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Oncogenic Nucleoporin CAN/Nup214 Interacts With Vitamin D Receptor and Modulates its Function

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ABSTRACT

Vitamin D receptor (VDR) is a ligand-dependent transcription factor and should be located in nucleus to transactivate target genes. To explore the molecules that interact with VDR and facilitate its nuclear localization, we screened a human kidney cDNA library using the yeast two-hybrid approach, and found that VDR binds to the carboxy-terminal region of an oncogenic nucleoporin, CAN/Nup214. CAN/Nup214 was originally identified through its involvement in a certain type of acute myeloid leukemia, and is a component of nuclear pore complex (NPC). Co-immunoprecipitation experiments confirmed the interaction between VDR and the carboxy-terminus of CAN/Nup214 containing a cluster of the phenylalanine-glycine (FG) repeat in mammalian cells. The exogenously expressed full-length CAN/Nup214 was localized predominantly at the nuclear envelope, suggesting its integration in the NPCs. We then examined the effects of exogenous expression of full-length CAN/Nup214 and its carboxy-terminal fragment on the VDR-mediated transactivation. The overexpression of full-length CAN/Nup214 facilitated the VDR-mediated transactivation, while the expression of the carboxy-terminal fragment suppressed it. The DNA-binding domain of VDR was required for the facilitation of the VDR-dependent transactivation by CAN/Nup214. Although the subcellular distribution of VDR was not obviously altered by the overexpression of full-length CAN/Nup214 or the carboxy-terminal fragment, the expression of the carboxy-terminal fragment inhibited the interaction between full-length CAN/Nup214 and VDR. These results indicate that CAN/Nup214 interacts with VDR and modulates its function as a transcription factor. J. Cell. Biochem. 106: 1090–1101, 2009.

KEY WORDS: VITAMIN D RECEPTOR; NUCLEAR PORE COMPLEX; NUCLEOPORIN; CAN/Nup214

Vitamin D receptor (VDR) acts as a ligand-dependent transcription factor by recognizing the vitamin D-responsive element (VDRE) with retinoid X receptor (RXR) and organizing the transcriptional control complex containing histone acetyltransferases and chromatin remodeling factors [Kato et al., 2004]. VDR mediates various biological functions of its cognate ligand 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], including the regulation of bone/calcium homeostasis, immunological regulation, bile acid metabolism, and cellular differentiation [Haussler et al., 1998; Makishima et al., 2002]. In particular, 1,25$(OH)_2$D is widely known to inhibit the proliferation and/or induce the differentiation of many types of malignant cells, including cancer of the breast [Calson et al., 1992], prostate [Skowronski et al., 1995; Yang et al., 2002], colon [Shabahang et al., 1994; Palmer et al., 2001], skin [Colston et al., 1981], and hematopoietic tissue [Abe et al., 1981; Rots et al., 1999].

Nuclear localization is an important biological event for transcription factors, because it is indispensable for accessing the genome. In eukaryotic cells, nucleoplasm is separated from cytoplasm by a nuclear envelope, and nuclear pore complexes (NPCs) built on nuclear pores bridge the nucleoplasm and cytoplasm [Rout and Aitchson, 2001]. NPCs have a huge symmetric cylindrical structure that consists of multiple copies of subunits, collectively called nucleoporins, and share a common architecture among eukaryotic organisms. NPCs serve as the sole gatekeeper controlling traffic between the two compartments regardless of the dependence on nuclear transport factors [Stewart et al., 2001; Bednenko et al.,

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2003]. Although NPCs are freely permeable to small molecules (such as water and ions) with passive diffusion, they restrict the movement of larger molecules (such as proteins and RNAs) across the nuclear envelope. To overcome the barrier, larger molecules harbor signal sequences that allow them to access the nuclear transport machinery [Mattaj and Englmeier, 1998; Jans et al., 2000; Bednenko et al., 2003]. The import of cargo molecules such as transcription factors and signal transducers through NPCs is mediated by nuclear transport factors such as importin β, which binds cargoes and nucleoporins simultaneously through separate domains [Cingolani et al., 2002]. Recent advances have demonstrated that the members of the importin β-like family can recognize cargoes harboring atypical NLSs, and transport them into nuclei independently of importin α [Cingolani et al., 2002]. Moreover, some molecules have been revealed to be able to cross the NPCs without the binding of importin family members, indicating the variable pathways of nuclear-cytoplasmic trafficking. As to VDR, we have previously revealed that importin 4 confers the ability for nuclear import in a ligand-independent manner [Miyauchi et al., 2005].

In the current study, to explore the molecules that interact with VDR and regulate its nuclear localization, we performed yeast two-hybrid screening and identified the interaction between VDR and the carboxy-terminus of CAN/Nup214, a protein containing FG repeats characteristic of nucleoporins. CAN/Nup214 is the product of the *CAN* gene and has also been identified as a target of recurrent chromosomal translocations associated with acute myeloblastic leukemia and myelodysplastic syndrome [Kandilci et al., 2004]. Interestingly, we have found that overexpression of the full-length CAN/Nup214 stimulated VDR-dependent transcription, while its carboxy-terminal fragment suppressed it. The findings suggest that the interaction with CAN/Nup214, a component of NPC, modulates the function of VDR.

MATERIALS AND METHODS

YEAST TWO-HYBRID SCREENING

Yeast two-hybrid screening was performed using the BD Matchmaker Two-Hybrid System (BD Biosciences Clontech, Inc., Palo Alto, CA), as previously described [Miyauchi et al., 2005]. The bait vector pAS2-1-VDR[a.a. 4–232] was constructed using the fragment of VDR obtained from pSG5-VDR (a gift from Dr. M. R. Haussler, University of Arizona) by *Eco*RI/*Tth*111I digestion. *S. cerevisiae* strain AH109 was transformed with the bait vector, and then subsequently mated with another strain Y187 pretransformed with a human kidney cDNA library. Transformants were plated onto a synthetic medium lacking adenine (Ade), leucine (Leu), histidine (His), and tryptophan (Trp). $Ade^{+}His^{+}$ growers were isolated, and subjected to a β-galactosidase filter assay. Blued clones were regarded as candidates, and the inserts were amplified by polymerase chain reaction using specific primers for the library plasmid, followed by sequencing.

CELL CULTURE AND TRANSFECTION

COS7 cells were maintained at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldlich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) stripped with dextran-coated charcoal to remove endogenous steroids. The human osteoblastic cell line SaOS-2 was cultured in alpha minimum essential medium (αMEM) supplemented with 10% FBS. Transient transfections were performed using FuGENE6™ reagent (Roche Diagnostics, Mannheim, Germany).

ANTIBODIES

Anti-FLAG® M2 mouse monoclonal antibody and rabbit polyclonal antibody were purchased from Sigma–Aldlich and Rockland (Gilbertsville, PA), respectively. Anti-VDR rat monoclonal antibody (9A7γ) and anti-nucleoporin mouse monoclonal antibody (MAb414) were obtained from Affinity Bioreagents (Golden, CO) and Covance (Berkeley, CA), respectively. Anti-cMyc mouse monoclonal antibody and rabbit polyclonal antibody were purchased from Santa Cruz (Santa Cruz, CA). Secondary antibodies for Western blotting and protein A/G agarose for immunoprecipitation were also obtained from Santa Cruz. Secondary antibodies labeled with Alexa 488 or Alexa 555 for immunofluorescence were obtained from Molecular Probes, Inc. (Eugene, OR).

PLASMID CONSTRUCTION

The cDNA fragment encoding the CAN/Nup214 carboxy-terminal region (a.a. 1,789–2,090) identified in the yeast two-hybrid screening was cloned into the pFLAG-CMV2 vector (Sigma–Aldrich) to express a fusion protein with the FLAG® epitope-tag at the amino-terminus in mammalian cells, which was designated as pFLAG-DH2650. An expression vector encoding the full-length cDNA of CAN/Nup214 was also constructed, using the pcDNA3.1 mammalian expression vector (Invitrogen), the cDNA encoding a CAN/Nup214 variant (KIAA0023) provided by Kazusa DNA Research Institute (Chiba, Japan) [Nomura et al., 1994], and the fragment obtained in the yeast two-hybrid screening. The carboxy-terminal variant region of KIAA0023 was replaced by the fragment obtained in the yeast two-hybrid screening, and the resultant full-length cDNA encoding CAN/Nup214 (accession No. NM_005085) was cloned into pcDNA3.1-Zeo(−) at *Apa*I/*Not*I site, which was designated as pcDNA-CAN. The full-length cDNA encoding CAN/Nup214 was also cloned into the pCMV-Myc vector (BD Biosciences Clontech, Inc.) to express the protein tagged with cMyc at the amino-terminus, which was designated as pCMV-Myc-CAN.

The fragment excised from pSG5-VDR by *Nae*I/*Eco*RI digestion was inserted into the pM vector (BD Biosciences Clontech, Inc.) to generate pM-VDR[a.a. 81–427], the expression vector for a chimeric transcription factor in which the GAL4 DNA-binding domain was fused in-frame with the VDR functional ligand-binding domain (a.a. 81–427) [Ozono et al., 1999]. Luciferase reporter constructs containing several types of VDREs were prepared as described previously [Ozono et al., 1999]. To generate a plasmid coding green fluorescent protein (GFP)-tagged DNA-binding domain of VDR (a.a.4–79), pGreenLantern-VDR[a.a. 4–79], the fragment obtained from pSG5-VDR by *Eco*RI/*Nae*I digestion was cloned to pGreenLantern-MCS, which was modified from pGreenLantern-1 vector (Invitrogen) to possess a multiple cloning site at the position of

original stop codon of GFP [Michigami et al., 1999]. The retinoic acid receptor (RAR) and glucocorticoid receptor (GR) expression vectors and reporter plasmids possessing a retinoic acid response element (RARE) or a glucocorticoid response element (GRE) were previously described [Makishima et al., 2002].

WESTERN BLOTTING

Whole cell extracts were harvested in RIPA buffer [1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris–Cl (pH 7.4), 5 mM EDTA, 1 mM orthovanadate and protease inhibitor cocktail (Complete™; Roche Diagnostics)]. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (BioRad, Hercules, CA). After blocking with Block Ace reagent (Dainippon Pharmaceuticals, Osaka, Japan), the membranes were incubated with anti-FLAG® M2 antibody, anti-cMyc antibody, 9A7γ or MAb414. Then, the membranes were washed and incubated with the corresponding HRP-conjugated secondary antibody, and the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

IMMUNOPRECIPITATION

Whole cell lysates were harvested in a lysis buffer (5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 10 mM Tris–HCl, pH 8.0) containing a protease inhibitor cocktail (Complete™, EDTA-free; Roche Diagnostics). The lysates were then incubated with anti-FLAG® M2 affinity gel (Sigma–Aldrich) at 4°C overnight. In some experiments, the lysates were incubated with anti-cMyc polyclonal antibody, and the immunocomplex was immobilized on protein-A/G agarose conjugate at 4°C overnight. The agarose beads were extensively washed, and the immunoprecipitates were subjected to Western blot analyses.

IMMUNOFLUORESCENT STAINING

Cells were plated on cover glasses placed over each well of six-well plates at a density of 1×10^5 cells per well. For fixation, cells were washed twice with phosphate-buffered saline (PBS) warmed at 37°C, and incubated in PBS containing 3.7% formaldehyde for 15 min at room temperature. After washing to remove formaldehyde for fixation, the cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 min at room temperature, then washed again. Blocking to prevent non-specific binding of antibodies was carried out with Block Ace (Dainippon Pharmaceuticals). Then the cells were incubated in anti-FLAG® M2 antibody (1:200 dilution), anti-cMyc antibody (1:200), 9A7γ (1:500) or MAb414 (1:1,000) overnight at 4°C, washed in PBS and incubated with the corresponding secondary antibody (1:500) for 90 min at room temperature, and subjected to indirect fluorescence microscopy.

REPORTER ASSAY

COS7 cells (1×10^5/well in six-well plates) were transfected with a luciferase reporter plasmid, a control plasmid encoding β-galactosidase, and pSG5-VDR together with pFLAG-DH2650, pcDNA-CAN, or the corresponding empty vector. When SaOS-2 cells were utilized, pSG5-VDR was not included. Twenty-four hours after transfection, appropriate stimuli, including $1,25(OH)_2D_3$ (10^{-8} M), dexamethasone (10^{-9} M), all-trans retinoic acid (10^{-7} M), or ethanol vehicle was added to the cells. The cell lysates were harvested after 24 h of treatment with the stimuli and subjected to the reporter assay using a luciferase assay kit (Toyo Ink Co., Ltd., Tokyo, Japan). The luciferase activities were standardized with β-galactosidase activities of the same cell lysates determined by using *o*-nitrophenyl-β-D-galactopyranoside as a substrate.

RESULTS

IDENTIFICATION OF CAN/Nup214 AS A NOVEL PROTEIN INTERACTING WITH VDR

In the current study, we performed yeast two-hybrid screening to explore the proteins interacting with VDR that might be involved in its nuclear transport. Since previously identified nuclear localization signals of VDR are located in the amino-terminal half of VDR [Luo et al., 1994; Hsieh et al., 1998; Michigami et al., 1999], we performed the screening using the fragment corresponding to VDR[a.a. 4–232] as bait. In the screening of a human kidney-derived cDNA library, one of the positive clones, DH2650, was identified to carry a cDNA fragment coding the carboxy-terminal region of CAN/Nup214 (a.a. 1,789–2,090) (Fig. 1A). To confirm the interaction between VDR and the identified carboxy-terminal fragment of CAN/Nup214, we performed a series of co-immunoprecipitation experiments. COS7 cells were transfected with pSG5-VDR and pFLAG-DH2650, and treated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle for 24 h, and the cell lysates were harvested and subjected to immunoprecipitation using anti-FLAG® M2 affinity gel. We observed the co-immunoprecipitation of VDR with FLAG®-tagged DH2650 in both the presence and absence of $1,25(OH)_2D_3$ treatment, confirming the interaction between VDR and CAN/Nup214 (Fig. 1B).

OVEREXPRESSION OF FULL-LENGTH CAN/Nup214 FACILITATES VDR-MEDIATED TRANSCRIPTIONAL ACTIVATION

In COS7 cells, exogenously expressed full-length CAN/Nup214 was predominantly detected in the nuclear periphery, which was consistent with the localization of endogenous CAN/Nup214 demonstrated by the immunostaining using MAb414 (Fig. 2A). MAb414 is an antibody that recognizes a related family of NPC proteins including CAN/Nup214, and is broadly utilized for studying the morphology and composition of the nucleus and nuclear envelope [Walther et al., 2002]. Co-immunoprecipitation experiments confirmed the interaction between full-length CAN/Nup214 and VDR (Fig. 2B). The exogenous expression of CAN/Nup214 did not obviously alter the expression level of VDR (Fig. 2C). The localization of CAN/Nup214 at NPC and its ability to interact with VDR suggest the possibility that the interaction with CAN/Nup214 might modulate the function of VDR as a transcription factor. To test this hypothesis, we examined the effects of overexpression of CAN/Nup214 on the VDR-mediated transcriptional activation. We performed a series of reporter assays to examine the effects of CAN/Nup214 overexpression, utilizing luciferase reporter plasmid pGVB2-r24OHase containing the rat 24-hydroxylase promoter region (−291/+9) with two VDREs [Ohyama et al., 1996]. COS7 cells were transfected with pGVB2-r24OHase, β-galactosidase control

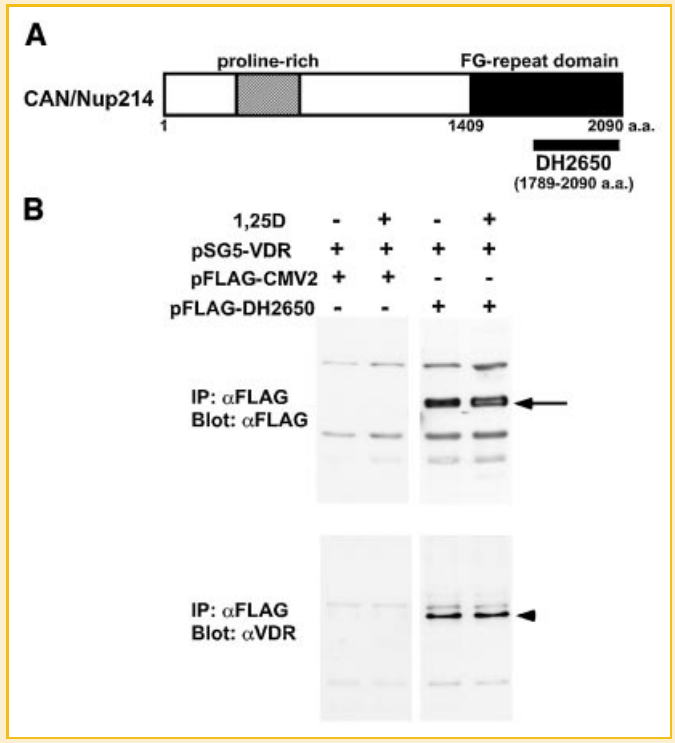

Fig. 1. Identification of CAN/Nup214 as a novel interacting protein of VDR. A: Schematic representation of the structure of human CAN/Nup214. The hatched square and the closed square represent the proline-rich domain and FG-repeat domain, respectively. The solid line represents the fragment named DH2650, which was identified in the yeast two-hybrid screening. B: Co-immunoprecipitation experiments confirmed the interaction between VDR and the carboxy-terminal fragment of CAN/Nup214. COS7 cells were transfected with pSG5-VDR together with pFLAG-DH2650 encoding the carboxy-terminal fragment of CAN/Nup214 that was tagged with FLAG® or pFLAG-CMV2 empty vector. The transfected cells were treated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle for 24 h, and the whole cell extracts were harvested and used for immunoprecipitation with anti-FLAG® M2 affinity gel. The immunoprecipitates were subjected to Western blot analyses using antibodies against FLAG®-tag (M2) or VDR (9A7γ). Arrow, signals corresponding to FLAG®-tagged DH2650; arrowhead, signals corresponding to VDR.

plasmid, pSG5-VDR, and various amounts of pcDNA-CAN, and then treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle for 24 h before cell lysates were harvested. In the cells treated with $1,25(OH)_2D_3$, the relative luciferase activities normalized based on β-galactosidase activities of the same lysates were increased by the overexpression of CAN/Nup214 (Fig. 2D). The fold-induction in the relative reporter activity on treatment with $1,25(OH)_2D_3$ was significantly enhanced by overexpression of CAN/Nup214 in a dose-dependent manner (Fig. 2E). When pCMV-Myc-CAN was utilized instead of pcDNA-CAN, similar results were obtained (data not shown). We also utilized a human osteocalcin promoter-luciferase construct (pGVB2-hOC) containing a single VDRE or the plasmid pGVP2-(DR3)2 carrying synthetic VDREs (two copies of consensus DR3) instead of pGVB2-r24OHase, and obtained similar results (data not shown). These results suggest that the interaction between VDR and full-length CAN/Nup214 facilitates the VDR-mediated transactivation.

OVEREXPRESSION OF THE CALBOXY-TEMINAL FRAGMENT OF CAN/Nup214 SUPPRESSED VDR-MEDIATED TRANSCRIPTIONAL ACTIVATION

We then examined the effects of the carboxy-terminal fragment of CAN/Nup214 on VDR-mediated transactivation using pFLAG-DH2650. In immunofluorescent staining, the fragment exhibited a predominantly nuclear localization (Fig. 3A). In contrast to the case of full-length CAN/Nup214, overexpression of FLAG®-tagged DH2650 clearly suppressed the ligand-induced transactivation of pGVB2-r24OHase in a dose-dependent manner in various cell-types including SaOS-2 cells that express endogenous VDR (Fig. 3C–F). These findings demonstrated that the expression of the carboxy-terminal fragment of CAN/Nup214 suppressed the VDR-mediated transactivation. The protein level of VDR was not affected by the increase in the amount of pFLAG-DH2650 as shown by Western blotting (Fig. 3B). Therefore, it is unlikely that the suppressive effect of DH2650 on VDR-mediated transactivation was due to the reduced protein amount of VDR.

EFFECTS OF CAN/Nup214 AND DH2650 ON TRANSACTIVATION MEDIATED BY RETINOIC ACID RECEPTOR AND GLUCOCORTICOID RECEPTOR

Next, we examined the effects of CAN/Nup214 and DH2650 on other nuclear receptors. Glucocorticoid receptor (GR) and retinoic acid receptor (RAR) belong to the steroid receptor superfamily as does VDR, and share a rather high similarity of sequence in the DNA-binding domains. To examine the effects of CAN/Nup214 and DH2650 on GR-mediated transactivation, we utilized the reporter

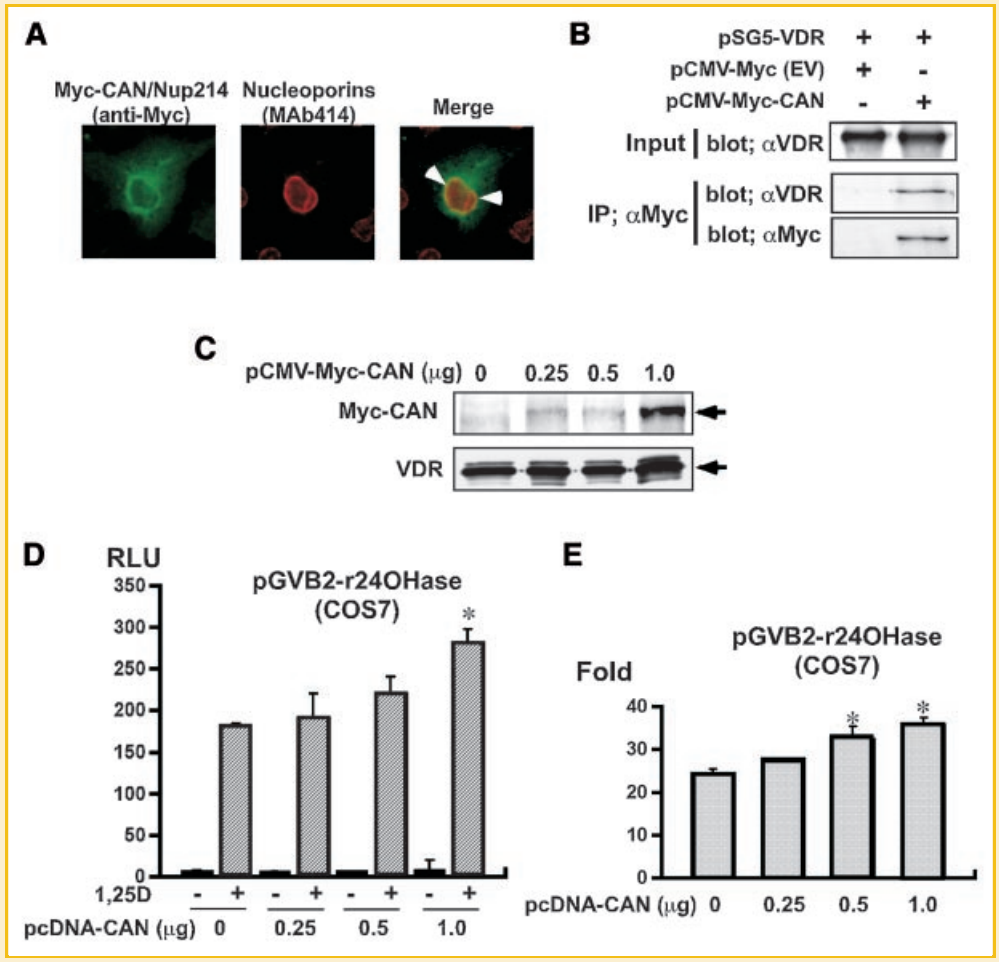

Fig. 2. Overexpression of full-length CAN/Nup214 facilitated VDR-mediated transactivation. A: Subcellular distribution of exogenously expressed CAN/Nup214. COS7 cells were transfected with the expression plasmid pCMV-Myc-CAN encoding full-length CAN/Nup214 tagged with cMyc, and subjected to immunofluorescent staining with anti-cMyc polyclonal antibody (green) and MAb414 (red). Arrowheads indicate the overlapping of the signals of the two antibodies. B: The interaction between full-length CAN/Nup214 and VDR. COS7 cells were transfected with pSG5-VDR together with pCMV-Myc-CAN or pCMV-Myc empty vector (EV), and cell lysates were harvested to serve for immunoprecipitation with anti-cMyc polyclonal antibody. The aliquoted immunoprecipitates and the input protein samples (5 μg) were subjected to Western blot using the indicated antibodies. The signal in the blot with anti-cMyc antibody corresponds to CAN/Nup214 tagged with cMyc. C: The exogenous expression of CAN/Nup214 did not alter the expression level of VDR. COS7 cells were transfected with pSG5-VDR and various amounts of pCMV-Myc-CAN, and cell lysates were harvested 48 h after transfection. To examine the expression levels of cMyc-tagged CAN/Nup214 and VDR, Western blot analyses were performed using antibodies against cMyc-tag or VDR, respectively. Arrows indicate the corresponding signals. D,E: Exogenous expression of full-length CAN/Nup214 facilitated the VDR-mediated transactivation of the rat 24-hydroxylase promoter. COS7 cells were transfected with pSG5-VDR, β-galactosidase control vector, and various amounts of pcDNA-CAN/Nup214 together with the rat 24-hydroxylase promoter-luciferase reporter plasmid pGVB2-r24OHase containing two VDREs. Transfectants were treated with 10^{-8} M 1,25$(OH)_2D_3$ or ethanol vehicle for 24 h, and the cell lysates were harvested and subjected to a luciferase assay. In (D), the data are shown as the relative luciferase activities standardized based on β-galactosidase activities. In (E), the data are shown as the fold-induction of the standardized luciferase activity by treatment with 1,25$(OH)_2D_3$. Data are shown as the mean ± SE ($n = 3$). *Significantly different from the value of the cells without exogenous CAN/Nup214 ($P < 0.01$). The experiments were performed three times, and similar results were obtained.

plasmid pMTV-Luc and the GRα expression vector pCMX-GRα. For RAR-mediated transactivation, we performed reporter assays using the luciferase reporter plasmid ptk-TREp2-Luc and the RARα expression vector pCMX-hRARα. Co-transfection of full-length CAN/Nup214 enhanced the GR-mediated ligand-dependent transcriptional activation while expression of DH2650 markedly suppressed it, which was similar to the case of VDR (Fig. 4A–D). As to the case of RAR-mediated transcriptional activation, co-transfection of DH2650 suppressed it, although that of full-length CAN/Nup214 did not exhibit significant effects on the fold-induction of the reporter activity on the treatment with the ligand (Fig. 4E–H). We confirmed the co-immunoprecipitation of GRα with FLAG®-tagged DH2650 both in the presence and absence of the ligand (Fig. 4I).

EFFECTS OF CAN/Nup214 ON VDR WERE DEPENDENT ON THE DNA-BINDING DOMAIN

In the current yeast two-hybrid screening, we utilized bait that corresponded to the amino-terminal half of VDR containing the DNA-binding domain. In addition, the overexpression of CAN/

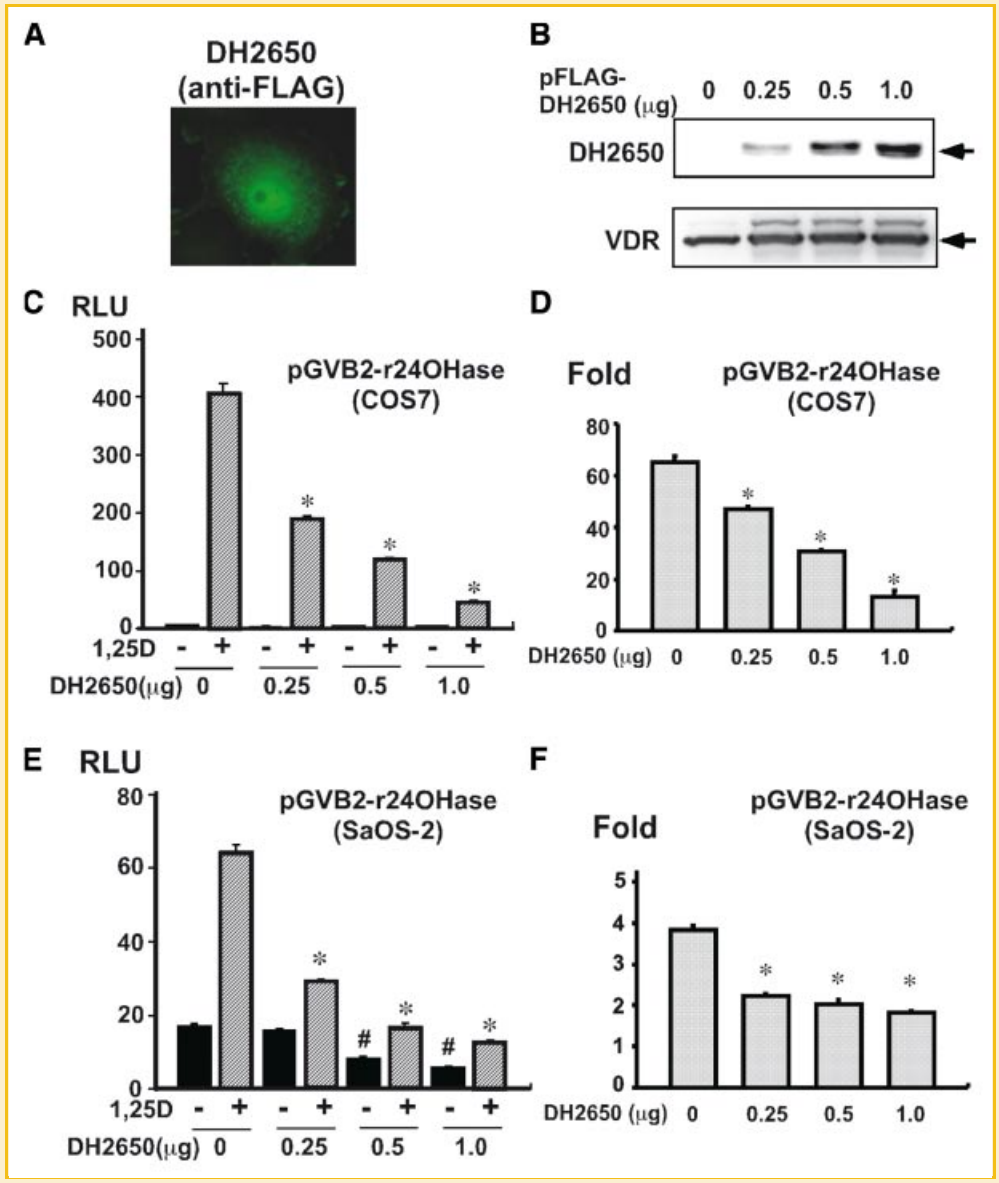

Fig. 3. Expression of the carboxy-terminal fragment of CAN/Nup214 (DH2650) suppressed VDR-mediated transactivation. A: Subcellular distribution of exogenously expressed DH2650. COS7 cells were transfected with the expression plasmid pFLAG-DH2650 encoding the carboxy-terminal fragment of CAN/Nup214 tagged with FLAG®, and subjected to immunofluorescent staining with anti-FLAG® monoclonal antibody (M2). DH2650 was predominantly located in the nucleus. B: The expression of DH2650 did not alter the protein expression level of VDR. COS7 cells were transfected with pSG5-VDR and various amounts of pFLAG-DH2650, and cell lysates were harvested 48 h after transfection. To examine the expression levels of FLA-DH2650 and VDR, Western blot analyses were performed using antibodies against FLAG®-tag or VDR, respectively. Arrows indicate the corresponding signals. C–F: Co-transfection of DH2650 suppressed the VDR-mediated transactivation of the rat 24-hydroxylase promoter. In (C) and (D), COS7 cells were transfected with pSG5-VDR, the β-galactosidase control vector, and various amounts of pFLAG-DH2650 together with the rat 24-hydroxylase promoter-luciferase reporter plasmid pGVB2-r24OHase containing two VDREs. In (E) and (F), the similar experiments were performed in SaOS-2 cells expressing endogenous VDR, without the expression plasmid pSG5-VDR. Transfectants were treated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle for 24 h, and the cell lysates were harvested and subjected to a luciferase assay. In (C) and (E), the data are shown as the relative luciferase activities standardized based on β-galactosidase activities. In (D) and (F), the data are shown as the fold-induction of the standardized luciferase activity by treatment with $1,25(OH)_2D_3$. Data are shown as the mean ± SE ($n = 3$). *Significantly different from the value of the cells without DH2650 ($P < 0.01$). The experiments were performed three times, and similar results were obtained. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Nup214 facilitated the transcriptional activation mediated by GR, which shares rather high similarity of sequence with VDR in the DNA-binding domains (Fig. 4A,B). Therefore, it is suggested that the DNA-binding domains might be responsible for the functional regulation by CAN/Nup214 in these nuclear receptors. To address this issue, we utilized pM-VDR[a.a. 81–427], encoding a chimeric transcription factor consisting of the DNA-binding domain of GAL4 and functional ligand-binding domain of VDR (Fig. 5A) [Ozono et al., 1999]. To evaluate the transcriptional activation of this chimera, a reporter construct possessing the SV40 promoter and the

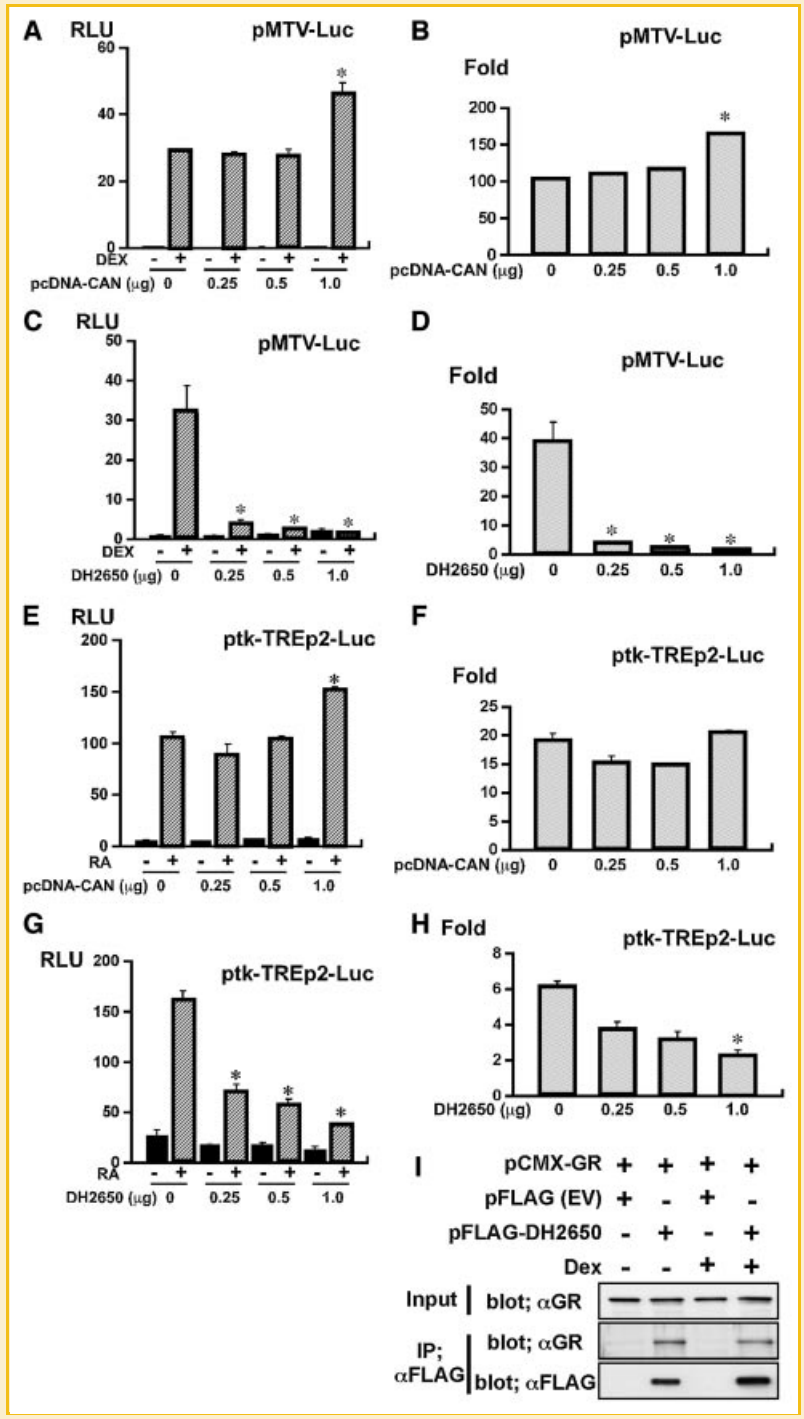

Fig. 4. Effects of full-length CAN/Nup214 and DH2650 on the transactivation mediated by nuclear receptors other than VDR. A–F: COS7 cells were transfected with pCMX-hGRα (A–D) or pCMX-hRARα (E–H) with the corresponding reporter plasmids: pMTV-Luc for GRα and ptk-TREp2-Luc for RARα, respectively. The β-galactosidase control vector and various amounts of pcDNA-CAN (A,B,E,F) or pFLAG-DH2650 (C,D,G,H) were included in the transfection. The cells transfected with pCMX-hGRα were treated with 10^{-9} M of dexamethasone or ethanol vehicle, and those transfected with pCMX-hRARα were treated with 10^{-7} M of all-trans retinoic acid or vehicle for 24 h, before cell lysates were harvested for luciferase assay. The panels (A,C,E,G) show the relative luciferase activities standardized based on β-galactosidase activities, and the panels (B,D,F,H) exhibit their respective calculated fold-induction of the standardized luciferase activities by treatment with the ligands. Data are shown as the mean ± SE ($n = 3$). *Significantly different from the value of the cells without exogenous expression of CAN/Nup214 or DH2650 ($P < 0.01$). The experiments were performed three times, and similar results were obtained. (I) Interaction between GR and FLAG-DH2650. COS7 cells were transfected with pCMX-hGRα together with pFLAG-DH2650 or pFLAG-CMV2 empty vector. The transfectants were treated with 10^{-9} M of dexamethasone (DEX) or ethanol vehicle for 24 h, and cell lysates were harvested to serve for immunoprecipitation using anti-FLAG® M2 affinity gel. The aliquoted immunoprecipitates and the input protein samples (5 μg) were subjected to Western blot using the indicated antibodies. The signal in the blot with anti-FLAG antibody corresponds to FLAG®-tagged DH2650.

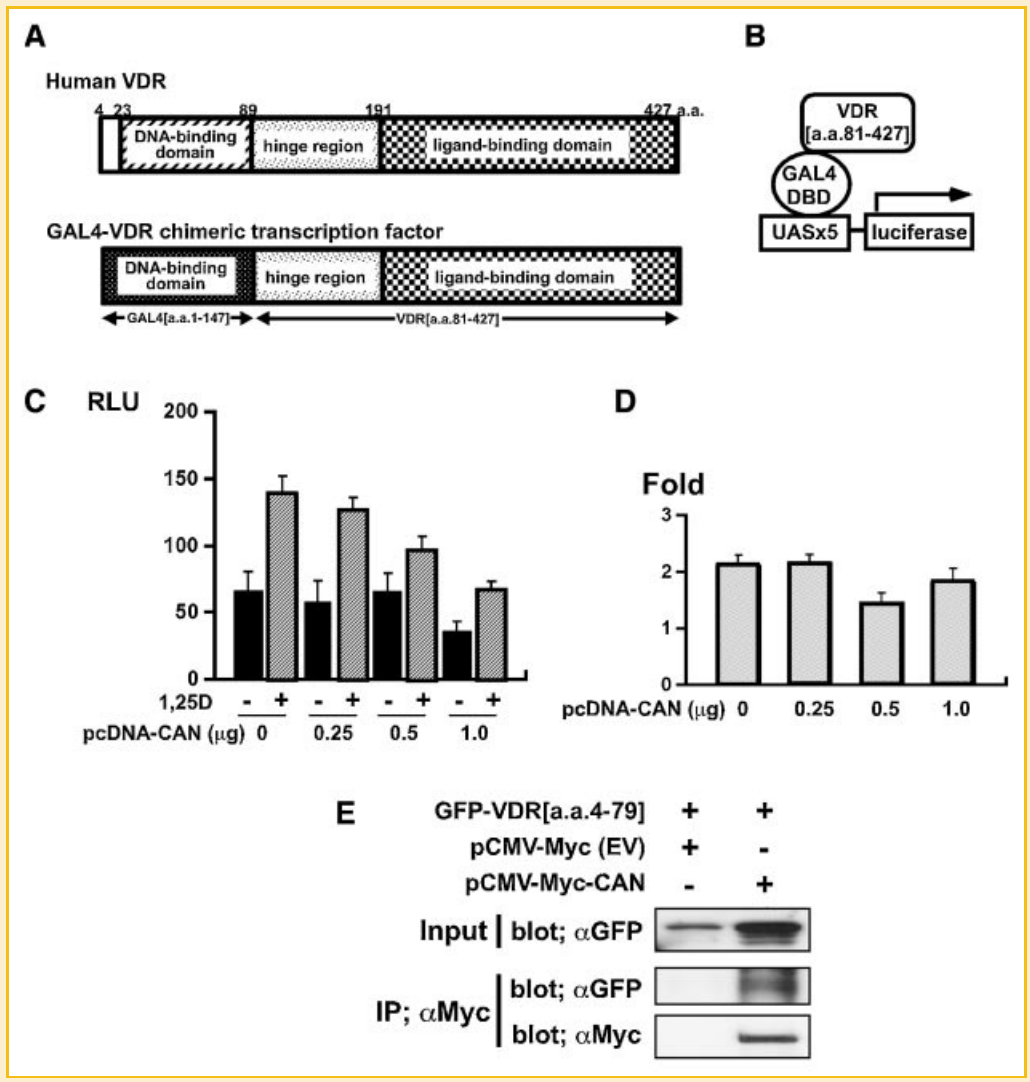

Fig. 5. The effects of CAN/Nup214 on VDR-mediated transcriptional activation are dependent on the DNA-binding domain of VDR. A: Structures of human VDR and the GAL4-VDR chimeric transcription factor, which are encoded by pSG5-VDR and pM-VDR [a.a. 81–427], respectively. The chimera possesses the DNA-binding domain of GAL4 instead of that of VDR. B: Schematic representation of the transactivation of the reporter plasmid containing GAL4-responsive elements (UAS × 5) by the GAL4-VDR chimeric transcription factor. C,D: Overexpression of the full-length CAN/Nup214 failed to facilitate the transactivation mediated by the GAL4-VDR chimera. COS7 cells were transfected with pM-VDR[a.a. 81–427], β-galactosidase control vector, and various amounts of pcDNA-CAN/Nup214 together with the reporter plasmid pGVP2-GAL4BS bearing 5 copies of the GAL4-binding site. Transfectants were treated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle for 24 h, and the cell lysates were harvested and subjected to luciferase assays. In (C), the data are shown as the relative luciferase activities standardized based on β-galactosidase activities. In (D), the data are shown as the fold-induction of the standardized luciferase activity by treatment with $1,25(OH)_2D_3$. Data are shown as the mean ± SE (n = 3). E: Co-immunoprecipitation experiments confirmed the interaction between DNA-binding domain of VDR and CAN/Nup214. COS7 cells were transfected with pGreenLantern-VDR [a.a. 4–79] encoding the DNA-binding domain of VDR tagged with GFP together with pCMV-Myc-CAN or the pCMV-Myc empty vector. Forty-eight hours later, cell lysates were harvested to serve for immunoprecipitation with anti-cMyc polyclonal antibody. The aliquoted immunoprecipitates and the input (10 μg protein) were subjected to Western blot using the indicated antibodies. The signals in the blots with anti-cMyc antibody and anti-GFP antibody correspond to Myc-CAN/Nup214 and the DNA-binding domain of VDR, respectively.

GAL4-binding site, pGVP2-GAL4BS, was employed. COS7 cells were co-transfected with pM-VDR [a.a. 81–427], pGVP2-GAL4BS, pSG5-VDR, and various amounts of pcDNA-CAN, and treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle for 24 h. As expected, treatment with $1,25(OH)_2D_3$ enhanced the reporter activity. However, the overexpression of CAN/Nup214 did not facilitate the induction of the reporter activity on the treatment with $1,25(OH)_2D_3$ (Fig. 5C,D), which was different from the case of VDR-mediated transactivation assayed using the rat 24-hydroxylase promoter (Fig. 3). Then, we examined the interaction between the DNA-binding domain of VDR and CAN/Nup214. To address this issue, we utilized the plasmid pGreenLantern-VDR [a.a.4–79] encoding the DNA-binding domain of VDR tagged with GFP, since the anti-VDR antibody 9A7γ does not recognize the DNA-binding domain of VDR. The DNA-binding domain of VDR tagged with GFP (GFP-VDR[a.a.4–79]) co-immunoprecipitated with CAN/Nup214, confirming the

involvement of the DNA-binding domain in the interaction with CAN/Nup214 (Fig. 5E).

CARBOXY-TERMINAL FRAGMENT DH2650 INHIBITED THE INTERACTION OF VDR WITH THE FULL-LENGTH CAN/Nup214

As described above, the exogenous expression of the full-length CAN/Nup214 facilitated the VDR-mediated transactivation, while that of DH2650 suppressed it (Figs. 2 and 3). To test the hypothesis that DH2650 suppresses VDR-mediated transactivation through the inhibition of the interaction of the endogenous CAN/214 with VDR, we examined the effects of DH2650 on the interaction between full-length CAN/Nup214 and VDR by co-immunoprecipitation experiments. Exogenous expression of FLAG®-tagged DH2650 reduced the co-immunoprecipitation of VDR with Myc-tagged CAN/Nup214 (Fig. 6), indicating the inhibitory effects of DH2650 on the interaction between VDR and the full-length CAN/Nup214.

EFFECTS OF CAN/Nup214 AND THE CARBOXY-TERMINAL FRAGMENT DH2650 ON THE NUCLEAR LOCALIZATION OF VDR

Since CAN/Nup214 is a component of NPC, it is reasonable to hypothesize that the interaction between VDR and CAN/Nup214 might modulate the nuclear accumulation of VDR. We examined the effects of overexpression of CAN/Nup214 or the carboxy-terminal fragment DH2650 on the nuclear localization of VDR. COS7 cells were transfected with pSG5-VDR together with pCMV-Myc-CAN or pFLAG-DH2650, and subjected to immunofluorescence microscopy to examine the subcellular distribution of VDR. The overexpression of full-length or carboxy-terminal CAN/Nup214 did not lead to the obvious alteration in the subcellular distribution of VDR (Fig. 7).

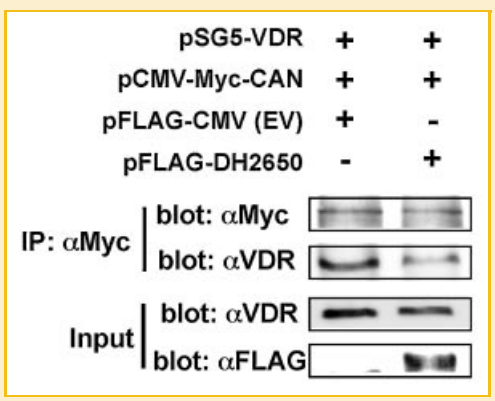

Fig. 6. The carboxy-terminal region of CAN/Nup214 inhibited the interaction between the full-length protein and VDR. COS7 cells were transfected with pSG5-VDR and pCMV-Myc-CAN, together with pFLAG-DH2650 or pFLAG-CMV2 empty vector. Forty-eight hours later, cell lysates were harvested to serve for immunoprecipitation with anti-cMyc polyclonal antibody. The aliquoted immunoprecipitates and the input protein (10 μg) were subjected to Western blot using the indicated antibodies. The signals in the blots with anti-cMyc antibody and anti-FLAG® antibody correspond to cMyc-tagged CAN/Nup214 and FLAG®-tagged DH2650, respectively.

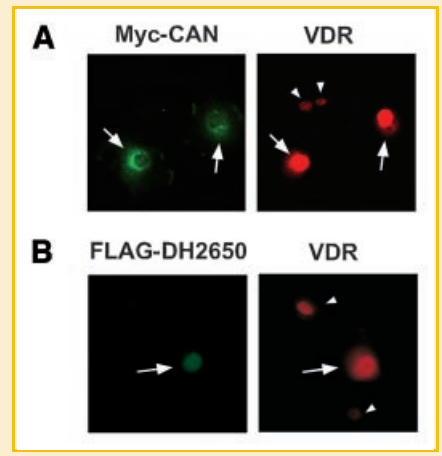

Fig. 7. Effects of overexpression of the full-length or carboxy-terminal region of CAN/Nup214 on the nuclear localization of VDR. A: COS7 cells were transfected with pSG5-VDR and pCMV-Myc-CAN, and subjected to immunofluorescence using antibodies against VDR (red) and cMyc-tag (green). The arrow shows the cell expressing both VDR and CAN/Nup214 tagged with cMyc, while the arrowhead represents the cell expressing VDR alone. B: COS7 cells were transfected with pSG5-VDR and pFLAG-DH2650, and subjected to immunofluorescence using antibodies against VDR (red) and FLAG®-tag (green) after treatment with 10^{-8} M $1,25(OH)_2D_3$ for 6 h. The arrow shows the cell expressing both VDR and FLAG®-DH2650, while the arrowheads represent the cells expressing VDR alone.

DISCUSSION

In the current study, we have identified the nucleoporin CAN/Nup214 as a novel protein interacting with VDR by yeast two-hybrid screening. The interaction between VDR and the carboxy-terminal FG-repeat domain of CAN/Nup214 was confirmed by co-immunoprecipitation (Fig. 1B). CAN/Nup214 is a component of the NPC and involved in both nuclear protein import and mRNA export [van Deursen et al., 1996; Matsubayashi et al., 2001; Xu et al., 2002], and the FG-repeat domain in its carboxy-terminus functions as a potential binding site for transport receptors (Fig. 1A). CAN/Nup214 was previously mapped to the cytoplasmic surface of NPC. However, by immuno-electron microscopy using domain-specific antibodies, Pailillo et al. [2005] have recently demonstrated that CAN/Nup214 is anchored to the cytoplasmic side of NPC via the amino-terminal and central domain, and that its carboxy-terminal FG-repeat domain appears flexible, residing on both sides of NPC. They also found that the spatial distribution of the FG-repeat domain of CAN/Nup214 shifts in a transport-dependent manner, suggesting that the location of this domain within the NPC correlates with cargo/receptor interactions [Pailillo et al., 2005].

In a series of reporter assays, we have revealed that the exogenous expression of full-length CAN/Nup214 facilitated the ligand-dependent transactivation mediated by VDR (Fig. 2), suggesting that the interaction with CAN/Nup214 modulates the function of VDR as a ligand-dependent transcription factor. Since immunofluorescent staining revealed the exogenous full-length CAN/Nup214 to be predominantly distributed at the nuclear envelope

(Fig. 2A), we assume that some of the exogenously expressed CAN/Nup214 was assembled into NPCs and interacted with VDR via its FG-repeat domain in the vicinity of NPCs. In contrast to the case of full-length CAN/Nup214, the expression of the carboxy-terminal fragment of CAN/Nup214, DH2650, which contains the FG-repeat domain, inhibited the ligand-dependent transactivation mediated by VDR (Fig. 3). Although CAN/Nup214 was reported to be involved in mRNA export, the protein level of VDR was not obviously altered by the exogenous expression of full-length CAN/Nup214 or DH2650 (Figs. 2C and 3B). The expression of DH2650 did not cause a reduction in the amount of VDR protein probably because the residual function of CAN/Nup214 was enough for the mRNA export. It has been reported that overexpression of the carboxy-terminal FG-repeat domain of CAN/Nup214 inhibits the nuclear transport of the proteins interacting with it such as CRM1 and Smad2 [Boer et al., 1998; Xu et al., 2002]. In the case of the interaction with VDR, we found that the exogenous expression of the carboxy-terminal fragment DH2650 containing the FG-repeat domain inhibited the interaction between the full-length CAN/Nup214 and VDR (Fig. 6). These results indicate that DH2650 suppressed the VDR-mediated transactivation through the inhibition of the interaction between VDR and endogenous full-length CAN/Nup214. We also examined the effects of overexpression of the full-length CAN/Nup214 or DH2650 on the subcellular distribution of VDR by immunofluorescent staining. Although we could not detect the obvious alteration in subcellular distribution of VDR (Fig. 7), we cannot exclude the possibility that the kinetics of the nuclear translocation of VDR might be changed by the overexpression of the full-length CAN/Nup214 or DH2650 or that the subtle change of VDR localization is enough to affect the function of VDR. CAN/Nup214 might involve some unknown mechanisms other than the regulation of subcellular distribution of VDR in modulation of VDR-mediated transcriptional activation. However, considering the direct interaction of the full-length CAN/Nup214 with VDR and the inhibitory effect of DH2650 on the interaction (Fig. 6), we prefer to think that CAN/Nup214 directly modulates the function of VDR, rather than assume the function through some other molecules such as co-factors.

Since the structure of the bait used in the yeast two-hybrid screening suggested that the amino-terminal region of VDR is involved in the interaction with CAN/Nup214, we also examined the effect of expression of exogenous CAN/Nup214 on the transcriptional activation mediated by other nuclear receptors, GR and RAR. These receptors share similarity with VDR in the sequence of DNA-binding domains. The overexpression of full-length CAN/Nup214 markedly facilitated the GR-mediated ligand-dependent transcription (Fig. 4A,B). The interaction between GR and the carboxy-terminal region of CAN/Nup214 was also confirmed (Fig. 4I), indicating that CAN/Nup214 regulates the GR-mediated transcription through the similar mechanism to the case of VDR. Since the overexpression of full-length CAN/Nup214 failed to enhance the transcriptional activity of the GAL4–VDR [a.a. 81–427] chimera and the DNA-binding domain of VDR co-immunoprecipitated with CAN/Nup214 (Fig. 5), the effects of CAN/Nup214 on the transcriptional activities are likely to be exerted through the DNA-binding domains of these nuclear receptors.

The interaction between CAN/Nup214 and VDR or GR was ligand-independent. Our previous work clearly demonstrated that VDR predominantly has a nuclear localization even in the absence of ligand, and that VDR possesses two distinct mechanisms for nuclear transport: ligand-dependent and -independent pathways [Michigami et al., 1999; Miyauchi et al., 2005]. We also identified importin 4 as a molecule that facilitates the ligand-independent nuclear import of VDR [Miyauchi et al., 2005]. Based on these findings, we assume that there might be significant amounts of unliganded VDRs as well as liganded VDRs in the nucleus. Indeed, Fujiki et al. [2005] recently reported that unliganded VDR binds to the Williams syndrome transcription factor (WSTF) and associates with the promoter of the 1α-hydroxylase gene, indicating that unliganded VDR also plays some physiological roles in the nucleus. The functional relationship between CAN/Nup214 and importin 4 in the ligand-independent nuclear import of VDR remains to be elucidated.

CAN/Nup214 is also known to be involved in myeloblastic leukemogenesis by producing fusion proteins such as DEK-CAN through chromosomal translocation. On the other hand, it has been described that VDR mediates the differentiation of some leukemia cell lines, and active vitamin D has been utilized to treat patients with leukemia [Nagpal et al., 2005]. The stimulation of VDR with a selective agonist without a vitamin D skeleton inhibits proliferation and induces the differentiation of human monocytoma [Adachi et al., 2005]. It is intriguing that the molecules with contrasting effects on the disease form a complex. More noteworthy is that the carboxy-terminal portion of CAN/Nup214 is frequently part of the products of chromosomal rearrangements associated with the malignant transformation of hematopoietic cells [von Lindern et al., 1992; Kraemer et al., 1994; Graux et al., 2004]. It has also been demonstrated that fusion proteins are exclusively located in the nucleus, and they suppress the $1,25(OH)_2D_3$-induced differentiation of human promonocytic cells [Fornerod et al., 1995; Kandilci et al., 2004]. In addition, a recent report has demonstrated the impaired nuclear localization of VDR in leukemia cells resistant to $1,25(OH)_2D_3$-induced differentiation [Humeniuk-Polaczek and Marcinkowska, 2004]. In the future, more attention should be focused on the functional loss of some transcription factors caused by oncogenic products, including the carboxy-terminal segment of CAN/Nup214, whereas influence on the functions of fusion partner proteins such as DEK previously attracted attention as one of the factors contributing to hematopoietic malignancy.

In conclusion, we have discovered a novel interaction between VDR and CAN/Nup214. The interaction with CAN/Nup214 facilitated, while its carboxy-terminal fragment inhibited the VDR-mediated transactivation. This is the first report demonstrating that the interaction with the component of NPC modulates the function of VDR as a transcription factor.

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