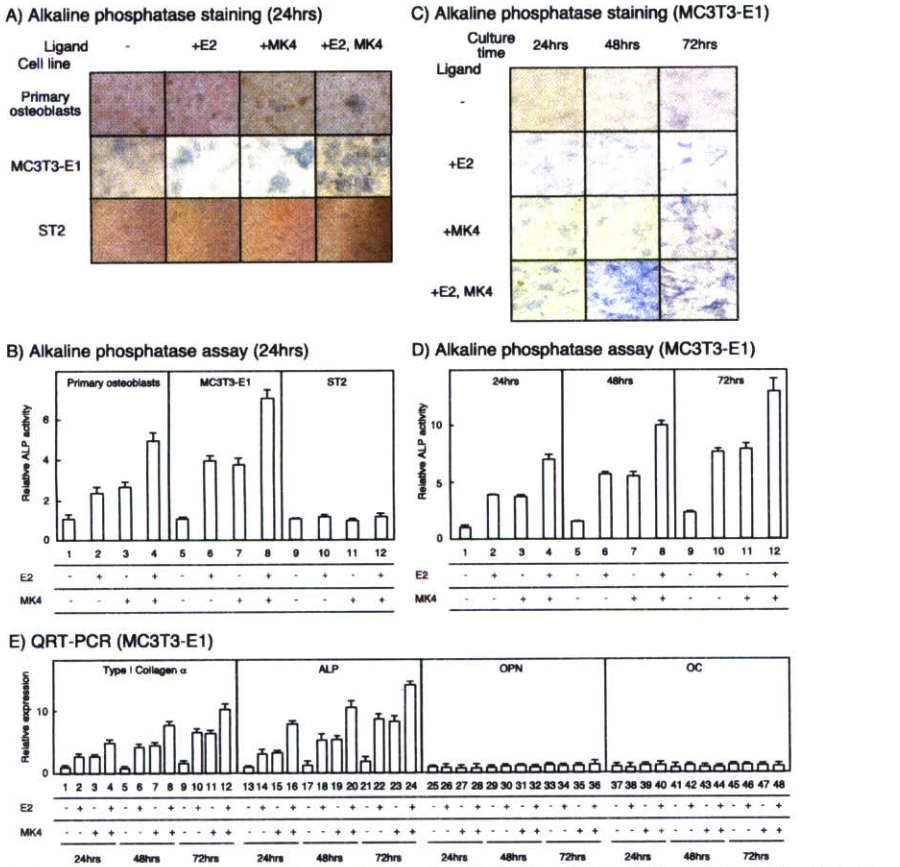

FIG. 1. VK and estrogen cooperatively promote mature osteoblast formation. (A) The effect of MK4 and E2 on osteoblastic differentiation in the presence or absence of MK4 (1 μM) and E2 (1 nM). After 24 h, osteoblast differentiation was detected by ALP staining. Upper panel, primary osteoblasts from mouse calvaria; middle panel, MC3T3-E1 cells; bottom panel, ST2 cells. (B) The effect of MK4 and E2 on osteoblastic differentiation in the presence or absence of MK4 (10 μM) and E2 (10 nM). After 24 h, ALP activity was measured using the optical density at 405 nm. All values are means ± standard deviations for at least three independent experiments. (C) The effect of MK4 and E2 in the early stage of osteoblastic differentiation in MC3T3-E1 cells. After 24-, 48-, and 72-h treatments, ALP staining was performed. (D) The effect of MK4 and E2 in the early stage of osteoblastic differentiation in MC3T3-E1 cells. After 24-, 48-, and 72-h treatments, ALP activity was measured. (E) Relative expression levels of the marker genes of osteoblastic differentiation. The total RNA for the qRT-PCR was extracted using the ISOGEN kit (Nippon Gene) from MC3T3-E1 cells treated with MK4 and E2 or left untreated. OPN, osteopontin; OC, osteocalcin.

at the N terminus. The mouse Msx2 promoters, the sequences of which were derived from the Ensembl genome browser (http://www.ensembl.org/index.html), were subcloned into a pGL3-Basic vector (Promega).

Animals. Estrogen receptor α knockout (ERαKO) mice were kindly provided by P. Chambon (19). Genotyping for ERαKO mice was routinely performed on DNA isolated from tail snips by a PCR procedure (19).

Cell culture, transient transfection, and luciferase assay. MC3T3-E1 and ST2 cells were cultured with alpha minimum essential medium (α-MEM) (GIBCO) containing 10% fetal bovine serum (FBS) (GIBCO) at 37°C and 5% CO_2 (31). HEK 293T cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (GIBCO) at 37°C and 5% CO_2. For transfection, cells were plated in the corresponding medium supplemented with 10% charcoal-stripped FBS in 12-well plates 1 day before transfection. Transfection was performed with Lipofectamine (Invitrogen) with Plus reagent (Invitrogen) or Polyfect (QIAGEN) as directed by the manufacturer's protocol. After 3 h, 17β-estradiol (E2) (10 nM) and MK4 (10 μM) were added to α-MEM containing 2% FBS, and the cells were incubated continuously at 37°C for 24 h. As a reference to normalize transfection efficiency, 2.5 ng/well of pRL-CMV plasmid (Promega) was cotransfected in all experiments. Luciferase activity was determined using the luciferase assay system (Promega) (5).

Osteoblast primary culture. Calvaria of newborn mice were digested for 80 min at 37°C in phosphate-buffered saline (PBS) containing 0.1% collagenase A

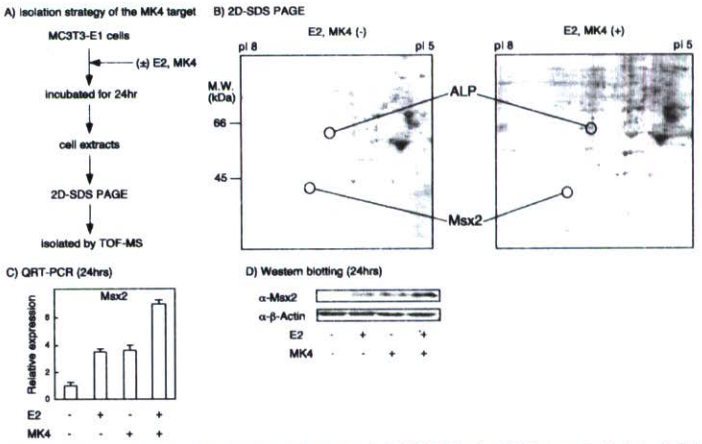

FIG. 2. Identification of Msx2 as a regulatory target of VK and estrogen in MC3T3-E1 cells. (A) Strategy for isolating MK4 targets. MC3T3-E1 cells were incubated for 24 h in the presence or absence of MK4 (1 μM) and E2 (1 nM). Total cell lysates then were separated by 2D SDS-PAGE, and the bands were examined with matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy (labeled TOF-MS) for protein identification. (B) Comparison of protein expression by 2D SDS-PAGE in the presence or absence of MK4 and E2. M.W., molecular size. (C) The effect of MK4 and E2 on Msx2 gene expression. Msx2 mRNA expression was analyzed by qRT-PCR. (D) The effect of MK4 and E2 on Msx2 protein expression. Msx2 protein levels were determined by immunoblotting. β-Actin was used as a control.

(Roche). Cells were plated with α-MEM containing 10% FBS. Media were changed every 3 days (32).

ALP staining. For alkaline phosphatase (ALP) staining, primary osteoblasts, MC3T3-E1 and ST2 cells, were washed twice with PBS, fixed in 3.7% formaldehyde, and stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.6 mg/ml fast-blue BB salt (Sigma), 2 mM $MgCl_2$, 5 μl/ml *N,N*-dimethylforamide (Wako), and 100 mM Tris-HCl (pH 8.8) buffer at 37°C for 5 to 10 min. When the cells turned blue, the cells were washed twice with PBS (32).

ALP assay. The ALP assay was performed using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. In brief, primary osteoblasts, MC3T3-E1 and ST2 cells, were washed twice with ice-cold PBS and then were solubilized with lysis buffer (20 mM Tris-HCl [pH 7.9], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol) containing protease inhibitors, followed by determination of the ALP activity in lysates. The protein concentration was determined with a Bio-Rad protein assay kit.

qRT-PCR. For quantitative real-time reverse transcription-PCR (qRT-PCR), 1 μg of total RNA from each sample was reverse transcribed into first-strand cDNA with random hexamers using Superscript III reverse transcriptase (Invitrogen). Primer sets for all genes were purchased from Takara Bio Inc. (Tokyo, Japan). Real-time RT-PCR was performed using SYBR premix EX *Taq* (Takara) with the thermal cycler Dice RealTime System TP800 (Takara) according to the manufacturer's instructions. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. To correct for variability in RNA recovery and the efficiency of reverse transcription, glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified and quantified in each cDNA preparation. Normalization and calculation steps were performed as reported previously (34).

2D SDS-PAGE analysis. For 2D SDS-PAGE analysis, MC3T3-E1 cells were washed with ice-cold PBS, collected by centrifugation at 2,000 × *g*, resuspended in 50 μl lysis buffer (20 mM Tris-HCl [pH 7.9], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol) containing protease inhibitors, incubated on ice for 30 min, and then centrifuged for 30 min at 12,000 × *g*. After centrifugation, the supernatants were cleaned with a 2D-Clean-Up kit (Amersham). These samples were separated with the 2D SDS-PAGE system (Bio-Rad). The 2D SDS-PAGE gels were visualized with the SilverQuest silver staining kit (Invitrogen).

ABCD assay. For the avidin-biotin complex DNA (ABCD) assay, sense and antisense oligonucleotide DNAs that were biotinylated at the 3′ terminus were incubated at 100°C for DNA annealing and then were cooled slowly at room temperature. To prepare the beads-DNA complex, biotin-conjugated double-stranded oligonucleotide DNA was mixed with a 50% slurry of avidin beads (Tetralink tetrameric avidin resin; Promega). Cells were lysed with lysis buffer (10 mM Tris-Cl [pH 7.8], 1 mM EDTA, 150 mM NaCl, 0.1% NP-40) containing protease inhibitors for 1 h at 4°C. Whole-cell lysates were clarified by centrifugation, mixed with the 50% slurry of avidin beads, and pelleted at 3,000 × *g*. Supernatants were added to the beads-DNA complex and rotated for 1 h at 4°C to mix. The beads were collected by centrifugation at 3,000 × *g* and were washed successively in the lysis buffer. Proteins were resolved with SDS-PAGE, and Western blotting was performed with the corresponding antibody (12).

ChIP analysis. Chromatin immunoprecipitation (ChIP) analysis was performed using the ChIP assay kit (Upstate Biotechnology Inc.) (13, 38) according to the manufacturer's instructions. MC3T3-E1 cells were cultured in the presence or absence of MK4 and E2. Soluble chromatin prepared from 1 × 10^6 cells was immunoprecipitated with antibodies against the indicated proteins.

Generation of adenovirus. Recombinant adenoviruses carrying RNAi of Msx2 and PXR were constructed using the RNAi-Ready pSIREN-Shuttle vector (BD Biosciences Clontech) first and then were moved to the Adeno-X vector (Clontech) by being spliced into the I-CeuI and PI-SceI site. The parental virus genomes in HEK 293 cells (ATCC) were constructed according to the manufacturer's protocol (20). MC3T3-E1 cells then were infected by incubation with the recombinant adenovirus.

RESULTS

Vitamin K2 promotes osteoblast differentiation. We explored the osteoprotective effects of K2 on a molecular level to better understand how K2 clinically prevents osteoporosis. We first tested the effects of MK4, a K2. The effect of MK4 was measured by ALP assay of primary cultured mice osteoblastic cells, derived from calvaria, to detect osteoblastic differentiation. As shown in the lower panels of Fig. 1 A and B (lanes 1 to 4), ALP activity was induced in the primary cultured osteoblastic cells treated with MK4 for 24 h. Likewise, E2 was stimulatory, and the actions of MK4 and E2 were additive. To clarify the stages of differentiation at which MK4 and E2 act,

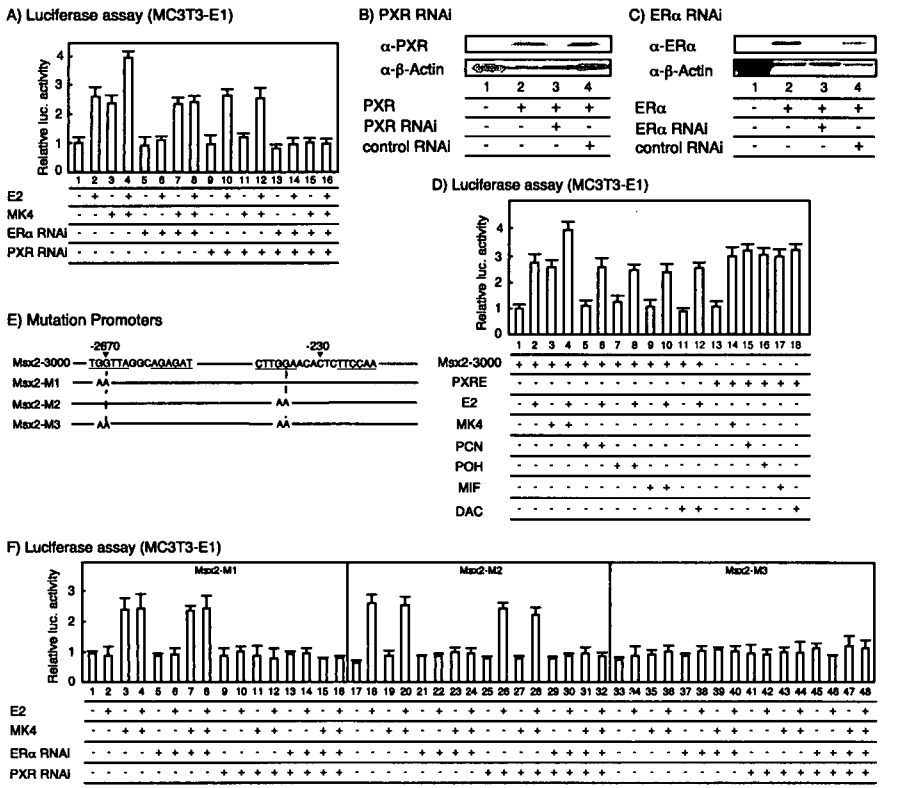

FIG. 3. Identification of PXRE-like and ERE-like elements in the Msx2 gene promoter region. (A) Functional analysis of the Msx2 gene promoter. Plasmid constructs pSIREN-Shuttle-PXR (100 ng) and pSIREN-Shuttle-ERα (100 ng), together with the Msx2-3000 reporter (400 ng), were transfected in MC3T3-E1 cells, and the transfected cells were cultured for 24 h. Promoter activity was measured by luciferase (luc.) activity. All values are means ± standard deviations for at least three independent experiments. (B and C) Gene-specific knockdown using PXR (B) and ERα (C) RNAi vectors. The RNAi vectors were transfected into MC3T3-E1 cells for 48 h, and the protein levels were determined by immunoblotting. β-Actin was used as a control. (D) Regulation of the Msx2-3000 reporter by known PXR ligands, such as pregnenolone 16α-carbonitrile (PCN), 17α-hydroxypregnenolone (POH), mifepristone (MIF), and dexamethasone 21-acetate (DAC), in MC3T3-E1 cells. The amounts of each transfected plasmid are listed above for panel A. (E) Mutation of the Msx2 gene promoters. These mutant promoters were generated with a mutagenesis kit (Stratagene). (F) Analysis of the mutated Msx2 gene promoters by MK4 and E2 using endogenous expression receptors in MC3T3-E1 cells. The amounts of each transfected plasmid are described above for panel A.

we tested ST2 cells and MC3T3-E1 cells. ST2 cells are bipotential cells derived from mouse bone marrow mesenchymal stem cells that differentiate into either osteoblasts or adipocytes under the proper conditions (32). MC3T3-E1 cells are derived from mouse calvaria and are preosteoblastic cells that can be induced to differentiate into mature osteoblasts (30). As shown in the middle panels of Fig. 1A and B (lanes-5 to 8), ALP induction following treatment with MK4 and/or E2 for 24 h was observed in MC3T3-E1 cells but not in ST2 cells, as shown in the bottom panels of Fig. 1A and B (lanes 9 to 12). The additive action of MK4 and E2 in MC3T3-E1 cells was more pronounced following a longer culture period (Fig. 1C and D). Our results suggest that MK4 and E2 act from the stage at which preosteoblasts differentiate into mature osteoblasts but not at the earlier stage of the cell fate decision. To confirm this, we measured the expression of osteoblast genesis marker genes in the MC3T3-E1 cells treated with MK4 and E2 by qRT-PCR. Early osteoblast genesis marker genes (encoding type I collagen α and ALP) were induced by both MK4 and E2 treatments, while mRNA levels of late osteoblast genesis marker genes (encoding osteopontin and osteocalcin) were unaltered following 24 h of treatment (Fig. 1E).

Msx2 is a target gene for MK4 and E2 in MC3T3-E1 cells. To elucidate the molecular pathway through which MK4 and E2 induce osteoblast genesis, we screened for MK4 target proteins in untreated and treated MC3T3-E1 cells with 2D SDS-PAGE (Fig. 2A and B). E2 was used for cotreatment, since it potentiated MK4 action in osteoblastic differentiation. As shown in Fig. 2B, expression levels of several candidate proteins were up-regulated or down-regulated by the treatment, and we tried to identify them by matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy analysis. One of these was Msx2, a protein that was shown to be a critical factor for inducing mesenchymal stem cells to differentiate into preosteoblasts (26). We reasoned that Msx2 mediates MK4 action as an MK4 target gene. Indeed, induction of Msx2 gene expression by MK4 alone was visible, and the additive induction with E2 was at both the transcriptional level (qRT-PCR) and the translational level (Western blotting) (Fig. 2C and D).

Msx2 promoter confers responsiveness to MK4 and E2 through cognate NRs. To test the idea that MK4 acts as a PXR ligand in the transcriptional control of the mouse Msx2 gene, we performed a promoter analysis of the Msx2 gene by using a luciferase reporter assay on MC3T3-E1 cells. The responsiveness to MK4 and E2 was tested with a luciferase gene reporter driven by the mouse Msx2 gene promoter region (−1 to −3000) (designated Msx2-3000). Either MK4 or E2 was sufficient to activate transcription alone, and synergy of MK4 with E2 was observed (Fig. 3A). Knockdown of PXR and ERα by RNAi (Fig. 3B and C) abolished the MK4- and E2-induced transactivation of the Msx2 gene promoter (Fig. 3A, lanes 5 to 16). Other known PXR ligands also were tested with this promoter. Though MK4 was effective in stimulating the transcription of the Msx2 gene promoter, none of the other tested ligands (pregnenolone 16α-carbonitrile, 17α-hydroxypregnenolone, mifepristone, and dexamethazone 21-acetate) stimulated transcription alone. Additionally, none demonstrated synergy with E2 (Fig. 3D). We used a series of promoter deletion mutants to map an MK4-responsive element in the proximal promoter (−1 to −390) (data not shown) that contained a consensus PXR/RXR binding-related element (designated Msx2-PXRE) at around bp −230 (Fig. 3E). Additionally, a consensus element (designated Msx2-ERE) related to the ER-responsive element (ERE) was found in the distal promoter region (−2672 to −2658) (see Fig. 3E and 4A). Short elements containing either Msx2-PXRE or Msx2-ERE conferred the expected response to the cognate NR ligand (data not shown). To verify the impact of the response elements, we introduced two point mutations into each of the elements (Fig. 3E). As shown in Fig. 3F, Msx2-M1, which was mutated at Msx2-ERE, lost the E2 response; likewise, the MK4 response was abolished by the mutation (Msx2-M2) in Msx2-PXRE. As expected, no NR ligand response was seen in the mutant (Msx2-M3) that was mutated at both Msx2-PXRE and Msx2-ERE (Fig. 3F).

Direct DNA binding of PXR on the Msx2 promoter. To determine whether PXR bound to the proposed PXRE (Fig. 4A), we examined the DNA binding ability of PXR and ERα on the Msx2 gene promoter with an ABCD assay. We tested whole-cell extracts of MC3T3-E1 cells transiently overexpressing PXR, RXRα, or ERα. PXR/RXRα bound to Msx2-PXRE, while ERα binding was not seen for Msx2-PXRE (Fig. 4B). Conversely, ERα bound, as expected, to Msx2-ERE (Fig. 4A and B). Moreover, in this assay, p300 also associated with the receptors. To verify if the observed DNA binding of PXR and ERα in vitro reflected the physiological events in the Msx2 promoter, we performed a ChIP analysis on the endogenous promoter of the Msx2 gene. E2-induced recruitment of the ERα, but not PXR, was observed in the upstream region of Msx2-ERE. In contrast, the ligand-independent association of PXR took place in the region of the distal promoter containing Msx2-PXRE (Fig. 4C). Consistent with the type of recruited receptors, the known NR coactivator p300 also was recruited to the promoter (Fig. 4C).

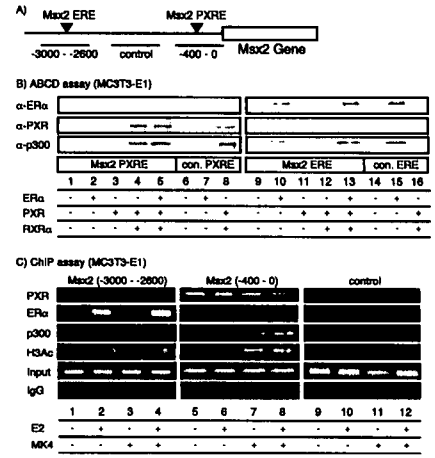

FIG. 4. Direct recruitment of PXR/RXRα to Msx2-PXRE in the Msx2 gene promoter. (A) The position of Msx2-PXRE and Msx2-ERE in the Msx2 gene promoter. (B) The recruitment of PXR/RXRα and ERα to Msx2-PXRE and Msx2-ERE in the Msx2 gene promoter in vitro. The ABCD assay was performed with two copies of Msx2-PXRE and Msx2-ERE that were conjugated to the beads and incubated with whole-cell extracts from HEK 293T cells that overexpressed PXR, RXRα, and ERα together with the respective ligands for the NRs. The proteins interacting with DNA-conjugated beads were detected by immunoblotting. α-ER, anti-ER antibody; α-PXR, anti-PXR antibody; α-p300, anti-p300 antibody, con., consensus. (C) The recruitment of PXR/RXRα and ERα to the Msx2 gene promoter in vivo. ChIP analysis was performed with soluble chromatin prepared from MC3T3-E1 cells treated with MK4 and E2 for 1 h and immunoprecipitated with the indicated antibodies. Extracted DNA samples were amplified using primer pairs to detect the regions containing either Msx2-PXRE or Msx2-ERE. IgG, immunoglobulin G.

MK4-induced osteoblast genesis is mediated by PXR. To confirm the physiological impact of PXR function on VK-induced osteoblast genesis, we knocked down either Msx2 or PXR in MC3T3-E1 cells by RNAi with adenovirus (Fig. 5A and B). Expression of endogenous Msx2 and PXR was significantly attenuated by RNAi (Fig. 5A and B). As expected, osteoblast genesis induced by MK4 was attenuated when cells were infected with Msx2 RNAi adenovirus (the middle panels of Fig. 5C and D [lanes 5 to 8]). Moreover, in primary osteoblasts derived from ERαKO mice, osteoblast genesis induced by E2 also was attenuated (Fig. 5E). The induction of marker

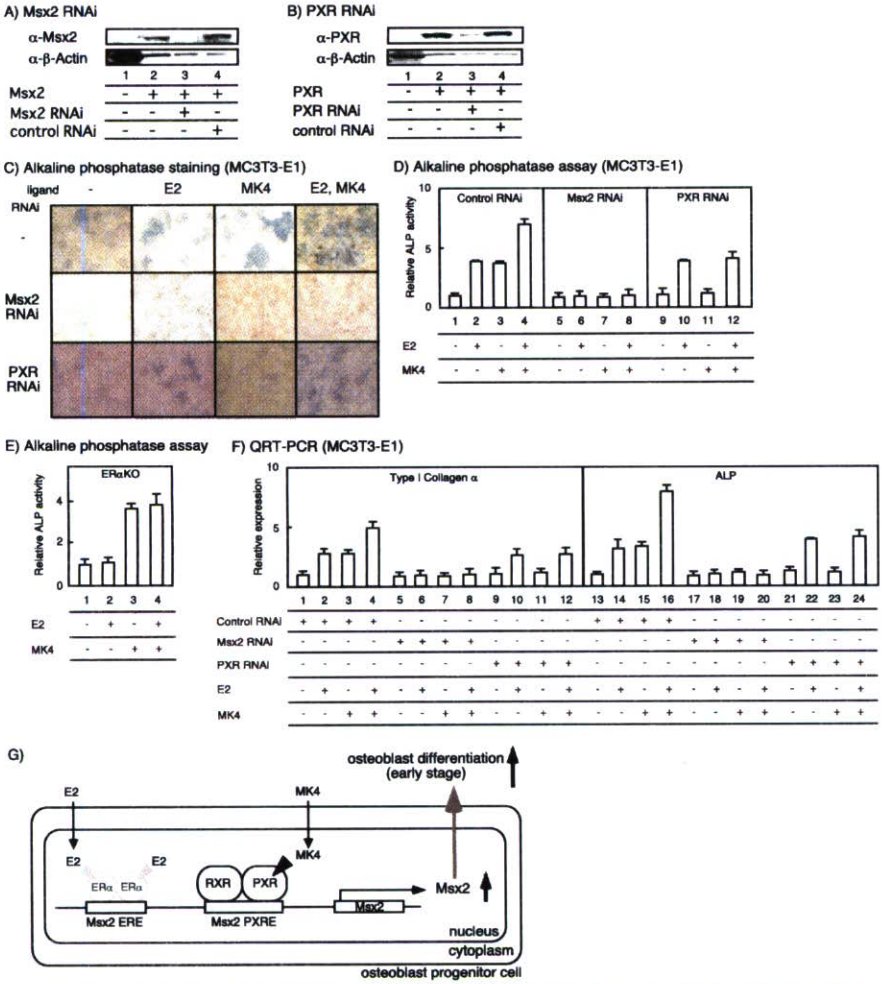

FIG. 5. VK and estrogen cooperatively promote osteoblast differentiation through Msx2 gene induction. (A and B) Gene-specific knockdown using Msx2 (A) and PXR (B) RNAi adenovirus. MC3T3-E1 cells were cultured with the RNAi adenovirus for 48 h, and the protein levels were determined by immunoblotting with each antibody. β-Actin antibody (α-β-actin) was used as a control. α-Msx2, anti-Msx2 antibody; α-PXR, anti-PXR antibody. (C) Msx2 mediates the effect of MK4 and E2 in osteoblast differentiation. MC3T3-E1 cells infected with the RNAi adenovirus for 48 h were incubated with MK4 and E2 for 24 h. Osteoblast differentiation was detected by ALP staining. (D) Msx2 mediates the effect of MK4 and E2 in osteoblast differentiation. MC3T3-E1 cells infected with the RNAi adenovirus for 48 h were incubated with MK4 and E2 for 24 h, and then ALP activity was measured. (E) The effect of E2 and MK4 in primary ERαKO osteoblast differentiation. Primary osteoblasts derived from ERαKO mice were incubated with MK4 and E2 for 24 h. The osteoblast differentiation was measured by ALP assay. (F) The expression levels of osteoblast differentiation marker genes induced by MK4 and E2 with Msx2 or PXR RNAi. MC3T3-E1 cells were infected with the RNAi (control, Msx2, or PXR) adenovirus for 48 h, followed by either no treatment or treatment with MK4 and E2 for 24 h. The total RNA then was extracted and used for qRT-PCR. (G) A schematic view of the VK and E2 actions at the Msx2 gene promoter.

genes (encoding type I collagen α and ALP) was, as expected, also abrogated with Msx2 RNAi adenovirus (Fig. 5F, lanes 5 to 8 and 17 to 20). Moreover, PXR RNAi also inhibited MK4-dependent osteoblast genesis, as shown in the bottom panels of Fig. 5C, D (lanes 9 to 12), and F (lanes 9 to 12 and 21 to 24).

DISCUSSION

This study demonstrates that Msx2 contains a PXRE in its gene promoter and that it is a direct target gene for MK4-bound PXR. Reflecting the similar effects of E2 and MK4, an ERE also was mapped in the Msx2 gene promoter. Although each of the response elements alone was not remarkable, the combined effects of MK4 and E2 were evident in transactivation. In osteoblastic differentiation induced by MK4, E2, or both, the knockdown of endogenous Msx2 abrogated the effects of the NR ligands. Thus, Msx2 likely mediates, at least in part, the effects of both MK4 and E2 on osteoblastic differentiation. Msx2 is one of the prime osteoblastogenic factors in an intact animal (26). Our observation that osteoblast genesis is induced by MK4 in cultured preosteoblastic cells suggests that MK4 exerts its osteoprotective action by upregulating Msx2 gene expression and increasing osteoblast genesis (Fig. 5G). Since extracellular matrix Tsukushi genes also are VK target genes (8), the osteoprotective effects of VK in the human are mediated both by the classical pathway of protein γ-carboxylation and through genomic action via PXR/SXR.

PXR/SXR is activated by a number of endogenous and exogenous ligands (23). PXR/SXR plays a prominent role in the detoxification of chemicals (36). PXR/SXR is considered a global sensor for low-molecular-mass fat-soluble drugs that also is responsible for the timely degradation of these compounds (14). Thus, in contrast to steroid hormone receptors, PXR/SXR responds to a variety of ligands with unrelated structures. The three-dimensional structure of the PXR/SXR ligand binding domain (LBD) is known (35). The LBD is flexible, with a wider cave than those of steroid receptors (37). This permits PXR/SXR to capture a variety of ligands, including MK4. Shifting of the C-terminal transactivation helix H12 in the ERα LBD alters the conformation of the receptor, depending on whether an agonist or antagonist is bound. This plasticity enables ERα to respond to a variety of different ligands (27). It is possible that MK4 binding induces an H12 shift that is distinct from those induced by the other ligands, since the transactivation of PXR in the Msx2 promoters was induced by MK4 but not by the other known PXR/SXR ligands in the osteoblastic cell lines. Thus, the MK4-induced angle in the H12 shifting of PXR might be preferential to recruit coactivator/coactivator complexes to the Msx2 gene promoter in preosteoblasts. In fact, the MK4-induced association of PXR with the well-characterized coactivator (p300) was detectable in both an ABCD assay and a ChIP analysis of the Msx2 gene promoter. Our observations are a starting point for investigating coregulators (17, 24) associating with MK4-bound PXR in osteoblastic cells. The identification of PXR coregulators will enhance the understanding of the osteoprotective effects of VK at a molecular level.

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A Regulatory Circuit Mediating Convergence between Nurr1 Transcriptional Regulation and Wnt Signaling▽

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The orphan nuclear receptor Nurr1 is essential for the development and maintenance of midbrain dopaminergic neurons, the cells that degenerate during Parkinson's disease, by promoting the transcription of genes involved in dopaminergic neurotransmission. Since Nurr1 lacks a classical ligand-binding pocket, it is not clear which factors regulate its activity and how these factors are affected during disease pathogenesis. Since Wnt signaling via β-catenin promotes the differentiation of Nurr1$^+$ dopaminergic precursors in vitro, we tested for functional interactions between these systems. We found that β-catenin and Nurr1 functionally interact at multiple levels. In the absence of β-catenin, Nurr1 is associated with Lef-1 in corepressor complexes. β-Catenin binds Nurr1 and disrupts these corepressor complexes, leading to coactivator recruitment and induction of Wnt- and Nurr1-responsive genes. We then identified KCNIP4/calsenilin-like protein as being responsive to concurrent activation by Nurr1 and β-catenin. Since KCNIP4 interacts with presenilins, the Alzheimer's disease-associated proteins that promote β-catenin degradation, we tested the possibility that KCNIP4 induction regulates β-catenin signaling. KCNIP4 induction limited β-catenin activity in a presenilin-dependent manner, thereby serving as a negative feedback loop; furthermore, Nurr1 inhibition of β-catenin activity was absent in PS1$^{-/-}$ cells or in the presence of small interfering RNAs specific to KCNIP4. These data describe regulatory convergence between Nurr1 and β-catenin, providing a mechanism by which Nurr1 could be regulated by Wnt signaling.

Expression and maintenance of the dopaminergic phenotype in the ventral midbrain (VM) require the orphan nuclear receptor (NR) Nurr1 (NR4A2) (48, 59). Genetic ablation of *Nurr1* produces embryonic lethality due to a nearly complete absence specifically of mesencephalic dopaminergic neurons, which are critical for motor function. Nurr1 regulates both the differentiation and the maintenance of these dopaminergic cells, as *Nurr1*$^{+/-}$ mice appear normal at birth but develop motor deficits resulting from reduced numbers of dopaminergic neurons and lower dopamine levels in the striatum (22). At the molecular level, Nurr1 binds specific response elements in the promoters of genes involved in dopaminergic neurotransmission, such as the genes encoding tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase, and the dopamine transporter (20, 26, 44, 45). In Parkinson's disease, mesencephalic dopaminergic neurons degenerate, ultimately leading to severe motor deficits; correspondingly, Nurr1 levels appear to be reduced (10, 11). Therapeutic strategies that promote Nurr1 function in Parkinson's disease might therefore restore dopaminergic function or even increase the number of dopaminergic neurons. However, Nurr1 has a closed ligand-binding pocket and thus appears to be regulated by ligand-independent mechanisms (55). These mechanisms include changes in the expression of its RNA and protein (36, 47, 54) and changes by second messenger signaling systems and coactivators that modify its transcriptional activity (15, 21, 23, 27, 43). Identifying factors that govern Nurr1 activity is thus important for understanding the development and pathophysiology of VM dopaminergic neurons.

Among the potential factors regulating Nurr1 function, a strong candidate is signaling downstream of the Wnt family of secreted glycoproteins. Wnt signaling is required for the establishment of the midbrain/hindbrain region of the developing nervous system, including VM dopaminergic neurons (reviewed in reference 5), in part by promoting the expression of transcription factors that specify regional identity, such as engrailed (13). In the mouse VM region, the Wnt signaling molecule β-catenin is highly expressed and active in Nurr1$^+$ precursor cells, as evidenced by local expression of TOPGAL, a β-catenin-responsive reporter (7). Specific Wnt molecules also promote the proliferation and differentiation of Nurr1$^+$ dopaminergic precursor cells cultured from the VM (7), and Wnt-5a is expressed in VM glial cells, potentially explaining their ability to induce the dopaminergic phenotype in vivo (6). Thus, there is clear evidence that Wnt signaling via β-catenin regulates the development of Nurr1$^+$ precursors in vivo, but the molecular mechanisms underlying this effect are unknown.

Canonical Wnt signaling is triggered by binding of a secreted Wnt family member to the membrane receptor complex of

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low-density lipoprotein-related protein 5 (LRP-5) or LRP-6 and frizzled, leading to the accumulation of cellular β-catenin protein through inhibition of protein degradation of β-catenin (reviewed in reference 4). β-Catenin is constitutively degraded through sequential phosphorylation events promoted within at least two distinct molecular complexes. The major complex consists of axin and its associated proteins, and the second complex involves presenilin-1 (PS1) and PS2, the membrane proteins responsible for the intramembranous processing of the β-amyloid precursor protein in Alzheimer's disease (24, 25, 39, 51, 57). β-Catenin phosphorylation is inhibited by Wnt activation of frizzled/LRP receptors, leading to protein accumulation and translocation to the nucleus, where it activates TCF/LEF transcriptional complexes in the promoters of target genes. Additionally, β-catenin can act as a coactivator for several NRs and modify transcription in that manner (29, 32, 50, 52; reviewed in reference 35).

In the present study, we examined the potential relationship between Nurr1 and Wnt signaling. We found that Nurr1 is present in corepressor complexes on TCF/LEF elements prior to Wnt signaling, after which β-catenin binds to Nurr1 and acts as a transcriptional cofactor. Furthermore, we identified KCNIP4 as a β-catenin/Nurr1 target that modulates Wnt signaling by interacting with the PS complex.

MATERIALS AND METHODS

Reagents. Full-length human Nurr1, human β-catenin, and rat LEF-1 cDNAs were cloned into pcDNA3.1 (Invitrogen) and, for Nurr1, also inserted into FLAG- and hemagglutinin (HA)-tagged pIRES2-EGFP Q-vector (Clontech). β-Catenin S33A and mutant promoter vectors were made with a site-directed mutagenesis kit (Stratagene). For glutathione *S*-transferase (GST) pull-down assays, each deletion mutant was inserted into pGEX4-1 5-2 vector (Amersham Biosciences). TOPFLASH was purchased from Upstate Biotechnology. The 2×-NBRE (AAAGGTCA) and promoter regions of cyclin D1 (positions −872 to +8), TH (positions −1110 to 0), and KCNIP4 (positions −1043 to −2) were cloned from a human genomic library (Clontech) and inserted into the PGL3-basic vector containing thymidine kinase to generate luciferase reporters.

The following commercially available antibodies were used: anti-Nurr1, anti-C-terminal binding protein (anti-CtBP), anti-transducin-like enhancer (anti-TLE), anti-protein inhibitor of activated STATy (anti-PIASy), and anti-axin from Santa Cruz Biotechnology; anti-PS1 from Chemicon; anti-CREB-binding protein (anti-CBP) and anti-LEF-1 from Upstate Biotechnology; anti-histone deacetylase 1 (anti-HDAC-1) and anti-HDAC-3 from Affinity Bioreagents; and anti-β-catenin, anti-p45 β-catenin, and anti-p33/37/41 β-catenin from Cell Signaling. Anti-KCNIP4 antibody was obtained from Takeshi Iwatsubo (University of Tokyo).

The following small interfering RNA (siRNA) pools (SMARTpool) were purchased from Dharmacon: Nurr1, M-003427-00; CtBP1, M-008609-01; TLE1, M-015528-00; HDAC-1, M-003493-02; HDAC-3, M-003496-00; PIASy, M-006445-00; β-catenin, M-003482-00; CBP, M-003477-01; KCNIP4, M-021472-00; PS1, M-004998-01; and nonspecific control, D-001210-02-05.

Cell culture. PS1$^{-/-}$ murine embryonic fibroblasts were kindly given by Bart de Strooper (Leuven, Belgium). All cells were routinely maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To establish stable transformants, 293F cells were infected with retrovirus made with Amphopac 293F cells as the packaging cell line. The packaging cell line was made by transfection of pQCXIN retroviral vectors and was cultured for 2 weeks with 750 μg/ml G418 for transformant selection. For large-scale purification, 293F cells were cultured in 293F SFM (Life Technologies) supplemented with Glutamax (Life Technolgies) in a bioreactor.

Nuclear extraction and purification of Nurr1 interactants. Nuclear extracts (28) from 293F stable transformants expressing tagged Nurr1 and from SK-N-MC cells were loaded onto an anti-FLAG M2 affinity resin column and washed extensively (20 mM Tris-HCl [pH 8.0], 300 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Bound proteins were eluted from the column by incubation with 133 g/ml FLAG peptide in washing buffer for 30 min at room temperature. The eluted solution was similarly applied to an HA resin column and washed, and Nurr1 complexes were eluted with the HA peptide.

Immunoprecipitation and GST pull-down assay. Cells were transfected with 5 μg of each expression vector and immunoprecipitated with anti-FLAG antibody (Sigma) for Western blotting with specific antibodies (28). For the GST pull-down assay, full-length human Nurr1, LEF-1, and β-catenin were translated in vitro and incubated with GST-fused mutants of Nurr1, LEF-1, and β-catenin immobilized on glutathione-Sepharose beads prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Chromatin immunoprecipitation (ChIP). Soluble chromatin from 293F or SK-N-MC cells was prepared with an acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) and immunoprecipitated with antibodies against the indicated proteins in the presence and absence of LiCl (28). LiCl stimulation was started 45 min before fixing the cells with formaldehyde (49). Specific primer pairs were designed to amplify the promoter region of cyclin D1 (5′-GGCTCCAGGACTTTGCAACTTC-3′ and 5′-GGCGCCTCAGGGATG G-3′), TH (5′-GCTGTCTCAGCCCCCC-3′ and 5′-CTGGGTCCCCCACCTTC C-3′), the KCNIP4 TCF/LEF site (5′-GTATTCTGCACCTCGGCCCT-3′ and 5′-TACTGCTGCACAAAGTTAGGCTGAG-3′), and the KCNIP4 Nurr-responsive element (NBRE) (5′-CAGCCATAGGGAAGGCAAATAG-3′ and 5′-AGAAGTCAAAATTAAAATGCAGATTTCTGTGTCC-3′) from human genomic DNA. PCR conditions were optimized to allow semiquantitative measurement, and PCR products were visualized on 2% agarose–Tris-acetate-EDTA gels.

Microarray and quantitative RT-PCR analysis. Microarray and quantitative reverse transcription-PCR (RT-PCR) were performed as previously described (37, 42). Gene-specific primers and probes for human KCNIP4 and TH were purchased from Applied Biosystems. For cyclin D1, the following primers and probe were used: probe, 5′-AAGGAGACCATCCCCCTGACGGC-3′; forward primer, 5′-GCATGTTCGTGGCCTCTAAGA-3′; and reverse primer, 5′-CGG TGTAGATGCACAGCTTCTC-3′.

Immunofluorescence. SK-N-MC cells were seeded (40,000/cm^2) on glass coverslips in six-well plates. Twenty-four hours after LiCl treatment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 45 min at 4°C. Primary antibodies were used at a dilution of 1:100 to 1:250 with blocking reagent (Roche) and were incubated for 1 h at room temperature. After four PBS washes (5 min each), secondary antibodies coupled to fluorescein isothiocyanate, tetramethyl rhodamine isocyanate (both from Sigma), or Alexa Fluor488 (Molecular Probes) at a 1:100 dilution were incubated with the cells for 30 min. Cell nuclei were stained with Hoechst 33342 (Molecular Probes), and coverslips were mounted using Slow-fade reagent (Molecular Probes). Immunofluorescence signals were evaluated with a Nikon T300 microscope.

Transfection and luciferase assay. Human 293F cells and SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and, at 40 to 50% confluence, were transfected with plasmids, using Lipofectamine-Plus reagents (Invitrogen) in 12-well dishes. Total amounts of DNA were adjusted by supplementation with up to 1.0 μg of empty vector. Luciferase activities were determined using a luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 2 ng pRL-CMV plasmid (Promega) was cotransfected in all experiments (28, 58). All values are means ± standard deviations for at least three independent experiments. For RNA interference (RNAi), two-step transfection was performed with Trans IT-TKO and Trans IT-NT1 transfection reagents (Mirus) following the manufacturer's recommendations. All siRNAs were evaluated for efficacy by immunoblotting (data not shown).

RESULTS

Transcriptional cross talk between Nurr1 and Wnt signal transduction. We first determined if Wnt signaling regulates the function of Nurr1. Either Wnt-1 or LiCl was used to activate β-catenin-mediated canonical Wnt signaling, and effects on Nurr1 transcriptional activity were measured. 293F cells stably overexpressing Nurr1 or parental control cells, which do not express detectable Nurr1, were transiently transfected with a luciferase reporter plasmid containing a consensus DNA binding site for Nurr1 (NBRE) (17). Overexpression of Nurr1 increased the activity of this reporter, as expected (Fig. 1A). Nurr1 transactivation function was enhanced when Wnt signaling was activated by either Wnt-1, LiCl, or a constitutively

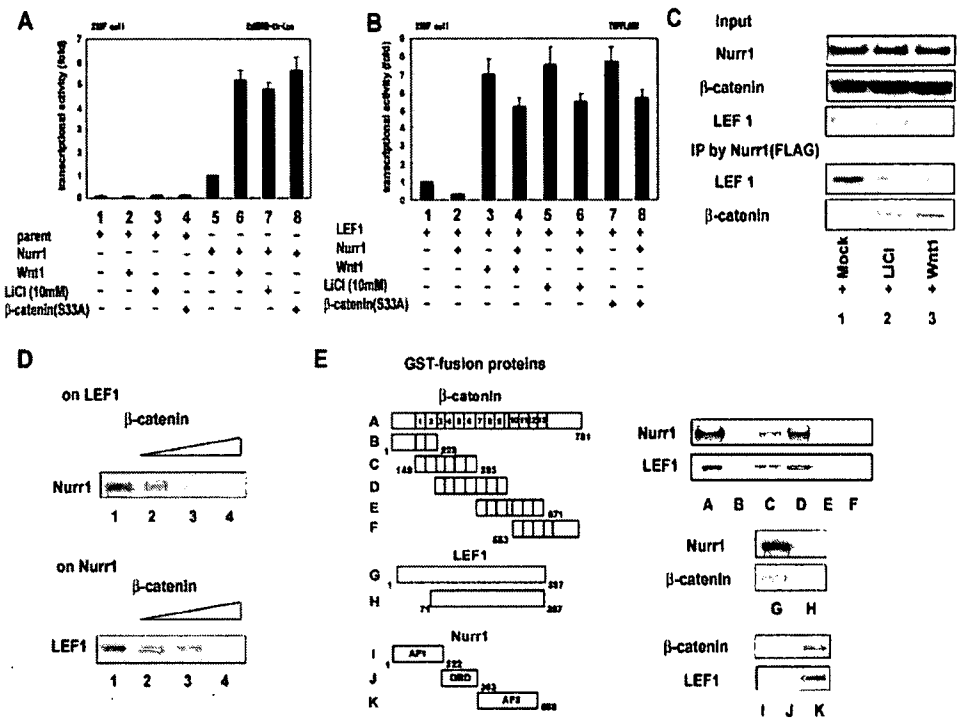

FIG. 1. Convergence of Nurr1 and Wnt signaling. (A) Coactivation of Nurr1 transactivation by Wnt signaling. Luciferase assays were performed with 293F cells transfected with a consensus 2×-NBRE-containing luciferase reporter plasmid (2× NBRE-tk-luc) (400 ng), with or without 200 ng of the indicated expression vector (Nurr1, a constitutively active form of β-catenin [β-catenin S33A], or Wnt-1), in the presence or absence of LiCl (10 mM) or Wnt-1. (B) Nurr1 repression of TCF/LEF transactivation. Luciferase assays were performed with 293F cells transfected with a TOPFLASH luciferase reporter plasmid (containing a consensus TCF/LEF binding site) (400 ng), with or without the indicated expression vectors, in the presence or absence of LiCl (10 mM) or Wnt-1. (C) Signaling-dependent interaction between FLAG-Nurr1, LEF-1, and β-catenin. Exogenous proteins were expressed in 293F cells, which were treated with vehicle, LiCl, or Wnt-1 for 24 h. Transfected cell extracts were subjected to IP with mouse anti-FLAG antibody and then immunoblotted. (D) Competitive binding between β-catenin and Nurr1 for LEF-1. GST-tagged LEF-1 (top) or Nurr1 (bottom) was mixed with in vitro-translated Nurr1 (top) or LEF-1 (bottom) in the presence of increasing levels of unlabeled, in vitro-translated β-catenin. Following GST pull-down, in vitro-translated Nurr1 and LEF-1 were visualized by immunoblotting. (E) Physical interaction and mapping of interaction domains of Nurr1, β-catenin, and LEF-1. Associations of GST-fused, in vitro-translated Nurr1, LEF-1, and β-catenin proteins with the indicated deletions were tested in a GST pull-down assay.

active mutant (S33A) of β-catenin (41) (Fig. 1A). Similar results were observed in other clones from the same transfection (data not shown). We then tested for the opposite modulation by using a reporter construct harboring a consensus DNA binding site for TCF/LEF (TOPFLASH), which is a target of canonical Wnt signaling. Nurr1 overexpression produced a modest but reproducible inhibition of TOPFLASH activity in either the absence or presence of Wnt-1, LiCl, or S33A β-catenin (Fig. 1B). These data suggested that Wnt signaling, specifically through β-catenin, modulates Nurr1 activity and, conversely, that Nurr1 influences β-catenin activity.

Direct physical interaction of Nurr1 with β-catenin and Lef-1. To determine the molecular basis of this mutual coregulation, the physical interaction of Nurr1 with β-catenin and LEF-1 was tested by coimmunoprecipitation of 293F cells overexpressing all three proteins (Fig. 1C). Lef-1, but not β-catenin, was coimmunoprecipitated with FLAG epitope-tagged Nurr1 in nuclear extracts from unstimulated 293F cells. Following stimulation with Wnt-1 or LiCl, β-catenin was recovered with anti-FLAG antibodies, while the amount of co-precipitating Lef-1 was reduced, suggesting that β-catenin competes with Nurr1 for Lef-1 binding (Fig. 1C). GST pull-down experiments showed that this competition is direct. GST–Lef-1 and in vitro-translated β-catenin were aliquoted into four assay tubes with increasing levels of recombinant β-catenin protein. In the absence of recombinant β-catenin,

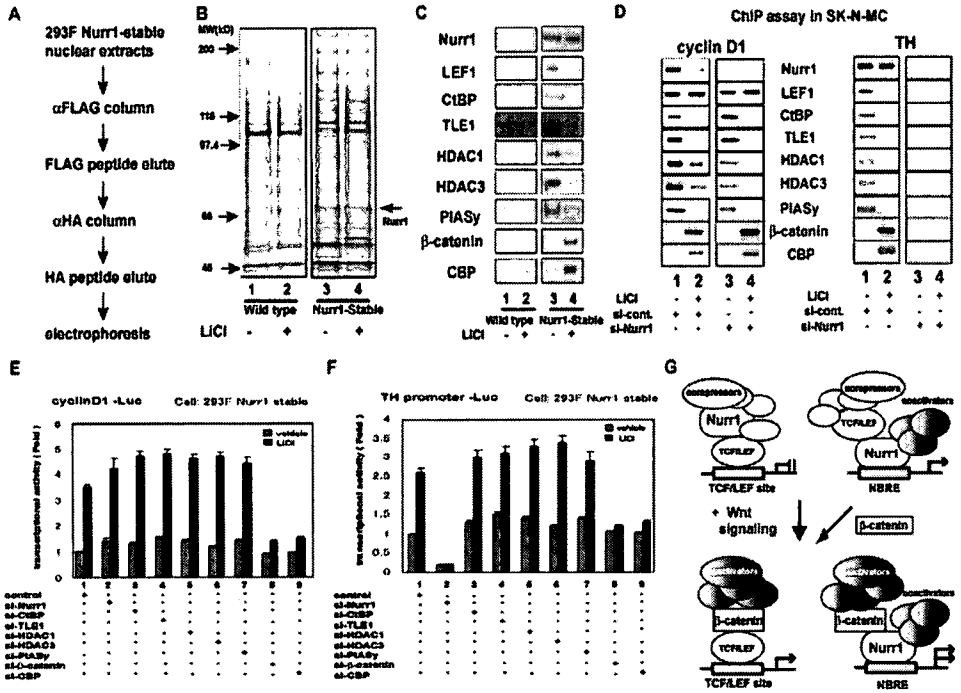

FIG. 2. Functional interplay between Nurr1, β-catenin, and LEF-1. (A) Schematic diagram of the biochemical purification procedure of FLAG/HA-Nurr1 interactants from a 293F cell stable transformant treated with and without LiCl. (B) Fractions eluted from anti-HA resin were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. The arrow on the right indicates the Nurr1 protein. (C) Nurr1 and potential interacting proteins were detected by immunoblotting in eluates from parental and Nurr1-expressing cells treated with or without LiCl. (D) ChIP assays examining the association of the genomic regions containing the TCF/LEF site in cyclin D1 (left) and the NBRE site in TH (right) with Nurr1 and interacting proteins (indicated in the middle) in SK-N-MC cells. siRNAs (100 nM) were transfected prior to a 24-h treatment with LiCl or vehicle. Following cross-linking, sonication, and immunoprecipitation, genomic regions recovered with the indicated antibodies were detected by semiquantitative RT-PCR. (E) Effects of siRNAs on transcriptional regulation of the cyclin D1 promoter in Nurr1-expressing 293F cells. Each siRNA (100 nM) was transfected with a luciferase reporter containing the cyclin D1 promoter (cyclin D1-Luc) in the presence and absence of LiCl. (F) Effects of siRNAs on transcriptional regulation of the TH promoter in Nurr1-expressing 293F cells. siRNAs were transfected as described above with a luciferase reporter containing the TH promoter linked to luciferase (TH promoter-Luc) in the presence and absence of LiCl. (G) Schematic representation of the relationships among Nurr1, LEF-1, and β-catenin under the control of activated canonical Wnt signaling at the TCF/LEF site (cyclin D1) and the NBRE (TH).

GST–Lef-1 pull-down recovered in vitro-translated Nurr1; the addition of β-catenin protein to the reaction mix dose-dependently reduced the amount of Nurr1 recovered. Identical results were seen when GST-Nurr1 was used as the bait for Lef-1 (Fig. 1C).

To determine which of the protein-protein interaction sites in β-catenin possess an affinity for Nurr1, the interacting domains were mapped by GST pull-down assay (Fig. 1E). A series of β-catenin deletion mutants fused to GST were produced, diluted to equal concentrations, and incubated with either in vitro-translated Nurr1 or Lef-1. Constructs containing armadillo repeats 3 to 10 retained the binding affinity for both Nurr1 and Lef-1, suggesting that both proteins interact with β-catenin in this region. Deletion of the Lef-1 N-terminal 70 amino acids caused a loss of binding of Lef-1 to both Nurr1 and β-catenin. Finally, only amino acids 363 to 598 of Nurr1, encompassing the closed ligand-binding domain and AF2, interacted with Lef-1 or β-catenin. These data suggest that the C terminus of Nurr1 binds to the N terminus of Lef-1 and that, in the presence of β-catenin, these interaction sites compete for binding to the armadillo domains of β-catenin.

Characterization of the Nurr1/β-catenin complex. To further characterize the molecular properties of the Nurr1/β-catenin complex, Nurr1 was purified from nuclear extracts of 293F cells stably expressing FLAG/HA-tagged Nurr1 in the presence of LiCl, with untransfected wild-type cells serving as a control (Fig. 2A). Endogenous proteins interacting with Nurr1 were purified through anti-FLAG and then anti-HA

affinity columns, visualized by silver staining (Fig. 2B), and characterized by Western blotting for transcriptional cofactors (Fig. 2C). Silver staining showed that immunocapture of FLAG/HA-Nurr1 resulted in the recovery of Nurr1, at approximately 70 kDa (validated by mass spectrometry [data not shown]), as well as the copurification of many proteins, most of which were equally recovered in the presence or absence of LiCl. The protein mixture was then analyzed by Western blotting for cofactors selected from the literature. In the absence of LiCl, Lef-1 as well as the corepressors CtBP, TLE-1, HDAC-1, HDAC-3, and PIASy (18) were purified with Nurr1 but were not present in controls. LiCl treatment reduced the amounts of these proteins associated with Nurr1 and instead led to the recovery of endogenous β-catenin and CBP, a key component of histone acetyltransferase complexes (9) (Fig. 2C). Thus, β-catenin interaction with Nurr1 coincides with a switch from Nurr1 association with Lef-1 and corepressors to complex formation with CBP.

Identification and function of endogenous Nurr1/β-catenin complexes. The proposed assembly of these factors on endogenous target promoters for TCF/LEF-1 (cyclin D1) and Nurr1 (TH) was then tested by ChIP with SK-N-MC cells, which express endogenous Nurr1 (26) (Fig. 2D). Cross-linked chromatin fragments averaging 250 bp in length were generated from SK-N-MC cells treated with nonsilencing control siRNA or Nurr1 siRNA and were immunoprecipitated with antibodies to Nurr1, Lef-1, and the cofactors identified above as being associated with Nurr1 in 293F cells. Semiquantitative PCR was used to detect the presence of the genomic region containing the TCF/LEF element in the cyclin D1 promoter. As expected, LEF-1 and its corepressors were associated with the cyclin D1 promoter in the absence of LiCl. Nurr1 was also associated with the TCF/LEF region in the cyclin D1 promoter in unstimulated cells. LiCl treatment reduced the amount of Nurr1 associated with the TCF/LEF region as well as the amounts of corepressor proteins, while β-catenin and CBP association increased. These data suggest that the LiCl-mediated cofactor switch observed during purification of overexpressed Nurr1 in 293F cells also occurs on native promoters with endogenous proteins. Since Nurr1 overexpression modestly inhibits TCF/LEF responsiveness to Wnt-1 and LiCl (Fig. 1A), the same analysis was performed with cells transfected with siRNA targeting Nurr1. Nurr1 siRNA treatment caused a loss of Nurr1 association with the TCF/LEF element but did not alter the association of Lef-1 with the cyclin D1 promoter, indicating that Nurr1 is not essential for Lef-1 binding to TCF/LEF elements, as expected. Nurr1 siRNA slightly reduced the amounts of HDAC-1, HDAC-3, and PIASy associated with the TCF/LEF region under unstimulated conditions, but did not abolish them, consistent with Nurr1 modulating TCF/LEF repression. Following LiCl treatment, β-catenin and CBP again were associated with the TCF/LEF element, with more β-catenin association detected than that with control siRNA-treated cells. Like the case under basal conditions, less association between the TCF/LEF region and the corepressors was observed in the presence of Nurr1 siRNA. Thus, Nurr1 siRNA appears to enhance the association of β-catenin with the TCF/LEF region of the cyclin D1 promoter and to reduce the amounts of associated corepressors.

Similar experiments were then performed to analyze endogenous Nurr1 association with the genomic region containing the NBRE in the TH promoter. In the absence of LiCl, Nurr1, Lef-1, and the corepressors were associated with the NBRE-containing region. LiCl promoted the association of β-catenin and CBP and the loss of corepressors without altering the amount of associated Nurr1. Consistent with the requirement for Nurr1 for binding the NBRE, siRNAs targeting Nurr1 abolished Nurr1, corepressor, CBP, and β-catenin association with the NBRE in the TH promoter. Thus, β-catenin interaction with Nurr1 at the NBRE in the TH promoter is associated with a loss of corepressors and recruitment of CBP.

To determine the functional relevance of this β-catenin-associated change in cofactor recruitment, promoter-reporter assays were performed to assess the effects of siRNAs targeting Nurr1, β-catenin, and the cofactors on the response of the cyclin D1 and TH promoters in 293F Nurr1 stable transformants (Fig. 2E and F). siRNAs targeting Nurr1 and the corepressors caused a modest but reproducible increase in cyclin D1 promoter responsiveness to LiCl, consistent with the data obtained using the TOPFLASH reporter (Fig. 1B). Knockdown of β-catenin or CBP nearly completely abolished LiCl responsiveness, confirming the known role for these factors in Wnt-stimulated cyclin D1 expression. As shown in Fig. 2F, TH promoter activity was stimulated by LiCl, similar to the effect observed using the 2×-NBRE reporter construct (Fig. 1A). siRNAs targeting Nurr1 inhibited promoter activity approximately fivefold and abolished LiCl responsiveness, while siRNAs targeting the corepressors tended to cause a small increase in activity of the TH promoter. In contrast, siRNAs targeting β-catenin and CBP abrogated the LiCl responsiveness of the TH promoter. Together, these data confirm the observations that Nurr1 modestly inhibits the cyclin D1 promoter and that the TH promoter is stimulated by LiCl in a process requiring Nurr1, β-catenin, and CBP. These data are summarized graphically in Fig. 2G.

Identification of KCNIP4 gene as a target gene coregulated by Nurr1 and β-catenin. In order to determine if genes other than those for cyclin D1 and TH are mutually regulated by Nurr1/β-catenin, we used microarrays to screen for genes that respond to both Nurr1 overexpression and Wnt-1 stimulation, particularly when both are present. Two clones of 293F cells stably expressing Nurr1 or wild-type parental cells were treated with LiCl or Wnt-1, and resulting changes in RNA levels were scrutinized for genes that responded to Nurr1 and LiCl treatment conditions more robustly than to either condition alone; in parallel, the same experiment was performed following LiCl or Wnt-1 stimulation of SK-N-MC cells transfected with Nurr1 or control vector (data not shown). One candidate gene, that encoding KCNIP4, was selected for further analysis based on these criteria and because we hypothesized it could be regulating Wnt signaling (see below). When analyzed by quantitative real-time RT-PCR, KCNIP4 RNA was approximately twofold lower in Nurr1$^+$ 293F stable transformants than in parental cells, suggesting Nurr1 repression of KCNIP4 expression (Fig. 3A). Following LiCl treatment, KNCIP4 RNA was induced 2.5-fold more in the Nurr1 stable transformants than in controls by 24 h. In SK-N-MC cells, LiCl induced the expression of cyclin D1 RNA, as expected, but also significantly induced TH (2-fold) and KCNIP4 (5.5-fold) RNAs. Similar results were obtained using Wnt-1 (data not shown). Thus,

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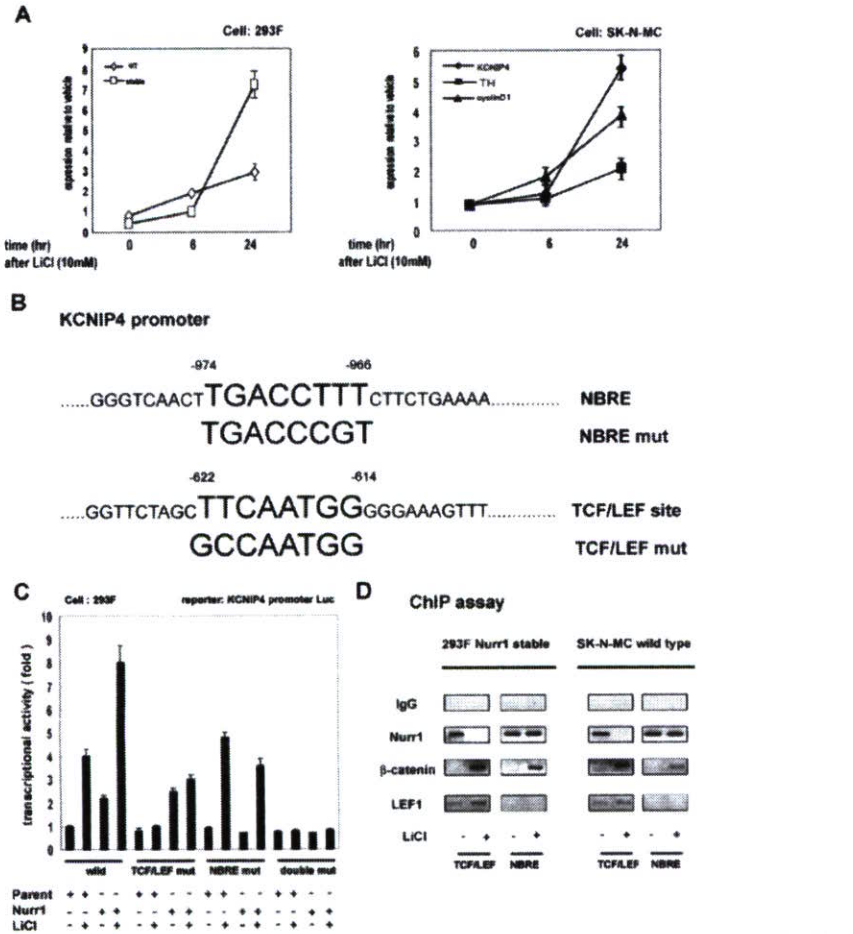

FIG. 3. Identification of KCNIP4 as a direct target of both Nurr1 and Wnt signaling. (A) Induction of the KCNIP4 gene by LiCl in 293F cells and SK-N-MC cells. The gene expression levels were measured in triplicate by quantitative RT-PCR and normalized to those of vehicle-treated parental cells. (B) Schematic presentation of putative binding sites for Nurr1 and TCF/LEF in the KCNIP4 promoter. Binding sites for Nurr1 (NBRE) and TCF/LEF/LY12 (TCF/LEF site) are shown. Two point mutations (NBRE mut and TCF/LEF mut) are displayed for reference. (C) The promoter region of the KCNIP4 gene is regulated by Nurr1 and TCF/LEF elements. Luciferase assays were performed with 293F cells transfected with reporters containing the KCNIP4 promoter or mutants (400 ng), with or without the indicated expression vectors (200 ng) (full-length Nurr1 or parent vector), in the presence and absence of LiCl (10 mM). Data were normalized to values for unstimulated parental cells expressing the wild-type KCNIP4-luciferase construct (first bar). (D) Recruitment of Nurr1 and TCF/LEF to the endogenous KCNIP4 promoter. ChIP analyses were performed with SK-N-MC cells, using specific antibodies for the indicated factors following 10 mM LiCl or vehicle treatment for 24 h. Genomic regions were tested for association with the indicated proteins by semiquantitative PCR.

KNCIP4 gene expression is regulated by Wnt-1/LiCl, and its expression is influenced by Nurr1.

To determine if the mechanism underlying these regulations involved direct transcriptional induction, the putative promoter region for KCNIP4 was searched, revealing potential binding sites for Nurr1 and β-catenin/LEF-1 (Fig. 3B). In a promoter-reporter assay with 293F cells, the promoter region of the KCNIP4 gene (positions −1043 to −2) was responsive to both LiCl treatment and Nurr1 overexpression and, as with the native RNA, showed a greater response when both were

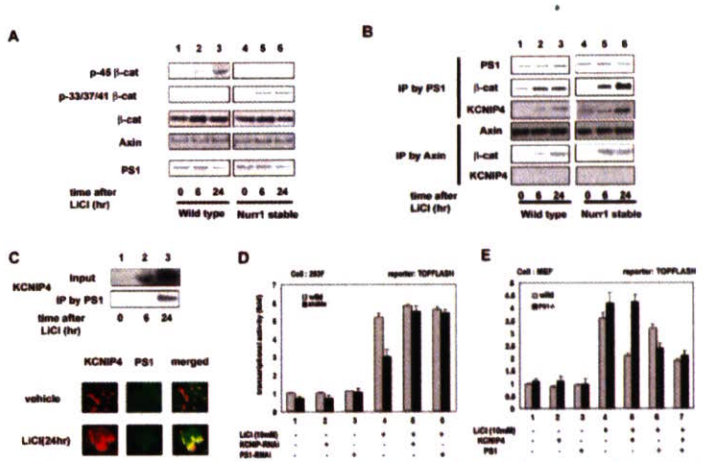

FIG. 4. Modulation of Wnt signaling by KCNIP4 depends on PS1. (A) Nurr1 regulation of the phosphorylation state of β-catenin. Nurr1-expressing or parental 293F cells were treated with 10 mM LiCl and subjected to Western blotting with specific antibodies for the indicated proteins. (B) LiCl-induced assembly of KCNIP4 with PS1 and β-catenin. Coimmunoprecipitation was performed with parental 293F cells or Nurr1 stable transformants treated with 10 mM LiCl. (C) Intracellular colocalization of PS1 with KCNIP4. Native coimmunoprecipitation was performed with SK-N-MC cells before and after LiCl stimulation (upper panels). Immunofluorescence was also performed with antibodies against KCNIP4 and PS1 (lower panels). (D) KCNIP4 and PS1 coregulate transcriptional activity of TCF/LEF in 293F cells. Luciferase assays were performed with 293F parental or Nurr1-expressing cells transfected with TOPFLASH (400 ng) and with the indicated siRNA (100 nM) for 24 h prior to treatment with 10 mM LiCl. (E) Effect of KCNIP4 on TCF/LEF-mediated transcription requires PS1. Luciferase assays were performed with wild-type or $PS1^{-/-}$ mouse embryonic fibroblasts (MEF) transfected with TOPFLASH (400 ng) and with the indicated expression vector (200 ng) in the presence or absence of LiCl (10 mM).

present. Mutational analysis of the KCNIP4 promoter revealed that the putative TCF/LEF and NBRE are required for induction by LiCl and Nurr1, respectively (Fig. 3C). Mutation of the TCF/LEF-like sequence significantly inhibited the response to LiCl, whereas mutation of the potential NBRE inhibited LiCl responsiveness only in the Nurr1 stably transformed cells. Importantly, mutating both sites abolished the response to both LiCl and Nurr1 expression, suggesting that together these sites account for Nurr1 and LiCl sensitivity of the KCNIP4 promoter.

The TCF/LEF and NBRE regions of the KCNIP4 gene promoter were then analyzed by ChIP assays with both 293F and SK-N-MC cells (Fig. 3D). In both cell types, Nurr1 was associated with the genomic regions containing the TCF/LEF and NBRE sequences in the absence of LiCl. β-Catenin was not associated with either sequence, and Lef-1 was associated only with the TCF/LEF-containing region, confirming that the assay distinguished between these elements, which are situated 344 bp apart. As observed with the cyclin D1 promoter, LiCl caused β-catenin to associate with and Nurr1 to dissociate from the TCF/LEF region, while Lef-1 association remained constant. The response of the putative NBRE was similar to that observed with the TH promoter: LiCl caused a recruitment of β-catenin with no change in Nurr1 association. These data, together with the promoter-reporter assay data, suggest that KCNIP4 gene expression is regulated by Nurr1 and β-catenin interaction at or near sequences resembling TCF/LEF and NBRE sites.

PS1-mediated degradation of β-catenin is regulated by KCNIP4. KCNIP4 is a protein that has been reported to interact with PS1 (34), a polytopic integral membrane protein that regulates β-catenin degradation in the cytoplasm (24). To determine if KCNIP4 induction by Nurr1/β-catenin influences PS1-mediated β-catenin phosphorylation, we first examined the casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β) phosphorylation sites of β-catenin (p45 and p33/37/41, respectively) in the presence and absence of Nurr1. CKIα phosphorylation precedes GSK3β phosphorylation, which in turn triggers β-catenin degradation; PS1 regulates the GSK3β phosphorylation steps specifically (24). When cells were treated with the GSK3β inhibitor LiCl in the absence of Nurr1, β-catenin phosphorylated at the CKIα p45 site accumulated, whereas no accumulation of p33/37/41 was observed (Fig. 4A). However, in the presence of Nurr1 cDNA, p33/37/41 forms of β-catenin accumulated and p45 forms did not. Thus, Nurr1 expression alters the GSK3β-dependent phosphorylation status of β-catenin.

Next, coimmunoprecipitation experiments were used to examine the effects of Nurr1 expression and LiCl stimulation on the assembly of cytoplasmic β-catenin complexes (Fig. 4B). 293F Nurr1 stable transformants or parental control cells were treated with LiCl, and the two major regulators of β-catenin degradation, PS1 and axin, were collected by immunoprecipitation. In parental cells, LiCl caused the expected increase in β-catenin coimmunoprecipitated with PS1 and axin. In contrast, the KCNIP4 protein displayed a time-dependent associ-

ation specifically with PS1 but not with axin. Nurr1 expression increased the relative abundance of β-catenin and KCNIP4 associated with PS1 but had no detectable effect on the axin/β-catenin complex. Further evidence for LiCl-mediated KCNIP4 interaction with PS1 was obtained by examining the intracellular localization of KCNIP4 protein in SK-N-MC cells. LiCl treatment induced KCNIP4 protein expression, as seen by Western blotting and immunofluorescence (Fig. 4C). After 24 h, the KCNIP4 protein was coimmunoprecipitated with PS1 and appeared to be colocalized partially with PS1, as seen in the merged images of the KCNIP4 and PS1 fluorescent signals. Thus, Nurr1/β-catenin induction of KCNIP4 is associated with remodeling of the PS1 signaling complex that regulates β-catenin phosphorylation.

PS-dependent Nurr1 repression of β-catenin signaling. The significance of PS1-KCNIP4 interaction was tested in 293F cells by RNAi and in mouse embryonic fibroblasts derived from PS1 knockout mice ($PS1^{-/-}$) (3). As shown above, Nurr1 overexpression modestly reduced TOPFLASH reporter activity in 293F cells (Fig. 4D). siRNA targeting either PS1 or KCNIP4 restored TOPFLASH activity to that seen in parental cells not overexpressing Nurr1, suggesting that KCNIP4 and PS1 are required for Nurr1-mediated TOPFLASH repression. This hypothesis was then tested in $PS1^{-/-}$ fibroblasts transfected with KCNIP4 or PS1 cDNA (Fig. 4E). Transfection with either plasmid had no effect on TOPFLASH activity in unstimulated wild-type or $PS1^{-/-}$ cells. In wild-type fibroblasts, as in 293F cells, KCNIP4 overexpression repressed LiCl-induced TOPFLASH activity, confirming that KCNIP4 inhibits β-catenin signaling. In $PS1^{-/-}$ embryonic fibroblasts, cells showed a slightly greater TOPFLASH response to LiCl than did wild-type cells, as reported previously (24), reflecting the relatively minor but biologically significant (57) contribution of PS1 to inhibiting β-catenin activity. Interestingly, the inhibitory effect of KCNIP4 cDNA was absent in $PS1^{-/-}$ cells, suggesting that KCNIP4 repression of β-catenin signaling requires PS1. Consistent with this hypothesis, PS1 cDNA cotransfection into $PS1^{-/-}$ cells inhibited TOPFLASH activity and restored the inhibitory effect of KCNIP4. These data together indicate that Nurr1/β-catenin induction of KCNIP4 serves to inhibit β-catenin signaling in a PS1-dependent feedback loop.

Nurr1 regulation of nuclear β-catenin levels. A prediction of the above model is that Nurr1 regulates the accumulation of β-catenin in the nucleus. To test this possibility, nuclei were prepared from cells treated with LiCl, with or without Nurr1 expression. In SK-N-MC cells (Fig. 5A, top two panels), LiCl caused a transient increase in nuclear β-catenin; cotransfection of siRNA directed towards Nurr1 elevated the amount and duration of nuclear β-catenin. Conversely, transfection of Nurr1 cDNA into 293F parental cells (Fig. 5A, bottom five panels) inhibited nuclear β-catenin accumulation. Interestingly, functional human variants of Nurr1 that possess reduced transcriptional activity (31) also alter the amount of nuclear β-catenin. We then tested the effect of these mutations on the Wnt signaling pathway by transiently transfecting Nurr1 cDNAs into 293F parental cells. As expected, the mutant forms of Nurr1 were less able to activate the NBRE-luciferase reporter than was wild-type Nurr1, but all were responsive to the additional stimulatory effects of LiCl (Fig. 5A). Nurr1 mutants had a generally reduced ability to inhibit LiCl-mediated stimulation of TOPFLASH activity, such that those most impaired in transactivation were also less able to suppress TOPFLASH activity. In contrast, the mutations did not affect KCNIP4 promoter activity in a consistent manner. Thus, human mutations in Nurr1 appear to affect not only transcriptional activation from an NBRE but also Nurr1 regulation of Wnt signaling pathways in a complex manner.

DISCUSSION

Nurr1 is an orphan NR best known for its essential role in the development and maintenance of the midbrain dopaminergic neurons that regulate motor control and degenerate during Parkinson's disease. Here we tested the possibility that Wnt signaling regulates Nurr1, as Wnt is critical for the regional specification of the midbrain (5) and influences the proliferation and differentiation of $Nurr1^{+}$ neuronal precursors in cell culture (6, 7). We found that β-catenin acts as a transcriptional cofactor for Nurr1, most likely by direct physical association (although other possibilities, including interaction of β-catenin with RXR or other Nurr1-associated factors, are not excluded by the current data). This interaction changes the transcriptional machinery associated with Nurr1 from a corepressor to a coactivator complex. Furthermore, we found that Nurr1 regulates Wnt signaling and propose that the major mechanism of inhibition is by inducing KCNIP4, a Nurr1/β-catenin target that functions as a regulatory subunit of the PS1 complex promoting β-catenin degradation. These findings are summarized in a model that incorporates these data (Fig. 6). It is important that the contribution of this proposed signaling system to dopaminergic cell differentiation and maintenance remains to be explored in vivo. It will be interesting to analyze mice that have a dopaminergic cell-specific deletion of β-catenin, PS1, or KCNIP4.

Several extracellular signals have been reported to modulate the transcriptional function of NRs through exchanging coregulators and cointegrators (14, 40). In this study, we show that Nurr1 can exist in corepressor complexes that are remodeled to CBP-containing coactivator complexes by β-catenin. This finding extends the role that β-catenin plays in NR signaling, as synergistic interactions with the androgen receptor and several other NRs have been described (35).

In vitro β-catenin interacts predominantly with the C-terminal domain of Nurr1 (Fig. 1), which is significant considering that Nurr1, as well as the other members of the NR4A group, rely heavily on the N-terminal AF1 domain for transcriptional regulation, unlike many NRs (33, 38, 56). The Nurr1 C-terminal domain possesses a cell type-specific transactivation function that does not normally interact with common NR coactivators, such as SRC-1 (8), as it lacks the common NR coactivator binding site (12, 55). This C-terminal activity was proposed to be regulated instead by tissue-specific ligands or cofactors (8). Our data suggest that β-catenin is one of these factors and that it acts somewhat similarly to an endogenous ligand in that it activates by interacting with the C-terminal domain. Given the unique function and structure of the Nurr1 C-terminal domain, it would be interesting to determine the structural changes in Nurr1 brought about by β-catenin binding. The precise binding site for β-catenin within the Nurr1 C-terminal domain has not been determined, but the recent

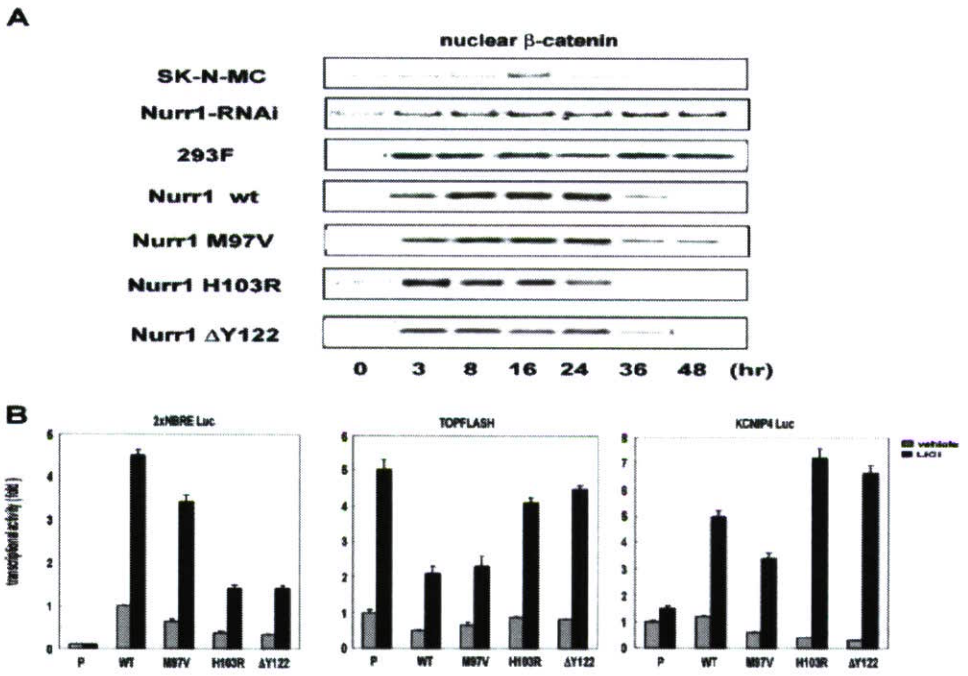

FIG. 5. Nurr1 modulates the degradation of β-catenin. (A) Duration of nuclear accumulation of β-catenin in SK-N-MC cells transfected with control or Nurr1 siRNA and in 293F cells stably expressing wild-type or mutant Nurr1. Nuclear extracts were subjected to Western blotting at each time point after 10 mM LiCl stimulation. (B) Transcriptional properties of each Nurr1 mutant on NBRE, TOPFLASH, and KCNIP4 promoter. Luciferase assays were performed with 293F cells transfected with the indicated reporters (400 ng) and stably expressing wild-type or mutated Nurr1 in the presence or absence of LiCl (10 mM).

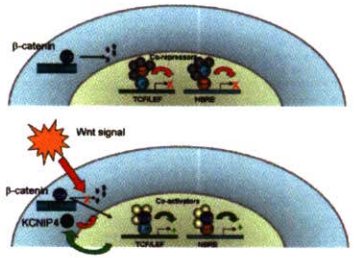

FIG. 6. Model for Nurr1 and Wnt cross-regulation. In the absence of Wnt signaling (top), Nurr1 is associated with corepressors in genes containing TCF/LEF binding sites, and Lef-1 is associated with Nurr1 on Nurr-responsive elements. Following Wnt-mediated β-catenin accumulation in the nucleus (bottom), TCF/LEF genes become derepressed and β-catenin switches Nurr1-associated proteins from corepressors to coactivators. Both β-catenin and Nurr1 induce KCNIP4, which associates with a membrane-associated PS1 complex and promotes β-catenin degradation in a negative feedback loop.

discovery of a large, functionally important hydrophobic pocket opposite the place where the classic coactivator binding site would normally be (53) raises this location as a possibility.

Using microarrays to identify an endogenous gene controlled by this convergent signaling pathway, we found the KCNIP4 gene. The KCNIP4 promoter contains apparent Lef-1 and Nurr1 response elements, and interestingly, the KCNIP4 protein interacts with the cytoplasmic domain of PS1, which acts as a scaffold for β-catenin signaling and degradation (24, 25, 39, 51, 57). We found that KCNIP4 promotes PS-mediated degradation of β-catenin and thus appears to be a regulatory subunit for the PS complex. Transcriptional induction of KCNIP4 appears to be a primary mechanism by which Nurr1 inhibits β-catenin activity, since the absence of KCNIP4 or PS1 abrogated the ability of Nurr1 to inhibit TOPFLASH. Although, as shown in Fig. 2, Nurr1 expression increases and Nurr1 siRNA decreases the levels of corepressors associated with the TCF/LEF region of the cyclin D1 promoter, these effects could also be indirect through the modulation of β-catenin cytoplasmic stability via KCNIP4. Further experiments will be required to determine if Nurr1 plays a significant role in

directly inhibiting the transcription of Wnt target genes or, rather, acts largely through the induction of KCNIP4. The physiological relevance of the modest inhibitor effect of Nurr1 on Wnt-1 signaling remains to be studied, but for cell culture our preliminary microarray data revealed that Wnt-1-regulated genes were, on average, 22% less responsive in 293F cells overexpressing Nurr1 than in parental controls (mean of two replicates with different subclones [$n = 135$ RNAs]; $P < 0.001$) (unpublished observations). It will be interesting to determine if Nurr1$^+$ neuronal precursors respond differentially to Wnt signaling compared to Nurr1$^-$ cells in vivo.

In addition to the well-studied role for Wnt signaling during CNS development of the VM dopaminergic system, aberrant Wnt signaling has been implicated in several psychiatric and neurological disorders, such as bipolar disorder (19) and schizophrenia (16). Given the importance of the dopaminergic system in these diseases, it is conceivable that Wnt signaling could, in part, affect the activity of Nurr1. Interestingly, both LiCl and valproic acid, drugs useful in a number of mental disorders, inhibit GSK3β, and recent data suggest that certain antipsychotics modulate this system as well (1, 2, 30). We show here that naturally occurring Nurr1 mutants have a reduced ability to inhibit β-catenin transcription from a TCF/LEF element and appear to differentially regulate β-catenin accumulation and KCNIP4 promoter activation. Future studies that determine if the functional interplay between β-catenin and Nurr1 varies with disease state and is modulated by genetic factors will be of interest.

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The Pituitary Function of Androgen Receptor Constitutes a Glucocorticoid Production Circuit▽

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Androgen receptor (AR) mediates diverse androgen actions, particularly reproductive processes in males and females. AR-mediated androgen signaling is considered to also control metabolic processes; however, the molecular basis remains elusive. In the present study, we explored the molecular mechanism of late-onset obesity in male AR null mutant (ARKO) mice. We determined that the obesity was caused by a hypercorticoid state. The negative feedback system regulating glucocorticoid production was impaired in ARKO mice. Male and female ARKO mice exhibited hypertrophic adrenal glands and glucocorticoid overproduction, presumably due to high levels of adrenal corticotropic hormone. The pituitary glands of the ARKO males had increased expression of proopiomelanocortin and decreased expression of the glucocorticoid receptor (GR). There were no overt structural abnormalities and no alteration in the distribution of cell types in the pituitaries of male ARKO mice. Additionally, there was normal production of the other hormones within the glucocorticoid feedback system in both the pituitary and hypothalamus. In a cell line derived from pituitary glands, GR expression was under the positive control of the activated AR. Thus, this study suggests that the activated AR supports the negative feedback regulation of glucocorticoid production via up-regulation of GR expression in the pituitary gland.

Sex steroid hormones exert a wide variety of biological actions. They are also involved in pathological events, such as the development of hormone-dependent cancers in reproductive organs (5, 37). In vertebrates, sex hormones play a pivotal role in male reproductive function and metabolic control. Most sex steroid actions are mediated through transcriptional control of target genes by nuclear receptors (NRs). NRs form a gene superfamily and act as transcriptional factors (9, 20). Sex hormone receptors have been shown to transactivate particular sets of target genes in a hormone-dependent manner through direct DNA binding to specific elements in target gene promoters. Hormone receptors activated by hormone binding recruit a number of coregulator-coregulator complexes for transactivation (28). These complexes then affect transcription through chromatin remodeling (12, 17, 22) and histone modification (1, 7). Hormone binding to the receptors may also transrepress target genes. The mechanisms of hormone-dependent transrepression of steroid receptors likely involve protein-protein interactions and are thus more diverse than that of transactivation (8, 10, 13, 21).

The molecular mechanisms behind the regulation of gene transcription by hormones and their NRs are complicated. Gene disruption studies have clarified the role of various NRs in steroid hormone action. By combining a Cre-loxP system with a canonical gene disruption approach, we succeeded in disrupting the androgen receptor (AR) on the X chromosome in mice in a manner that did not result in male infertility (14). Male AR null mutant (ARKO) mice exhibit abnormalities typical of testicular feminization mutants, including female external genitalia with atrophic testis and impaired sex behavior (29). Growth of the male ARKO mice is partially retarded, with impaired bone growth coupled with high bone turnover (16). The male mice also develop late-onset obesity (30). In contrast, no clear phenotypic abnormalities are present in female ARKO mice. However, normal folliculogenesis does require the AR, which suggests that androgen/AR signaling is also physiologically important in females (32).

To study how and why obesity develops in ARKO males, we began by examining the adrenal glands, which were hypertrophic in both males and females. In the present study, we explored the molecular basis of this observation. Dissection of the gland revealed that the layers of the zona fasciculata were thicker and coupled to the remaining layers of the X-zone (fetal zone). The hypertrophy resulted from a hypercorticoid state. Adrenal corticotropic hormone (ACTH) overproduction was driven by impaired negative feedback through the hypothalamus-pituitary-adrenal (HPA) axis. No clear alteration in the numbers of hormone-producing cells in the pituitary glands and hypothalamus was detected, but there were increased proopiomelanocortin (POMC) and decreased glucocorticoid receptor (GR) expression levels of transcripts in the ARKO pituitary glands. Androgen-induced GR gene activation was further confirmed in a pituitary gland-derived cell line (AtT-20 cells). These findings suggest that androgen/AR signaling in the pituitary gland supports the normal feedback system of glucocorticoid production through the HPA axis.

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MATERIALS AND METHODS

Animals. ARKO mice were generated by targeted disruption of the AR gene by means of a Cre-loxP system (19) and maintained as described previously (16, 29, 30, 32). Experiments were performed with 2- to 25-week-old male mice. All mice protocols were approved by the Animal Care and Use Committee of the University of Tokyo (31, 40).

Cell culture. Adherent AtT-20 cells, a murine corticotropic tumor cell line, were cultured in a 5% CO_2 atmosphere at 37°C with Dulbecco's modified Eagle's medium-Ham's F12 at 1:1 containing 15% fetal calf serum (FCS) and penicillin-streptomycin. 3T3-L1 cells, a murine preadipocyte cell line, were cultured with Dulbecco's modified Eagle's medium containing 10% FCS. FCS in the culture media was replaced with charcoal-treated FCS for 1 week prior to the administration of 5α-dihydrotestosterone (DHT). For Northern and Western blot analyses, the cultured AtT-20 cells were subcultured in six-well plates. After incubation for 24 h, DHT (10^{-7} M) was added to the medium.

Histology and immunohistochemistry. Adrenal glands and pituitary glands were fixed by immersion with 4% paraformaldehyde for 24 h at 4°C. They were then embedded in paraffin, sliced into 4-μm sections by standard methods, and mounted onto silane-coated slides. After perfusion by 0.9% saline followed by 4% paraformaldehyde, brains were postfixed in the same fixative for 2 h at 4°C and soaked in phosphate-buffered saline containing 20% sucrose. Frontal sections were cut at 30-μm thickness using a cryostat. Serial sections were divided into four groups and used for single-labeling immunohistochemistry for the GR, corticotropin-releasing hormone (CRH), α-melanocyte-stimulating hormone (α-MSH), or thionin to allow determination of the areas to be measured.

Immunostaining was carried out using antibodies as described below (34). The primary antibodies included rabbit polyclonal anti-human AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-GR (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human ACTH (DAKO, Carpinteria, CA), mouse anti-human luteinizing hormone β (Immunotech, Marseille, France), mouse anti-human follicle-stimulating hormone β (DAKO, Carpinteria, CA), mouse anti-human thyroid-stimulating hormone β (Advanced Immunochemical Inc., CA), rabbit anti-rat glycoprotein hormone (kindly supplied by A. F. Parlow, the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, MD), rabbit anti-human growth hormone (DAKO, Carpinteria, CA), and rabbit anti-rat prolactin (kindly supplied by A. F. Parlow, NIDDK).

After treatment with 0.5% H_2O_2 (30 min) and 5% normal serum (1 h), the sections were incubated for 24 h at 4°C with specific primary antibodies. The sections were then incubated with secondary antibodies and an avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories). The signals were visualized with diaminobenzidine and the nuclei were counterstained with hematoxylin.

For dual labeling of ACTH and the AR or GR, a single staining of the AR or GR was first performed as described above. After the primary antibodies were removed by treatment with 0.1 M glycine, sections were incubated with anti-ACTH antibodies followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G (DAKO, Carpinteria, CA). The signals were visualized with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

Detection of proliferation and apoptosis of adrenal gland cells. Eight-week-old mice were injected intraperitoneally (i.p.) with the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU) (30 mg/g body weight [BW]) every 12 h five times (25). Mice were fully anesthetized and their adrenal glands removed 12 h after the last injection. Incorporated BrdU was detected immunohistochemically using a mouse monoclonal anti-BrdU antibody. The proliferative index was defined as the number of BrdU-positive cells per microscopic field. Five fields per mouse were counted for each of three wild-type (WT) and three ARKO mice.

Cells undergoing apoptosis were identified by digoxigenin labeling of the free 3′OH ends of fragmented DNA by use of terminal deoxynucleotidyltransferase (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] assay). Assays were performed on sections from the same tissue blocks used for BrdU immunohistochemistry. Sections were counterstained with hematoxylin to facilitate cell counting. The fraction of apoptotic cells was defined as the fraction of diaminobenzidine-positive cells per total number of cells. Five fields per mouse were counted for each of three WT and three ARKO mice.

Serum endocrine parameters. A circadian rhythm experiment and dexamethasone suppression tests were performed on 8-week-old male mice as previously described (2). For the circadian rhythm experiment, blood was collected at 08:00 or 18:00 h. For the dexamethasone suppression tests, mice were injected i.p. with different doses of dexamethasone (0, 2, or 5 μg/20 g BW) in 0.3 ml of 0.9% saline. Injections were performed between 08:00 and 08:30 h and blood was collected 6 h later. Mice were fully anesthetized and blood was collected by cardiac puncture. Plasma ACTH and serum corticosterone were measured using radioimmunoassay kits (IRMA; Mitsubishi, Tokyo, Japan) at SRL (Tokyo, Japan), according to the manufacturers' instructions. Measurements were independently duplicated, and interassay variability and buffer dilution were corrected for by using internal correction factors.

RNA extraction and mRNA quantitation. Total mRNA was extracted from pituitary glands with TRIzol (Invitrogen) for reverse transcription-PCR (RT-PCR) and Northern blotting (35). To remove any possible DNA contamination prior to semiquantitative RT-PCR, the DNA was digested with RNase-free DNase. The digested total mRNA (2 μg) was subjected to RT using SuperScript reverse transcriptase (Invitrogen) primed by oligo(dT) primers. After first-strand cDNA synthesis, 1 ml from a 5% reaction mixture was diluted serially (2- to 128-fold). Amplification was performed with *rTaq*DNA polymerase (Takara) using primer pairs for GAPDH as an internal control to allow for concentration estimation (38). Expression levels of transcripts were measured using the standardized cDNA and specific primer pairs. The validity of the PCR products was confirmed by direct sequencing.

Western blot analysis. The lysates of mouse tissue and AtT-20 cells were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (15, 39). Membranes were probed with rabbit polyclonal anti-GR antibody (M-20; Santa Cruz Biotechnology) and goat polyclonal anti-β-actin antibody (I-19; Santa Cruz Biotechnology) as an internal control. The blots were visualized using peroxidase-conjugated anti-rabbit antibody and anti-goat antibody, together with an ECL detection kit (Amersham Biosciences). The small interfering RNA analysis used AR and control small interfering RNA (Ambion), and transfection was accomplished with the Lipofectamine 2000 system (Invitrogen).

Luciferase reporter assay. GR promoter regions (upstream regions of exons 1B, 1C, and 2) were cloned by PCR and subcloned into a luciferase reporter gene driven by a tk promoter (tk-luc). PCR primers were as follows: for 1B Fw, 5′-GGCATAGTTAGGCCACTAAAGAGA-3′; for 1B Rv, 5′-GGGAGAAGT TGCAAAGCAGA-3′; for 1C Fw, 5′-CTGGAGCAGCAAATGTCAAG-3′; for 1C Rv, 5′-AGCTCGCAAAATGGAGGAG-3′; for 2 Fw, 5′-GGATCTGGCGT CCTTTTC-3′; and for 2 Rv, 5′-CCACATTATCTCTGATCCGATT-3′.

For the luciferase reporter assay, cultured cells were transfected with the indicated plasmids using the Lipofectamine Plus reagent (Invitrogen) into 24-well plates at 40 to 50% confluence. The total amount of DNA was adjusted by supplementing with empty vector up to 1.0 μg/well. Luciferase activity was determined using a dual luciferase assay system (Promega). As a reference plasmid to normalize the transfection efficiency, 1.5 ng/well of pRL-CMV plasmid (Promega) was cotransfected in all experiments.

Statistical analysis. Values are given as the means ± standard deviations. Comparisons between two groups were made by Student's *t* test. *P* values of <0.05 were accepted as statistically significant.

RESULTS

High serum levels of ACTH and corticosterone in male ARKO mice. The male ARKO ($AR^{1,-/Y}$) mice exhibited growth retardation in comparison to WT male mice until 10 weeks of age but then showed catch-up growth over the next few weeks. Thereafter, the male ARKO mice weighed more than the WT mice and developed severe obesity (Fig. 1A) as previously reported (6, 30). Obesity to this extent was not seen for female ARKO ($AR^{1,-/1,-}$) mice. To identify causes for the late-onset obesity in male ARKO mice, serum endocrine parameters were measured. We found that serum corticosterone levels in male ARKO mice were elevated at 8 weeks of age and became significantly higher at 13 and 20 weeks ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 1B).

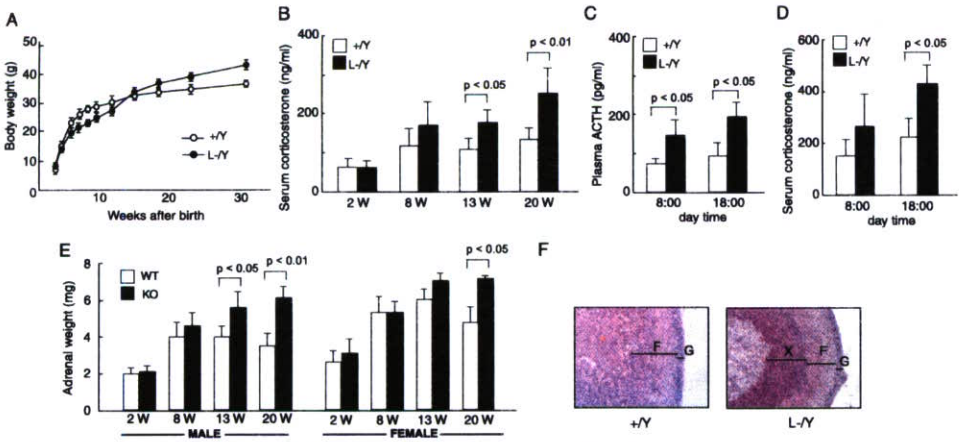

FIG. 1. Hypertrophic adrenal glands with high serum levels of ACTH and corticosterone in ARKO mice. (A) Growth curves of ARKO and WT littermate mice. The floxed AR mice (female, $AR^{L+/L+}$; male, $AR^{L+/Y}$) were crossed with Cre-CMV transgenic mice to generate ARKO male ($AR^{L-/Y}$) and female ($AR^{L-/L-}$) mice (16, 30). (B) Serum corticosterone levels of ARKO and WT mice at 2, 8, 13, and 20 weeks (W) of age. (C) Plasma ACTH levels of ARKO and WT mice measured in the morning (8:00) and evening (18:00). (D) Serum corticosterone levels of ARKO and WT mice in the morning (8:00) and evening (18:00). (E) Adrenal gland weights of male and female ARKO and WT mice at 2, 8, 13, and 20 weeks of age. (F) Histology of ARKO and WT adrenal glands. All sections were stained with hematoxylin and eosin. F, zona fasciculata; G, zona glomerulosa; X, X-zone.

To more carefully examine the hypercorticoid state in male ARKO mice, we measured the serum levels of corticosterone and its upstream hormone ACTH. Measurements were taken both in the morning and in the evening, as these hormones exhibit a circadian rhythm. As expected, the 8-week-old WT and ARKO mice had low levels of both hormones in the morning and higher levels in the evening (Fig. 1C and D). Overall, the ARKO males tended to have high levels of ACTH and corticosterone at any time compared to WT mice. They had significantly high levels of plasma ACTH at both 8:00 and 18:00 and high levels of corticosterone at 18:00 compared to WT mice ($P < 0.05$) (Fig. 1C and D). However, for female ARKO mice, though serum levels of these hormones tended to be higher than in WT littermates, the differences were not statistically significant (data not shown). The obesity seen for these mice was likely the result of their hypercorticoid state, as centripetal obesity is a typical symptom of Cushing's syndrome. In the following experiments, we explored the etiology of the hypercorticoid state in the ARKO mice.

Hypertrophic adrenal glands in ARKO mice. To investigate the hypercorticoid state in the ARKO mice, we first examined the adrenal glands. The adrenal glands in the ARKO males clearly weighed more than the glands of WT mice at 13 weeks of age (Fig. 1E). This coincided with the onset of obesity and the hypercorticoid state. Likewise, in ARKO females, the adrenal glands also increased in size in comparison to what was seen for WT littermate females; however, the growth was not as pronounced as that in ARKO males (Fig. 1E). The adrenal glands of male ARKO mice were then used for subsequent experiments.

The adrenal cortex forms the major part of the gland and is divided into three layers in mammals: the zona glomerulosa, immediately beneath the capsule, followed by the zona fasciculata and the zona reticularis. The zona reticularis is replaced in rodents by the X-zone, which develops prenatally and begins to degenerate at pubertal maturity in males. In mice, corticosterone, the major glucocorticoid in rodents, is produced in the zona fasciculata, while aldosterone, the most potent mineralocorticoid, is formed in the zona glomerulosa. Hematoxylin and eosin staining of adrenal glands in 13-week-old mice revealed that the enlargement of the adrenal glands in ARKO males was caused by cellular hypertrophy of the zona fasciculata as well as by a failure of X-zone (fetal zone) regression (Fig. 1F). Since glucocorticoids are produced in the zona fasciculata, it is likely that the overproduction of corticosterone is the result of the hypertrophy in this area.

Increased proliferation and decreased apoptosis in the adrenal cortex of ARKO males. The failed regression of the X-zone in 13-week-old ARKO males raised the possibility of impaired cell death or decreased apoptosis in the adrenal cortex. Indeed, the percentage of apoptotic cells in the zona fasciculata, detected by TUNEL assay in the ARKO males, was clearly less (19.5%) than that for WT mice (33.1%) (Fig. 2A). When actively proliferating cells of the adrenal glands were counted by BrdU labeling in WT and ARKO males, 2.5 times more BrdU-labeled cells/section were found in ARKO mice. This suggests increased proliferation in the adrenal cortex of ARKO mice (Fig. 2B). Thus, the hypercorticoid state likely results from the overproduction of glucocorticoid by the hypertrophic zona fasciculata. This hypertrophy is caused by chronic exposure to high levels of ACTH.

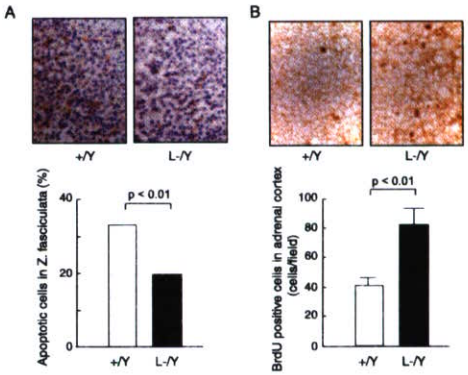

FIG. 2. Increased proliferation and decreased apoptosis in ARKO adrenal glands. (A) Decreased apoptosis in the ARKO adrenal glands. Histogram showing the number of TUNEL-positive cells in the zona fasciculata (Z. fasciculata). (B) Increased proliferation in the ARKO adrenal cortex. Histogram showing the number of BrdU-positive cells.

The HPA negative feedback system for glucocorticoid production is impaired in ARKO males. Glucocorticoid synthesis is regulated by a negative feedback loop via the HPA axis involving CRH and ACTH, produced by the hypothalamus and pituitary glands, respectively. We assessed whether the axis was intact and functioning normally in the ARKO males with a dexamethasone suppression test. As expected, serum corticosterone levels were down-regulated in 8-week-old WT mice 6 hours after i.p. injection of either 2 μg/20 g BW or 5 μg/20 g BW of dexamethasone (Fig. 3A). In ARKO mice, injection with 2 μg/20 g BW of dexamethasone did not suppress the serum levels of corticosterone. However, a dose of 5 μg/20 g BW was effective in lowering the serum levels of corticosterone in ARKO mice (Fig. 3A). This is similar to the high-dose dexamethasone suppression seen for patients with central Cushing's syndrome. Plasma ACTH levels in both the ARKO and WT males were decreased 6 h after dexamethasone injection at both the low and high doses; however, suppression was less sensitive in the ARKO males than in the WT males (Fig. 3B). No statistical difference in ACTH levels was detected between ARKO and WT males in this suppression test.

No overt abnormalities were present in the hypothalami or pituitary glands of ARKO males. The results of the suppression tests suggested that the adrenal hypertrophy of the ARKO males resulted from the hyperfunction of the hypothalami and/or pituitary glands. To address this issue, the hypothalami and pituitary glands of 8-week-old ARKO males were histologically examined. No overt abnormalities were detected in sections of the ARKO mice stained with hematoxylin and eosin (Fig. 4A). Immunohistochemical staining of the pituitary glands demonstrated similar numbers of cells expressing pituitary hormones in WT and ARKO mice (Fig. 4B). AR protein expression was detectable in several types of hormone-producing cells in the WT males but was absent in the ARKO mice (data not shown).

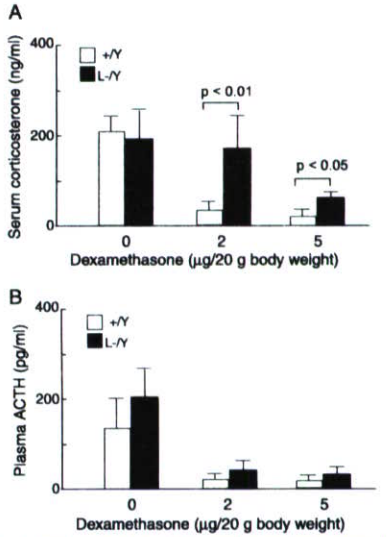

FIG. 3. Impairment of the HPA negative feedback system of glucocorticoid production in ARKO mice. (A) Serum corticosterone levels of ARKO and WT mice in the dexamethasone suppression test. Trunk blood was collected from ARKO and WT mice 6 hours after injection with increasing doses of dexamethasone. (B) Plasma ACTH levels of ARKO and WT mice in the dexamethasone suppression test.

Increased POMC expression and decreased GR expression in the pituitary glands is caused by AR deficiency. To address if hormone production was intact following AR inactivation, we examined the mRNA expression levels of pituitary hormones by RT-PCR. As shown in Fig. 5A, the expression of luteinizing hormone β, follicle-stimulating hormone β, and thyroid-stimulating hormone β, as well as that of the orphan NRs (Nur77 and Nurr1), appeared unaltered by AR deficiency. The POMC mRNA levels, however, were clearly up-regulated in males (Fig. 5A) but not in females (Fig. 5B). The up-regulation of POMC mRNA was confirmed by Northern blot analysis (Fig. 5C). This finding is consistent with the high ACTH levels observed for the ARKO mice. In contrast, pituitary GR expression was decreased at both the mRNA and protein levels (Fig. 5A and D). Decreased GR gene expression in ARKO males was also seen for the spleen but not for the other tested tissues (Fig. 5E), suggesting tissue-specific regulation of GR expression by the AR. Interestingly, a clear decrease in the GR mRNA levels was not detected in the total brain RNA of ARKO males (Fig. 5E). Additionally, there was no alteration in the numbers of GR and CRH immunoreactive cells in the hypothalamic paraventricular nucleus in the male ARKO brain (Fig. 4D). These results suggest that the androgen/AR signaling system affects the negative feedback regulation of glucocorticoid production via pituitary GR expression. This view is further supported by the observation that colocalization of ACTH with the AR and/or GR in the pituitary

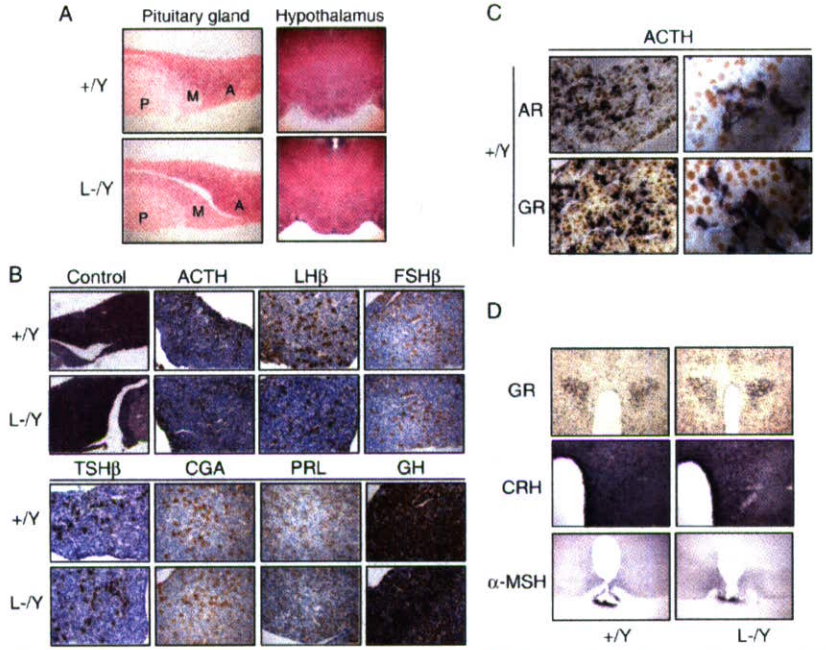

FIG. 4. Histological appearance of the hypothalamus and pituitary gland in ARKO mice. (A) No clear alteration in morphology of the hypothalami or pituitary glands of ARKO mice. Sections of pituitary glands and hypothalami were stained with hematoxylin and eosin. A, anterior lobe; M, intermediate lobe; P, posterior lobe. (B) No overt abnormality in the distribution of cells expressing pituitary hormones in ARKO mice by immunohistochemical staining. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β; CGA, glycoprotein hormone; PRL, prolactin; GH, growth hormone. (C) Pituitary ACTH (black/gray) colocalized with AR (brown) or GR (brown) (left) and its higher magnification (right) in WT mice as detected by immunostaining with specific antibodies. (D) No clear alterations in the GR and CRH (in the paraventricular nucleus) and α-MSH (in the arcuate nucleus) immunoreactive neurons in the hypothalami of ARKO mice.

glands of WT mice was detected by double immunostaining with specific antibodies (Fig. 4C).

Induction of the GR gene by DHT in a pituitary cell line. Finally, to confirm that GR expression levels in the pituitary glands of ARKO mice were reduced, we tested whether DHT induced GR gene expression in cultured cells. DHT treatment of the pituitary cell line AtT-20 for 4 h induced expression of the GR gene (Fig. 6A) and protein (Fig. 6B). Unexpectedly, the expression levels of POMC mRNA (Fig. 6A) and protein (Fig. 6B) were reduced. The AR effect was confirmed following treatment with an AR antagonist (Flutamide) (Fig. 6A and B) and RNA interference (Fig. 6C). Reflecting tissue-specific regulation of GR expression by the AR in intact animals, no response to DHT in the expression levels of either GR or POMC was seen in 3T3-L1 preadipocytes (Fig. 6A to C, lower panels). No consensus androgen response elements or closely related sequences are present up to −3 kb in the GR promoter. However, the intron between exons 1A and 1B of the GR gene (33) was found to counter androgen responsiveness in a transient expression assay in AtT-20 cells (Fig. 6D). This suggests that this element is responsible for androgen-induced GR expression in the pituitary.

DISCUSSION

Hypertrophic and hyperplastic adrenal glands are associated with a hypercorticoid state in male mice deficient for the AR. A hypercorticoid state was observed in sexually mature male mice deficient for the AR. It likely resulted from glucocorticoid overproduction by the hypertrophic and hyperplastic zona fasciculata of the adrenal gland. The ARKO mice demonstrated hyperplasia of the X-zone, which normally regresses by the time sexual maturity is attained (11). TUNEL assays and BrdU labeling confirmed that the hypertrophy and hyperplasia of adrenal glands resulted from decreased apoptosis and increased cell proliferation. Chronic ACTH stimulation causes zona fasciculata cell hypertrophy and hyperplasia, and ACTH is a potent inhibitor of apoptosis in the adrenal cortex (36). Thus, the findings for the ARKO males are consistent with exposure to high levels of ACTH.

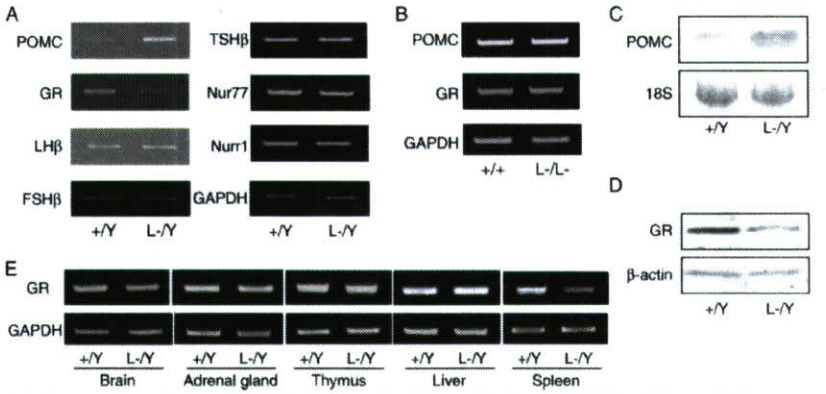

FIG. 5. Altered expression levels of gene transcripts involved in the HPA axis. (A) Increased POMC and decreased GR expression levels of transcripts in ARKO pituitary by semiquantitative RT-PCR. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β. (B) No significant alterations of POMC and GR mRNA levels in the pituitary glands of female ARKO ($AR^{1-/\Delta-}$) mice. (C and D) Northern blot analyses showing clear up-regulation of POMC mRNA levels and down-regulation of GR mRNA levels in the ARKO pituitary. (E) Tissue-specific reduction of GR transcripts in ARKO mice. GR expression levels are down-regulated only in the spleen and pituitary in male ARKO mice.

The hypertrophic and hyperplastic adrenal glands in the ARKO mice probably resulted from high levels of serum ACTH, derived from high POMC transcript levels in the pituitaries of ARKO mice. Studies with transgenic mice expressing antisense RNA against the GR in the brain and anterior pituitary demonstrate that the GR mediates the negative feedback regulation of glucocorticoid production through HPA axis activity (26, 36). Consistent with this observation, the male ARKO mice had low pituitary GR mRNA levels but no difference in the distribution of pituitary hormone-producing cells compared to WT animals. Thus, our findings suggest that the activated AR in the pituitary gland is needed to express pituitary GR at a sufficiently high level to participate in the negative feedback regulation of glucocorticoid production. The X-zone, which is considered a fetal zone, regresses during sexual maturation and reappears after gonadectomy (11). The molecular basis underlying X-zone regression during sexual maturation remains to be investigated. However, our results raise the possibility that the activated AR in adrenal glands induces X-zone repression by the induction of apoptosis. Consequently, the identification of AR target genes expressed in the X-zone is another interesting direction to pursue.

Liganded AR augments GR gene expression in the pituitary gland. We found that GR gene expression was impaired in the pituitary glands of ARKO males. We presumed that the reduced GR levels led to increased expression of the POMC gene, with subsequent high levels of serum ACTH. This idea was supported by the observation that the suppression of ACTH production by exogenous glucocorticoids was partially impaired in the ARKO mice. Moreover, the DHT-activated AR enhanced the GR mRNA levels in a pituitary cell line but not in 3T3-L1 preadipocytes. The effect of DHT was most likely mediated by a response element in an upstream region of the GR promoter exon 1B (33). Thus, the activated AR directly induces the pituitary GR in a cell-specific manner. How this is accomplished on a molecular level remains to be elucidated.

Do androgen/AR signaling disorders link with an ACTH-dependent hypercorticoid state? A hypercorticoid state in humans is well known to cause Cushing's syndrome, in which patients suffer from a number of disorders such as centripetal obesity, facial rounding, glucose intolerance, hyperinsulinemia, and impaired lipid and bone metabolism (23). Most of these lesions are a reflection of glucocorticoid-driven gluconeogenesis. The hypercorticoid state may result from either endogenous disorders or chronic treatment with exogenous glucocorticoid. Endogenous causes of Cushing's syndrome are further classified as ACTH dependent or independent (18). The ACTH-dependent syndrome is characterized by up-regulated levels of ACTH; however, the molecular basis underlying the ACTH overproduction remains to be investigated. It is possible that sex steroids are involved, but this has not yet been fully addressed.

The male ARKO mice exhibited abnormalities similar to those seen for ACTH-dependent Cushing's syndrome patients. Since we detected up-regulation of the pituitary POMC transcript, other POMC-derived peptides might have contributed to the onset of obesity in male ARKO mice. For example, α-MSH in the neurons of the hypothalamus plays a central role in appetite control and energy homeostasis (3, 4). Although we detected no clear alteration in α-MSH immunoreactivity in the arcuate nuclei of the hypothalami of male ARKO mice, it will be of interest in future experiments to examine the melanocortin receptor system in ARKO brain. In contrast to the male ARKO mice, ARKO females did not display some of the abnormalities, such as obesity. It is possible that the lack of obesity in female ARKO mice may result from activation of estrogen receptors (ERs). ERs activated by high physiological

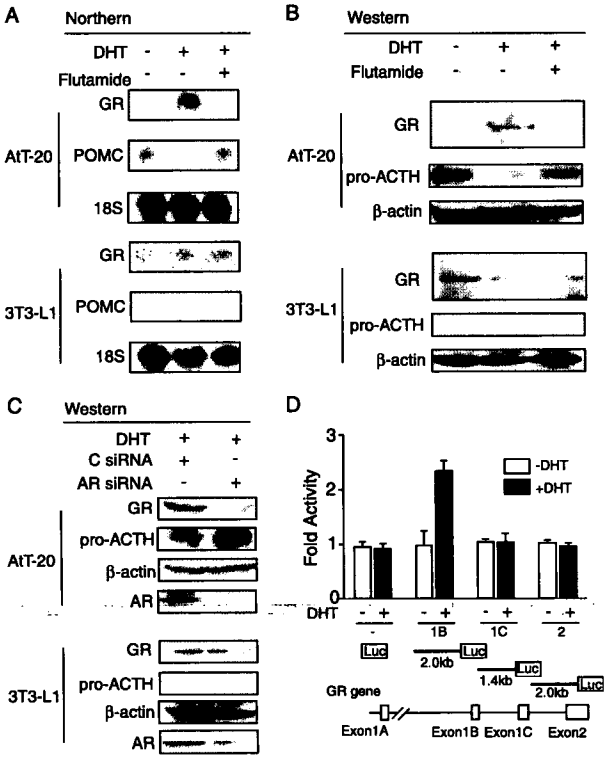

FIG. 6. Cell-type-specific regulation of the GR by activated AR. (A) Regulation of GR and POMC gene expression by treatment with either DHT or an AR antagonist (Flutamide) in the cultured cells as analyzed by Northern blot analysis. (B) Expression of the GR and pro-ACTH proteins was analyzed by Western blot analysis. (C) The significance of AR in the GR gene regulation was tested by AR RNA interference (with small interfering RNA [siRNA]) in the cultured cells. C siRNA, control siRNA. (D) Luciferase assay was performed with a series of the GR promoter regions in AtT-20 cells. After transfection with each of the promoter tk-luciferase vectors, the transfected cells were incubated with or without 10^{-7} M DHT.

levels of endogenous estrogens are effective in maintaining the proper levels of pituitary GR mRNAs needed to control POMC gene expression. This idea is indeed supported by the finding of unaltered levels of GR and POMC transcripts in the pituitary glands of the female ARKO mice. Moreover, estrogen treatment in female rats is shown to suppress serum levels of ACTH (27, 41). The common but gender-specific putative functions of the AR and ER in the brain have already been described in the context of mouse sexual behavior (24, 29). Though the possible ER functions remain to be studied for female ERKO mice, the present study suggests that the activated AR potentiates the negative HPA feedback regulation of glucocorticoid production through up-regulation of GR expression levels. Our study implies that the AR may be a potential therapeutic target for ACTH-dependent Cushing's syndrome.

In conclusion, the present study suggests that the androgen/AR signaling system is a negative pathway for glucocorticoid secretion in adult male mice. ARKO mice showed decreased GR expression in the pituitary glands and increased circulating ACTH and glucocorticoid. Androgens may increase the sensitivity of the HPA negative feedback loop to glucocorticoids by increasing GR expression in the pituitary gland, leading to suppression of adrenal cortical function. Thus, we presume that activated AR in the pituitary gland is a component of the negative feedback system for glucocorticoid production.

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Review

$1\alpha,25(OH)_2D_3$-induced DNA methylation suppresses the human CYP27B1 gene

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Abstract

CYP27B1 is a critical enzyme of Vitamin D biosynthesis that hydroxylates $25(OH)D_3$ at the final step of the biosynthetic pathway. The CYP27B1 gene is expressed primarily in kidney and negatively controlled by Vitamin D receptor. We have characterized the negative vitamin D response element and its binding protein, a bHLH transcription factor. This factor directly binds to the 1αnVDRE and activates transcription, but its transcriptional activity is suppressed by the ligand-activated Vitamin D receptor through recruitment of histone deacetylase. We have shown that histone deacetylation is a critical step for chromatin structure remodeling in suppression of the CYP27B1 gene. We have further demonstrated that, in addition to histone acetylation, this transrepression by VDR requires DNA methylation in the CYP27B1 gene promoter. Thus, transcriptional regulation of the CYP27B1 gene appears to be mediated by dual epigenetic modifications.

Keywords: CYP27B1; Transrepression; VDIR; VDR; WSTF

Contents

1. Introduction

Vitamin D_3 1α-hydroxylase (CYP27B1) is a critical enzyme of Vitamin D biosynthesis that hydroxylates $25(OH)D_3$ at the final step of the biosynthetic pathway (Kato et al., 1998; Takeyama et al., 1997). Lipophilic ligands, such as Vitamin D, as well as thyroid/steroid hormones, are thought to exert their physiological effects through transcriptional control of target genes via cognate nuclear receptors (NRs) (Mangelsdorf et al., 1995). The Vitamin D receptor (VDR, NR1I1) is a member of this NR superfamily that is activated by its ligand Vitamin D_3. A number of co-regulator complexes that support ligand-dependent transcriptional control have been identified, and these complexes can be classified into three categories according to the function (Glass and Rosenfeld, 2000). Members of the first co-regulator complex class regulate transcription directly, through a physical interaction with general transcription factors and RNA polymerase II (Gu et al., 1999; Rachez et al., 1998). Members of the second co-regulator complex class covalently modify histone tails, for example by acetylation, in promoter nucleosomal arrays (Heinzel et al., 1997; Kamei et al., 1996; Onate et al., 1995; Yanagisawa et al., 2002). The major function of the third class of complexes is chromatin remodeling, which involves the ATP-dependent dynamic remodeling of chromatin structure (Ito et al., 1997; Kitagawa et al., 2003; Narlikar et al., 2002). Chromatin remodeling complexes utilize energy from ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. As chromosomal DNA is generally packed as nucleosomal arrays, chromatin-remodeling complexes are thought to render specific promoter regions accessible to other co-regulator complex classes and sequence-specific regulators.

Recently, we identified a novel multifunctional ATP-dependent chromatin remodeling complex, designated WINAC, which consists of 13 subunits (Kitagawa et al., 2003). It contains SWI/SNF chromatin remodeling complex components and DNA replication-related factors. VDR interacts with WINAC in a ligand-independent manner through the Williams syndrome transcription factor (WSTF). WSTF contains a bromodomain that is adjacent to a zinc-finger motif common for the (BAZ) protein family. Members of this family harbor both a PHD finger and bromodomain in their C-terminal domain (Jones et al., 2000). As bromodomains have been recently shown to bind acetylated histones, it is possible that WSTF serves as an adaptor protein for acetylated histones (Hassan et al., 2002; Margueron et al., 2005; Martin and Zhang, 2005), facilitating the association between WINAC and chromatin.

2. Vitamin D negatively regulates expression of the $25(OH)D_3$ 1α-hydroxylase gene

Expression of CYP27B1 is negatively regulated by Vitamin D[$1\alpha,25(OH)_2D_3$] (Murayama et al., 2004; Murayama et al., 1998; Takeyama et al., 1997). We recently reported that a bHLH-type activator, VDR-interacting repressor (VDIR), directly binds to the negative Vitamin D response element (1αnVDRE) in the human CYP27B1 gene promoter, thus activating its transcription (Fig. 1) (Murayama et al., 2004). However, ligand-induced association between VDR and VDIR results in ligand-induced repression (transrepression) of the CYP27B1 gene expression. This transrepression is associated with VDIR switching from a co-activator complex containing histone acetyltransferase (HAT) to a co-repressor complex containing histone deacetylase (HDAC) (Murayama et al., 2004).

3. WSTF potentiates the ligand-induced transrepression by VDR of the human CYP27B1 gene promoter

We have previously shown that WINAC supports ligand-induced transactivation through chromatin remodeling (Kitagawa et al., 2003). However, it remained unclear if ligand-induced transrepression by VDR requires WINAC. To address this question, the role of WSTF in ligand-induced VDR/VDIR transrepression was studied in MCF7 cells, which express the endogenous CYP27B1 gene. Transrepression at the CYP27B1 promoter was monitored in transfection assays using a luciferase reporter plasmid under the control of a human CYP27B1 gene promoter segment (~700 bp upstream region of the transcription start site) containing two consensus 1αnVDRE sequences recognized by VDIR. Using gene-specific RNAi, we found that endogenous VDR, VDIR and WSTF are involved in the ligand-induced suppression of the CYP27B1 gene promoter (Fig. 2A) that is consistent with previously shown physical association of these three factors (Kitagawa et al., 2003; Murayama et al., 2004).

4. WSTF promotes recruitment of unliganded VDR to the CYP27B1 gene promoter

To test whether WSTF was recruited to VDIR via liganded VDR in the nuclei of living cells, we performed a ChIP assay on the endogenous CYP27B1 gene promoter. In agreement with

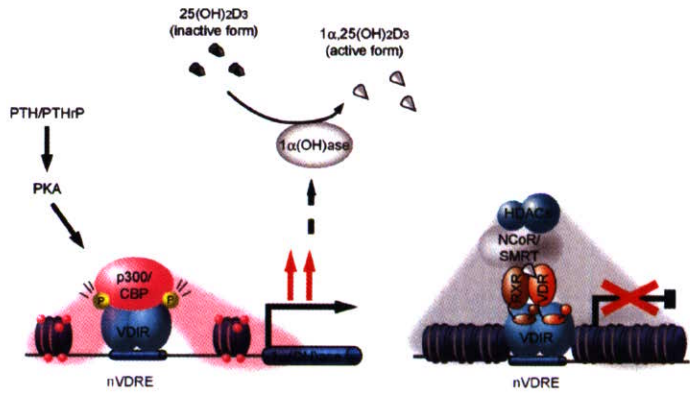

Fig. 1. Schematic view of ligand-induced transrepression by VDR in the human CYP27B1 gene promoter. A bHLH-type activator, VDIR, binds to the 1αnVDRE, and activates transcription. PKA, signaling downstream of PTH/PTHrP, phosphorylates VDIR leading to the association of VDIR with HAT co-activator p300/CBP. Upon binding $1\alpha,25(OH)_2D_3$, the VDR associates with VDIR promoting dissociation of the HAT co-activator and recruitment of HDAC co-repressor.

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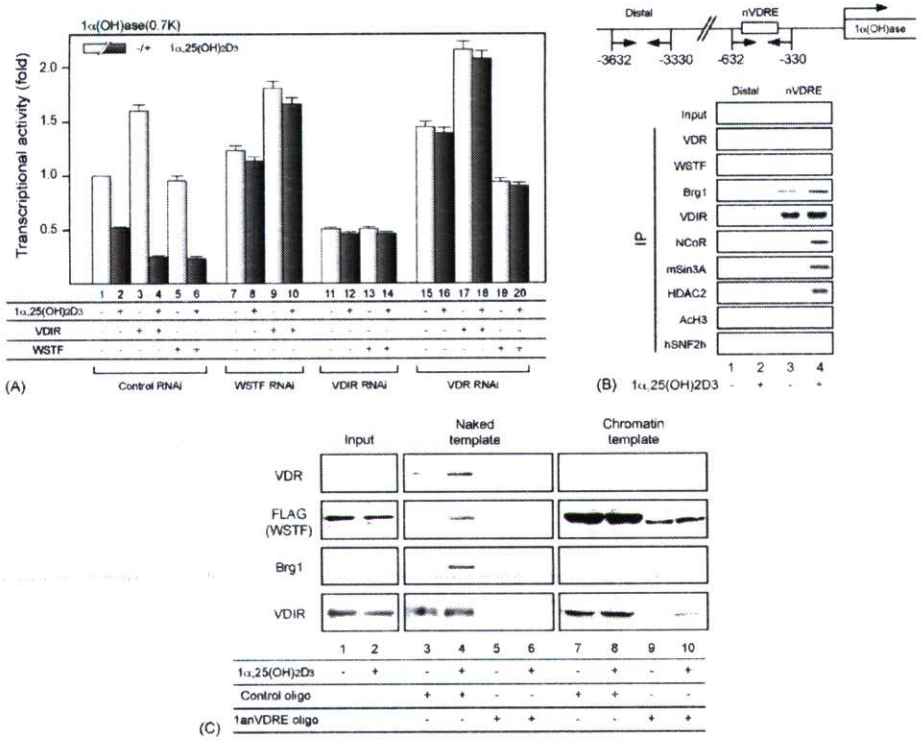

Fig. 2. WSTF enhances VDIR-mediated transrepression of CYP27B1 gene expression through chromatin remodeling. (A) The effect of gene-specific knockdown of endogenous WSTF, VDIR or VDR on CYP27B1 promoter expression. Gene-specific knockdown by factor-targeted RNAi was confirmed by Western blots using the respective antibodies (data not shown). MCF7 cells were transfected with 0.3 μg of the indicated siRNAs; 48 h later, a luciferase reporter gene containing a CYP27B1 promoter segment harboring the nVDRE was transfected into the cells. Luciferase activity was assessed after 12 h culture in the presence or absence of $1\alpha,25(OH)_2D_3$ (10^{-8} M). (B) Recruitment of VDR, WSTF, VDIR, and other co-regulators to the endogenous CYP27B1 gene promoter. For ChIP analysis, soluble chromatin was prepared from MCF7 cells treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 45 min and immunoprecipitated with the indicated antibodies. Extracted DNA samples were amplified using primer pairs that covered the CYP27B1 negative VDRE region (1αnVDRE) (Kato et al., 2003; Murayama et al., 2004). As a control, DNA samples were amplified with primer pairs covering a region 3 kb upstream from 1αnVDRE. (C) Stabilization of the ligand-free VDR/WSTF complex on the CYP27B1 promoter required chromatin-structured DNA *in vitro*. Whole cell extracts from MCF7 cells stably expressing FLAG-WSTF treated with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M) were mixed with immobilized templates. The template beads were then concentrated using a magnet and analyzed by Western blotting using the indicated antibodies. The data of panels A–C are basically similar to those in our previous report (Fujiki et al., 2005).

previous reports (Kitagawa et al., 2003; Murayama et al., 2004), VDIR was constitutively bound to the 1αnVDRE (Fig. 2B). As WSTF RNAi remarkably attenuated the promoter occupancy by VDR in the absence of ligand, WSTF appeared to facilitate the binding of unliganded VDR at the 1αnVDRE (Fig. 2B).

To examine the mechanism by which WSTF targets unliganded VDR to the promoter *in vitro*, we determined that factors are indispensable for the promoter targeting of unliganded VDR by employing an immobilized DNA/chromatin template recruitment assay. DNA fragments containing either 1αnVDRE (−60 to −615) or the CYP27B1 distal region (−3632 to −3032) were end-biotinylated to allow their immobilization onto streptavidin beads. Whole cell extracts from MCF-7 cells that stably expressed FLAG-tagged WSTF and treated with or without $1\alpha,25(OH)_2D_3$ were incubated with either naked or chromatin fragments of the promoter DNA. Proteins bound to the promoter DNA were then analyzed by immunoblotting (Fig. 2C). WSTF and VDR bound to naked DNA templates only in the presence of ligand, while VDIR stably associated with naked DNA templates even in the absence of ligand (Fig. 2C). In contrast, for the chromatin templates with HeLa histone octamers, recruitment of WSTF and VDR was ligand-independent (Fig. 2C), indicating a role for DNA-bound VDIR in the stable association of VDR/WSTF with chromatin. Thus, considering all of these results, we conclude that WINAC facilitates VDR-mediated transrepression of the CYP27B1 gene through a physical interaction between the WSTF bromodomain and an acetylated nucleosomal array (Fujiki et al., 2005) (Fig. 3).

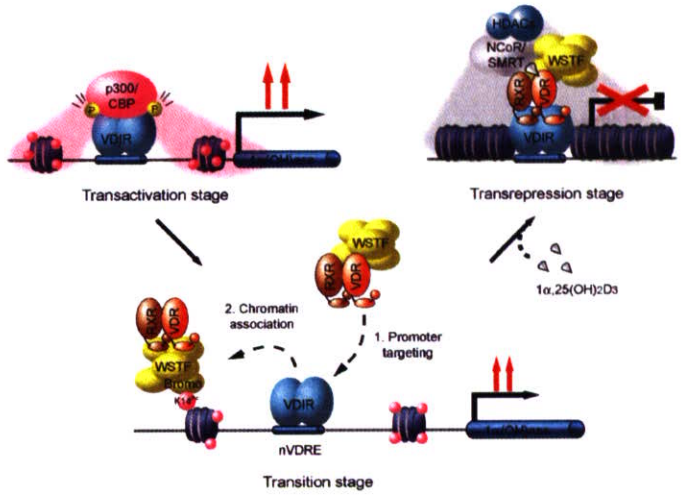

Fig. 3. Model demonstrating the role of WINAC in the ligand-induced transrepression function of VDR at the CYP27B1 gene promoter. p300 is recruited to VDIR, which was phosphorylated via PKA signaling, and acetylates the nucleosomes at the VDRI in the CYP27B1 gene promoter (Transactivation stage). WINAC, along with VDR, sequentially targets VDIR through interaction between unliganded VDR and VDIR, and is retained on the acetylated promoter via the WSTF bromodomain (Transition stage). Upon $1\alpha,25(OH)_2D_3$ binding, HDAC co-repressor complexes are recruited to the ligand-bound VDR/VDIR complex and deacetylate the nucleosomes. WINAC then exerts its ATP-dependent chromatin remodeling activity (Transrepression stage) (see also the figure in Fujiki et al., 2005).

5. DNA methylation followed histone deacetylation in the VDR-meditaed transrepression of the CYP27B1 gene

Histone deacetylation by recruited HDACs to VDR/VDIR at the 1αnVDRE is a critical step for remodeling chromatin structure at the CYP27B1 gene promoter in VDR-mediated transrepression (Murayama et al., 2004). However, TSA (a HDAC inhibitor) could not fully abrogate $1\alpha,25(OH)_2D_3$-induced transrepression (Fig. 4A), suggesting that an additional mechanism may contribute to this transcriptional repression. To explore this unknown mechanism, we purified a $1\alpha,25(OH)_2D_3$-dependent VDIR-VDR co-repressor complex. By biochemical purification and consequent mass spectrometric sequence analyses, we identified a DNA methyltrasferase as a part of this co-repressor complex. The DNA methyltrasferase interacted with both VDR and HDAC2 in a ligand-dependent manner (Fig. 4B). Indeed, we found that treatment with $1\alpha,25(OH)_2D_3$ induced DNA methylation at CpG sites in the promoter and exon regions of the CYP27B1 gene (data not shown). All together, these findings suggest that ligand-induced DNA methylation in the promoter may contribute, at least in part, to continuous transcriptional repression by VDR, and provide a novel mechanism of a ligand-dependent transrepression by NR that links repressive histone modification with epigenetic repression through DNA methylation (Kim et al., unpublished results).

6. Discussion

6.1. Transrepression of the CYP27B1 by liganded VDR requires two epigenetic modifications

We have shown that the CYP27B1 gene nVDRE is composed of two E-box like motifs (Murayama et al., 2004). This E-box like motif(s), serving as a VDIR binding site, also appears to confer a negative response to $1\alpha,25(OH)_2D_3$ at other VDR target gene promoters (Kim et al., in press). Thus, it appears that this E-box type motif acts as a nVDRE, and that DNA-bound VDIR is directly targeted by the VDR. In the present study, we have provided evidence that, in addition to hitsone deacetylation by VDR/VDIR-recruited HDAC, transrepression by VDR/VDIR also requires DNA methylation. It is conceivable that both these steps of epigenetic modifications may required for the transrepression of other VDR negative target genes by VDR/VDIR. Therefore, characterization of nVDREs in other VDR negative target genes together with identification of their binding factors would provide a better understanding of the action of Vitamin D.

6.2. WINAC supports ligand-induced transrepression by VDR on the human CYP27B1 gene promoter region

A large number of co-regulator complexes appear to support transcription control by NRs at multiple but sequential steps (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Pascual et al., 2005). ATP-dependent chromatin remodeling complexes are considered to facilitate the promoter-specific

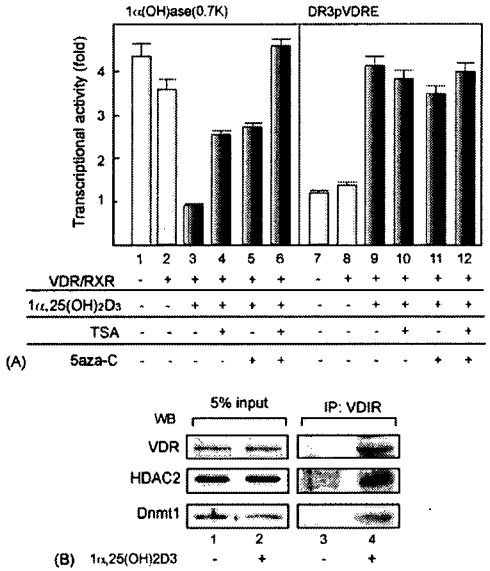

Fig. 4. Ligand-induced transrepression of the CYP27B1 gene by VDR mediates DNA methylation. (A) The effects of TSA (HDAC inhibitor) or 5aza-C (DNA methylation inhibitor) on transrepression of a luciferase reporter under control by a human CYP27B1 gene promoter segment containing 1αnVDRE. 293F cells (human embryonic kidney) were treated with or without TSA (10^{-6} M), 5aza-C (10^{-5} M) or both. After 36 h, the lucifease reporter plasmid and VDR/RXR expression vectors were transfected into the cells. Luciferase activity was assessed as described in Fig. 2. (B) 1α25(OH)$_2$D$_3$-dependent association between endogenous Dnmt1 (DNA methyltransferase) and VDIR *in vivo*. Whole cell extracts from 293F cells treated with or without 1α,25(OH)$_2$D$_3$ were immunoprecipitated with anti-VDIR antibodies, followed by Western blot analysis using anti-VDR, anti-Dnmt1 or anti-HDAC2 antibodies.

recruitment of other co-regulator complexes (Emerson, 2002; Narlikar et al., 2002). We have previously reported that WINAC dysfunction resulted in a failure of proper transcriptional regulation by VDR, possibly because of impairment of co-regulator recruitment to VDR-target gene promoters (Kato et al., 2003). These findings strongly suggested that ATP-dependent chromatin remodeling activity is indispensable for subsequent co-regulator recruitment in response to ligand binding (Kitagawa et al., 2003).

It has been suggested that ligand-unbound VDR/RXR on the VDRE mainly associates with a HDAC complex to repress target genes (Murayama et al., 2004). According to this model of a positive VDRE, ligand binding leads to co-repressor dissociation from VDR. In contrast, the WINAC assists recruitment of HDAC co-repressor complex in VDIR-mediated transrepression on a negative VDRE (Fujiki et al., 2005). These imply that the set of factors/complexes associated with unliganded VDR on negative VDREs differ from those associated with positive VDREs in the VDR target gene promoters. Indeed, ligand binding significantly increased the interaction of VDR/WINAC with a HDAC complex (Fujiki et al., 2005) Hence, in addition to ligand-induced transactivation by VDR, WINAC also has an important role in mechanisms of VDR-mediated transrepression. The proposed mechanism of the ligand-induced transrepression by VDR in the present study appears to be dependent on the promoter-content, since it is unlikely that all of the VDR target gene promoters for Vitamin D-induced transrepression harbor VDIR binding sites. Another mechanism of ligand-induced transrepression may be found for other promoters negatively controlled by VDR or some other NRs, like recently reported data on the transrespressive function of PPARγ (Pascual et al., 2005).

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A reduction state potentiates the glucocorticoid response through receptor protein stabilization

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The intracellular redox state regulates all biological processes including gene expression. The glucocorticoid receptor (GR), a hormone-dependent transcription factor, is affected by the redox state. GR translocation from the cytoplasm to the nucleus is regulated by oxidative stress. The molecular mechanism of how the redox state affects GR transcriptional regulation, however, has not been clarified. We identified a deoxidizing agent, cobalt chloride ($CoCl_2$), that potentiates the GR transcriptional effects by stabilizing endogenously expressed GR protein as well as exogenously over-expressed one without affecting GR mRNA level. Consequent GR protein stabilization enhanced co-factor recruitments on the target gene promoters. These results support the existence of a novel redox-dependent mechanism of GR transcriptional regulation mediated by receptor protein stabilization.

Introduction

The intracellular environment results from the coordination of numerous signaling pathways. These pathways control the most basic biological events, including gene transcription. One component of the intracellular environment is the redox state, which is a reflection of the intracellular concentrations of reactive oxygen species. The intracellular redox state plays a key role in all cellular events including the modulation of gene expression. How the redox state affects gene expression is poorly understood (Kim *et al.* 2002; Rahman *et al.* 2004, 2006; Pouyssegur & Mechta-Grigoriou 2006).

Transcription regulatory factors mediate chromatin reorganization and histone modification, and are involved in the key steps of gene expression. These factors require several classes of co-regulator/co-regulator complexes (Kishimoto *et al.* 2006; Rosenfeld *et al.* 2006). Protein modification of the transcription factors themselves also affects transcription (Kumar *et al.* 2004). The glucocorticoid receptor (GR), a member of the nuclear receptor gene superfamily, is one transcription factor regulated in this manner (Rhen & Cidlowski 2005; Qiu *et al.* 2006).

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The GR engages in both positive and negative regulation of transcription. Its roles are diverse and include participation in glucose homeostasis and the suppression of inflammation (Smoak & Cidlowski 2004). Hormone binding induces translocation of the GR from the cytosol into the nucleus. In hormone-dependent transactivation, the GR, activated by hormone binding, binds as a homodimer to the consensus glucocorticoid responsive element (GRE) in the target gene promoters. Hormone-induced transrepression of inflammatory genes by the GR is mediated mainly by indirect GR association with the binding sites for AP-1 and NF-κB (Jonat *et al.* 1990; Heck *et al.* 1994; Ogawa *et al.* 2005). Thus, the intracellular translocation of the GR is a critical step to initiate gene regulation and appears to be modulated through the other intracellular signals (Saklatvala 2002; Rogatsky & Ivashkiv 2006).

In the present study, we tested how redox conditions modulate the function of the hormone-bound GR in hormone-induced gene regulation. H_2O_2-induced oxidative stress retained hormone-bound GR in the cytosol. In contrast, a reduced condition induced by cobalt chloride ($CoCl_2$) potentiates the function of the hormone-bound GR in transactivation through protein stabilization, but not in transrepression. Thus, the present findings indicate that a reduction state potentiates the response to glucocorticoids through GR protein stabilization.

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Results

To test if the redox state regulated GR functions, the effects of oxidative stress induced by H_2O_2 and reduction with $CoCl_2$ were examined with a luciferase reporter assay. H_2O_2 is a strong oxidative agent commonly used for the induction of cellular oxidative stress. $CoCl_2$ is a hypoxia mimetic reagent which reduces the oxidative environment in living cells (Zhang *et al.* 2002). $CoCl_2$ changes cellular conditions such as the NAD^+ : NADH ratio and modulates the structure of several proteins. Three types of reporters [Glucocorticoid responsive element (GRE), AP-1 responsive element (AP1-RE) and NF-κB responsive element (NFκB-RE)] were utilized with the reporter plasmids. A GR point mutant (C481S), known to remain in the nucleus after oxidative stress, was also used (Okamoto *et al.* 1999; Tanaka *et al.* 2000).

As expected, dexamethasone (Dex), a synthetic GR agonist stimulated GR-mediated transactivation through the GRE. It potently repressed transcription through the AP1-RE and NFκB-RE in the luciferase assay. H_2O_2 treatment attenuated GR function, while $CoCl_2$ potentiates it. Similar effects were seen on Dex-induced transrepression by the GR, but the effects were not significant (Fig. 1A). However, the Dex-induced function of the GR mutant (C481S) was sensitive to the treatment of $CoCl_2$, but not H_2O_2. This indicated that H_2O_2-induced oxidative stress caused the retention of Dex-bound GR in the cytosol (Fig. 1A). Redox state-mediated transcriptional regulation was also confirmed with endogenously expressed GR in A549 cells using the same luciferase assays without GR transfection (Fig. 1B; Wang *et al.* 2004). Another deoxidizing agent *N*-acetyl-L-cysteine (NAC) had the same effect as $CoCl_2$ in all assays, confirming the effect of reduction on the GR function (data not shown). Reflecting the GR protein stabilization by $CoCl_2$, the expression of the endogenous GR target genes were up-regulated, although the mRNA level of GR was unaltered (Fig. 1C).

Next, we confirmed the cellular localization of the GR in a reduction state by expressing a chimeric human GR protein fused to GFP in 293F cells. In the absence of ligands, the GR was located mainly in the cytosol and H_2O_2 treatment induced clear cytosolic localization (Fig. 2A, top panel). Dex treatment induced the nuclear localization of the GR, but H_2O_2 inhibited GR translocation as previously reported (Okamoto *et al.* 1999). However, H_2O_2 treatment failed to block the nuclear translocation of the C481S mutant (Fig. 2A, left panels). These results indicated that the inhibitory action of H_2O_2 on the transcriptional activity of the GR was correlated with the retention of the GR in the cytosol. In contrast, $CoCl_2$ treatment did not alter the locations of Dex-bound and unbound GR (Fig. 2, right panel). These results suggested that another regulatory mechanism potentiates the function of Dex-bound GR.

To explore the molecular mechanism underlying potentiation of the GR by $CoCl_2$, we measured the expression levels of the GR over time. Measurements were made upon Dex addition since several steroid hormone receptors like ERs or AR are destabilized by ligand binding (Dennis & O'Malley 2005; Garside *et al.* 2006; Ohtake *et al.* 2007). In the 293F GR expressing stable transformant, the expression level of the GR was down-regulated in a time-dependent manner after Dex treatment (Fig. 3B). Furthermore, Dex-induced down-regulation of the GR was also seen with endogenous GR protein in A549 cells (Fig 3A,C). In the presence of $CoCl_2$, Dex-induced down-regulation of GR protein levels was not detected. This suggested that $CoCl_2$ interfered with Dex-induced destabilization of the GR protein. As a proteasome inhibitor MG132 was also effective in attenuating Dex-induced destabilization, Dex-induced destabilization of the GR protein appeared to be mediated by a ubiquitin-proteasome cascade that was presumably blocked by $CoCl_2$ (Fig. 3B,C).

To address if stabilization of the GR protein by $CoCl_2$ reflected the transcriptional potentiation of Dex-bound GR at the promoter level, GR recruitment to its target gene promoters was tested by a ChIP assay. An ATPase chromatin remodeling factor, Brg-1, was utilized as a representative co-factor for the GR as previously reported (Nagaich *et al.* 2004). The presence of $CoCl_2$ enhanced the recruitment of the GR as well as Brg-1 to three different gene promoters which contained either GRE, AP1-RE or NFκB-RE (Fig. 3D). Since the protein stability of Brg-1 was unaltered by either Dex or $CoCl_2$ (data not shown), the potentiation of GR function by $CoCl_2$ most likely resulted from increased co-factor recruitment by the stabilized GR protein in the target gene promoters.

Discussion

During the decade, the regulation of nuclear receptor transcription was extensively analyzed with the development of biochemical approach and the improvement of protein identification methods using mass spectrometry (Yanagisawa *et al.* 2002; Kitagawa *et al.* 2003; Takezawa *et al.* 2007). Various types of transcriptional co-factors have been already identified, most of which are related to chromatin reorganization (Perissi & Rosenfeld 2005; Rosenfeld *et al.* 2006). Accumulating knowledge suggests

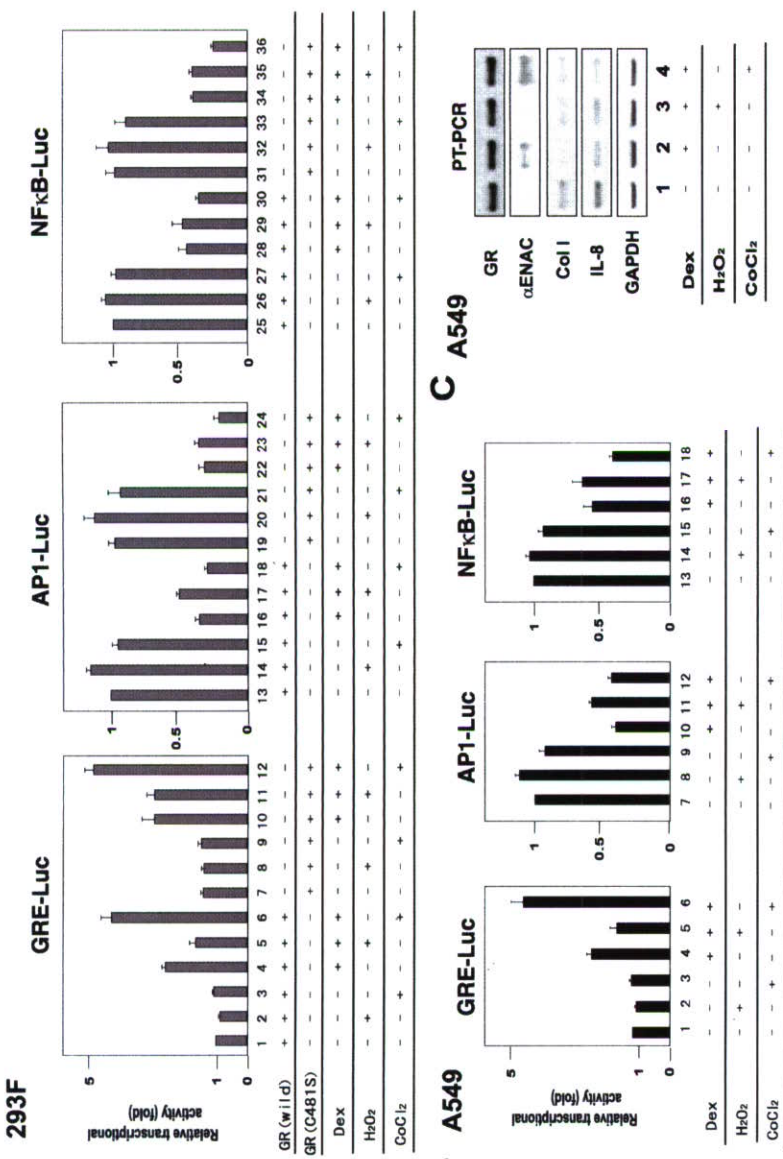

Figure 1 Ligand-dependent transcriptional regulation of the glucocorticoid receptor (GR) is redox state-dependent. (A) Luciferase assays were performed in 293F cells transfected with each of the indicated reporter plasmids (300 ng) and human GR or GR mutant (C481S) expression vectors (50 ng). For AP1-Luc, expression vectors were for c-Jun and c-Fos, and for NFκB-Luc, expression vectors were for p50 and p65. Vectors were simultaneously transfected (50 ng each). Dexamethasone (10^{-7} M), H_2O_2 (0.1 mM) and $CoCl_2$ (0.2 mM) were added 3 h after transfection. (B) Luciferase assays were performed in A549 cells as explained in (A) without transfection of the human GR or GR mutant (C481S). (C) GR target genes were really regulated by redox state in A549 cells. Total RNA was extracted 16 h after indicated stimulations. RT-PCR of GR and its target genes were performed as described in Experimental procedures.

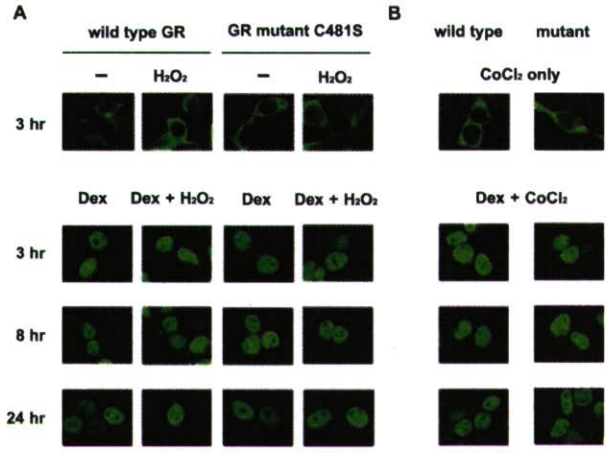

Figure 2 The ligand induced change in cellular localization of the glucocorticoid receptor (GR) is redox state-dependent. (A) Oxidative stress affects the cellular localization of the GR. 293F cells were transfected with expression vectors of GFP-GR or GFP GR mutant (C481S). Dex (10^{-7} M) and H_2O_2 (0.1 mM) were added at the indicated times before fixing. (B) Cobalt chloride ($CoCl_2$) did not affect GR localization. Dex (10^{-7} M) and $CoCl_2$ (0.2 mM) were added at the indicated times before fixing. After 24 h of transfection, the cells were scanned using a Zeiss confocal laser scanning system 510, and results were assessed with Adobe Photoshop 5.0 (Adobe) (Miyamoto *et al.* 2007).

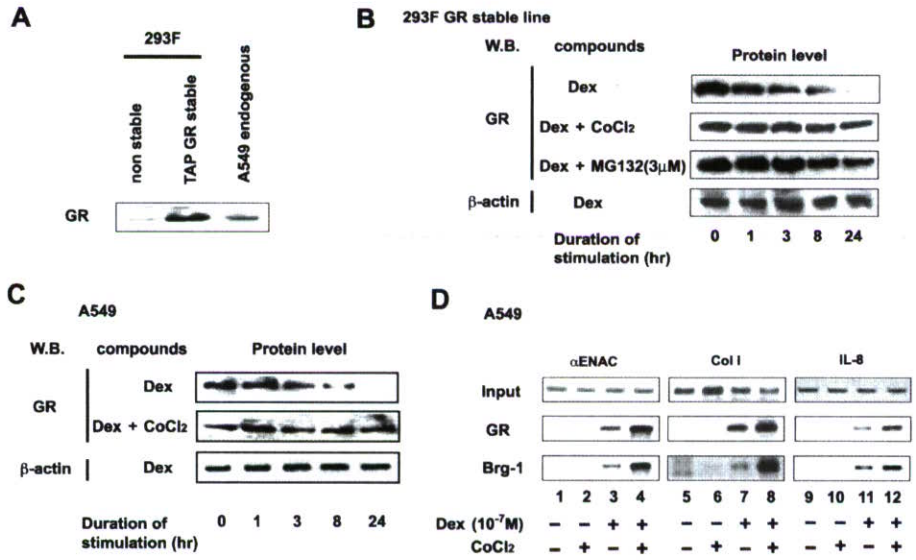

Figure 3 $CoCl_2$ stabilizes the GR protein and co-factor recruitment. (A) Expression of the glucocorticoid receptor (GR) in indicated cells. Cell extracts were immunoprecipitated with GR antibody and subjected to Western blotting. (B) $CoCl_2$ stabilized the GR protein. Whole cell extract from 293F cells with stable expression of the GR were immunoprecipitated at an indicated time upon stimulation with Dex or Dex + $CoCl_2$ and Western blotted as shown in (A). MG132 (3 μM) was also used for cell stimulation. (C) Endogenous GR protein was also stabilized by $CoCl_2$ in A549 cells. A549 cells were treated and processed in the same way as (B). (D) Co-factor recruitment was also stabilized by $CoCl_2$. A ChIP assay was performed as described in the Experimental procedures.

that the chromatin reorganization related to the transcriptional regulation occurred in spatiotemporally-specific manner and is supported by a number of specific protein complexes (Ju *et al.* 2006; Garcia-Bassets *et al.* 2007). Moreover, specific combinations of the complex components appear to be highly regulated responding to extracellular signals. Redox condition is presumed as such a key determinant, and affects the biological conditions affecting the cell fate. Although it is generally accepted that redox condition modulates the property of transcription factors, it remains to be understood how it regulates the conditions of promoter regions around the target gene promoters of transcription factors, particularly in terms of complex formation (Pouyssegur & Mechta-Grigoriou 2006).

Three types of redox state-dependent transcriptional regulation have been described so far. One is a direct oxidation/reduction of cysteines on the transcription factor itself (e.g. AP-1). The second is a change of the factor's subcellular localization as in the case of NF-κB. The last are alterations of the redox buffers (e.g. NAD^+/NADH exchange) which change the properties of transcriptional repressors such as CtBP or SirT1 (Zhang *et al.* 2002; Liu *et al.* 2005). In this study using the GR, we found a novel mechanism of redox-dependent transcriptional regulation (Tanaka *et al.* 2000). In this model, blockade of GR protein degradation by the deoxiding agent $CoCl_2$ increased co-factor recruitment and consequently potentiates the GR transactivation property. We used a representative GR interacting co-factor Brg-1, an ATP-dependent chromatin remodeling factor, as an example of co-factor recruitment in our experiments. We also presume that various types of co-factor complexes may be associated with GR at different timing after redox stimulus. A detailed time course analysis is essential to completely understand this form of redox-dependent transcriptional regulation. Moreover, further extensive biochemical analysis is needed to fully describe how the regulation of protein stabilization affects the transcriptional regulation of various nuclear receptors in different biological situations (Lonard & O'Malley 2006; Ohtake *et al.* 2007).

Regarding the redox-dependent regulation of GR transcriptional function, another regulatory mechanism has already been reported in a certain cell line (Leonard *et al.* 2005). However, such up-regulation of GR mRNA levels by hypoxia was not observed in the tested cell lines in the present study. Such regulatory mechanisms by hypoxia as well as reduction state may be diverse and may appear in cell context-dependent manner.

It is well known that some nuclear receptors including the GR have an anti-inflammatory effect resulting from the repression of AP-1 or NF-κB mediated transcription in ligand-dependent manner (Smoak & Cidlowski 2004; Valledor & Ricote 2004). The mechanism for this form of transrepression is largely unknown (Ogawa *et al.* 2005; Reily *et al.* 2006). Additionally, increasing co-factor (Brg-1) recruitment on target gene promoters by receptor protein stabilization was not sufficient to potentate the transrepressional property of the GR in the reduced state (see Figs 1 and 3B). These results suggest that distinct co-regulators or protein modifications may affect these transrepressional mechanisms (Kodama *et al.* 2003). To investigate this further, we are establishing a biochemical purification system for analyzing GR transcriptional regulation under various conditions.

Experimental procedures

Reagents and plasmids

Plasmids

The full-length human GR was inserted into the pNTAP expression vector using the InterPlay™ Mammalian TAP system (Stratagene, Cedar Creek, TX). It was also inserted into the pEGFP-C2 vector (BD Bioscience, San Jose, CA). Human GR mutants (Cys 481 for Ser) for both vectors were made with the QuikChange site-directed mutagenesis kit (Stratagene). pGRE-Luc was purchased from BD Bioscience. pAP1-Luc and pNF-κB-Luc were purchased from Stratagene. Expression vectors with c-Jun, c-Fos, p50 and p65 were cloned from a 293T cDNA library and inserted into the pcDNA3 vector (Invitrogen, Carlsbad, CA).

Antibodies

Anti-human GR antibody (PA1-512) was purchased from Affinity BioReagents (ABR, Golden, CO). Anti-Brg-1 Antibody (H-88) and β-actin (I-19) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Reagents

Dexamethasone was purchased from Sigma (St. Louis, MO). H_2O_2 and $CoCl_2$ were purchased from Wako Chemicals, and MG132 was purchased from Peptide Institute Inc (Osaka, Japan).

Reporter assay

293F cells were transfected using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Luciferase reporter plasmids were co-transfected with the GR-expression vectors as indicated in the figure legends together with 2 ng/well of pRL-CMV plasmid (Promega, Madison, WI). After 3 h of transfection, the media were replaced with fresh media containing 0.2% fetal bovine serum. Dexamethasone (10^{-7} M), H_2O_2 (0.1 mM) or $CoCl_2$ (0.2 mM) were then added to the cells and incubated for an additional 12 h. Cell extract preparation and dual luciferase assays were performed according to the manufacturer's protocols (Promega). Individual transfections performed in triplicate wells were repeated at least 3 times.

Preparation of stably transfected cell lines

TAP-tagged human GR-expressing retroviruses were produced using a pQCXIN vector (BD Bioscience). The 293F cells were infected by incubating them with the virus and 6 μg/mL hexadimethrine bromide (Sigma). Cells stably expressing the GR were combined and cultured with 700 μg/mL G418 (Promega) prior to colony selection.

RNA extraction and RT-PCR

Total cellular RNA was isolated from A549 cells by ISOGEN (Wako). RT reaction was performed using SuperScript (Invitrogen) and the indicated mRNAs were amplified by PCR as previously reported (Kitagawa *et al.* 2003).

Immunoprecipitation and Western blotting

After treating 293F cells or A549 cells with either H_2O_2 or $CoCl_2$ for the indicated time, cells were washed twice with ice-cold phosphate-buffered saline, resuspended in 1 mL ice-cold lysis buffer [10 mM Tris–HCl (pH 4.7), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (vol/vol) NP-40] and incubated on ice for 30 min. Cells were then centrifuged again for 5 min at 500*g* and the sedimented nuclear fractions resuspended in TNE buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 1% NP-40] and incubated for 30 min on ice. After centrifugation, supernatants were used as cell extracts for immunoprecipitation using anti-GR antibody with protein G Sepharose and then Western blotted with an anti-GR polyclonal antibody or anti-Brg-1 antibody.

ChIP assay

Soluble chromatin from A549 cells was prepared with the acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology, Billerica, MA) and was immunoprecipitated with antibodies against the indicated proteins (Kitagawa *et al.* 2003). Specific primer pairs were designed to amplify the promoter region of human αENAC (5′-TTCCTTTCCAGCGCTGGCCAC-3′ and 5′-CCTCCAACCTTGTCCAGACCC-3′), human collagenase 1 (5′-GCAGAGTGTGTCTCTTTCGCACAC-3′ and 5′-GCCCTTCCAGAAAGCCAGAGGCTG-3′), human IL-8 (5′-GGGCCATCAGTTGCAAATC-3′ and 5′-TTCCTTCCGGTGGTTTCTTC-3′) from genomic DNA (Kitagawa *et al.* 2002; Kassel *et al.* 2004). PCR conditions were optimized to allow semi-quantitative measurement and PCR products were visualized on 2% agarose/TAE gels.

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

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Abstract

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

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

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

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

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

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