Importin 4 Is Responsible for Ligand-independent Nuclear Translocation of Vitamin D Receptor*

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Vitamin D receptor (VDR) is localized in nuclei and acts as a ligand-dependent transcription factor. To clarify the molecular mechanisms underlying the nuclear translocation of VDR, we utilized an in vitro nuclear transport assay using digitonin-permeabilized semi-intact cells. In this assay, recombinant whole VDR-(4–427) and a truncated mutant VDR-(4–232) lacking the carboxyl terminus of VDR were imported to nuclei even in the absence of ligand. In contrast, VDR-(91–427) lacking the amino-terminal DNA-binding domain was not imported to nuclei in the absence of ligand, and was efficiently imported in its liganded form. These results suggested that there are two distinct mechanisms underlying the nuclear transport of VDR; ligand-dependent and -independent pathways, and that the different regions of VDR are responsible for these processes. Therefore, we performed the yeast two-hybrid screening using VDR-(4–232) as the bait to explore the molecules responsible for ligand-independent nuclear translocation of VDR, and have identified importin 4 as an interacting protein. In the reconstruction experiments where transport factors were applied as recombinant proteins, recombinant importin 4 facilitated nuclear translocation of VDR regardless of its ligand, whereas importin β failed in transporting VDR even in the presence of ligand. In conclusion, importin 4, not importin β, is responsible for the ligand-independent nuclear translocation of VDR.

The active form of vitamin D, 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) regulates various biological events through its direct actions on gene expression in its target organs such as intestine, kidney, and bone (1, 2). Its cognate receptor, vitamin D receptor (VDR), belongs to the nuclear receptor superfamily, and plays crucial roles in the physiological actions of 1α,25(OH)₂D₃ including calcium homeostasis and bone metabolism (1, 2). Nuclear receptors including VDR act as ligand-inducible transcription factors, and consist of several functional domains to regulate the expression of the target genes (1–3). Utilizing its DNA-binding domain, VDR recognizes a vitamin D-responsive element (VDRE) in the target genes, and regulates the expressions via forming complexes with retinoid X receptor (RXR) and transcriptional co-factors on its ligand-binding domain. To fulfill these events on the genome, nuclear receptors must be localized in nuclei. However, it seems that the subcellular distributions of the nuclear receptors do not resemble each other, unlike their transcriptional regulatory systems. Several reports have demonstrated that VDR is located in nuclei even in the absence of 1α,25(OH)₂D₃, and is accumulated in nuclei to a greater extent by addition of the ligand (4–6). These observations are in contrast with the cases of other nuclear receptors, glucocorticoid receptor or estrogen receptor; the former translocates from the cytoplasm into nuclei only in the presence of the ligand, and the latter is located in nuclei regardless of the ligand (7, 8). The obvious differences in their subcellular distribution might be derived from different systems adopted to control nuclear localization.

In cells of eukaryotes, nucleoplasm is separated from cytoplasm with nuclear envelope, which is perforated by numbers of large apparatus consisting of specific proteins. The assembled structures, termed nuclear pore complexes, are composed of subunits of nucleoporins, and act as the sole gatekeepers controlling the exchange of materials between the two compartments (9, 10). Although nuclear pore complexes 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 (11–13). The import of cargo molecules such as transcription factors and signal transducers through nuclear pore complexes is mediated by the nuclear transport factors such as importin β, which binds cargoes and nucleoporins simultaneously through separate domains (14). In the case of classical nuclear localization signal (NLS)-containing proteins, importin α bridges the interaction between the NLS motif and importin β (14). Another common element in the process is the small GTPase Ran in its GTP-bound form (Ran-GTP), whose binding to importin β triggers the dissociation of the complex consisting of importin α, importin β, and the cargoes. Ran exists mostly in its GTP-bound form in nuclei and in a GDP-bound form in cytoplasm. Such a Ran-GTP gradient across the nuclear envelope prompts the release of cargoes only in nucleoplasm, ensuring the accumulation of the cargoes in nuclei. 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 (14). The importin α-independent pathways also function in a Ran-dependent manner. Although the general mech-
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Anisms of nuclear transport are rather well understood as described above, the specific role of the importin β family other than importin β remains to be determined. In addition, the nuclear transport system for VDR has not yet been clarified.

In the current study, we have investigated the nuclear import process of VDR utilizing an in vitro nuclear transport assay. Our results have demonstrated that VDR possesses two distinct nuclear transport pathways, i.e. the ligand-dependent and -independent pathways, and that the different regions of VDR are responsible for these processes. The exploration of the molecules involved in the nuclear import of VDR utilizing the yeast two-hybrid screening have revealed the interaction of VDR and importin 4, and the recombinant importin 4 was capable of transporting VDR into nuclei regardless of the presence or absence of the ligand.

MATERIALS AND METHODS

Cell Culture and Transfections—Monkey kidney epithelial cell line COS 7 was maintained at 37 °C under a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Hyclone, Logan, UT) stripped with dextran-coated charcoal to remove endogenous steroids. Human fibroblastic cell line HeLa was cultured under the same conditions as described above except that non-stripped fetal bovine serum was used. Transient transfections were performed using FuGENE 6® (Roche Diagnostics).

Antibodies—Antibodies against VDR, 9A7, and D-6 were purchased from Affinity Bioreagents, Inc. (Neshanic Station, NJ) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Anti-RXR antibody (D-20) and anti-glutathione S-transferase (GST) antibody (B-14) were obtained from Santa Cruz, and anti-FLAG® antibody (M2) was purchased from Sigma.

Bacterial Expression and Purification of Recombinant Proteins—The expression vector of the human VDR, pSG5-hVDR, was kindly provided by Dr. M. R. Haussler, University of Arizona (15). Although the amino acid number is indicated according to the original paper concerning the human VDR cDNA (GenBank® accession number J03258), pSG5-hVDR itself contains the VDR cDNA that starts from the second translation start point because of the existence of polymorphism, resulting in 424 amino acids (aa 4 – 427). To construct the bacterial expression vector, pGEX2T-hVDR, the fragment excised with EcoRI from pSG5-hVDR was subcloned into pGEX-2T (Amersham Biosciences). The truncated mutants of VDR-(4–232) and VDR-(91–427) were obtained by digestion of pSG5-hVDR with appropriate restriction endonucleases and inserted into pGEX-2T vector. The GST fusion proteins were then expressed in Escherichia coli BL21 (DE3). Cells were then collected and disrupted in sonication buffer (phosphate-buffered saline containing 10% glycerol, 5 mM DTT, 1% Triton X-100, and Complete® protease inhibitor mixture (Roche)). After centrifugation, the supernatant was incubated with glutathione-Sepharose (Amersham Biosciences) at 4 °C. GST fusions bound on glutathione-Sepharose were washed extensively with the sonication buffer. For GST pull-down experiments, the fusion proteins were eluted from glutathione-Sepharose and cleaved to remove GST with thrombin. The soluble proteins passed through the desalting columns were equilibrated with required buffers, followed by concentration using a Vivaspin® Concentrator (Vivascience, Hannover, Germany). The purity of all recombinant proteins was confirmed by SDS-PAGE.

Human importin 4 cDNA containing full-length open reading frame (GenBank™ accession number AK057277; equal to NM_024658) was purchased from the Helix Institute (Kisarazu, Japan). To prepare recombinant importin 4, bacterial expression vector was constructed by insertion of importin 4 cDNA into pGEX-6P-3 followed by expression, extraction, and purification of the recombinant protein as described above. The vectors pGEX-HA-PTAC97 encoding mouse importin β with hemagglutinin tag at the amino terminus and pGEX-2T-SV40NLS-GFP containing NLS and green fluorescent protein (Ref. 16; designated as GST-NLS(SV40-GFP) in this paper) were also utilized to prepare the corresponding recombinant proteins. Ran-GDP and NTF2 were prepared as described previously (17, 18). Mammalian expression vectors for FLAG®-tagged importins were also constructed using pFLAG-CMV-2 (Sigma).

Yeast Two-Hybrid Screening—Yeast two-hybrid screening was performed using the BD Matchmaker Two-Hybrid System (Clontech) following the manufacturer’s protocols. The bait vector pAS2-1-VDR-(4–232) was constructed by inserting the same fragment of VDR with that of pGEX-VDR-(4–232) into pAS2-1 (Clontech). Saccharomyces cerevisiae strain AH109 was transformed with the bait vector, and then subsequently mated with another strain Y187 pretreated with the human kidney cDNA library. Tranformants were plated onto the synthetic media lacking adenine (Ade), leucine (Leu), histidine (His), and tryptophan (Trp). Ade” His” growers were isolated, and subjected to β-galactosidase filter assay. Blue clones were regarded as candidates, and the inserts were amplified by polymerase chain reaction using specific primers for the library plasmid, followed by sequencing.
Mammalian Two-hybrid Assay—To construct mammalian two-hybrid vectors, the carboxyl-terminal fragment of importin 4 (aa 846–1081) was released by SacII/XbaI digestion and fused in-frame into pM vector (Clontech), resulting in pM-IMP4. Whole cDNA of human VDR was released by EcoRI digestion from pSG5-hVDR and inserted in-frame into pVP16 vector (pVP16-hVDR), and further cDNA fragments of human VDR were excised by digestion with appropriate restriction endonucleases and cloned to pVP16 vector. The fragment obtained by Nael/EcoRI digestion of pSG5-hVDR was also cloned into pVP16 vector, resulting in pVP16-hVDR-(81–427). A luciferase reporter plasmid was generated by the insertion of 4 copies of consensus GAL4 binding sites into pGVP2 vector (Toyo Ink Co. Ltd., Tokyo, Japan) and designated as pGVP2-GAL4BS.

Various combinations of the mammalian two-hybrid vectors constructed as above were transfected with pGVP2-GAL4BS and β-galactosidase expression vector into COS7 cells. If necessary, cells were treated with 10⁻⁸ M 1α,25(OH)₂D₃ (Merck, Darmstadt, Germany) or vehicle (0.1% ethanol) 24 h after transfection. Cells were harvested 48 h after transfection, and the luciferase activities of the lysates were then evaluated using a luciferase assay kit (Toyo Ink Co. Ltd.). The reporter activities were standardized by the β-galactosidase activities of the same lysates.

In Vitro Binding Assay—To prepare whole cell extracts, cells were resuspended in 300 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 20% glycerol, 5 mM MgCl₂, 2 mM DTT, and Complete EDTA-free protease inhibitor mixture (Roche)) and gently rocked at 4 °C for 10 min. The mixture was sonicated briefly and centrifuged at 16,000 × g for 10 min. The supernatant was then diluted to 5-fold with dilution buffer (20 mM HEPES, pH 7.9, 5 mM MgCl₂, 2 mM DTT, and Complete EDTA-free). The diluted extracts were preincubated twice with glutathione-Sepharose at 4 °C for 30 min to remove nonspecific binding proteins, and were then incubated with various GST fusion proteins bound to glutathione-Sepharose at 4 °C for 2 h. Sepharose beads were washed extensively with dilution buffer, and interacted proteins were analyzed by SDS-PAGE followed by immunoblotting.

In Vitro Nuclear Transport Assay—HeLa cells were plated at a density of 5 × 10⁵ cells/ml on 8-well multitest slides (ICN Biomedicals, Aurora, OH) 24 h before use. The cells grown on the slides were rinsed twice in ice-cold transport buffer (20 mM HEPES, pH 7.3, 110 mM CH₃COOK, 2 mM (CH₃COO)₂Mg, 5 mM CH₃COONa, and 0.5 mM EGTA) and permeabized for 5 min in ice-cold transport buffer containing 40 μg/ml digitonin, 2 mM DTT, and Complete EDTA-free (Roche). After washed and immersed in ice-cold transport buffer containing 2 mM DTT and Complete EDTA-free for 10 min, the slides were blotted to remove excess buffer, and the reaction mixtures were applied to each well. Import reactions were performed for 30 min at 30 °C. Then the slides were rinsed twice in the transport buffer, and fixed with 3.7% formaldehyde in phosphate-buffered saline. After being rinsed in phosphate-buffered saline, the fixed slides were permeabized with 0.2% Triton X-100 in phosphate-buffered saline for 7 min at room temperature in the presence of an ATP-regeneration system. Then the reaction mixtures were added to digitonin-permeabilized HeLa cells, and the cells were incubated for 30 min at 30 °C. In some reactions, the digitonin-permeabilized cells were pretreated with 0.4 mg/ml WGA for 10 min at 30 °C (WGA (+)). The subcellular distribution of cargoes was visualized by indirect immunofluorescence using antibodies against GST. As to the GST-VDR-(4–427), both WT and R274A were imported independently of ligand. On the other hand, GST-VDR-(91–427) without the mutation was imported in a ligand-dependent manner, whereas that with R274A failed to enter nuclei even in the presence of ligand. Pretreatment of the digitonin-permeabilized HeLa cells with WGA abolished all the nuclear import.

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FIGURE 2. Nuclear transport of various truncated mutants of VDR. A, recombinant whole VDR (aa 4–427) and various truncated mutant VDRs tagged with GST were subjected as cargoes to the nuclear transport assay using digitonin-permeabilized HeLa cells after incubation with or without HeLa cytoplasmic extract (cytosol) and 10⁻⁸ M 1α,25(OH)₂D₃ for 20 min at room temperature in the presence of an ATP-regeneration system. The subcellular distribution of the cargoes was visualized by indirect immunofluorescence using anti-GST antibody. B, the import reactions of the indicated cargoes were performed with or without the pretreatment of digitonin-permeabilized HeLa cells with 0.4 mg/ml WGA for 10 min at 30 °C. The subcellular distribution of the cargoes was visualized by indirect immunofluorescence using anti-GST antibody.

FIGURE 3. Ligand-dependent and -independent pathways in the nuclear translocation of VDR. Recombinant GST-VDR-(4–427) and GST-VDR-(91–427) with (R274A) or without (WT) the R274A mutation, which results in the defect in ligand binding, were utilized as the cargoes in the import reactions. These recombinants were incubated with or without HeLa cytoplasmic extract (cytosol) and 10⁻⁸ M 1α,25(OH)₂D₃ for 20 min at room temperature in the presence of an ATP-regeneration system. Then the reaction mixtures were added to digitonin-permeabilized HeLa cells, and the cells were incubated for 30 min at 30 °C. In some reactions, the digitonin-permeabilized cells were pretreated with 0.4 mg/ml WGA for 10 min at 30 °C (WGA (+)). The subcellular distribution of cargoes was visualized by indirect immunofluorescence using antibodies against GST. As to the GST-VDR-(4–427), both WT and R274A were imported independently of ligand. On the other hand, GST-VDR-(91–427) without the mutation was imported in a ligand-dependent manner, whereas that with R274A failed to enter nuclei even in the presence of ligand. Pretreatment of the digitonin-permeabilized HeLa cells with WGA abolished all the nuclear import.
FIGURE 4. Importin 4 interacts with VDR. A, schematic representation of the concept of the mammalian two-hybrid analysis to examine the interaction between VDR and importin 4. Interaction between VDR and importin 4 (aa 846–1081) is assumed to result in the transactivation of the reporter through the GAL4 binding sites (UAS×5). GAL4DBD, the DNA-binding domain of GAL4; VP16AD, the activation domain of a herpes virus. B, the indicated combinations of mammalian two-hybrid plasmids were co-transfected into COS7 cells with pGVP2-GAL4BS that had luciferase reporter gene bearing 5 copies of GAL4-binding sites and β-galactosidase expression vector. Twenty-four hours later, 10⁻⁶ M 1α,25(OH)₂D₃ or vehicle (0.1% ethanol) was added to the cells. Cells were harvested 48 h after transfection, and then, the luciferase activities of their lysates were then measured with a luciferase assay kit (Toyo Ink Co.) according to the manufacturer’s procedures. The reporter activities were standardized by the β-galactosidase activities of the same lysates. The mean value in the cells transfected with a combination of pM and pVP16 empty vectors and treated with vehicle (0.1% ethanol) was designated the mean value in the cells transfected with a combination of pM and pVP16 empty vectors and treated with vehicle (p < 0.0001). C, in vitro binding between importin 4 and VDR. COS7 cells were transfected with the expression vector for the indicated molecules; RXRα (pSGS-RXRα), FLAG-tagged full-length importin 4 (pFLAG-importin 4), or FLAG-tagged mouse importin β (pFLAG-PTAC). Whole cell extracts were then prepared and subjected to GST pull-down assay using GST-VDR(WT) or GST-VDR(R274A) on glutathione-Sepharose. The pull-down reactions were performed in the presence or absence of 10⁻⁸ M 1α,25(OH)₂D₃ as the ligand. The pulled down proteins were separated by SDS-PAGE, and then RXR and importins were detected by anti-RXR and anti-FLAG antibodies, respectively. The identical whole cell extracts without pull-down were also applied to the gel to show the protein amount to be input (shown as input). D, importin 4 interacts with the DNA-binding domain (aa 4–90) of VDR. Whole cell extracts obtained from COS7 cells transfected with pFLAG-importin 4 were subjected to GST pull-down assay using GST alone or GST fusions with various truncated mutants of VDR as indicated.

RESULTS

Characterization of Nuclear Transport of VDR by an in Vitro Nuclear Transport Assay—The nuclear translocation of VDR was characterized in an in vitro nuclear transport assay that uses digitonin-permeabilized cells (20). When the recombinant protein for whole VDR (aa 4–427: see “Materials and Methods”) was applied to this system, it was imported to nuclei in the presence of cytosolic extracts prepared from HeLa cells as a source of nuclear transport factors, whereas VDR failed to enter nuclei without cytosolic extracts (Fig. 1A). Utilizing two specific antibodies that recognize the different epitopes of VDR, we obtained completely identical results. Treatment with 1α,25(OH)₂D₃ did not further enhance the transport activity of the cytosolic extracts. A recombinant protein that contains the classical NLS of simian virus 40 (SV40) large T antigen (21), designated as GST-NLS₁₅₋₄₋₄₋₋, was also used as the cargo protein to confirm the quality and fidelity of the assay system. As previously proved, GST-NLS₁₅₋₄₋₋ was imported to nuclei in the presence of cytosolic extracts and ATP (Fig. 1B). The nuclear accumulation of VDR was entirely blocked by pretreatment with wheat germ agglutinin (WGA), suggesting that the nuclear import of VDR was a temperature and subjected to indirect immunofluorescence using specific antibody against VDR or GST. The second antibodies were labeled with Alexa 488 (Molecular Probes). The import of GST-NLS₁₅₋₄₋₋ was detected by direct fluorescence. Each transport reaction mixture contained an import substrate combined with HeLa cytosolic fraction as a source of nuclear transport factors or a combination of recombinant transport factors with an ATP regeneration system (1 mM ATP, 5 mM creatine phosphate, and 20 units/ml creatine phosphokinase). HeLa cytosolic fraction was prepared as previously described (19).

Plasmid Constructions of Green Fluorescent Protein (GFP)-VDR Fusion Proteins—The construction of the expression plasmid coding GFP-tagged whole hVDR (4–427) was previously described (6). Briefly, the termination codon in a GFP fusion vector pGreen Lantern-1 (Invitrogen) was mutared to a new HindIII site, where the full-length hVDR cDNA obtained from pSG5-hVDR was inserted to yield a fusion protein in-frame. We also modified pGreen Lantern vector to possess a multiple cloning site at the position of original termination codon of GFP (designated as pGreen Lantern-MCS) (6), and constructed pGreen Lantern-hVDR-(81–427) by cloning the NaeI/EcoRI fragment of pSG5-hVDR into pGreenLantern-MCS in-frame.
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FIGURE 5. Carboxyl-terminal region of importin 4 is responsible for the interaction with VDR. A, schematic representation of truncated mutants of human importin 4 utilized in the pull-down experiments. DH1150 represents the fragment identified in the yeast two-hybrid screening. B, in vitro binding between various truncated mutants of human importin 4 and VDR. COS7 cells were transfected with the expression vectors for full-length or various truncated mutants of importin 4 tagged with FLAG®. Whole cell extracts were then prepared and subjected to GST pull-down assay using GST-VDR-(4–427)WT on glutathione-Sepharose. The pulled-down proteins were separated by SDS-PAGE and detected with anti-FLAG antibody. The identical whole cell extracts without pull-down were also applied to the gel to show the protein amount to be input (shown as input). Asterisk, nonspecific bands.

facilitated procedure involving contacts with nucleoporins that construct the nuclear pore complex (Fig. 1C).

The Ligand-dependent and -independent Nuclear Import of VDR—To further characterize the mechanisms underlying the nuclear transport of VDR, we performed a nuclear transport assay using truncated forms of VDR (Fig. 2). In the series of these experiments, GST-VDR fusion protein was utilized as the control, and the obtained results were identical to those of the whole VDR-(4–427) without GST tag, demonstrating that GST does not affect the import process of VDR. Two truncated forms of VDR fused to GST were then constructed, and were subjected to the assay. One mutant contained the amino terminus of VDR-(4–232), and the other contained the carboxyl terminus of VDR and lacked the DNA-binding domain (aa 91–427). GST-VDR-(4–232) displayed cytosol-dependent, ligand-independent nuclear translocation in this system, which was similar to the case of GST-VDR-(4–427) (Fig. 2A). On the other hand, GST-VDR-(91–427) was not transported in the absence of ligand, and the addition of 1α,25(OH)2D3 resulted in the efficient nuclear import of GST-VDR-(91–427) (Fig. 2A). All nuclear import observed in this assay was inhibited completely by WGA treatment, suggesting that the cargoes were transported into nuclei through nuclear pore complexes (Fig. 2B). To confirm the effect of the ligand on the process, we carried out the assay utilizing GST-VDR-(4–427) and GST-VDR-(91–427) carrying the R274A mutation, which results in ligand binding deficiency. GST-VDR-(4–427) carrying R274A (GST-VDR-(4–427)R274A) entered nuclei via the nuclear pore as well as wild-type GST-VDR-(4–427) (GST-VDR-(4–427)WT). On the other hand, GST-VDR-(91–427) carrying R274A (GST-VDR-(91–427)R274A) failed to enter nuclei even in the presence of the ligand, which is different from the case of GST-VDR-(91–427)WT (Fig. 3). These results supported the notion that VDR has two distinct types of nuclear import signals, i.e. ligand-dependent and -independent pathways, and that the different regions of VDR may be responsible for these processes.

Identification of Novel Interacting Proteins of VDR—We next attempted to identify the molecules responsible for ligand-independent nuclear transport of VDR based on protein-protein interaction. Because GST-VDR-(4–232) exhibited ligand-independent nuclear import as well as whole VDR, we screened a human kidney cDNA library with a yeast two-hybrid method using the fragment corresponding to VDR-(4–232) as bait. The obtained transformants were selected for growth on the synthetic media that lacked specific nutrients, followed by β-galactosidase filter assay. The selected clones were then analyzed by PCR using vector-specific primers followed by sequencing to identify the inserted genes. Among them, there was a clone carrying the gene fragment encoding the carboxyl-terminal region (aa 824–1081) of human importin 4 (GenBank™ accession number NM_024658), a relatively new member of the importin β-family, and we selected the molecule for further analysis.

Interaction between VDR and the carboxyl-terminal region of importin 4 was further confirmed by a mammalian two-hybrid assay, which detected protein-protein interaction and ligand dependence in vivo. The interaction between the carboxyl terminus of importin 4 (aa 845–1081; pM-IMP4) and VDR occurred independently of ligand (Fig. 4B). It is well known that the binding of 1α,25(OH)2D3 enhances the heterodimerization of VDR with RXR, which was confirmed by an in vitro binding assay using the GST-VDR-(4–427) recombinant protein (Fig. 4C). We then examined whether ligand might affect the association of importin 4 and VDR utilizing the in vitro binding assay. It was revealed that GST-VDR-(4–427)WT pulled down FLAG-tagged full-length importin 4 regardless of treatment with 1α,25(OH)2D3, whereas importin β, a typical nuclear transport factor, did not bind to VDR in either its liganded and unliganded form (Fig. 4C). These results suggested that the interaction between VDR and importin 4 did not require the ligand. In parallel with this observation, recombinant GST-VDR-(4–427)R274A lacking the capability to bind ligand also pulled down importin 4 as well as GST-VDR-(4–427)WT (Fig. 4C).

Next, the pull-down analyses utilizing a series of truncated VDR proteins were performed to investigate which region of VDR is responsible for the interaction with importin 4. In addition to GST-VDR-(4–232), we prepared two more GST fusion proteins containing VDR-(4–90) and VDR-(91–232), respectively, which made up the yeast two-hybrid bait, and then applied them to the in vitro binding assay to map the interacting region of VDR. Although GST-VDR-(4–232) and GST-VDR-(4–90) were able to bind importin 4, only the background level of binding was detected between importin 4 and GST-VDR-(91–232) (Fig. 4D). These results indicated that aa 4–90 corresponding to the DNA-binding domain was essential for the interaction with importin 4. In addition, the in vitro binding assay also demonstrated that the carboxyl-terminal region of importin 4 was important for the interaction (Fig. 5). GST-VDR-(4–427) pulled down the various forms of importin 4 from each cell lysate except for the importin 4ΔC, a carboxyl-terminal truncated form (Fig. 5).

Importin 4 Mediates the Ligand-independent Nuclear Import of VDR—Finally, to investigate whether importin 4 is the molecule that carries the nuclear translocation of VDR, we performed the in vitro nuclear transport assay where all the required components were supplied as recombinant proteins. It has already been established that nuclear transport can be reconstructed using the in vitro nuclear transport assay with the recombinant proteins of Ran-GDP, NTF2, and nuclear transport factors instead of
cytosolic extracts (22). If importin 4 is the molecule responsible for nuclear transport of VDR, VDR can be imported to nuclei in the presence of recombinant importin 4, such as in the system driven with the HeLa cytosol. Indeed, whole VDR (aa 4–427) was steadily imported to nuclei in the presence of recombinant importin 4, and the effect of importin 4 was abolished by elimination of Ran-GDP and NTF2 from the system (Fig. 6A). Although recombinant importin \( \alpha \) together with importin \( \beta \) effectively transported GST-NLSSV40-GFP into nuclei as previously established, the active importin \( \alpha \) was ineffective for nuclear translocation of VDR even in the presence of all the other required components (Fig. 6B). In contrast, importin 4 failed to import GST-NLSSV40-GFP even in the presence of all the required factors (Fig. 6A). The nuclear accumulation of VDR mediated by importin 4 was not affected by treatment with 1\( \alpha \).25(OH)\(_2\)D\(_3\) as well as the cytosol-dependent nuclear import of VDR. It was also validated that the ineffectiveness of importin \( \beta \) was not improved with the liganded form of VDR (Fig. 6B). These results obviously indicated that importin 4, not importin \( \beta \), played a crucial role in the ligand-independent import of VDR to nuclei. The dispensability of the ligand-binding step was also supported by further observations that importin 4 steadily transported not only GST-VDR-(4–232) that lacked the most part of the ligand-binding domain but also GST-VDR-(4–427)\(_{274AA}\), which was a ligand-binding deficient form (Fig. 6C).

To further examine whether the interaction between importin 4 and VDR is required for the importin 4-mediated nuclear transport of VDR, we performed in vitro nuclear transport assay using recombinant importin 4\( \Delta C \) lacking the interaction with VDR. As shown in Fig. 5, importin 4\( \Delta C \) does not interact with VDR. Whereas full-length importin 4 conferred the nuclear translocation of whole VDR-(4–427) in the absence of ligand, importin 4\( \Delta C \) failed (Fig. 7A). We also examined subcellular distribution of VDR-(81–427) lacking the interaction with importin 4. In the mammalian two-hybrid assay, it was demonstrated that the truncated mutant VDR-(81–427) failed to interact with importin 4 (Fig. 7B). Therefore, we constructed the mammalian expression vectors of GFP-tagged whole VDR-(4–427) and truncated VDR-(81–427), and performed the transient transfection experiments. As previously reported (6), GFP-tagged whole VDR-(4–427) were predominantly located in nuclei even in the absence of ligand. On the other hand, GFP-VDR-(81–427) exhibited less accumulation in nuclei, and was distributed throughout the cells (Fig. 7C). Western blot analysis using anti-VDR antibody revealed that importin 4 conferred the ligand-independent nuclear translocation of VDR even in the absence of ligand.
HeLa cells, nuclear translocation of VDR was reconstructed using the indicated recombination with VDR (Fig. 5) failed to transport VDR into nuclei. Using digitonin-premeabilizeddent nuclear translocation of VDR by importin 4.

DISCUSSION

Interaction between VDR and importin 4 is critical for ligand-independent nuclear transport of VDR by importin 4. A, importin ΔC lacking the interaction with VDR (Fig. 5) failed to transport VDR into nuclei. Using digitonin-premeabilized HeLa cells, nuclear translocation of VDR was reconstructed using the indicated recombinant proteins. VDR was visualized by indirect immunofluorescence using anti-VDR antibody (9A7). B, VDR (81–427) does not interact with importin 4. The indicated combinations of mammalian two-hybrid plasmids were co-transfected into COS7 cells with pGVP2-GAL48S and β-galactosidase expression vector. Cells were harvested 48 h after transfection, and then the luciferase activities of their lysates were measured. The reporter activities were standardized by the β-galactosidase activities of the same lysates. The mean value in the cells transfected with a combination of pM empty vector and pVP16-VDR (4–427) was designated 1, and the relative luciferase activities were determined. The data are expressed as mean ± S.E. Asterisk, significantly different from the value in the cells transfected with the combination of pM and pVP16-VDR (4–427) (p < 0.0001). C, subcellular distribution of GFP-tagged VDR (4–427) and VDR (81–427) in the absence of ligand. COS7 cells were transfected with the expression vectors of GFP-VDR (4–427) or GFP-VDR (81–427), and 72 h later, the GFP-tagged VDRs were visualized by direct fluorescent microscopy.

GFP monoclonal antibody (Roche) confirmed the appropriate full-length expression of the GFP-tagged whole and truncated VDRs (data not shown). These results suggest that the interaction between VDR and importin 4 is critical for the ligand-independent nuclear transport of VDR.

DISCUSSION

VDR is one of the ligand-dependent transcription factors, and must be localized in nuclei to exert its function. Although several NLSs have been reported by ourselves as well as others, the molecular mechanisms underlying the nuclear translocation of VDR have been unclear (6, 23, 24). The in vitro nuclear transport assay is useful to characterize the nuclear transport system of certain proteins, and we previously examined the process in cases of several kinds of nuclear proteins such as p27Kip1, Smad3, and sterol regulatory element-binding protein 2 (SREBP-2) using this assay (22, 25–27). Therefore, in the current study, we have carefully dissected the nuclear entry process of VDR utilizing this assay, and have substantiated at least two nuclear import signals of VDR. A truncated mutant of VDR, GST-VDR (4–232), entered nuclei in the presence of cytosol prepared from HeLa cells in a ligand-independent manner, whereas GST-VDR (91–427) entered nuclei only in the presence of ligand. Although researchers including ourselves previously reported that VDR is located in nuclei even in the absence of ligand and that the addition of ligand causes more accumulation of VDR in nuclei, this is the first report that has clearly distinguished ligand-dependent and -independent nuclear transport of VDR. To our knowledge, three NLSs of VDR have been identified to date: aa 49–55, 76–102, and 158–173, respectively (6, 23, 24). Among them, aa 158–173 that we have reported is the only signal located in the region that exhibited ligand-dependent nuclear localization in the present study, and might be responsible for the ligand-dependent nuclear import.

To identify the molecule(s) responsible for ligand-independent nuclear transport of VDR, we screened a human kidney cDNA library based on protein-protein interaction, and have identified importin 4 as a novel interactive partner of VDR. Of note, in the in vitro nuclear transport assay, recombinant human importin 4 effectively imports VDR to nuclei both in the presence and absence of the ligand, whereas importin β failed even in the presence of the ligand (Fig. 6). The interaction of VDR and importin 4 was further confirmed by other methods (Figs. 4 and 5), whereas the interaction with importin β, which is reported to be involved in the nuclear import of other nuclear receptors (28), was not detected by both two-hybrid methods and biochemical analyses. These results together strongly indicate that importin 4, not importin β is the molecule responsible for the ligand-independent nuclear entry of VDR. Although the structure of importin β bound to SREBP-2 has been identified (27), the detailed study on the difference in the three-dimensional structures of importin β and importin 4 is the next issue to be addressed because the difference may lead to an explanation of the difference in the affinity with VDR.

Importin 4 has been identified recently from express sequence tags of mouse and human by bioinformatical approaches, and characterized as a nuclear transport factor for a component of ribosome, rpS3a (29). Mouse importin 4 (Imp4a), which transports rp3a and human importin 4 (Imp4b), share only ~80% identical residues and seem to be functionally distinct (29). In addition, it has also been shown that mouse importin 4 has another function as a chaperone for exposed domains with basic charges (29). However, no other substrate for importin 4 both in mouse and human has been identified to date, and this is the first report demonstrating that importin 4 is responsible for well characterized molecules such as VDR. The primary structure of rp3a is not apparently similar to that of DNA-binding domain of VDR, although both of them are rich in basic amino acids. VDR may keep solubility in a cytoplasmic environment prior to DNA binding through interaction with importin 4. Although our analysis of the interaction between VDR and the carboxyl-terminal portion of importin 4 utilizing a mammalian two-hybrid method suggested that the complex of them might be rather unstable (Fig. 4B), the weak interaction seems to be critical and sufficiently functional as confirmed by the reconstruction experiment of the nuclear transport (Figs. 6 and 7A). Nuclear transport is dominated by complex formation between nuclear transport factors and their cargoes, and the complex must rapidly dissociate in the nucleoplasm to terminate the transport. Therefore, the instability of the complex between
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VDR and importin 4 was rather reasonable, and may suggest that the interaction through nucleocytoplasmic traffic is intrinsically unstable unlike that in other biological processes such as transcriptional regulation.

Recently, it has been demonstrated that dimerization of some cargo proteins triggers complex formation with nuclear transport factors, followed by nuclear entry (27). SREBP-2, a nuclear transcription factor that belongs to a basic helix-loop-helix leucine zipper superfamily, needs homodimerization to form a complex with importin β (27). In the case of SREBP-2, homodimerization is required not only to recognize the response element on the genome, but also to construct a structural basis for direct interaction with importin β (27). This is a very suggestive observation, because VDR must form a heterodimer with RXR to bind the enhancer region on the genome. It has been reported that RXR promotes nuclear accumulation of VDR by influencing both nuclear import and retention (30). However, we have demonstrated that VDR enters the nuclei in the absence of RXR by reconstructing nuclear import with recombinant proteins. Although RXR may be one of regulatory factors for nuclear localization of VDR, it is unlikely to be a dominant factor.

Although the results of the present study demonstrate that importin 4 is involved in nuclear transfer of VDR in a ligand-independent manner, the molecule responsible for the ligand-dependent nuclear transfer remains to be determined. The significance of the ligand-dependent nuclear transport of VDR might be uncovered when the ligand-independent pathway is suppressed as in our experiment where the DNA-binding domain was deleted (Fig. 2). The conformational changes induced by binding of 1α,25(OH)2D3 might have the ligand-dependent NLS fulfilling the ligand-independent one suppressed as in our experiment where the DNA-binding domain was deleted (Fig. 2). The conformational changes induced by binding of 1α,25(OH)2D3 might have the ligand-dependent NLS accessible to the corresponding transport factor(s). This notion has been further supported by our experiments utilizing the R274A mutant lacking ligand binding (Fig. 3). In pathophysiological conditions such as the limited amount of importin 4, the ligand-dependent nuclear transport of VDR may play an essential role for its function as a transcriptional factor. It remains to be elucidated how these two distinct pathways, the ligand-dependent and -independent pathways, cooperate to regulate the nuclear transport of VDR in various tissues.

In conclusion, we have demonstrated that VDR has two nuclear import systems: ligand-dependent and ligand-independent pathways, and that importin 4, not importin β, fulfills the ligand-independent nuclear transport through the interaction with the amino terminus of VDR. This is the first report that indicates that importin 4 acts as the dominant transport factor of well known functional molecules such as VDR. Our findings strongly suggest the possibility that importin 4 instead of importin β might mediate nuclear translocation in more cases than expected.

Acknowledgments—We thank Dr. Hiroki Kondou for helpful discussions. We also thank Tomoko Hayashi for secretarial assistance.

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