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Deletion of Vitamin D Receptor Gene in Mice Results in Abnormal Skeletal Muscle Development with Deregulated Expression of Myoregulatory Transcription Factors

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Although rachitic/osteomalacic myopathy caused by impaired vitamin D actions has long been described, the molecular pathogenesis remains elusive. To determine physiological roles of vitamin D actions through vitamin D receptor (VDR) in skeletal muscle development, we examined skeletal muscle in VDR gene deleted (VDR −/−) mice, an animal model of vitamin D-dependent rickets type II, for morphological changes and expression of myoregulatory transcription factors and myosin heavy chain isoforms. We found that each muscle fiber was small and variable in size in hindlimb skeletal muscle from VDR −/− mice, although overall myocyte differentiation occurred normally. These abnormalities were independent of secondary metabolic changes such as hypocalcemia and hypophosphatemia, and were accompanied by aberrantly high and persistent expression of myf5, myogenin, E2A, and early myosin heavy chain isoforms, which are normally down-regulated at earlier stages. Moreover, treatment of VDR-positive myoblastic cells with $1,25(OH)_2D_3$ *in vitro* caused down-regulation of these factors. These results suggest that VDR plays a physiological role in skeletal muscle development, participating in temporally strict down-regulation of myoregulatory transcription factors. The present study can form a molecular basis of VDR actions on muscle and should help further establish the physiological roles of VDR in muscle development as well as pharmacological effects of vitamin D on muscle functions. (*Endocrinology* 144: 5138–5144, 2003)

THE ACTIVE FORM of vitamin D, 1α,25-dihydroxyvitamin D [$1,25(OH)_2D$], is a major calcium-regulating hormone that is indispensable for maintenance of calcium and bone homeostasis and acts through binding to the vitamin D receptor (VDR) that belongs to the nuclear receptor superfamily. Various disorders with impaired vitamin D actions, including vitamin D deficiency, genetic defects in the vitamin D-activating enzyme, 25-hydroxyvitamin D 1α-hydroxylase, or in the vitamin D receptor (VDR) lead to rickets or osteomalacia characterized by hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and bone abnormalities due to mineralizing defects (1–3). Moreover, VDR is known to be expressed in a wide spectrum of tissues unrelated to calcium and bone metabolism, and accordingly, vitamin D has been shown to modulate fundamental cellular processes such as proliferation, differentiation, and survival of various cell lineages *in vitro* (4, 5). However, physiological relevance of such vitamin D effects *in vivo* has not yet been established, nor has the role of VDR.

Clinical evidence suggests that vitamin D may play a role in muscle metabolism and function. Progressive weakness and wasting of skeletal muscle have been demonstrated in patients with rickets or osteomalacia (6, 7). In addition, it has been shown that VDR is expressed at particular stages of differentiation from myoblasts to myotubes (8–10), implying that skeletal muscle may potentially be a physiological target of $1,25(OH)_2D$. However, the mechanism of rachitic/osteomalacic myopathy is not fully understood, and it is currently unclear whether muscle abnormalities in those patients are a direct consequence of impaired vitamin D actions in muscle or a result of secondary systemic changes such as hypocalcemia, hypophosphatemia, and elevated PTH levels in the circulation.

To address these issues *in vivo*, we examined morphological abnormalities of skeletal muscle in VDR gene-null mutant (VDR −/−) mice that recapitulated a human disease of vitamin D resistance, vitamin D-dependent rickets type II (11). At the same time, we investigated expression of myogenic regulatory factors such as Myf5, myogenin, MyoD, MRF4, and E2A (12, 13) that play critical roles in myoblast differentiation and skeletal muscle development. We also examined myosin heavy chain (MHC) isoforms including embryonic, neonatal, and adult fast types (14) as differentiation markers that are expressed in a stage-specific manner during muscle development. In addition, we examined $1,25(OH)_2D$ effects on expression of these genes by a mouse myoblast cell line, C2C12, to analyze direct vitamin D actions on muscle cells *in vitro*. We hereby present evidence that VDR plays a pivotal role in the maintenance of homeostasis in fully differentiated skeletal muscle cells, supporting our hypothesis that muscle is a direct physiological target of VDR-dependent vitamin D actions.

Abbreviations: $1,25(OH)_2D$, 1α,25-Dihydroxyvitamin D; MHC, myosin heavy chain; SSC, sodium chloride-sodium citrate; VDR, vitamin D receptor.

Materials and Methods

Animals and cell cultures

Generation of VDR gene deleted mice has been described (11). C2C12, a mouse myoblast cell line, was purchased from Riken cell bank (Tsukuba, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies, Rockville, MD). For experiments, 80% confluent C2C12 cells were treated with vehicles alone or 10 nM $1,25(OH)_2D_3$ for 48–96 h and harvested for further analysis.

Chemicals and antibodies

All the reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Antibodies against Myf5, MyoD, MRF4, E2A, Id1, and Id2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), those against myogenin and MHC of embryonic type from American Research Products, Inc. (Belmont, MA), and those against MHC of neonatal and adult fast type from Medac Diagnostika (Hamburg, Germany).

Histological analysis

Samples were isolated from hindlimb skeletal muscle of 3- and 8-wk-old VDR knockout (−/−) mice and wild-type control littermates, rapidly frozen in liquid nitrogen-cooled isopentane (2-methylbutane), sectioned, and stored in liquid nitrogen. Muscle tissue sections were subjected to hematoxylin/eosin staining as follows. Serial sections of frozen muscle with 6-μm thickness were first incubated with Mayer's hematoxylin solution for 5 min, washed in distilled deionized water, and then incubated with 0.5% eosin solution for 3 min. Sections were washed three times in distilled deionized water and once in ethanol, dehydrated, and mounted. Diameters of muscle fibers were measured in photomicrographs of hematoxylin/eosin-stained muscle tissue sections.

Immunostaining

Serial sections of frozen muscle with 6-μm thickness were fixed for 20 min with PBS containing 4% paraformaldehyde and first incubated with a primary antibody at room temperature for 1 h. Sections were washed three times in PBS, incubated with biotin-conjugated secondary antibodies for 1 h, and then with ABC solution (Vector Laboratories, Burlingame, CA) diluted 100-fold in PBS. For detection, the samples were incubated for 5–20 min with 0.5 mg/ml diaminobenzidine solution or p-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Life Technologies, Rockville, MD), washed twice in distilled deionized water, mounted, and observed under a microscope.

RT-PCR analysis

Total RNA from skeletal muscle tissues and C2C12 cells were isolated by using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) or TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram total RNA was reverse-transcribed by incubating in a 20-μl reaction containing random primers (Promega; 0.5 mg/ml, 2 μl), reverse transcriptase buffer (Promega; 2 μl), deoxynucleoside triphosphates (2.5 mM each), 20 U RNase inhibitor (Promega), and 20 U reverse transcriptase (Promega) for 10 min at room temperature, 60 min at 42 C, and 5 min at 95 C. PCR was performed using various sets of primers shown in Table 1. One microliter of 20 μl reverse transcription reactions was denatured for 2 min at 95 C, followed by 28–35 cycles (except for 23 cycles with glyceraldehyde-3-phosphate dehydrogenase) of amplification: 2 min at 95 C, 30 sec at 57–61 C, and 30 sec at 72 C. PCR products were electrophoretically separated on 2% agarose gels and visualized with ethidium bromide staining.

Northern blot analysis

Total RNA (20 μg) was separated on a 1% agarose gel containing 6% formaldehyde and transferred to HYBOND+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK) by capillary action in 20× sodium chloride-sodium citrate (SSC) buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0). RNA was cross-linked to the membrane using the UV Cross-Linker (model CX-2000, UVP, Upland, CA). An equal amount of RNA loading and transfer was confirmed by ethidium bromide staining and UV visualization of ribosomal RNAs (data not shown). Hybridization was performed with a nonisotopic digoxigenin labeling system using DIG PCR Probe Synthesis Kit and DIG Easy Hyb (Roche Diagnostics, Indianapolis, IN). Briefly, the membranes were first prehybridized in the DIG Easy Hyb buffer for 30 min at 42 C, then hybridized in DIG Easy Hyb buffer with an appropriate probe generated by DIG PCR Probe Synthesis Kit for 16 h at 42 C, washed twice in 2× SSC/0.1% sodium dodecyl sulfate at room temperature for 5 min and twice in 0.2% SSC/0.1% sodium dodecyl sulfate at 50 C for 15 min, and exposed to films. PCR primers used to generate cDNA probes for Northern blot analysis were as follows: 5′-gcatgcaaggtgtgtaagaggaag-3′ and 5′-ggctgttttctggacatcaggaca-3′ for myogenin (593 base); 5′-aagagaggtatcctgaccctgaag-3′ and 5′-cttgatcttcatggtgctaggagc-3′ for β-actin (801 base); and 5′-aaccaagctttcgagacgctcaag-3′ and 5′-aaaagaacaggcagaggagaaccc-3′ for Myf5 (664 base). The expected size of the obtained cDNA probes is shown in *parentheses*. In some experiments, poly A^+ RNA was obtained with PolyATract mRNA Isolation systems (Promega) following the manufacturer's instruction and analyzed by Northern blot analysis.

TABLE 1. List of PCR primers used in this study

Gene	Primer	Accession no.
Myf5	F: 5′-tgtatcccctcaccagaggat-3′	58-78; XM192677
	R: 5′-ggctgtaatagttctccacctgtt-3′	442-419; XM192677
Myogenin	F: 5′-gagcgcgatctccgctacagagg-3′	470-492; NM031189
	R: 5′-ctggcttgtggcagcccagg-3′	849-830; NM031189
E2A (E12)	F: 5′-agacgaggacgaggacgaccttc-3′	130-152; D29919
E2A (E47)	F: 5′-ccagcagtacagatgaggtgctg-3′	1660-1682; AF352579
E2A (common)	R: 5′-acgccagacaccttctcctcctc-3′	426-404; D29919/ 1954-1932; AF352579
Id	F: 5′-gcctgttctcaggatcatgaaggt-3′	71-94; XM203819
	R: 5′-tgcaggtccctgatgtagtcgatt-3′	382-359; XM203819
MyoD	F: 5′-ctcctttgagacagcagacgactt-3′	254-277; M84918
	R: 5′-aaatcgcattggggtttgagcctg-3′	1134-1111; M84918
MRF4	F: 5′-gagggtgcggatttcctgcgcacc-3′	571-548; NM008657
	R: 5′-aagggctgaggcatccacgtttgc-3′	664-641; NM008657
MHCneonatal	F: 5′-acgcaatgctgaggctgttaaagg-3′	5729-5753; XM204651
	R: 5′-agtaaacccagagaggcaagtgac-3′	370-346; M12289
VDR	F: 5′-cctcactggacatgatggaaccg-3′	668-690; NM009504
	R: 5′-gatgtaggtctgcagcgtgttgg-3′	1194-1172; NM009504
G3PDH	F: 5′-tgaaggtcggtgtgaacggatttggc-3′	51-76; NM008084
	R: 5′-catgtaggccatgaggtccaccac-3′	1033-1010; NM008084

F, Forward; R, reverse.

Results

Abnormal skeletal muscle development in VDR −/− mice

To test a hypothesis that VDR has a physiological role in skeletal muscle development, we first examined skeletal muscle tissues from VDR −/− mice for morphological abnormalities. As previously described (11), the VDR −/− mice grow normally until weaning and thereafter develop various metabolic abnormalities including hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and bone deformity as typical features of rickets. At the age of 3 wk, there were no significant differences between VDR−/− and VDR+/+ mice in body weight or serum concentrations of calcium, phosphate, alkaline phosphatase, 25(OH)D, 24,25(OH)D or $1,25(OH)_2D$. To exclude any deleterious effects of such secondary systemic metabolic changes on muscle, we analyzed 3-wk-old mice just before weaning that showed no apparent biochemical or morphological abnormalities. As shown in Fig. 1, each muscle fiber obtained from quadriceps femoris muscle of VDR −/− mice (Fig. 1A) appeared smaller than that of wild-type (VDR+/+) mice (Fig. 1B) at 3 wk. Quantitative analysis showed that skeletal muscle cell diameters in VDR −/− mice at 3 wk were significantly decreased by approximately 20% on the average and appeared to be more widely distributed compared with those in wild-type mice (Fig. 2). The morphological changes were more prominent in 8-wk-old VDR −/− mice (Fig. 1C) compared with VDR+/+ controls of the same age (Fig. 1D), suggesting either a progressive nature of the abnormalities caused by the absence of VDR or additive effects of systemic metabolic changes already present at this age. Neither degenerative nor necrotic changes were observed in VDR −/− skeletal muscle. Similar results were obtained with biceps femoris, medial gastrocnemius, anterior tibial, and soleus muscles, indicating that the muscle abnormalities in VDR −/− mice occurred diffusely without any preference to type I or type II fibers. These results demonstrate that VDR is involved in physiological regulation of skeletal muscle development. Our observations further suggested that although overall differentiation steps into myocytes occurred normally, the absence of VDR caused abnormalities probably in late stages of myocyte maturation and/or in metabolism of mature myocytes.

Deregulated expression of myogenic regulatory factors in VDR −/− mice

To obtain insight into the mechanism of muscle abnormalities observed in VDR −/− mice, we examined expression of myogenic differentiation factors including MyoD family of transcription factors with muscle contractile proteins, *i.e.* embryonic, neonatal, and adult fast (type II) isoforms of MHC. Immunohistochemical analysis of quadriceps femoris muscle from 3-wk-old mice revealed persistently increased expression of myf5 (Fig. 3A), E2A (Fig. 3B), and myogenin (Fig. 3C), all of which were minimally expressed in muscle from VDR +/+ mice at this age (Fig. 3, F–H). No apparent differences were observed in expression of MyoD (Fig. 3, D and I) and MRF4 (Fig. 3, E and J). Consistent with the deregulated expression of myogenic transcription factors that control muscle phenotype, we also observed aberrantly increased expression of embryonic (Fig. 4A) and neonatal type MHC (Fig. 4B) in the cytoplasm of small muscle fibers of quadriceps femoris muscle from 3-wk-old VDR −/− mice, whereas type II (adult fast) MHC expression in VDR −/− muscle was the same as VDR +/+ controls (Fig. 4, C and F). At the age of 8 wk, although the embryonic MHC had disappeared, persistent expression of neonatal MHC was still detectable in VDR −/− mice (data not shown). There were no differences in expression levels of Id1 and Id2, known targets of vitamin D (15) in either 3- or 8-wk-old mice (data not shown). All the above findings were confirmed at the mRNA level as shown in Fig. 5: expression of myf5, myogenin, E12 and E47, both of which are produced from the same E2A gene, and neonatal MHC mRNA was higher in VDR −/− mice than that of VDR +/+ mice at 3 and 8 wk. VDR mRNA was only detectable in 3-wk-old wild-type muscle under our experimental conditions. These results are consistent with the notion that the absence of VDR disturbs the coordinate pattern of expression of myogenic transcription factors during myocyte development, causing altered levels of differentiation-associated, lineage-specific gene expression and thereby morphological abnormalities. The possibility that these alterations were secondary effects of systemic changes appeared unlikely, because abnormalities in both morphology and MyoD family expression were observed already in 3-wk-old VDR −/− mice and also in mice

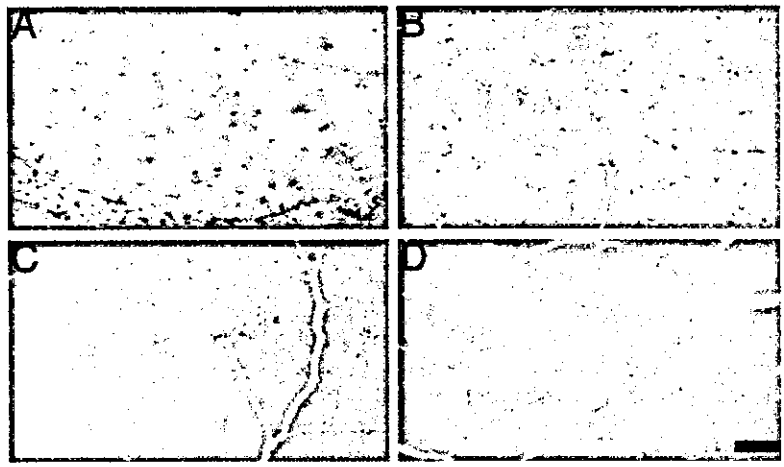

FIG. 1. Morphological abnormalities of skeletal muscle tissue from VDR −/− mice. Three- or 8-wk-old VDR −/− and +/+ (wild-type littermate) mice were euthanized, and quadriceps femoris muscle tissues were obtained. Fresh-frozen sections were stained with hematoxylin/eosin as described in *Materials and Methods* and observed under microscope. *Scale bar*, 20 μm. A, Three-week-old VDR −/− mice; B, 3-wk-old VDR +/+ mice; C, 8-wk-old VDR −/− mice; D, 8-wk-old VDR +/+ mice. Similar changes in muscle fiber size were also observed in biceps femoris, medial gastrocnemius, anterior tibial, and soleus muscles (data not shown).

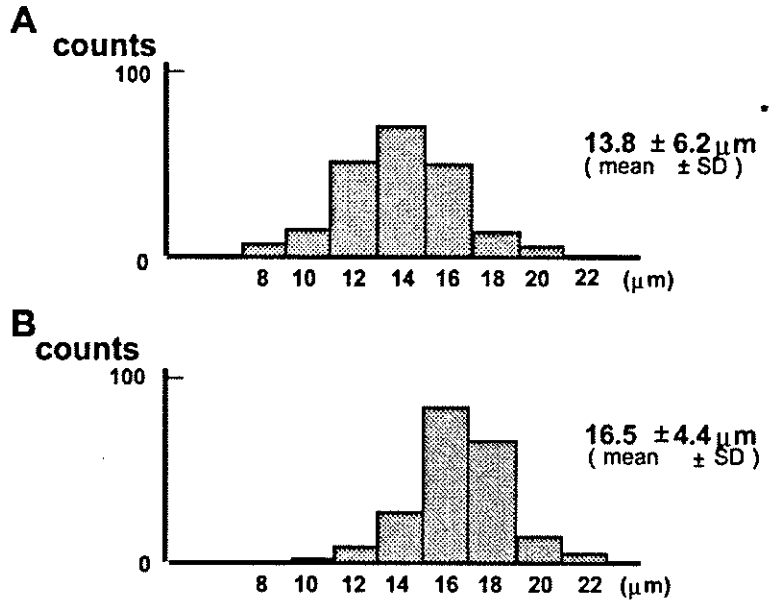

FIG. 2. VDR −/− muscle fibers are small and variable in size. Diameter of muscle fibers in 3-wk-old VDR −/− (A) and VDR +/+ (B) mice was measured in microphotograph of the hematoxylin/eosin-stained tissue sections. Two hundred cells were randomly counted, and data were expressed in histogram and as mean size ± SD. *, Significantly different from VDR +/+ wild-type littermates.

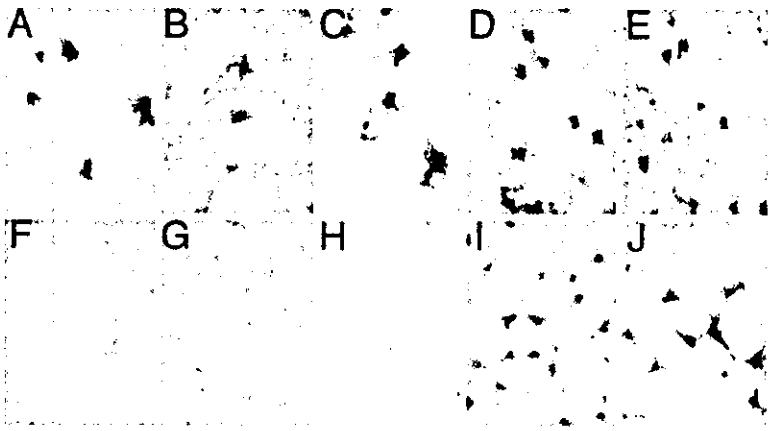

FIG. 3. Immunohistochemical analysis of MyoD family transcription factors in skeletal muscle tissues of 3-wk-old mice. Quadriceps femoris muscle tissue sections of 3-wk-old VDR −/− (A–E) and VDR +/+ (F–J) mice were analyzed by immunohistochemistry for expression of Myf5 (A and F), E2A (B and G), myogenin (C and H), MyoD (D and I), and MRF4 (E and J) as described in *Materials and Methods*. Expression of myf5, E2A, and myogenin was only detectable in VDR −/− muscles, which was mostly localized in nuclear and perinuclear regions. Virtually the same results were obtained in biceps femoris, medial gastrocnemius, anterior tibial, and soleus muscles (data not shown).

fed with high-calcium diet to rescue the phenotype of rickets that showed normal circulating levels of calcium, phosphate, and PTH (Ref. 11 and data not shown).

1,25(OH)$_2$D$_3$ down-regulates myf5 and myogenin expression by myoblasts in vitro

Our results suggest that temporally strict down-regulation of myogenic differentiation factors requires the presence of VDR. Therefore, we finally examined whether or not 1,25(OH)$_2$D$_3$ was able to directly down-regulate MyoD family gene expression in myocyte-lineage cells *in vitro*. C2C12 myoblasts were grown to 80% confluence and then treated with 10 nM 1,25(OH)$_2$D$_3$ or a vehicle alone in the presence of 10% charcoal-treated fetal bovine serum for 48–96 h. During this phase of initial differentiation, less than 5% of myotubes appeared (data not shown). There were no apparent differences in the overall differentiational process between vehicle-treated and 1,25(OH)$_2$D$_3$-treated cells. As shown in Fig. 6A, VDR mRNA was found to be expressed at constant levels throughout the experimental period. In control cells, myf5 was already expressed in growing cells, and the level of expression stayed the same. In contrast, expression of myogenin and neonatal type MHC showed a gradual increase in a time-dependent manner. Treatment with 10 nM 1,25(OH)$_2$D$_3$ for 48 h caused a decrease in the steady-state levels of myf5, myogenin, and neonatal MHC, and the 1,25(OH)$_2$D$_3$ effects lasted up to 96 h (Fig. 6A). The effects on myogenin and myf5 expression were also confirmed quantitatively by Northern blot analysis (Fig. 6, B and C). Thus, these *in vitro* results using myoblast cultures have in large part recapitulated our *in vivo* findings and are in agreement with our hypothesis that 1,25(OH)$_2$D participates in physiological regulation of muscle development, particularly playing a role in temporally strict down-regulation of some myogenic differentiation factors through VDR.

Discussion

Defects in VDR-dependent vitamin D actions cause rickets or osteomalacia. Although rachitic or osteomalacic myopathy has long been described, the molecular pathogenesis remains elusive. One of the central questions to be answered is whether the myopathy is caused by impairment of direct VDR-dependent actions of active vitamin D on muscle or by secondary metabolic changes including hypocalcemia, hy-

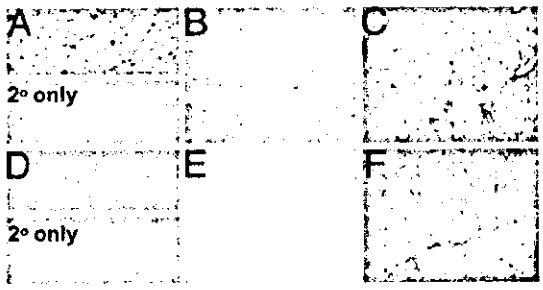

FIG. 4. Immunohistochemical analysis of MHC isoforms in skeletal muscle tissues of 3-wk-old mice. Quadriceps femoris muscle tissue sections of 3-wk-old VDR −/− (A–C) and VDR +/+ (D–F) mice were analyzed by immunohistochemistry for expression of MHC of embryonic type (A and D), neonatal type (B and E), and adult fast type (type II) (C and F), as described in *Materials and Methods*. In the lower half of panels A and D, nonspecific staining of the intercellular space without specific primary antibodies (2° only) is shown. Also note that in panels C and F, some type I fibers scattered in the field are devoid of staining in contrast to the diffuse cytoplasmic staining of surrounding type II fibers. *Scale bar*, 40 μm.

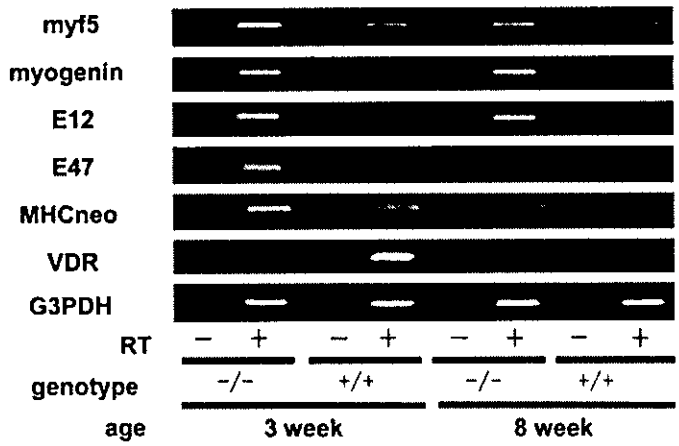

FIG. 5. Expression of MyoD family and MHC mRNA in skeletal muscle from VDR −/− and +/+ mice. Expression of myf5, myogenin, E2A, neonatal type MHC, and VDR mRNA in hindlimb skeletal muscle was analyzed by RT-PCR in 3- and 8-wk-old VDR −/− and +/+ mice as described in *Materials and Methods*. For each PCR, a negative control without reverse transcriptase is also shown.

pophosphatemia, and hyperparathyroidism. Some clinical studies have indicated that the extent of hypocalcemia and/or hypophosphatemia does not correlate well with the severity of myopathy and that correction of hypocalcemia does not lead to a cure of the muscle symptoms (16), which supports an involvement of direct VDR actions. However, others have demonstrated that PTH excess leads to similar muscle atrophy and weakness causing increased intracellular calcium (17) and impaired production of contractile proteins (18). Efforts to obtain conclusive results have been hampered by an inability to test direct effects of VDR in human muscles and absence of appropriate animal models of rickets for this purpose.

We have recently generated VDR gene deleted mice as an animal model of type II vitamin D-dependent rickets. VDR −/− mice almost completely recapitulated the human disease and showed most of the characteristic abnormalities including hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, increased serum levels of 1,25D and alkaline phosphatase, decreased 24,25-dihydroxyvitamin D, and osteopathy (11). A unique feature in these model mice is that they grow normally and show no bone or metabolic abnormalities until they are weaned, presumably due to high calcium content or other critical nutrients in the breast milk. In the present study, we took advantage of this feature and were able to demonstrate that the absence of VDR causes muscle abnormality independently of secondary effects of systemic metabolic changes. Three lines of evidence from the present study support physiological roles of direct VDR actions on skeletal muscle: firstly, VDR −/− mice developed apparent morphological abnormalities in skeletal muscle and a deregulated pattern of muscle gene expression before weaning; secondly, the same changes were still observed in older rescued VDR −/− mice fed with high calcium diet; and thirdly, direct negative regulatory effects of $1,25(OH)_2D$ on muscle gene expression were at least in part reproduced in cultured myoblasts *in vitro*. Thus, our results suggest that the skeletal muscle is a direct physiological target of VDR actions and that the absence of VDR *in situ* caused muscle abnormalities in VDR −/− mice, although secondary changes such as hypocalcemia, hypophosphatemia, and hyperparathyroidism may contribute in an additive and/or modulatory manner.

As a clue to the mechanism whereby the absence of VDR caused skeletal muscle abnormalities, we found prolonged up-regulation of a certain subset of myogenic regulatory factors: myf5, myogenin, and E2A. The transcription factors of the MyoD family play pivotal roles in muscle cell differentiation. During muscle differentiation, the four members of the family thus far identified, myf5, MyoD, myogenin, and MRF4, show a temporal and sequential pattern of expression that is subject to complex mutual regulation and exhibit distinct but overlapping functions that are not yet completely understood (12, 19). Expression of muscle-specific genes including MHC subtypes is under the control of the MyoD family members (20–23). Although we currently have no evidence for a direct link between deregulated expression of myogenic transcription factors and the muscle phenotype observed in VDR −/− mice, it is plausible to assume that aberrant up-regulation of myf5, myogenin, and E2A leads to abnormal expression of MHC and muscle atrophy, because it appears that a strictly regulated, coordinate pattern of expression of the MyoD family defines the program of myocyte differentiation and maturation. Such an assumption is further supported by a previous report that transgenic myogenin overexpression in differentiated postmitotic muscle fibers in mice resulted in grossly normal muscle development but higher rates of neonatal mortality, probably due to mildly impaired muscle function (24).

The molecular mechanism by which myogenic transcription factors including myf5, myogenin, and E2A are aberrantly and persistently up-regulated is currently unknown. However, it is of note that, in the course of muscle differentiation, these genes are normally down-regulated during the stages in which VDR is expressed (25). We therefore assume that VDR is involved in transcriptional down-regulation of these genes during the process of physiological muscle differentiation. Our *in vitro* observations that

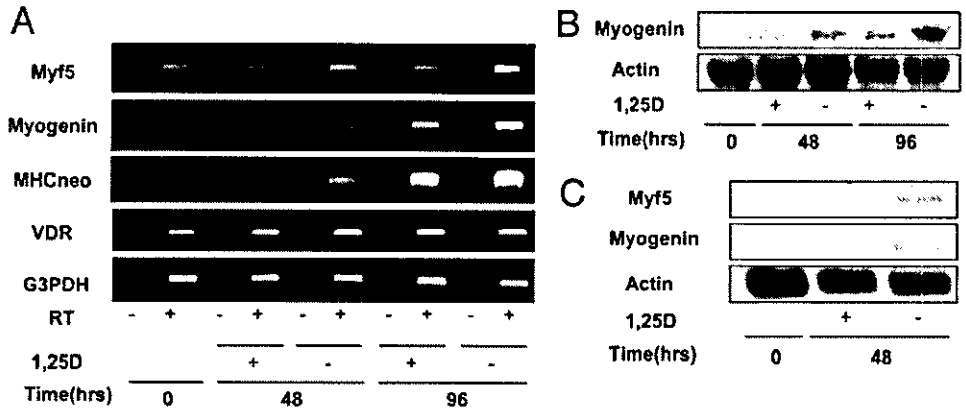

FIG. 6. Down-regulation of myf5, myogenin, and MHC neonatal type mRNA by 1,25D3 in C2C12 myoblastic cell line. A, 80% confluent C2C12 cells were treated with a vehicle alone or 10 nM 1,25D3 for indicated times and analyzed for mRNA expression of myf5, myogenin, MHC neonatal type, VDR and glyceraldehyde-3-phosphate dehydrogenase by RT-PCR as described in *Materials and Methods*. For each PCR, a negative control without reverse transcriptase is also shown. B, The same RNA samples as panel A were analyzed for myogenin and actin mRNA expression by Northern blot analysis as described under *Materials and Methods* to show quantitative difference. C, Poly A$^+$ RNA was purified as described under *Materials and Methods* and analyzed for expression of myf5, myogenin, and actin mRNA by Northern blot analysis.

1,25$(OH)_2$D was able to down-regulate myf5, myogenin, and neonatal MHC mRNA expression in C2C12 myoblasts further support this idea. However, we have not been able to identify known negative vitamin D response elements (26–28) in the promoter region of myf5 and myogenin genes. Further functional analysis of the promoters of MyoD family members may elucidate the down-regulatory mechanism of these genes through VDR.

Our findings may be clinically relevant to the musculoskeletal health in the aged, because vitamin D insufficiency has been shown to be associated with lower muscle strength and increased falling tendency in adults. Conversely, supplement of native vitamin D or treatment with active vitamin D has been reported to improve muscle functions and protect from falling events and falling-associated fractures (29–33). Whether the beneficial effects of vitamin D treatment occur via direct VDR actions on skeletal muscle cells or indirect mechanisms remains unclear. Interestingly, however, abnormal expression of MyoD family members and MHC isoforms has been reported in various models of immobilization and denervation (34–38). Considering the plasticity and highly adaptive nature of muscle fibers, it is conceivable that reprogramming and adaptations of muscle fibers may occur under various pathological conditions, particularly in elderly patients, and that these processes may be modulated by VDR-dependent vitamin D actions.

In summary, we have shown that VDR gene deleted mice exhibit abnormal skeletal muscle development. These abnormalities occur independently of secondary metabolic changes such as hypocalcemia and hypophosphatemia and are accompanied by deregulated expression of myogenic transcription factors and MHC isoforms. These effects appear to involve direct vitamin D actions on muscle through VDR, because similar effects were reproduced by treatment of VDR-positive myoblastic cells with 1,25$(OH)_2$D *in vitro*. The present study can form a molecular basis of VDR actions on muscle and should help further establish the physiological roles of VDR in muscle development as well as pharmacological effects of vitamin D on muscle functions.

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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

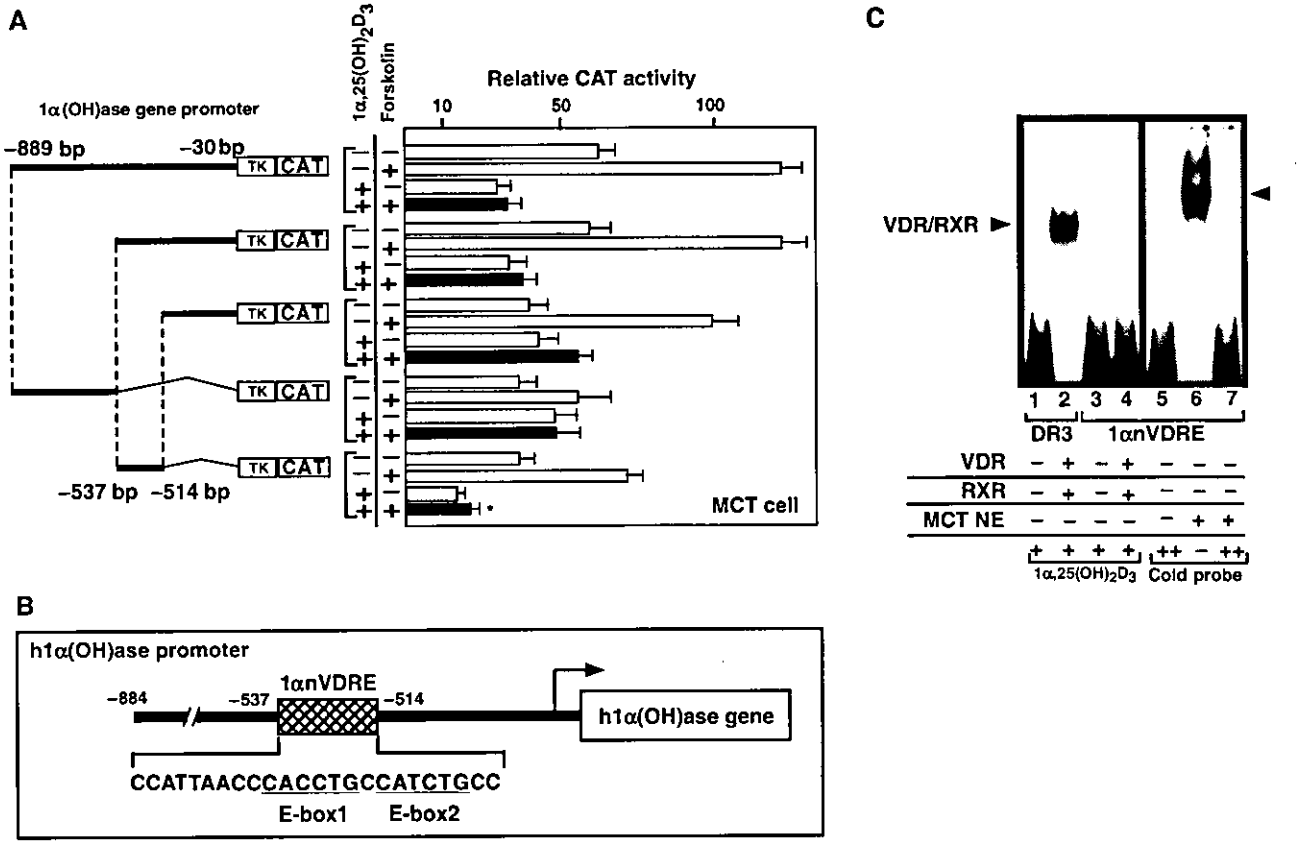

Figure 1 Identification of 1αnVDRE. (**A**) CAT assay using a series of human 1α(OH)ase gene promoter deletion mutants in MCT cells. After 3 h, forskolin (1×10^{-8} M), which activates PKA signaling, and 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) were added, respectively. 1α(OH)ase gene promoter deletion constructs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

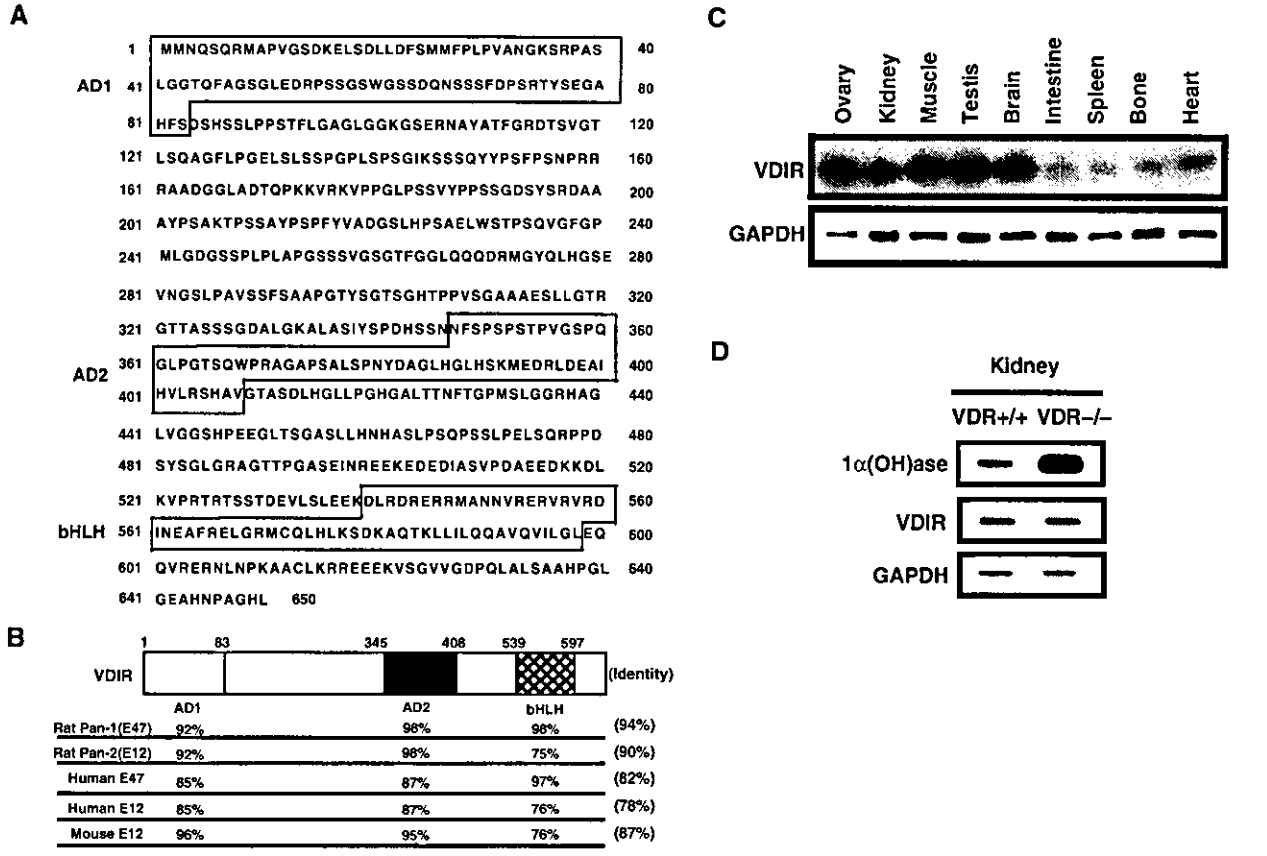

Figure 2 Cloning of the 1αnVDRE-binding factor, VDIR. (**A**) Sequence of VDIR. VDIR has two transactivation domains (AD1 and AD2), and a bHLH motif. (**B**) Functional domain sequence homology between VDIR and members of the bHLH-type activator family (rat Pan-1, E47; rat Pan-2, E12; human E47; human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

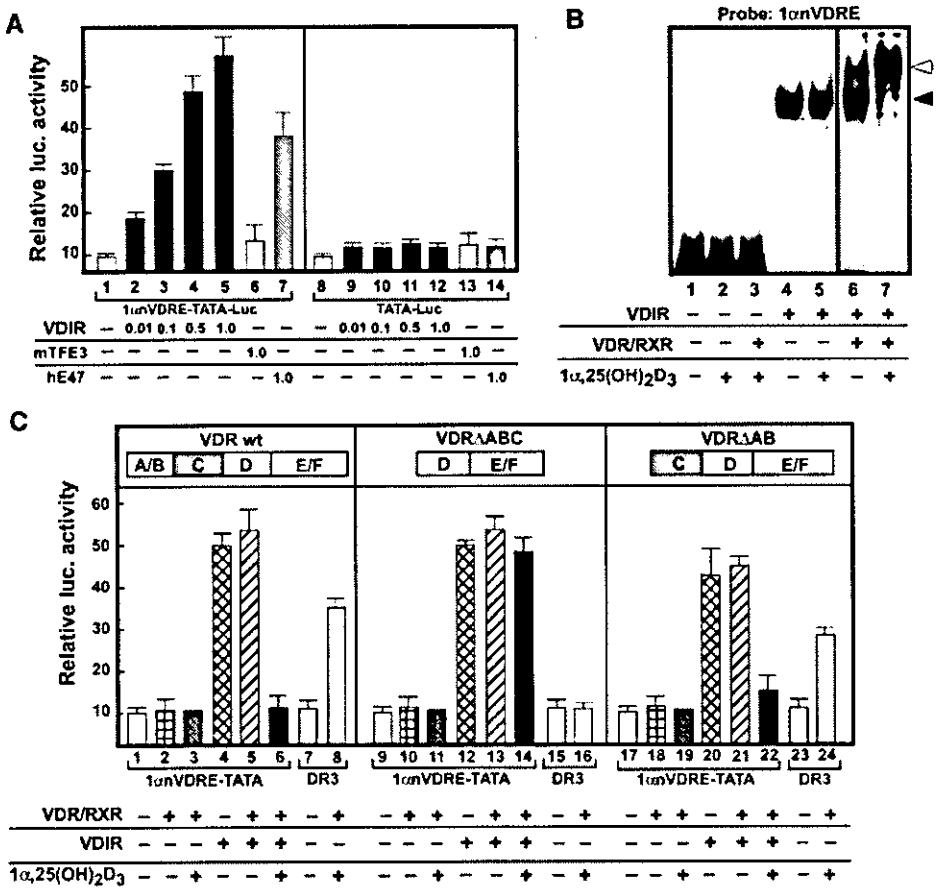

Figure 3 VDIR as an activator for 1αnVDRE. (**A**) Plasmid dose dependency of VDIR activation of nVDRE. Luciferase activity under the control of 1αnVDRE after the transfection of VDIR, mTFE3 or hE47 into MCT cells. MCT cells were cotransfected with LUC reporter plasmid (0.3 μg of nVDRE pGL3 TATA-LUC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

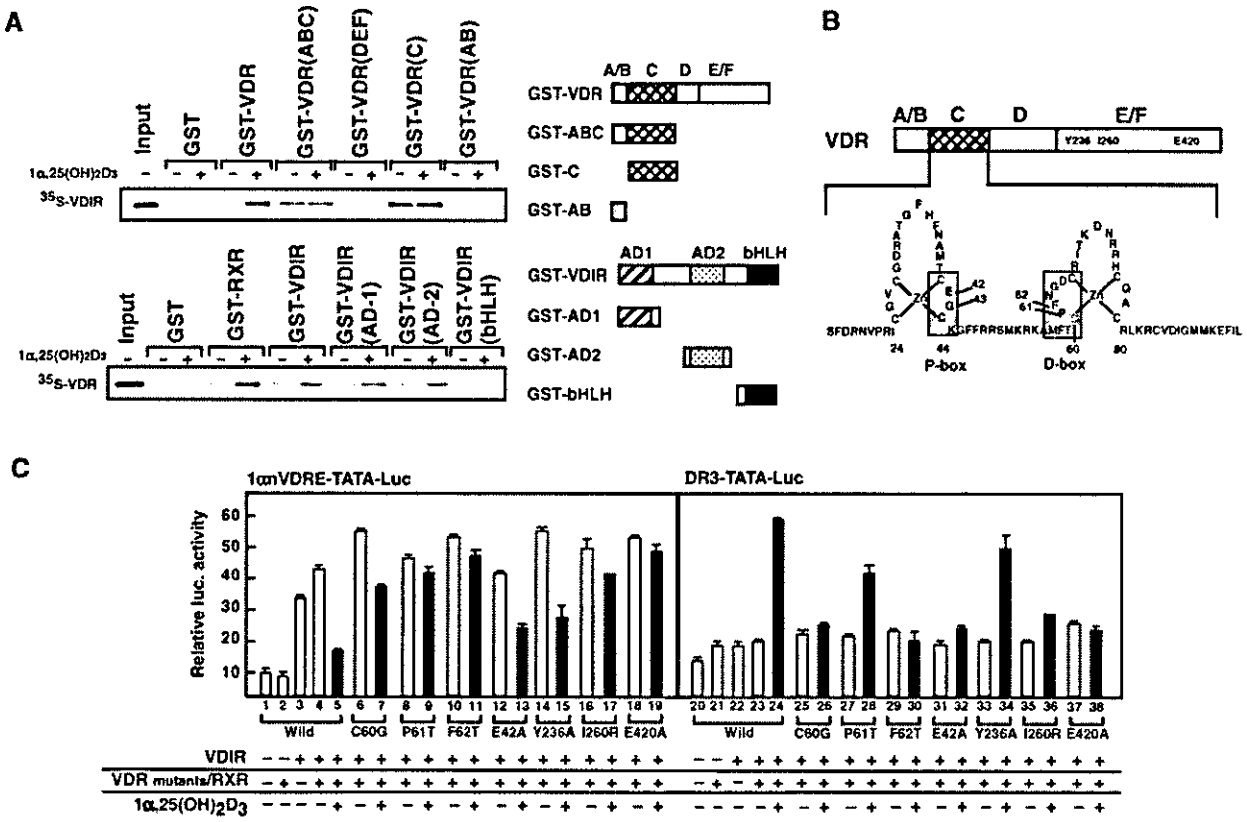

Figure 4 The DNA-binding domain (C-domain) of VDR leeds to the binding of VDIR. (**A**) GST pull-down assay using either GST alone, GST wild-type VDR or GST-fused VDRs deletion mutants together with [^{35}S]-labeled VDIR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (upper panel). GST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

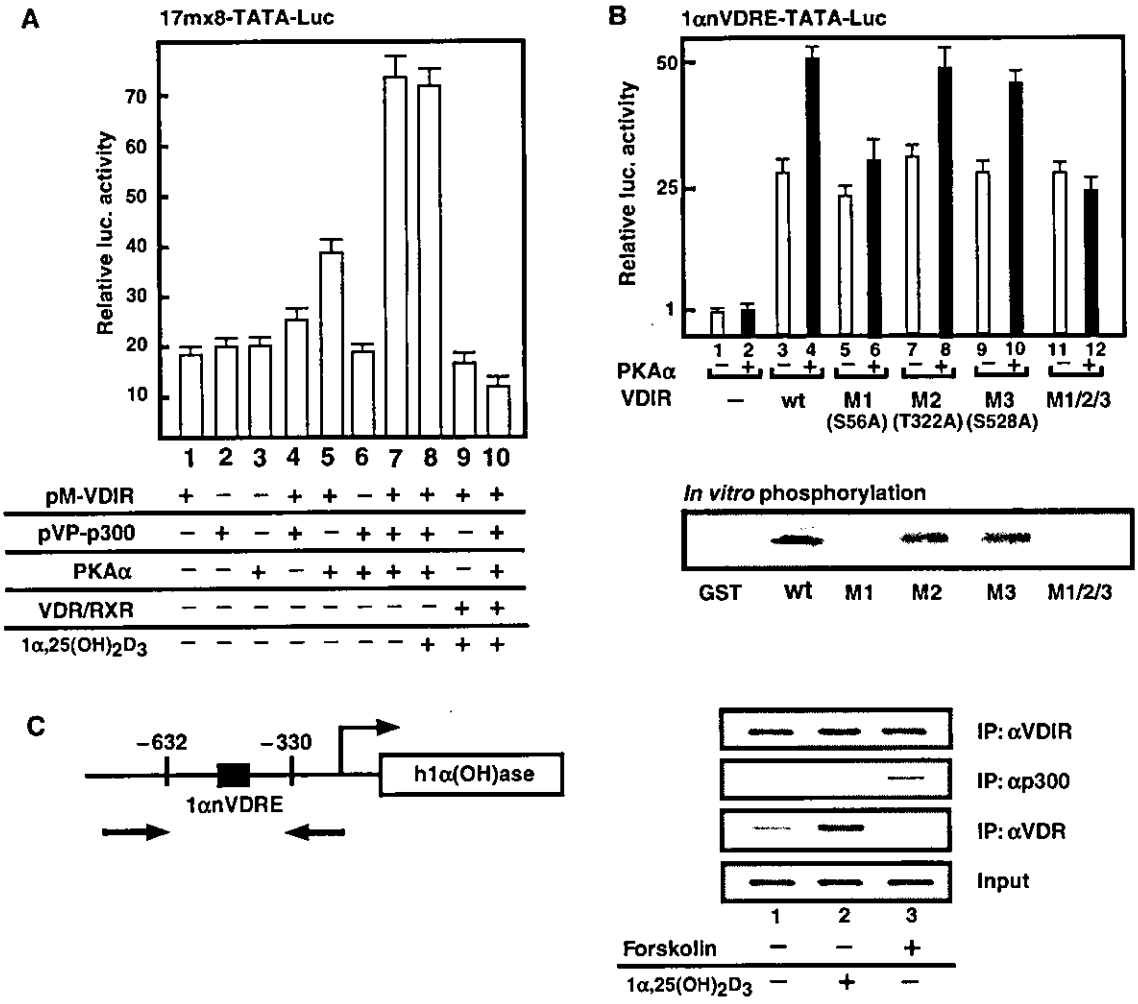

Figure 5 Phosphorylation of VDIR by PKA induced a p300 co-activator recruitment. (**A**) Association of VDIR and p300 in the mammalian two-hybrid assay. The expression plasmids of fusion proteins with GAL4-DBD (pM) and VP16-AD (pVP) were transiently transfected into MCT cells with a GAL4-DBD-regulated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

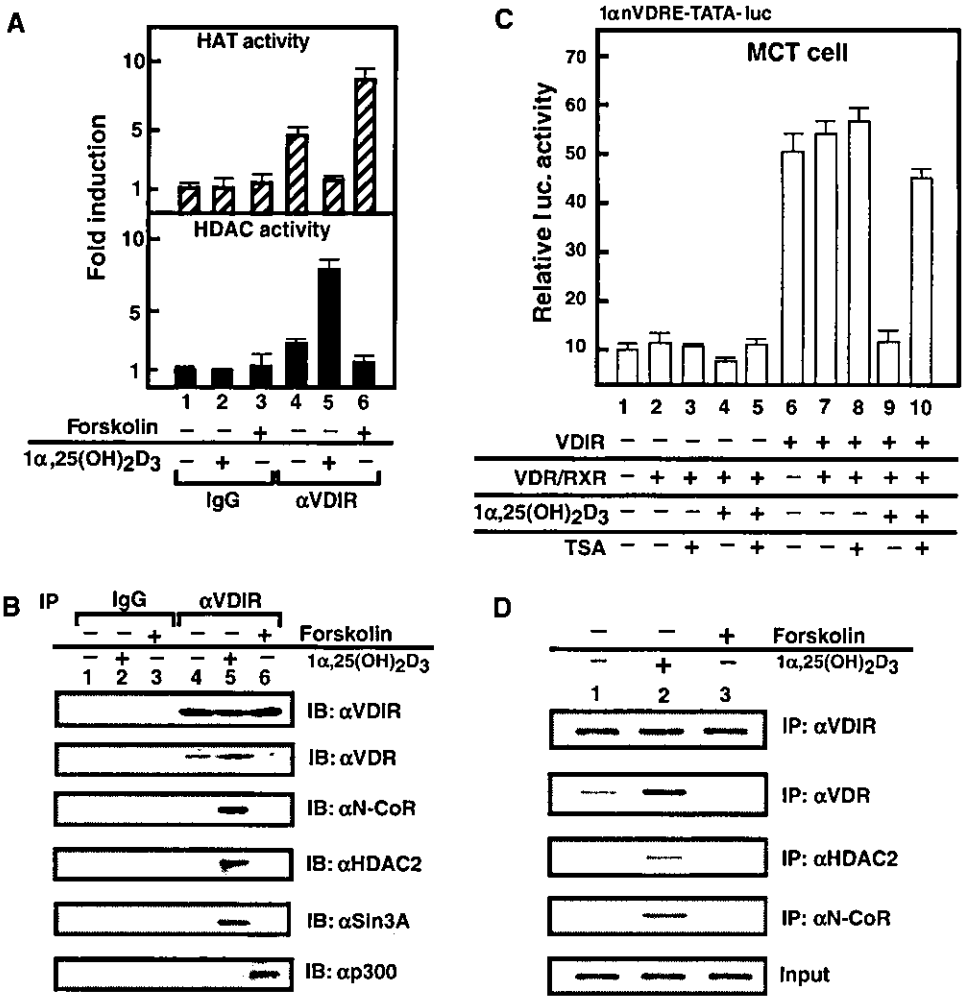

Figure 6 Co-regulator switching upon VDIR for the ligand-induced transrepression by VDR. (**A**) HAT and HDAC activities of the immunoprecipitated VDIR complexes in the MCT cells. Assays were determined in MCT cells after treatment, in the absence or presence of $1\alpha,25(OH)_2D_3$ and forskolin. Representative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

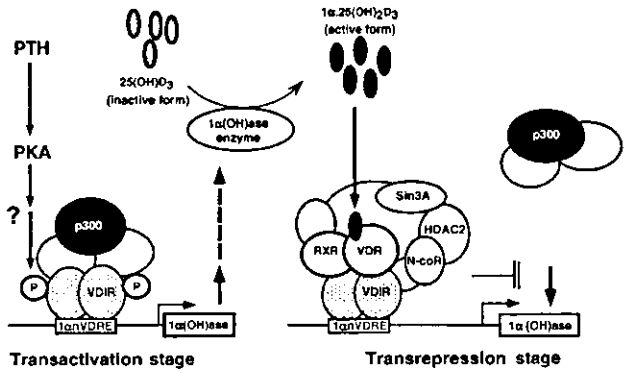

Figure 7 Schematic illustration of the proposed molecular mechanism of $1\alpha,25(OH)_2D_3$-induced transrepression in the 1α-hydroxylase gene promoter. Upon activated-PKA signaling due to PTH, the 1α-hydroxylase gene is transactivated through recruitment of a HAT co-activator complex to VDIR bound 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)$_2$D$_3$ (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)$_2$D$_3$ (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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Brain masculinization requires androgen receptor function

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Testicular testosterone produced during a critical perinatal period is thought to masculinize and defeminize the male brain from the inherent feminization program and induce male-typical behaviors in the adult. These actions of testosterone appear to be exerted not through its androgenic activity, but rather through its conversion by brain aromatase into estrogen, with the consequent activation of estrogen receptor (ER)-mediated signaling. Thus, the role of androgen receptor (AR) in perinatal brain masculinization underlying the expression of male-typical behaviors remains unclear because of the conversion of testosterone into estrogen in the brain. Here, we report a null AR mutation in mice generated by the Cre-loxP system. The AR-null mutation in males ($AR^{L-/Y}$) resulted in the ablation of male-typical sexual and aggressive behaviors, whereas female AR-null homozygote ($AR^{L-/L-}$) mice exhibited normal female sexual behaviors. Treatment with nonaromatizable androgen (5α-dihydrotestosterone, DHT) was ineffective in restoring the impaired male sexual behaviors, but it partially rescued impaired male aggressive behaviors in $AR^{L-/Y}$ mice. Impaired male-typical behaviors in $ER\alpha^{-/-}$ mice were restored on DHT treatment. The role of AR function in brain masculinization at a limited perinatal stage was studied in $AR^{L-/L-}$ mice. Perinatal DHT treatment of females led to adult females sensitive to both 17β-estradiol and DHT in the induction of male-typical behaviors. However, this female brain masculinization was abolished by AR inactivation. Our results suggested that perinatal brain masculinization requires AR function and that expression of male-typical behaviors in adults is mediated by both AR-dependent and -independent androgen signaling.

It is thought that local production of estradiol, converted from testicular testosterone by brain aromatase, and the subsequent activation of estrogen receptor (ER)-mediated signaling is sufficient to induce brain masculinizaton in the male fetus and eventual expression of male typical behaviors in the adult (1–3). This hypothesis is supported by findings that mice deficient in either ER or aromatase display severely reduced male-typical behaviors (2–4). However, studies have indicated that when androgens are given to the female fetus, male-typical behaviors can be induced on further androgen treatment in adulthood (5, 6). Thus, although androgen receptor (AR)-mediated androgen actions are thought to be important in the induction of male-typical behaviors, the activity of locally converted estrogen from aromatizable androgens in the brain has prevented the assessment of the role of AR in perinatal brain masculinization and expression of male-typical behaviors.

Naturally occurring mutations in mammalian AR genes, located on the X chromosome and therefore present as a single copy in males, result in AR dysfunction that can lead to androgen-insensitive testicular feminization mutation (Tfm) (7, 8). Tfm is characterized by a variety of phenotypic abnormalities along with species-specific effects (8). However, Tfm mice appear to express a truncated AR protein (9). Most male animals with severe androgen insensitivity exhibit female-like external sexual organs and are infertile (8). This fact makes it impossible to generate female animals homozygous for AR deficiency and precludes the establishment of AR-null mutant (AR knockout, ARKO) lines either in nature or by conventional gene disruption techniques. Therefore, to define AR function we used the Cre-loxP system to allow the AR-null mutation (10) to be passed to both male and female offspring (11, 12). We report here that perinatal brain masculinization requires AR function and that expression of male-typical behaviors in adults is mediated by both receptor-dependent and -independent androgen signaling.

Materials and Methods

Generation of ARKO Mice. AR genomic clones were isolated from a TT2 embryonic stem cell genomic library by using human AR A/B domain cDNA as a probe. The targeting vector consisted of a 7.6-kb 5′ homologous region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and the neo cassette with two loxP sites (10). Two targeted clones (FB-18 and FC-61), identified by Southern analysis using probes A and B (Fig. 1*b*), were aggregated with single eight-cell embryos from CD-1 mice. Floxed AR mice (C57BL/6) were then crossed with the CMV-Cre transgenic mice. The two lines exhibited the same phenotypic abnormalities. The chromosomal sex of each pup was determined by genomic PCR amplification of the *Sry* gene on the Y chromosome.

RNA Analysis. Total RNA was isolated by the improved acid-guanidine-phenol-chloroform method and poly(A)$^+$ RNA purified with Dynabeads (Dynal, Oslo). Total and poly(A)$^+$ RNA was separated in formaldehyde-containing agarose gels for Northern blot analysis as described (13, 14). An AR cDNA fragment (353 bp: 2368–2721) or neuronal nitric oxide synthase (nNOS) cDNA fragment (1,814 kbp: 182–1995) was used as a probe. RT-PCR analysis was conducted as described in *Supporting Materials and Methods* in *Supporting Text*, which is published as supporting information on the PNAS web site.

Protein Analysis. To detect AR protein expression (7), brain cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were probed with poly-

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Abbreviations: AR, androgen receptor; ARKO, AR knockout; DHT, 5α-dihydrotestosterone; ER, estrogen receptor; Tfm, testicular feminization mutation; PLA, placebo; E_2, 17β-estradiol; nNOS, NO synthase.

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