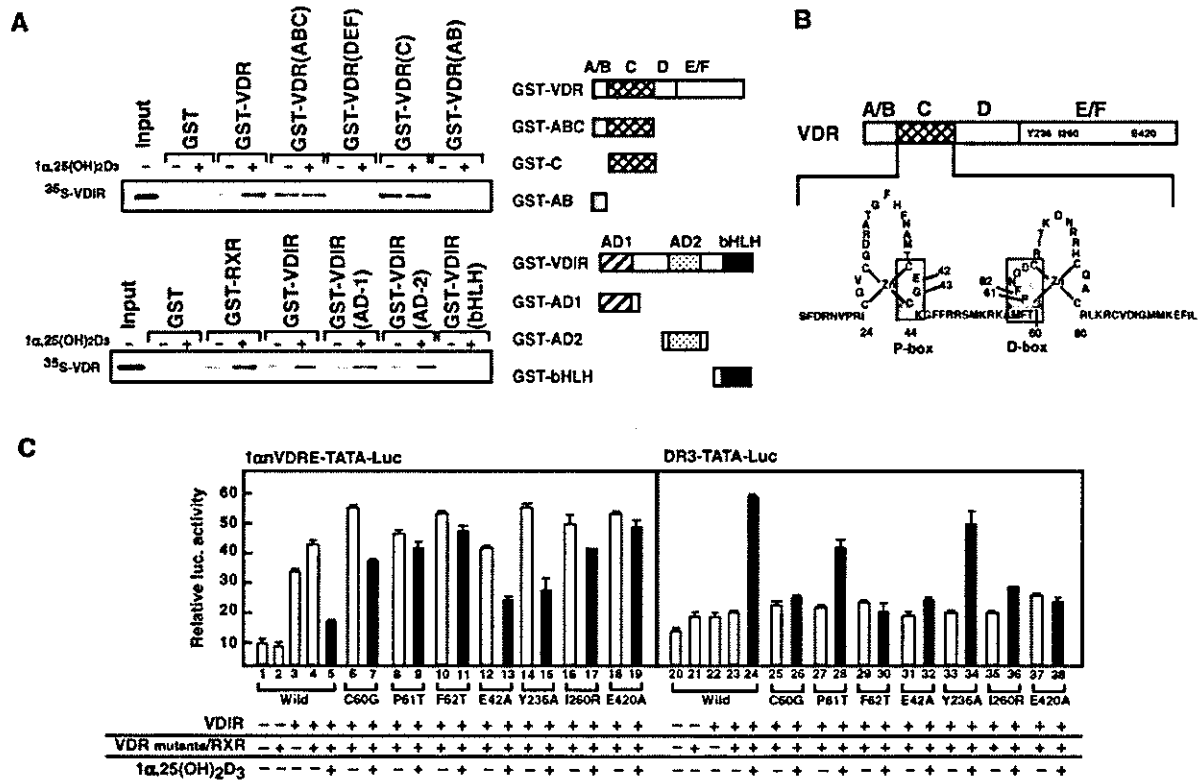

Figure 4 The DNA-binding domain (C-domain) of VDR leeds to the binding of VDIR. (**A**) GST pull-down assay using either GST alone, GST wild-type VDR or GST-fused VDRs deletion mutants together with [^{35}S]-labeled VDIR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (upper panel). GST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

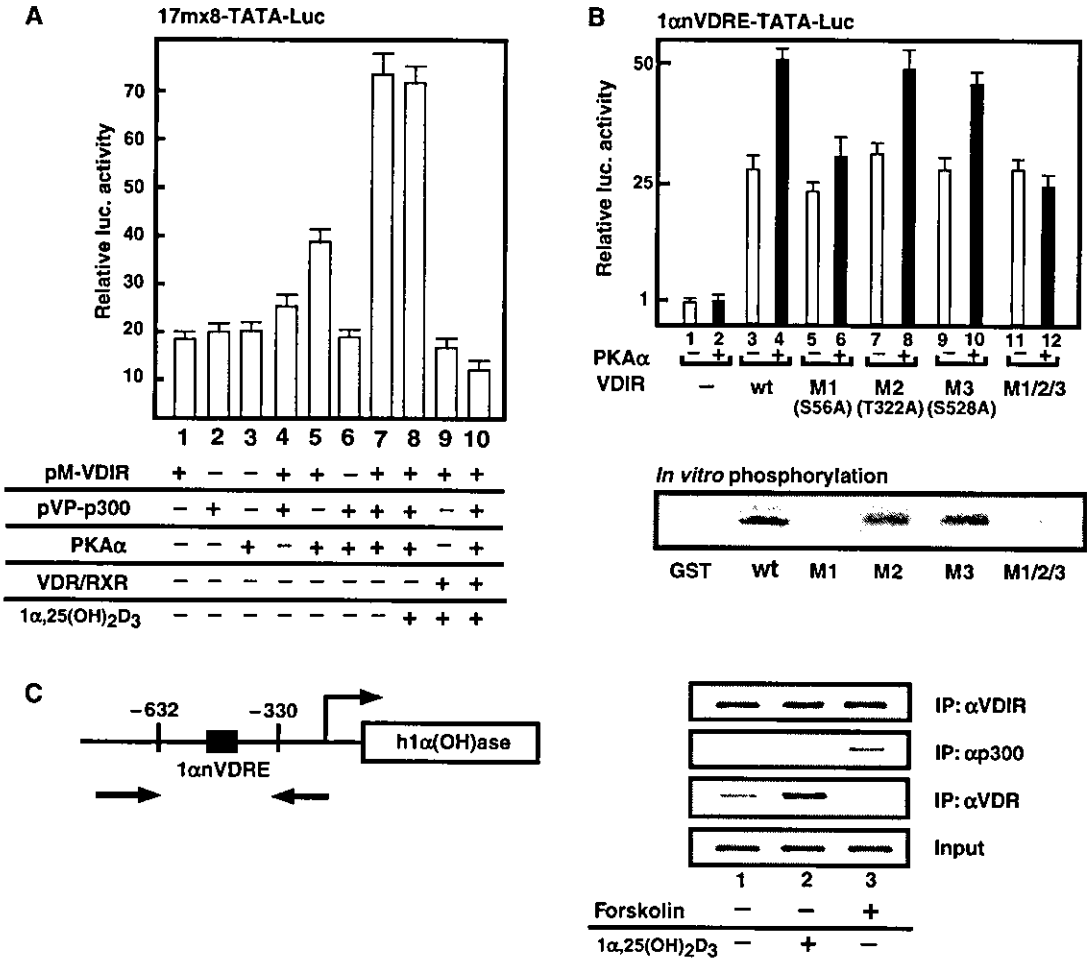

Figure 5 Phosphorylation of VDIR by PKA induced a p300 co-activator recruitment. (**A**) Association of VDIR and p300 in the mammalian two-hybrid assay. The expression plasmids of fusion proteins with GAL4-DBD (pM) and VP16-AD (pVP) were transiently transfected into MCT cells with a GAL4-DBD-regulated 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.

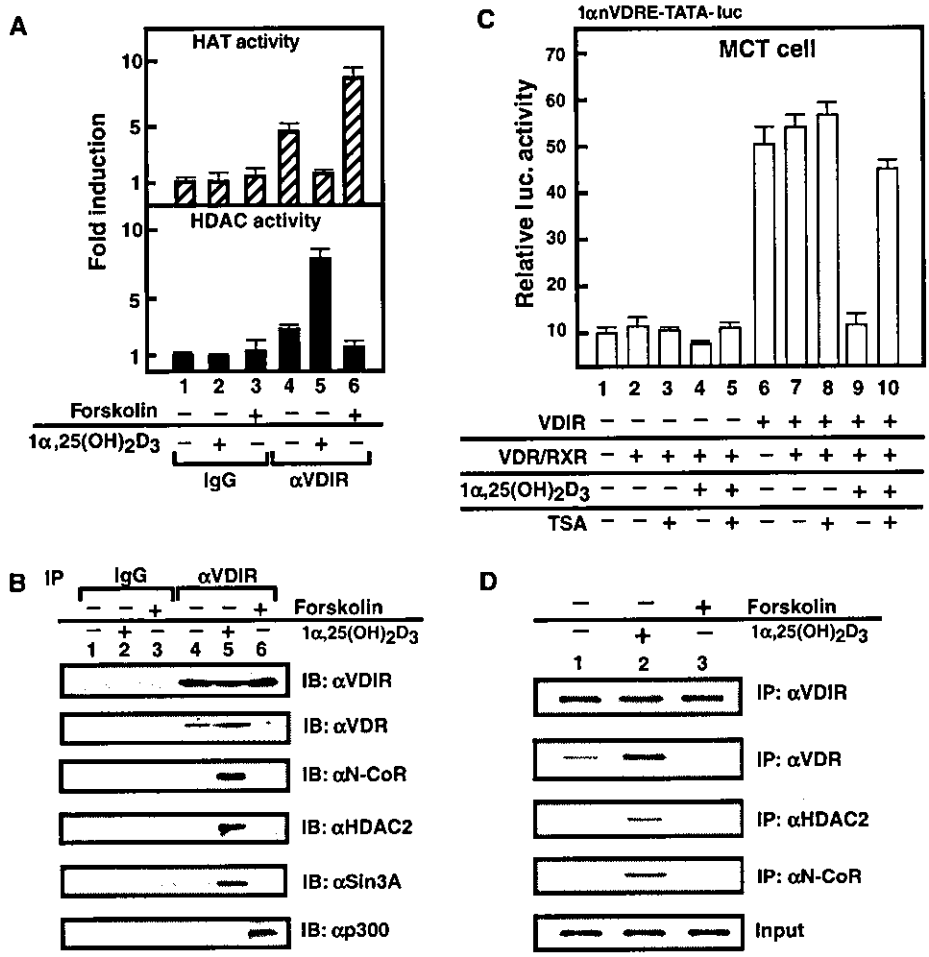

Figure 6 Co-regulator switching upon VDIR for the ligand-induced transrepression by VDR. (**A**) HAT and HDAC activities of the immunoprecipitated VDIR complexes in the MCT cells. Assays were determined in MCT cells after treatment, in the absence or presence of $1\alpha,25(OH)_2D_3$ and forskolin. Representative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

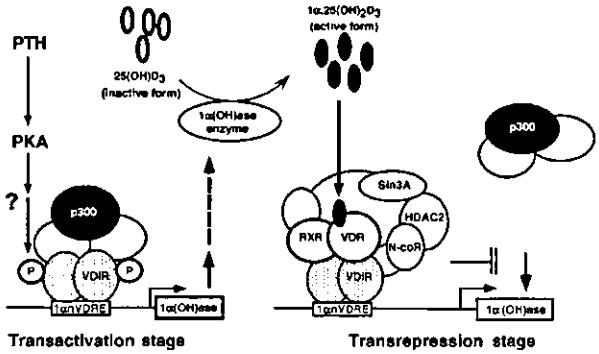

Figure 7 Schematic illustration of the proposed molecular mechanism of $1\alpha,25(OH)_2D_3$-induced transrepression in the 1α-hydroxylase gene promoter. Upon activated-PKA signaling due to PTH, the 1α-hydroxylase gene is transactivated through recruitment of a HAT co-activator complex to VDIR bound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