

# The Vitamin D Receptor Is Present in Caveolae-Enriched Plasma Membranes and Binds $1\alpha,25(\text{OH})_2$ -Vitamin $\text{D}_3$ *in Vivo* and *in Vitro*

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The steroid hormone  $1\alpha,25(\text{OH})_2$ -vitamin  $\text{D}_3$  (1,25D) regulates gene transcription through a nuclear receptor [vitamin D receptor (VDR)] and initiation of rapid cellular responses through a putative plasma membrane-associated receptor (VDR<sub>mem</sub>). This study characterized the VDR<sub>mem</sub> present in a caveolae-enriched membrane fraction (CMF), a site of accumulation of signal transduction agents. Saturable and specific [ $^3\text{H}$ ]-1,25D binding *in vitro* was found in CMF of chick, rat, and mouse intestine; mouse lung and kidney; and human NB4 leukemia and rat ROS 17/2.8 osteoblast-like cells; in all cases the 1,25D  $K_D$  binding dissociation constant = 1–3 nm. Our data collectively support the classical VDR being the VDR<sub>mem</sub> in caveolae: 1) VDR antibody immunoreactivity was detected in CMF of all tissues tested; 2) competitive binding of [ $^3\text{H}$ ]-1,25D by eight analogs of 1,25D was signifi-

cantly correlated between nuclei and CMF ( $r^2 = 0.95$ ) but not between vitamin D binding protein (has a different ligand binding specificity) and CMF; 3) confocal immunofluorescence microscopy of ROS 17/2.8 cells showed VDR in close association with the caveolae marker protein, caveolin-1, in the plasma membrane region; 4) *in vivo* 1,25D pretreatment reduced *in vitro* [ $^3\text{H}$ ]-1,25D binding by 30% in chick and rat intestinal CMF demonstrating *in vivo* occupancy of the CMF receptor by 1,25D; and 5) comparison of [ $^3\text{H}$ ]-1,25D binding in VDR KO and WT mouse kidney tissue showed 85% reduction in VDR KO CMF and 95% reduction in VDR KO nuclear fraction. This study supports the presence of VDR as the 1,25D-binding protein associated with plasma membrane caveolae. (*Molecular Endocrinology* 18: 2660–2671, 2004)

THE STEROID HORMONE  $1\alpha,25(\text{OH})_2$ -vitamin  $\text{D}_3$  [1,25D] activates multiple signaling pathways in its target cells (1, 2). The vitamin D receptor (VDR), a member of the superfamily of nuclear receptors (3), exerts 1,25D-dependent responses in the nucleus as a ligand-activated transcription factor. In addition to these relatively slow (hours to days) genomic effects, 1,25D generates rapid responses (minutes to hours) including  $\text{Ca}^{2+}$  uptake from intestine (transcaltachia) (4, 5), augmentation of insulin secretion from pancreatic  $\beta$ -cells (6, 7), modulation of cartilage chondrocyte growth plate (8) and matrix vesicles (9), growth and differentiation of vascular smooth muscle cells (10) and keratinocytes (11) and in osteoblasts opening of voltage-gated calcium and chloride channels (12) and stimulation of secretion (13).

Abbreviations: AR, Androgen receptor; BLM, basal lateral membrane fraction;  $B_{\text{Max}}$ , maximal binding; CMF, caveolae-enriched membrane fraction; 1,25D,  $1\alpha,25(\text{OH})_2$ -vitamin  $\text{D}_3$ ; DBP, vitamin D binding protein; ER, estrogen receptor;  $K_D$ , binding dissociation constant; KO, knockout; MARRS, membrane-associated rapid-response steroid; MF, membrane fraction; NF, nuclear fraction; PNS, post-nuclear supernatant; RCI, relative competitive index; VDR, vitamin D receptor; VDR<sub>mem</sub>, a membrane receptor for 1,25D that has not been characterized; WT, wild-type.

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Many of these rapid responses are believed to be mediated by 1,25D binding to a plasma membrane-associated receptor [VDR<sub>mem</sub>] (9, 14, 15). The initial signal is amplified by production of second messengers including inositol triphosphate and diacylglycerol in the plasma membrane by phospholipase C- $\gamma$ , phosphoinositol 3-kinase (10), activation of protein kinase A, and production of cAMP (16), and activation of the MAPK pathway (17–19).

Membrane receptors for most of the steroid hormone subclasses have been described (20). A novel class of progestin receptor recently cloned from sea-trout ovaries is a G protein-coupled receptor that regulates the meiotic maturation of fish oocytes (21). A subpopulation of the classical estrogen receptor  $\alpha$  (ER $\alpha$ ) has been localized in cell surface microdomains, caveolae, of the vascular endothelium (22–24). A classical androgen receptor (AR) has been detected in the plasma membrane of *Xenopus laevis* oocytes (25), and a nonclassical AR has been identified in the plasma membrane of a human prostate cancer cell line, LNCaP (26). It is likely that both the classical and novel classes of steroid hormone receptors in the plasma membrane can mediate divergent cellular responses (20).

The molecular structure of the VDR<sub>mem</sub>, its means of association with the plasma membrane, and its mechanism of action remain unresolved. The conformational flexibility of 1,25D is unique among the steroid

hormones and has enabled study of a structure-function relationship of conformationally restricted vitamin D analogs (2, 5). The 6-*s-cis* configuration of the ligand favors activation of the nongenomic pathway, whereas the 6-*s-trans* shape mediates preferentially genomic responses (5). It is not known whether the divergent responses result from binding of 1,25D to structurally unrelated VDRs localized in the nucleus and cell membrane, or alternatively, binding of 1,25D to distinct ligand binding pockets in the same classical VDR molecule (20). It is also possible that the membrane localization of the classical VDR favors nongenomic over genomic responses analogous to ER $\alpha$  and AR localization (25, 27).

Several candidates for a VDR<sub>mem</sub> distinct from the classical VDR have been proposed. 1) A membrane-associated calcium-binding protein that functions as a calcium-specific ion channel, annexin-II, was suggested to bind 1,25D directly in osteoblasts (28); however subsequent studies did not support this proposal (29). 2) A binding protein for 1,25D unrelated to the classical VDR was identified in chick intestinal basal lateral membranes (9, 14, 30). Because this protein demonstrated specificity for 1,25D binding and an antibody against its N terminus blocked 1,25D-dependent changes in PKC activity (9), the protein was named a membrane-associated rapid-response steroid binding protein (31). 3) It has also been postulated that protein kinase C is a VDR<sub>mem</sub> that could directly bind and be activated by 1,25D (32). On the other hand, evidence for the classical VDR being the VDR<sub>mem</sub> is provided by studies showing association of the VDR with rapid signaling molecules in human keratinocytes (11), binding of VDR-ligand complex with extranuclear acceptance sites in ROS 17/2.8 cells (33), and translocation of the VDR to the plasma membrane of chick skeletal muscle cells after 1,25D stimulation (34).

This study investigated the classical VDR as a candidate receptor for 1,25D-initiated rapid actions. Caveolae-enriched membrane fractions (CMF) were purified by density ultracentrifugation by a detergent-free method. Specific *in vitro* [<sup>3</sup>H]-1,25D binding in CMF was studied in chick and rat tissues, human leukemic NB4, and rat osteoblast-like ROS 17/2.8 cells and wild-type (WT) and VDR knockout (KO) mouse kidney tissue. Western blots were used to evaluate both VDR expression and VDR cellular localization. *In vivo* experiments in vitamin D-deficient chicks studied the ability of 1,25D to occupy the VDR<sub>mem</sub>. Collectively, our results support the conclusion that the VDR<sub>mem</sub> is the classical VDR associated with caveolae.

## RESULTS

### Specific [<sup>3</sup>H]-1,25D Binding *in Vitro* in Caveolae-Enriched Membrane Fraction of Vitamin D-Deficient Chick Duodenum and ROS 17/2.8 and NB4 Cells

The principal objective of this study was to examine the ability of [<sup>3</sup>H]-1,25D to bind under *in vitro* condi-

tions to CMFs isolated from key tissues that are known to have a nuclear VDR (35). The tissues were obtained from chickens, mice, rats; ROS 17/2.8 and NB4 cell lines were also used as a source of caveolae. The results from three typical experiments are shown in Fig. 1. Figure 1A shows vitamin D-deficient chick duodenal CMF binding of [<sup>3</sup>H]-1,25D. Incubations of the CMF with 0.1–10 nM [<sup>3</sup>H]-1,25D resulted in a low, but readily measurable, specific high-affinity binding. The data points fit a one-site rectangular hyperbola (Fig. 1B) that can be transformed into a linear Scatchard plot (inset in Fig. 1B). Similar results to Fig. 1A have been obtained in 23 CMF preparations; K<sub>D</sub> (binding dissociation constant) = 1.4 ± 0.6 nM and B<sub>Max</sub> (maximal binding) = 28 ± 11 fmol/1,25D/mg protein.

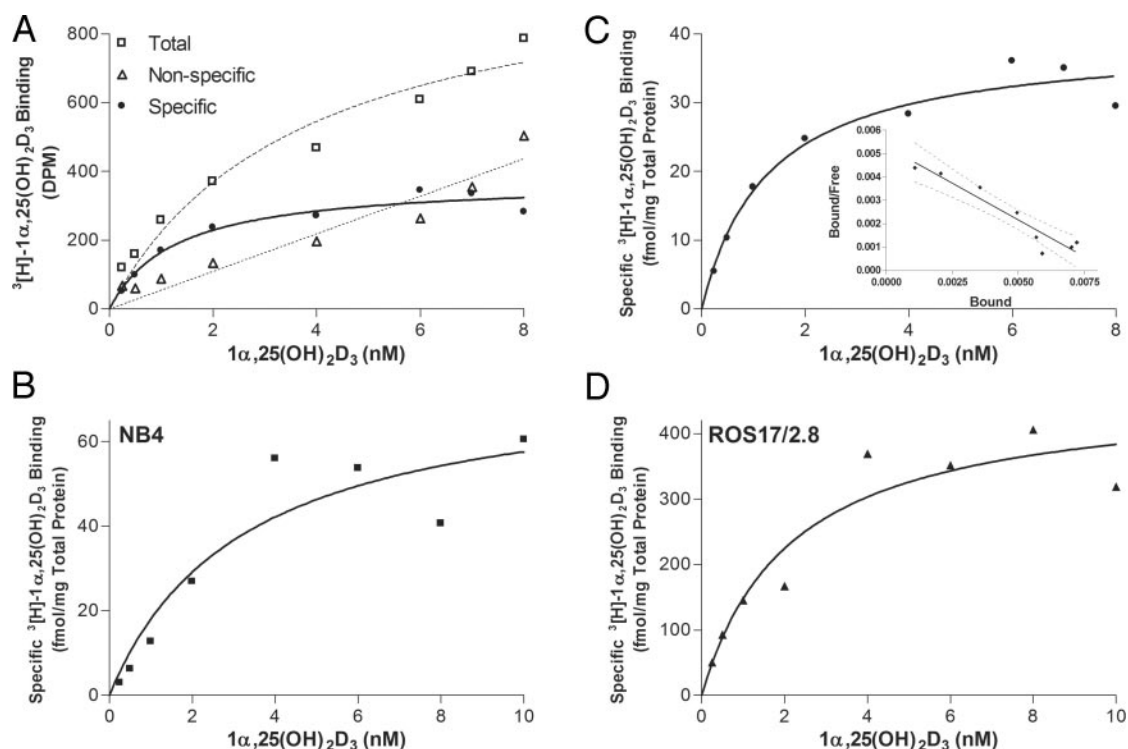
Specific, concentration-dependent and saturable binding of [<sup>3</sup>H]-1,25D *in vitro* was found in CMF from human leukemia NB4 cells (1C) and rat osteoblast-like ROS 17/2.8 cells (1D). In a representative saturation binding curve, the CMF of ROS 17/2.8 cells showed a higher concentration and equivalent affinity of specific [<sup>3</sup>H]-1,25D binding (B<sub>Max</sub> = 470 fmol/mg protein, K<sub>D</sub> = 2.2 nM) relative to NB4 cells (B<sub>Max</sub> = 76 fmol/mg protein, K<sub>D</sub> = 3.2 nM). Rat duodenal CMF bound [<sup>3</sup>H]-1,25D with an affinity of K<sub>D</sub> = 2.7 nM and B<sub>Max</sub> = 241 fmol/mg protein (Fig. 2B). Also, mouse intestine, kidney, and lung showed a saturable binding of [<sup>3</sup>H]-1,25D to the CMF, K<sub>D</sub> = 1.0, 4.1 and 1.0 nM, respectively, and a B<sub>Max</sub> of 9.5, 180, and 720 fmol/mg respectively (data not shown).

### Saturable Binding of [<sup>3</sup>H]-1,25D to Vitamin D-Deficient Chick or Rat Intestinal CMF Is Reduced by Prior Treatment *in Vivo* with 1,25D

Figure 2, A and B, shows that when either vitamin D-deficient chicks or rats are given a physiological dose of nonradioactive 1,25D *in vivo* (1.3 nmol), the subsequent saturable binding of [<sup>3</sup>H]-1,25D *in vitro* to duodenal mucosa CMF was reduced by approximately 30%. Thus, the B<sub>Max</sub> (femtomoles per milligram of protein) was reduced from 15 to 11 in the chick and from 241 to 171 in the rats.

### Caveolae Localization of [<sup>3</sup>H]-1,25D *in Vivo* in Vitamin D-Deficient Chicks

The ligand present in the chick intestinal CMF after *in vivo* dosing with 1,25D could be 1,25D or a derived metabolite. Figure 2C shows that when [<sup>3</sup>H]-1,25D is administered *in vivo* to vitamin D-deficient chicks, 1,25D, not a metabolite, is the bound ligand in the intestinal CMF. Two hours after a [<sup>3</sup>H]-1,25D dose of 1.3 nmol, lipids in the chick duodenal CMF fraction were extracted (36) and subjected to HPLC. Figure 2C illustrates the HPLC chromatogram with standards: 25OH-D<sub>3</sub>, 1,25D, 1 $\alpha$ ,25(OH)<sub>2</sub>-D<sub>3</sub>-26,23-lactone, 1 $\alpha$ ,24,25(OH)<sub>2</sub>-D<sub>3</sub>. At 2 h, 95% of the isolated tritium is 1,25D, whereas the metabolites



**Fig. 1.** Saturation Binding Analysis of Caveolae-Enriched Membrane Fractions

A and B, Chick duodenal CMF. A, Typical [ $^3\text{H}$ ]-1,25D binding (total, nonspecific, and specific) is expressed as DPM. The *in vitro* saturation analysis was performed on CFM over the concentration range of 0.25–8 nM 1,25D. Bound and free ligand were separated using hydroxylapatite (see *Materials and Methods*). B, The saturable specific binding data of [ $^3\text{H}$ ]-1,25D in panel A, was plotted as femtomoles per milligram of total protein (GraphPad 4.02, Prism, San Diego, CA) using a one-site rectangular hyperbola ( $r^2 = 0.95$ ); the  $K_D$  for 1,25D is 1.28 nM and the  $B_{\text{Max}} = 39.3$  fmol/mg protein. The *inset* is a Scatchard transformation of the specific binding data of the larger panel; the *curved lines* represent the 95% confidence limit to the linear line. The data are representative from 23 experiments. C, Specific [ $^3\text{H}$ ]-1,25D binding in caveolae (CMF) isolated from human leukemia NB4 cells and (D) rat osteosarcoma ROS 17/2.8 osteoblast-like cells. Altogether, 1.5 g of NB4 cells (twelve 1L flasks containing  $8.4 \times 10^5$  cells/ml) were harvested by centrifugation and 1.0 g of ROS 17/2.8 cells (40 confluent plates) by scraping (no trypsin). In these analyses, NB4 cell  $K_D = 3.2$  nM and  $B_{\text{Max}} = 76$  fmol/mg protein ( $r^2 = 0.88$ ); ROS 17/2.8 cell  $K_D = 2.2$  nM and  $B_{\text{Max}} = 470$  fmol/mg protein ( $r^2 = 0.90$ ). The data are representative of three experiments for each cell line.

$1\alpha,25(\text{OH})_2\text{-D}_3$ -26,23-lactone and  $1\alpha,24,25(\text{OH})_2\text{D}_3$  were undetectable.

Collectively, these results (Fig. 2) demonstrate that occupancy of the CMF binding protein can be achieved by *in vivo* dosing with the steroid hormone 1,25D and suggest that this membrane-associated receptor may be associated with physiological functions.

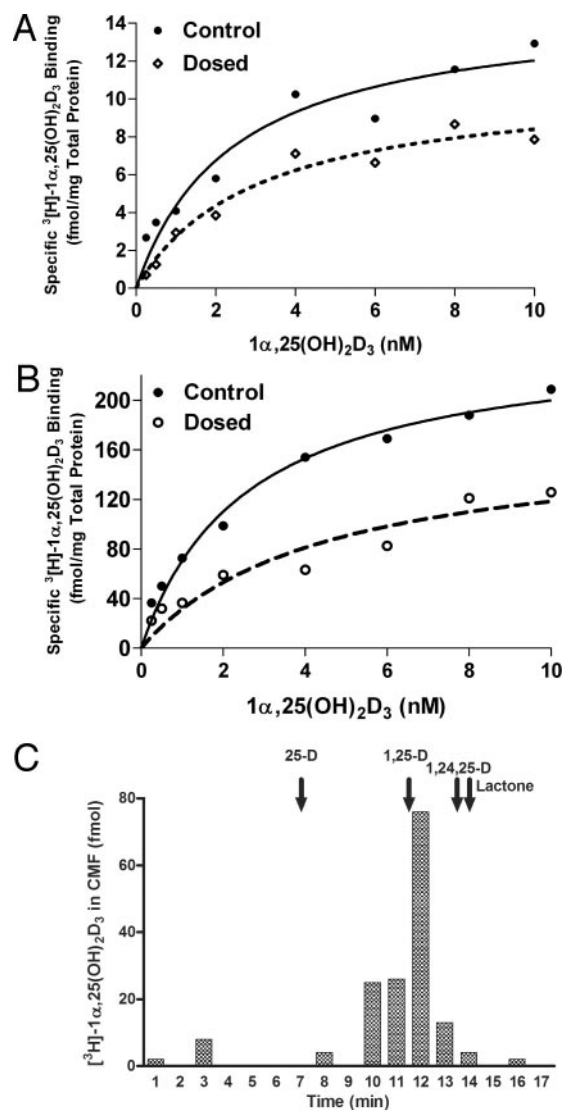
### Characterization of CMF

Detergent-free isolation of CMF was carried out using the unique buoyant density of CMF to separate them from other cell membrane structures (37). The lack of detergents allows isolation of CMF with their natural composition and reliable quantitation of [ $^3\text{H}$ ]-1,25D binding *in vitro*. In previous studies from this laboratory using the detergent CHAPSO (Sigma, St. Louis, MO) in the preparation of a basal lateral membrane (BLM) fraction from chick intestine (14), it was not possible to reliably observe saturable binding of [ $^3\text{H}$ ]-1,25D (5, 38). Despite the clear advantages of the present proce-

duce, density gradient centrifugation is unable to completely separate caveolae from other lipid rafts and vesicular structures that have a similar buoyant density. Whereas further purification of caveolae can be achieved by immunoisolation, this procedure does not generate enough caveolae to be used for *in vitro* ligand binding studies.

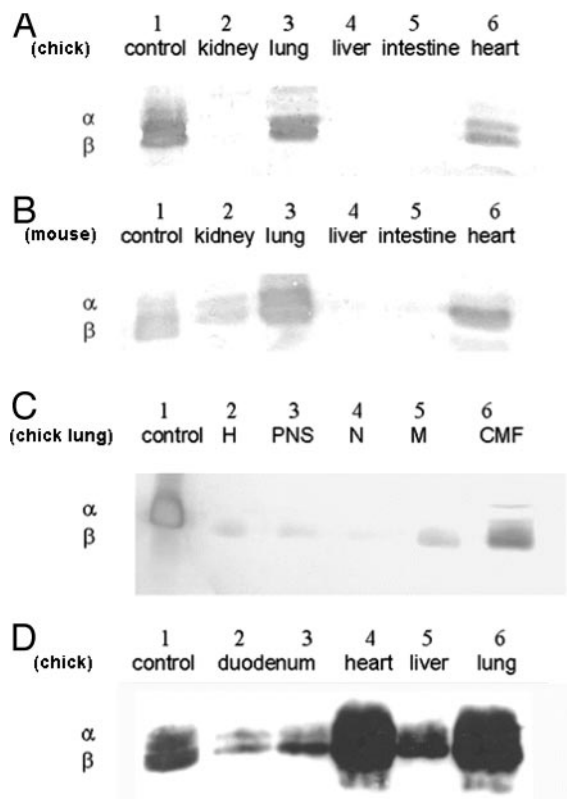
Expression of caveolin-1, an integral marker protein of caveolae, was present in CMF of chick and mouse tissues (Fig. 3, A–D). Caveolin-1 was expressed in CMF from chick duodena, lung, liver, and heart, but was low in kidney (Fig. 3, A and D). Mouse tissues showed a similar expression pattern of caveolin-1. A doublet of caveolin-1 ( $\alpha$ , 24 kDa;  $\beta$ , 21 kDa) was detected in all caveolin-1-positive tissues.

Because the chick lung had a high caveolae content determined by caveolin-1 expression, aliquots from homogenate, post nuclear supernatant, nuclear pellet, total membranes, and caveolae were collected and subjected to Western blot and marker enzyme analyses. Expression of caveolin-1 was found to be up to



**Fig. 2.** Evidence for Occupancy of Chick and Rat Duodenal Caveolae-Enriched Membrane Fraction Binding Protein *in Vivo* by 1,25D

A and B, Comparison of *in vitro* [ $^3\text{H}$ ]-1,25D binding in CMF of duodenal mucosa from vitamin D-deficient chicks (A) or vitamin D-deficient rats (B). Ten vitamin D-deficient (–D) chicks were given a single dose of 1.3 nmol of 1,25D im (in 100  $\mu\text{l}$  of ethanol:propanediol, 1:1) [dosed] and 10 were given 100  $\mu\text{l}$  of vehicle im [control] 2 h before CMF isolation (see *Materials and Methods*). The two pools of isolated CMF were incubated with [ $^3\text{H}$ ]-1,25D (0.25–10 nM) *in vitro*, for 16 h at 4 C, and the specifically bound tritium determined. In parallel, two groups of eight –D rats received either a single dose of 1.3 nmol of 1,25D or vehicle im; 2 h later, the intestinal CMF were isolated (see *Materials and Methods*). The  $K_{\text{D}}$ s for chick CMF were 2.5 nM and 3.0 nM (control and dosed) and  $B_{\text{Max}}$  15 and 11 fmol/mg protein (control and dosed). The rat intestinal CMF  $K_{\text{D}}$  were 2.7 and 2.5 nM (control and dosed), the  $B_{\text{Max}}$  241 and 171 fmol/mg protein (control and dosed). Pretreatment *in vivo* with 1,25D reduced by 26% (chicks) or 29% (rats) the amount of [ $^3\text{H}$ ]-1,25D bound to the CMF *in vitro*. C, Administration of [ $^3\text{H}$ ]-1,25D *in vivo* to vitamin D-deficient chicks results in localization of [ $^3\text{H}$ ]-1,25D in intestinal CMF. Three vitamin D-deficient chicks were dosed im with 1.3 nmol



**Fig. 3.** Western Analysis of Caveolin-1 in Chick (A, C, and D) and Mouse (B) CMF of Selected Tissues

Panel C, Enrichment of caveolin-1 in chick lung CMF over the homogenate (H), and the nuclei (N), PNS and membrane fraction (M). The  $\alpha$  (24 kDa) and  $\beta$  subunits (21 kDa) of caveolin-1 differ by an amino terminal 31 amino acids sequence (45). Equal amounts of total protein A and B (10  $\mu\text{g}$ ), or C (2  $\mu\text{g}$ ) were loaded in each well in a 10% SDS-PAGE gel. Panel D shows the presence of caveolin-1 in CMF from four chick tissues using an antibody against chick caveolin-1 followed by enhanced chemiluminescence autoradiography.

94-fold enriched in lung CMF over total homogenate (Fig. 3C). Plasma membrane marker enzymes alkaline phosphatase and  $\text{Na}^+, \text{K}^+$ -ATPase were enriched 15-fold in chick intestine CMF compared with the total cell homogenate (data not shown). Consistent with earlier reports we found marker enzyme evidence for some contaminants from endoplasmic reticulum, lysosomes, and mitochondria (39) in our CMF.

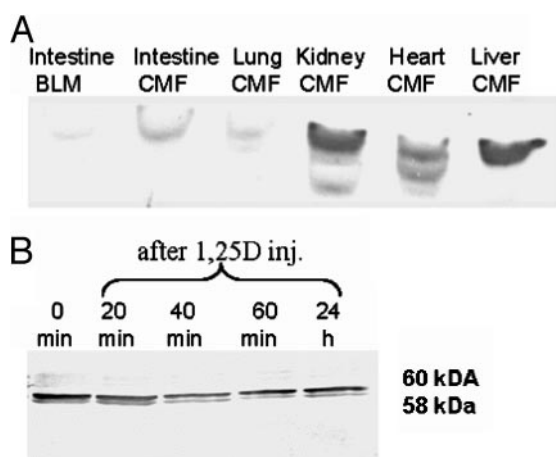
of [ $^3\text{H}$ ]-1,25D (4662 dpm/pmol in 100  $\mu\text{l}$  of ethanol:propanediol, 1:1) 2 h before CMF isolation. The total lipids in the aliquots of CMF were extracted (36) for HPLC. The chromatogram represents 17 min of collections at 2 ml/min. Tritium activity in each fraction was measured by liquid scintillation spectrometry. The histogram bars indicate the tritium isolated from the CMF. The arrows indicate the position of migration of standard 25(OH) $\text{D}_3$ , 23S,25R-1 $\alpha,25(\text{OH})_2\text{-D}_3$ -26,23-lactone (lactone), 1,25D and 1 $\alpha,24,25(\text{OH})_3\text{D}_3$ .

### Expression of VDR and Vitamin D Binding Protein in CMF

Expression of the classical VDR in CMF of chick tissues was studied by Western blot analysis (Fig. 4A). In three Western blot replicates, the VDR C-20 antibody recognized a double band at 58–60 kDa and no other bands. These two bands may represent the two alternative splicing variants of the human VDR gene (40) (41) or the alternative promoter start sites of the chick gene (42). VDR expression was highest in caveolae from kidney, liver, and heart, and lower, but clearly detectable in lung and intestine. The CMF showed enrichment of VDR over the basal lateral membrane fraction. Immunoreactive VDR did not vary from 20 min to 24 h after 1,25D stimulation (Fig. 4B). An immunoreaction with an antibody against the plasma vitamin D binding protein (DBP) was only seen in a positive control but not in CMF of any chick tissues (data not shown). Thus, the protein binding 1,25D in the CFM is not DBP as we also concluded in a preliminary report (43).

### Comparison of Binding Properties of the Receptors in Caveolae and Nuclear Fractions of Chick Duodenal Mucosa and DBP

We used steroid competition analysis to study the ability of eight structurally diverse analogs of 1,25D to compete with the binding of [<sup>3</sup>H]-1,25D to CMF and NF of chick duodenal mucosa and to the plasma DBP. The objective was to assess whether ligand binding to the CMF was more like that of the VDR or the DBP or was unique. Table 1 gives the relative competitive



**Fig. 4.** Western Blot Analysis of VDR with a C-Terminal Antibody (C-20; Santa Cruz Biotechnology) in Chick Tissues

Equal amounts of total protein (10  $\mu$ g) were loaded in each well. A, VDR expression in chick CMF or BLM in the indicated tissues. BLM shows a faint immunoreaction. B, VDR expression in chick CMF in the basal state (0 min) after administration of 1,25D (1.3 nmol im) to three vitamin D-deficient chicks. Alternative VDR splice variants of 58 and 60 kDa are better separated in panel A due to a longer running time.

index (RCI) values for the nine ligands evaluated using the CMF, NF, or DBP. The results (Fig. 5) clearly show that the ligand binding specificity in the CMF correlates very well to that of the VDR ( $r^2 = 0.99$ ) but not with the DBP ( $r^2 = 0.16$ ). These results are congruent with the Western blot analyses that showed the presence of the VDR, but not DBP, in caveolae (not shown).

### Specific [<sup>3</sup>H]-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> Binding *in Vitro* in WT and VDR KO Kidney Tissue Nuclear and Caveolae Membrane Fractions

As a consequence of the data presented in Figs. 1–5 that led to the conclusion that the 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> binding protein present in the CMF is the classic nuclear VDR, we carried out an experiment using VDR WT and KO mice. Because the cell nucleus is known to have a high VDR content (44), we used the nuclear fraction of kidney tissue as a positive control for [<sup>3</sup>H]-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> binding. Specifically, we compared the magnitude of saturable binding of [<sup>3</sup>H]-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (eight-point saturation curve) in the crude nuclear fraction and the CMF isolated from the two pools (WT and KO) of kidney tissue. The results in Fig. 6 report the  $B_{\text{Max}}$  values determined from the saturation curves. The mouse kidney nuclear fraction  $B_{\text{Max}}$  values were reduced 95%, whereas the mouse CMF  $B_{\text{Max}}$  values were reduced approximately 85%. These results show that the VDR KO mouse kidney has little or no functional 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> binding capability in either the cell nucleus or the CMF.

### Confocal Immunofluorescence Microscopy of VDR and Caveolin-1

ROS 17/2.8 cells, known to express high levels of the VDR, were used to study the cellular localization of VDR and caveolin-1 via confocal microscopy. Immunoreactive caveolin-1 (red) was largely distributed in the cytosol and cell membrane but was largely absent in the nucleus (Fig. 7). Strong immunoreactivity of the VDR (green) was seen in the cytosol and the perinuclear areas of ROS 17/2.8 cells (Fig. 7A). Interestingly, regions of strong VDR immunostaining were clearly visible in close proximity to the cell membrane, and colocalized with the plasma membrane protein caveolin-1 (yellow) in approximately 50% of the studied cells (Fig. 7, B and C). No difference was found in the cellular distribution of VDR with or without stimulation with  $10^{-8}$  M 1,25D (5–10 min, data not shown).

### DISCUSSION

Rapid responses of the steroid hormone 1,25D are postulated to be initiated by a plasma membrane-associated receptor (9, 14, 15). Candidates for the VDR<sub>mem</sub> have been suggested, including annexin II (28), the MARRS binding protein (14, 31), protein ki-

**Table 1.** Relative Binding of Analogs of 1 $\alpha$ ,25(OH) $_2$ D $_3$  to CMF, Nuclear VDR, and DBP Relative Competitive Index

| Analog Name   | Analog Code | CMF               |    | NF             |    | DBP                  |    |
|---|-------------|-------------------|----|----------------|----|----------------------|----|
|   |             | Mean $\pm$ SEM    | n  | Mean $\pm$ SEM | n  | Mean $\pm$ SEM       | n  |
| 1 $\alpha$ ,25(OH) $_2$ D $_3$  | C           | 100 $\pm$ 0       | 20 | 100 $\pm$ 0    | 20 | 100 $\pm$ 0          | 20 |
| 20-Epi-1 $\alpha$ ,25(OH) $_2$ D $_3$   | IE          | 120 $\pm$ 11      | 5  | 120 $\pm$ 35   | 3  | 2.6 $\pm$ 0.8        | 4  |
| 1,25(OH) $_2$ -16-ene-23-yne-D $_3$   | V           | 95 $\pm$ 5.3      | 3  | 110 $\pm$ 39   | 4  | 5.4 $\pm$ 2.3        | 4  |
| 21-(3'-Hydroxy-3'-methylbutyl)-1 $\alpha$ ,25(OH) $_2$ D $_3$                 | KH          | 81 $\pm$ 16       | 2  | 76 $\pm$ 32    | 2  | 2.6 $\pm$ 0.3        | 3  |
| 1,24,25(OH) $_3$ D $_3$   | W           | 66 $\pm$ 0        | 1  | 53 $\pm$ 0     | 1  | 20.9 $\pm$ 1.1       | 3  |
| 20,21-Methylene-23-yne-26,27-F $_6$ -5,6-trans-1 $\alpha$ ,25(OH) $_2$ D $_3$ | OA          | 15 $\pm$ 3.0      | 2  | 20 $\pm$ 1.5   | 2  | 9.6 $\pm$ 1.0        | 3  |
| 1 $\alpha$ ,25(OH) $_2$ -lumisterol $_3$                                      | JN          | 0.85 $\pm$ 0.05   | 3  | 2.8 $\pm$ 1.5  | 2  | -0.68 $\pm$ 0.2      | 4  |
| 1 $\alpha$ ,25(OH) $_2$ -7-dehydrocholesterol                                 | JM          | 0.21 $\pm$ 0      | 1  | 0.47 $\pm$ 0   | 1  | -0.34 $\pm$ 0.1      | 4  |
| 22(p-Hydroxyphenyl)-23,24,25,26,27-pentanol-D $_3$                            | JX          | 0.013 $\pm$ 0.009 | 2  | 0.002 $\pm$ 0  | 1  | 211,100 $\pm$ 31,550 | 4  |

Comparison of the relative ability of selected 1 $\alpha$ ,25(OH) $_2$ D $_3$  analogs to compete with [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  for binding to the VDR<sub>NUC</sub> in a chick duodenal crude NF, to a chick duodenal CMF, and to the plasma DBP. It is well established that the VDR and DBP have unique ligand binding specificities (35). The incubations of [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  with the indicated competitors and the separation of bound from free vitamin D steroid using hydroxylapatite were conducted as described in *Materials and Methods*. Each analog and its respective *letter code* are summarized as an average  $\pm$  SEM with replicates of n = 3 or higher. See also Fig. 5 for an alternative presentation of the data.

nase C (32), and the classical VDR (33). This study provides evidence that in various avian and mammalian tissues and cells, the classical VDR<sub>mem</sub> closely associates with CMF and that the CMF can bind [ $^3$ H]-1,25D with high affinity both *in vivo* and *in vitro*. We conclude from the results presented in this communication that the VDR, which is normally found in the nucleus, is also a plasma membrane-associated receptor that binds 1,25D *in vivo* to generate rapid responses.

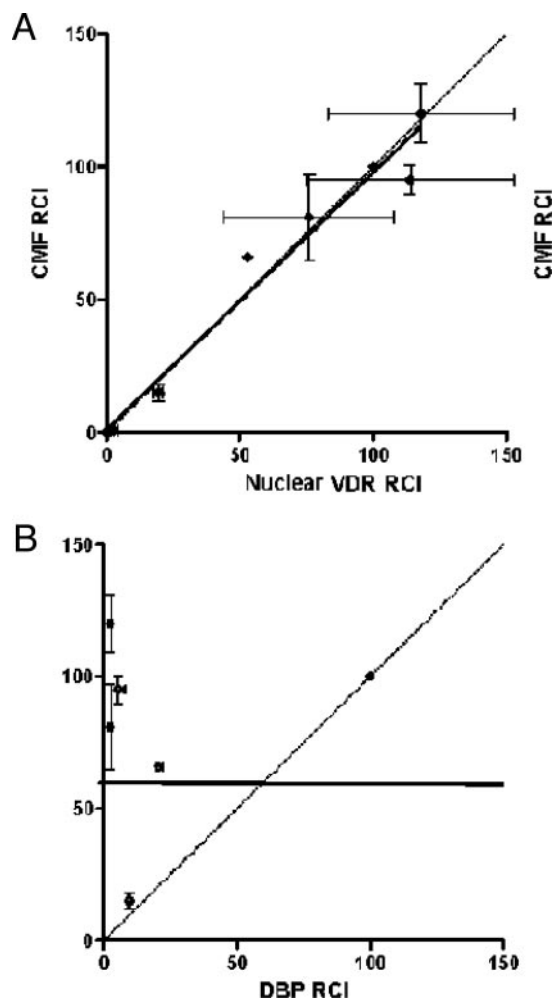
By Western blot, there was clear evidence for the caveolae marker caveolin-1 (45) in our CMF from chick and mouse tissues, with up to 94-fold enrichment in CMF over homogenate (Fig. 3). Thus, the purity of our CMF is comparable with that used for studies on estradiol binding (23), receptor membrane localization (22), or as initiators of rapid responses (24).

To our knowledge, we show here for the first time by Western blot analysis enrichment of immunoreactivity of VDR in CMF as compared with the less pure basal lateral membrane fraction of chick tissues (Fig. 4). Lability of the VDR<sub>mem</sub> or its low concentration may have hindered its detection in the basal lateral membrane fraction and caused false negative results in Western blots of earlier studies (14, 46). Because our CMF isolation method also enriched some components of the endoplasmic reticulum, lysosomes, and mitochondria that have similar buoyant density, it is possible that a part of the VDR immunoreactivity comes from subcellular structures other than CMF. However, contamination with the nuclear VDR is unlikely because less than 0.45% of the [ $^{35}$ S]-VDR added to a chick intestinal mucosa homogenate was found in the resulting CMF (data not shown).

To further investigate possible membrane localization of the classical VDR, we performed immunocytochemical confocal microscopy studies on fixed ROS 17/2.8 cells using fluorescein isothiocyanate-conjugated secondary antibodies for VDR and F(ab') $_2$  fragment-Cy3 for caveolin-1. These cells express the clas-

sical VDR (47) and showed a high concentration of [ $^3$ H]-1,25D binding sites in CMF in this study (Fig. 1D). We found conspicuous regions of VDR and caveolin-1 colocalization (overlapping *yellow* color, see Fig. 7C) in proximity to the plasma membrane in approximately 50% of cells. In accordance with binding studies with BODIPY-calcitriol (a fluorescently labeled form of 1,25D) in living cells (48), we also found VDR in the cytosol. However, we also detected VDR immunoreactivity in the cell membrane. Because in the study with BODIPY-calcitriol only a portion of the VDR was occupied by the ligand, it is possible that the concentration was VDR<sub>mem</sub> was below the detection limit in that study. We conclude from our microscopy studies that classical VDR or a closely related protein is localized in, or close to, the plasma membrane in association with caveolae. Ongoing Matrix-Assisted Laser Desorption Ionization Coupled to Time of Flight Separation studies may afford sequence information on the CMF protein that binds [ $^3$ H]-1,25D and cross-reacts with the VDR antibody. Our present results indicating that the VDR is associated with the CMF parallel the evidence for the plasma membrane localization of the ER $\alpha$  in MCF7 breast cancer cells obtained by similar methodology (22).

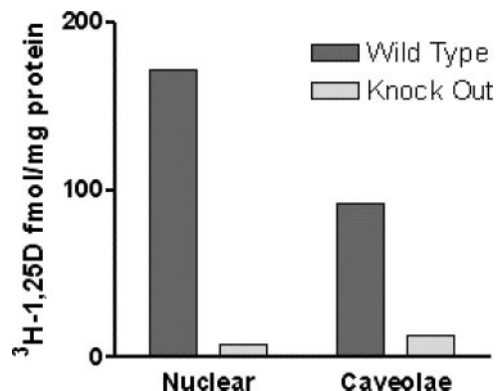
Using high specific activity [ $^3$ H]-1,25D, it has been possible to clearly show that CMF from four species (chick, rat, mouse, and human) and three tissues (intestinal mucosa, kidney, and lung) and two cell lines (rat osteoblast-derived ROS 17/2.8 cells and human monocyte-like NB4 leukemia cells) bind the steroid hormone in a saturable (hyperbolic) and reproducible fashion *in vitro*. The CMF K<sub>D</sub>  $\approx$  1–3 nM for 1,25D binding agrees well with the K<sub>D</sub> for 1,25D ligand binding to the VDR in nuclear or chromatin preparations (49, 50). It is notable that we have not encountered, to date, any cells with the nuclear VDR that did not have detectable specific [ $^3$ H]-1,25D binding or immunoblot detectable VDR in the CMF.



**Fig. 5.** Comparison of 1,25D Analog Competition Binding to Chick Duodenal CMF Correlated with the Binding to (A) the Chick Duodenal Nuclear Fraction (a Source of VDR) or (B) DBP

Steroid competition analysis (see *Materials and Methods*) of chick duodenal CMF, and nuclear fraction and serum DBP was used to determine the RCI of 1,25D (RCI set to 100%) and its structural analogs IE, JM JN, JX, KH, OA, V, and W. The chemical abbreviated names of the analogs and the data used for the plots in each panel are given in Table 1. The dashed line (· · · ·) indicates the theoretical line of identity for values on the ordinate (CMF RCI) and the abscissa (either Nuclear RCI or DBP RCI). The solid line (—) indicates the calculated linear regression line for the experimental data. The error bars are SEM; data are given in Table 1. There was a high linear correlation coefficient in the RCI values for CMF vs. nuclei ( $r^2 = 0.99$ ), but not CMF vs. DBP ( $r^2 = 0.16$ ) for the nine different ligands.

There are only two known biochemically characterized proteins with a specific ligand binding domain for 1,25D; these are the VDR ( $K_D = 1.2$  nM) and DBP ( $K_D = 60$  nM). X-ray crystallography shows no structural homology of the VDR ligand binding domain with the DBP ligand binding domain (51–53). We compared the ability of eight structurally diverse analogs of 1,25D to compete with [ $^3$ H]-1,25D for binding to the CMF



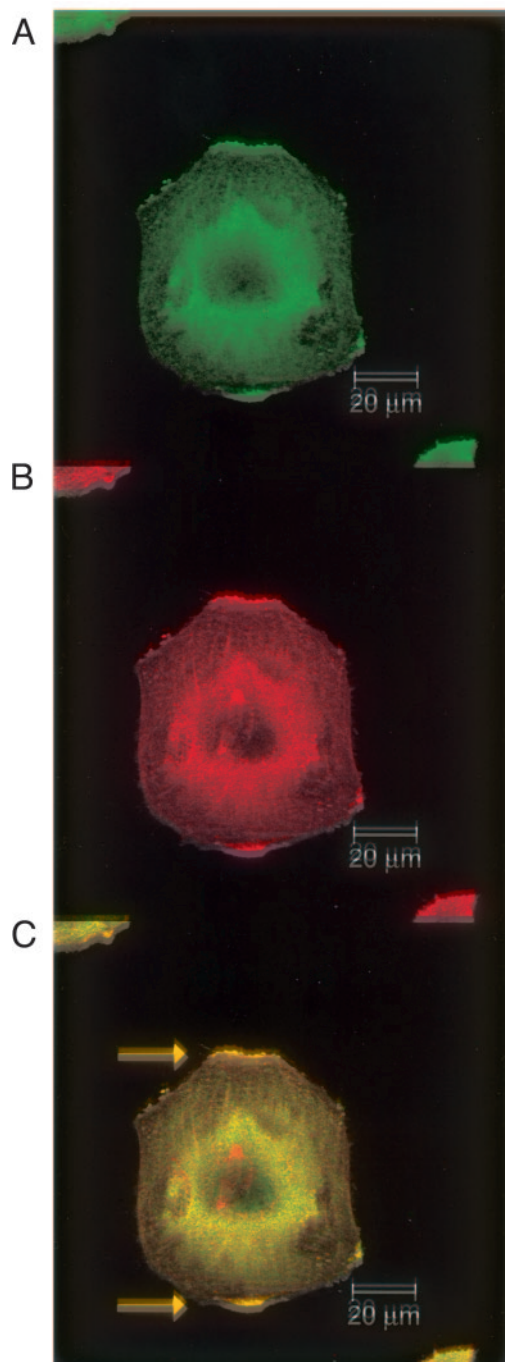
**Fig. 6.** Comparison of the Total Saturable [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  Binding in the Nuclear Fraction and CMF Prepared from Mouse Kidney Tissue Obtained from Either VDR WT or KO Animals

The data histogram compares  $B_{Max}$  values obtained using [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  in 8-point saturation binding curves in separate incubations of mouse kidney VDR WT (from 14 mice) and KO (from 16 mice) nuclear and CMF; the relative proportion of total and nonspecific DPM in each subcellular fraction saturation curve (data not presented) was comparable to that shown for the chick duodenum in Fig. 1. The  $B_{Max}$  values are expressed in fmol of [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  per mg of total protein. Saturation 1 $\alpha$ ,25(OH) $_2$ D $_3$  binding was observed in the physiological range (1–10 nM) with  $K_D$  approximately 1–3 nM similar to that shown in Fig. 1. Experiments were performed as described in *Materials and Methods*; the results are expressed as the average of two separate experiments.

VDR $_{mem}$ , VDR and DBP. The ligand specificity of the CMF VDR $_{mem}$  is virtually indistinguishable from that of VDR and bears no resemblance to that of DBP. DBP was not observed in CMF of any of the tissues by Western blot analysis. This is further support for the proposal that the CMF possesses the classical VDR or possibly a slightly modified VDR that is responsible for the ligand binding of [ $^3$ H]-1,25D.

We have also evaluated the binding of [ $^3$ H]-1,25D to the CMF and nuclear fractions of kidney tissue obtained from VDR WT and VDR KO mice (see Fig. 6). The VDR KO mice had greatly reduced [ $^3$ H]-1,25D-specific binding in kidney CMF and nuclei as compared with VDR WT. These results clearly support the conclusion that the [ $^3$ H]-1 $\alpha$ ,25(OH) $_2$ D $_3$  binding activity present in the CMF is attributable to the presence of the classic VDR. The results are also compatible with the necessity for the presence of the VDR to initiate rapid responses.

Giving a dose of 1,25D to vitamin D-deficient chicks followed by *in vitro* quantitation of a reduction in [ $^3$ H]-1,25D binding demonstrated that the 1,25D receptors in CMF of duodenal mucosa can be occupied *in vivo* (Fig. 2A). Because the time from the dose to isolation of CMF was only 2 h, down-regulation of the VDR $_{mem}$  expression is not likely to explain the reduced [ $^3$ H]-1,25D binding in the dosed chicks. Furthermore, we did not find in Western blot studies any indication of



**Fig. 7.** Confocal Immunofluorescence Images of a Single ROS 17/2.8 Cell Showing Subcellular Localization of VDR (Green, A) and Caveolin-1 (Red, B)

C, Merged image of A and B illustrating colocalization of both proteins (yellow). The arrow points at a region of the plasma membrane with a strong fluorescent colocalization signal. Bars, 20  $\mu$ M.

down-regulation or translocation of the VDR 20–60 min after a single dose of 1,25D (Fig. 4B).

It is known that 1,25D can be rapidly metabolized either to  $1\alpha,24,25(\text{OH})_3\text{D}_3$  and then to calcitroic acid or

to the 1,25D-lactone (35). HPLC analysis indicated that only [ $^3\text{H}$ ]-1,25D was present in the CMF after an *in vivo* dose of [ $^3\text{H}$ ]-1,25D. These results show that the binding protein for 1,25D present in the CMF can be occupied *in vivo*, which is consistent with the CMF VDR<sub>mem</sub> being linked to 1,25D-mediated responses. Experiments are in progress to evaluate this possibility.

An important issue concerns whether the presence of a VDR is essential for the generation of a 1,25D-mediated rapid response. This laboratory studied 1,25D modulation of chloride and  $\text{Ca}^{2+}$  ion channel electrical activities in the plasma membrane of calvarial osteoblasts isolated from VDR WT and KO mice (54). We found that the presence of a functional VDR was essential for ion channel activity as well as 1,25D-dependent rapid exocytosis of osteoblast secretory granules (54). In a different VDR KO mouse model (55), the absence of the VDR was found to abrogate rapid 1,25D mediated changes in intracellular  $\text{Ca}^{2+}$  in the calvarial osteoblasts. In studies using cultured skin fibroblasts from three patients with vitamin D-resistant rickets resulting from homozygous missense VDR mutations [which abolished either VDR binding to DNA (Lys45Glu) or stable binding of ligand binding (Trp286Arg)], the authors concluded that the 1,25D rapid effects require the presence of the VDR (56). Collectively, these studies are compatible with the presence of the VDR in the caveolae membrane fraction being available for initiation of rapid responses, and that show in the absence of the VDR a variety of 1,25D-mediated responses were not detectable.

In studies in the ER field, clear evidence has been presented for the presence of the classical ER associated with CMF isolated from endothelial cells (57, 58). The presence of the ER with caveolae has also been linked to the rapid activation of nitric oxide synthase [by estradiol (57)]. Furthermore, both the full-length ER, 66 kDa, and an amino terminal-truncated 46 kDa form of the ER, resulting from alternative splicing, have been reported in the plasma membrane of immortalized human endothelial cells (59).

In this study, we have demonstrated the presence of a specific, saturable binding protein/receptor for 1,25D in the CMF of various tissues in four different species (mouse, rat, human, and chicken). However in a VDR KO mouse, the [ $^3\text{H}$ ]- $1\alpha,25(\text{OH})_2\text{D}_3$  binding activity present in the CMF as well as the control nuclear fraction was dramatically reduced. These observations are all consistent with the conclusion that the CMF binder is the VDR. This CMF binder can be occupied *in vivo* or *in vitro* and shows similar ligand specificity to the classical VDR. DPB has a completely different ligand specificity profile, and we have shown that it is not localized in the CMF; therefore, DBP is very unlikely to be this binding protein/receptor. Immunofluorescence studies show colocalization of VDR and caveolin-1, indicating that classical VDR is present in the plasma membrane. Therefore, in summary, we show here clear evidence for the classical

VDR or possibly a modest N-terminal truncated version of the VDR in binding of 1,25D in the cell plasma membrane *in vivo* and suggest that it may mediate rapid responses to this steroid hormone.

## MATERIALS AND METHODS

### Animal Maintenance

White Leghorn male chicks (Hyline International, Lakeview, CA) were raised on a vitamin D<sub>3</sub>-free diet (0.6% Ca and 0.4% P) (60) up to 3 wk of age when they had fully developed rickets. Vitamin D-deficient chicks were used to reduce occupancy of the VDR by its natural ligand 1,25D to detect more binding of [<sup>3</sup>H]-1,25D in CMF and nuclear fractions. Sprague Dawley rats were fed Rodent Diet 5008, PMI International (Richmond, IN). Mice were fed Rodent Diet 5001, PMI. VDR Tokyo strain KO mice were generated by targeted ablation of exon 2 that encodes the first zinc finger of the DNA-binding domain (61). The breeding colony was a generous gift from Dr. S. Kato (University of Tokyo, Tokyo, Japan). VDR KO and WT were weaned at 3–4 wk of age, then maintained on a normal diet (1.0% Ca, 1.0% P, 0% lactose, 4.5 IU vitamin D<sub>3</sub>/g) (Laboratory Rodent Diet 50, PMI Nutrition International). The experimental protocols involving use of animals were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside.

### Cell Cultures

Human leukemia NB4 cells (K. Meckling-Gill, University of Guelph, Guelph, Ontario, Canada) were maintained in suspension culture in DMEM/Ham's F-12 (50:50, vol/vol) medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin and streptomycin. Rat osteosarcoma ROS 17/2.8 osteoblast-like cells (J. Stein, University of Massachusetts, Boston, MA) were cultured in the same medium. Both cell lines were grown in humidified atmosphere of 5% CO<sub>2</sub> at 37 C.

### Isolation of CMF

CMF in these studies were isolated using the procedure of Smart *et al.* (37). Buffers. Buffer A [0.25 M sucrose, 1 mM EDTA, 20 mM Tricine (pH 7.8)]; Buffer B [0.25 M Sucrose, 6 mM EDTA, 120 mM Tricine (pH 7.8)]; Buffer C [50% Optiprep (Greiner Bio-One, Longwood, FL) in Buffer B]; Buffer D (20% Optiprep in Buffer A); Buffer E (10% Optiprep in Buffer A); Buffer F (5% Optiprep in Buffer A); Buffer G (8% Buffer A in Buffer C); Buffer H [30% Percoll (Amersham Biosciences, Piscataway, NJ) in Buffer A].

Approximately 1 g of cultured cells or 5–20 g of mouse, chick, or rat tissue were used for the preparation of CMF. Immediately after euthanasia, tissues were perfused with 20–40 ml of ice-cold saline (0.9%) through the heart. Kidney, liver, lung, duodenum, and heart were removed and placed in ice-cold saline (0.9%). The duodenum was softly scraped (over ice) to separate mucosal cells from the serosal and muscular layers. Solid tissues were cut into small pieces. Cells and tissue pieces were washed twice in 20 ml Buffer A and centrifuged (1500 × *g*, 5 min, 4 C). The washed pellet was homogenized in 20 ml ice-cold Buffer A with 20 strokes (2000 rpm) using a Potter-Elvehjem tissue homogenizer. The homogenate (H) was centrifuged (1000 × *g*, 10 min, 4 C) to separate a post-nuclear supernatant (PNS) (8–16 ml/tube) from a nuclear pellet (NF). Eight milliliters of PNS were layered over 15 ml Buffer H and centrifuged (84,000 × *g*, 30 min, 4 C) in a Beckman 60Ti rotor (Beckman Instruments, Fullerton,

CA). The total membrane fraction (MF, 4–8 ml/tube), a clearly visible brownish layer in the middle, was collected, added to Buffer G 1:1 (vol/vol) and sonified six times with 15-sec bursts (50% duty cycle, output control setting 5) from a Sonifier Cell Disruptor 350 (WWR Scientific, West Chester, PA). Sonified MF (8 ml) was loaded under linear 10–20% Optiprep gradients (20 ml/tube) and centrifuged (52,000 × *g*, 90 min, 4 C) in a Beckman SW28Ti rotor. The uppermost layer was collected (8–10 ml/tube), mixed 5:4 (vol/vol) with Buffer C and poured into a SW28Ti tube under 7 ml Buffer F. After centrifugation (52,000 × *g*, 90 min, 4 C) in a Beckman SW28Ti rotor, CMF formed a white fluffy layer (2–4 ml/tube) at the interface.

### Marker Enzyme Assays

Marker enzymes for plasma membrane (alkaline phosphatase and Na<sup>+</sup>,K<sup>+</sup>-ATPase), endoplasmic reticulum (glucose-6-phosphatase), lysosomes (acid phosphatase), and mitochondria (succinate dehydrogenase) were analyzed during CMF isolation in H, PNS, NF, MF, and CMF to characterize each fraction (62).

### Western Analyses

Samples were run on 10 or 12% SDS/PAGE gels and transferred on to a PVDF membrane (Millipore, Bedford, MA). Figure 3, A–C, used antibodies against VDR (C-20) and caveolin-1 (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA), whereas Fig. 3D employed an antibody against caveolin-1 specific for chick (2234) (Transduction Laboratories, Los Angeles, CA). The VDR Western results (Fig. 4) used Santa Cruz Biotechnology C-20 antibody. Membranes were incubated with primary antibodies (1:500) in TBS buffer with 5% nonfat dried milk and 0.1% Tween overnight at 4 C. After five 5-min rinses, membranes were incubated with secondary antibodies (1:1000) conjugated either with alkaline phosphatase (Sigma-Aldrich) or horseradish peroxidase (Pierce, Rockford, IL) for 2 h. After five 5-min rinses, antigens in Fig. 3, A–C, were detected using BCIP-NBT-blue (Sigma-Aldrich); Fig. 3D was developed using enhanced chemiluminescence using SuperSignal West Pico Kit substrate reagents (Pierce).

### Vitamin D Metabolites and Analogs

[<sup>3</sup>H]-1,25D was purchased from Amersham Biosciences. Nonradioactive 1,25D analogs KH [21-(3'-hydroxy-3'-methylbutyl)-1 $\alpha$ ,25-(OH)<sub>2</sub>-D<sub>3</sub>], W [1 $\alpha$ ,24R,25(OH)<sub>3</sub>D<sub>3</sub>], V [1,25-(OH)<sub>2</sub>-16-ene-23-yne-D<sub>3</sub>], and OA [20,21-methylene-23-yne-26,27-F<sub>6</sub>-5,6-trans-1,25D] were gifts from Dr. Milan Uskokovic (Hoffman La Roche, Nutley, NJ). Analog IE [20-epi-1 $\alpha$ ,25-(OH)<sub>2</sub>-D<sub>3</sub>] was obtained from Leo-Pharma (Ballerup, Denmark). Analogs JX [22-(p-hydroxyphenyl)-23,24,25,26,27-pentanol-D<sub>3</sub>], JN [1 $\alpha$ ,25-(OH)<sub>2</sub>-lumisterol<sub>3</sub>], and JM [1 $\alpha$ ,25-(OH)<sub>2</sub>-7-dehydrocholesterol] were prepared by Dr. W. H. Okamura (Department of Chemistry, University of California, Riverside, CA).

### Ligand Binding Assays in Caveolae-Enriched Membrane and Crude Nuclear Fractions Chicks

Saturable binding of [<sup>3</sup>H]-1,25D (0.25–10 nM, 105 Ci/mmol) was assayed in CMF isolated from cultured cells and animal tissues with (two tubes) or without (three tubes) 100-fold excess of 1,25D. The tubes were incubated for 17 h at 4 C. Hydroxylapatite was used to separate protein-bound hormone from free (63). The tritium activity was determined by liquid scintillation spectrometry (LS6500, Beckman Instruments) with a 2% error. For *in vivo* occupancy studies, 10 vitamin D-deficient chicks were given 1.3 nmol 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or vehicle im 2 h before euthanasia and CMF isolation.

Steroid competition analysis was used to determine the RCI (64) of 1,25D (RCI normalized to 100%) and eight analogs IE, JM, JN, JX, KH, OA, V, and W. [ $^3\text{H}$ ]-1,25D (0.4 nM) was competed against increasing concentrations of nonradioactive ligands for binding chick intestinal CMF. Bound [ $^3\text{H}$ ]-1,25D was separated from free [ $^3\text{H}$ ]-1,25D using the hydroxylapatite procedure (63), and the tritium activity was determined by liquid scintillation spectrometry.

#### Determining *in Vivo* Distribution and Metabolism of [ $^3\text{H}$ ]-1,25D in Vitamin D-Deficient Chicks

Three rachitic chicks were dosed with 1.3 nmol (4660 dpm/pmol) in 100  $\mu\text{l}$  of ethanol:propanediol, 1:1) of [ $^3\text{H}$ ]-1,25D im 2 h before CMF and NF isolation. The total lipids of the CMF and NF were extracted (36) for HPLC separation (2 ml/min, 4–20% isopropanol gradient in hexane, NovaPak Silica column, Waters Instruments, Milford, MA) with standards [25OH-D<sub>3</sub>, 1,25D, 1 $\alpha$ ,25(OH)<sub>2</sub>-D<sub>3</sub>-26,23-lactone, and 1 $\alpha$ ,24,25(OH)<sub>3</sub>D<sub>3</sub>]. The tritium activity in each fraction was determined by liquid scintillation spectrometry.

#### Immunocytochemistry of VDR in ROS 17/2.8 Cells

Rat osteoblastic ROS 17/2.8 cells were cultured as described above (see *Cell Cultures*) on coverslips for 48 h, fixed for 20 min with 3.7% (vol/vol) formaldehyde at room temperature, and permeabilized with ice-cold ethanol for 5 min according to conventional protocols (65). Cells were then incubated with 5% goat serum at room temperature for 1 h to reduce background staining and treated overnight with primary antibodies against VDR and caveolin-1 (mouse monoclonal D-6 VDR and rabbit polyclonal N-20 Cav-1, respectively, Santa Cruz Biotechnology) at 4 C. Cells were then treated with secondary fluorescein isothiocyanate- and F(ab')<sub>2</sub> fragment Cy3-conjugated antimouse and antirabbit antibodies, respectively, (Sigma) in a 1:500 dilution for 2 h. Immunostained VDR and caveolin-1 were visualized with a laser scanning confocal Leica TCS SP2 microscope (Leica Microsystems, Inc., Exton, PA) using a  $\times 63$  immersion lens with aperture and PBS buffer as the imaging medium. Leica confocal software was used for acquisition of the data and merging of the digital images. Controls were performed with either no primary antibody or nonreactive secondary antibodies. Fluorescence immunostaining was also performed on nonpermeabilized cells.

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