All natural DR3-type vitamin D response elements show a similar functionality *in vitro*

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The vitamin D₃ receptor (VDR), which is the nuclear receptor for 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], acts primarily as a heterodimer with the retinoid X receptor (RXR) and binds preferentially to directly repeated arrangements of two hexameric binding sites with three spacing nucleotides [DR3-type vitamin D response elements (VDREs)]. In this study, all presently known natural DR3-type VDREs have been compared and classified on the basis of their complex-formation with VDR–RXR heterodimers and their ability to stabilize VDR–RXR heterodimer conformations. Based on the affinity of each VDRE for VDR–RXR heterodimers, the DR3-type VDREs were divided into three classes. The ligand sensitivity of this complex-formation and conformational stabilization was determined to be in the range of 0.1 nM. No significant differences in the 1 α ,25(OH)₂D₃-

modulated interactions of the DR3-type VDRE-complexed VDR–RXR heterodimer with the co-activator SRC-1 (steroid receptor co-activator-1) or the co-repressor NCoR (nuclear receptor co-repressor) were found. Taken together, the affinity for VDR–RXR heterodimers appears to be the major discriminating parameter between natural DR3-type VDREs. This will not only facilitate further investigation of the principles of DR3-type-VDRE-mediated gene regulation, but also strongly suggests that DR3-type VDREs alone cannot explain the pleiotropic genomic action of 1 α ,25(OH)₂D₃.

Key words: cofactors, gene regulation, VDR conformations, vitamin D receptor, vitamin D signalling.

INTRODUCTION

All genomic effects of the lipophilic nuclear hormone 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], which is the biologically active form of vitamin D₃, are mediated through its nuclear receptor, the vitamin D receptor (VDR) [1]. The VDR is a member of the nuclear receptor superfamily that also contains structurally related receptors for other nuclear hormones, such as steroids, retinoic acid and thyroid hormone, and many orphan nuclear receptors [2]. An essential prerequisite for mediating transactivation by 1 α ,25(OH)₂D₃ is the positioning of the VDR in close proximity to the basal transcription machinery. This is achieved initially through binding of the VDR to specific sequences in promoter regions of 1 α ,25(OH)₂D₃ target genes, commonly referred to as 1 α ,25(OH)₂D₃ (vitamin D) response elements (VDREs) [3]. The VDR preferentially functions as a heterodimer with the retinoid X receptor (RXR), for which simple VDREs consist of two hexameric nuclear receptor binding sites. Screening of differently spaced response elements [4] has indicated that a directly repeated arrangement of the hexameric binding sites with three spacing nucleotides (DR3-type VDREs) represents an optimal binding site for VDR–RXR heterodimers. Moreover, modelling of the DNA-binding domains of the VDR and RXR on DNA [5] also suggested that an asymmetrical arrangement, i.e. head-to-tail, with three intervening nucleotides provides the most efficient interface of the core DNA-binding domains. Finally, the vast majority of the presently known VDREs show a DR3-type structure [3], so that there is no doubt that DR3-type VDREs are the preferential and predominant form of binding sites for VDR–RXR heterodimers. However, other VDRE structures, such as direct repeats with four or six

spacing nucleotides (DR4- and DR6-type VDREs respectively) or inverted palindromes with nine intervening nucleotides (IP9-type VDREs), have been described [6].

The VDR knock-out mouse model confirmed that the VDR and its ligand 1 α ,25(OH)₂D₃ have an important role in calcium homeostasis and bone formation, but also suggested critical functions of the VDR in the hair cycle and in female fertility. Moreover, 1 α ,25(OH)₂D₃ has been shown to inhibit cell growth, to induce differentiation in several normal and malignant cell types and to modulate apoptosis (programmed cell death) in human breast cancer and leukaemic cell lines. In order to take advantage of the promising therapeutic potential of 1 α ,25(OH)₂D₃, the cell regulatory functions of 1 α ,25(OH)₂D₃ and its synthetic analogues [7,8] have to be dissected from side effects such as hypercalcaemia, hypercalciuria and soft tissue calcification, which are caused by exaggeration at high concentrations of the classical function of the nuclear hormone. The pleiotropic physiological effects of 1 α ,25(OH)₂D₃ are based on transcriptional regulation of primary 1 α ,25(OH)₂D₃-responsive genes, i.e. mainly on the activation of VDR–RXR heterodimers bound to DR3-type VDREs. Therefore there have been attempts to explain the pleiotropic effects of 1 α ,25(OH)₂D₃ by a multiplicity of 1 α ,25(OH)₂D₃ signalling pathways that are based on DR3-type VDREs [9]. Finally, this leads to the central question of the present study, i.e. whether each DR3-type VDRE has an individual functionality that may explain specific effects of 1 α ,25(OH)₂D₃, or whether all DR3-type VDREs function in the same way.

The understanding of the molecular mechanisms of 1 α ,25(OH)₂D₃ signalling has progressed significantly following the recent findings on VDR conformations and VDR-interacting

Abbreviations used: 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; ANF, atrial natriuretic factor; c1_{GSC} and c2_{GSC}, conformations of protein–DNA complexes from gel-shift clipping assays; CYP24, 24-hydroxylase; DR3 (etc.), direct repeat spaced by three nucleotides (etc.); GST, glutathione S-transferase; IP9, inverted palindrome spaced by nine nucleotides; NCoR, nuclear receptor co-repressor; OPN, osteopontin; PTH, parathyroid hormone; PTHrP, PTH-related peptide; RXR, retinoid X receptor; SRC-1, steroid receptor co-activator-1; VDR, vitamin D receptor; VDRE, 1 α ,25(OH)₂D₃ (vitamin D) response element.

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cofactors [1]. DNA-bound VDR–RXR heterodimers can be considered as the molecular switch of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling. Conformational changes of this molecular switch have been investigated by a very potent method, referred to as the gel-shift clipping assay [10,11]. This method combines the characterization of functional conformations of VDR monomers in solution through a limited protease digestion assay [12,13] with the ligand-dependent gel-shift assay, which assesses the interaction of VDR–RXR heterodimers with VDREs. The resulting truncated protein–DNA fragments are interpreted as representatives of individual ligand-stabilized VDR–RXR heterodimer conformations. The ligand-triggered conformational change of VDR–RXR heterodimers results in a dissociation of co-repressor proteins, such as NCoR (nuclear receptor co-repressor), and facilitates the interaction with members of the p160 co-activator family, such as SRC-1 (steroid receptor co-activator-1), TIF2 (transcriptional intermediary factor 2) and RAC3 (receptor-associated co-activator 3) [14]. The VDR–co-activator interaction then facilitates further recruitment of other factors to form a larger complex that modulates chromatin structure and initiates transcription [15]. This also involves the recently described DRIP (vitamin D receptor interacting protein) cofactor complexes [16,17], which appear to contact the VDR and other nuclear receptors preceding their interaction with the p160 family of co-activators [18]. The most straightforward investigation of a VDR–cofactor interaction is facilitated by ligand-dependent supershift assays, which are modifications of the traditional gel-shift assay.

In the present study, all presently known natural DR3-type VDREs have been compared and classified for their *in vitro* functionality, i.e. for their complex-formation with VDR–RXR heterodimers, their ability to stabilize VDR–RXR heterodimer conformations and their interactions with co-activators and co-repressors.

EXPERIMENTAL

Compounds

$1\alpha,25(\text{OH})_2\text{D}_3$ (provided by L. Binderup, Leo Pharmaceutical Products, Ballerup, Denmark) and the RXR-selective retinoid CD2425 (provided by U. Reichert, Galderma R&D, Sophia Antipolis, France) were dissolved and diluted in ethanol and DMSO respectively.

DNA constructs

In vitro translation/mammalian constructs

The cDNAs for human VDR [19] and human RXR α [20] were subcloned into the T₇/simian virus 40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany).

Glutathione S-transferase (GST) fusion protein constructs

The cDNAs of the nuclear receptor interaction domains of human SRC-1 (spanning amino acids 596–790) [21] and mouse NCoR (spanning amino acids 1679–2453) [22] were generated by PCR and subcloned into the GST fusion protein vector pGEX (Amersham-Pharmacia, Freiburg, Germany).

Protein production by *in vitro* translation and bacterial overexpression

In vitro-translated VDR and RXR proteins were generated by transcribing their linearized pSG5-based cDNA expression vectors with T₇ RNA polymerase and translating these RNAs *in vitro* using rabbit reticulocyte lysate as recommended by the

supplier (Promega, Mannheim, Germany), providing a concentration of approx. 4 ng/ μl specific protein. Bacterial overexpression of GST–SRC-1-(596–790) and GST–NCoR-(1679–2453) fusion proteins was facilitated in the *Escherichia coli* BL21(DE3)pLysS strain (Stratagene) by induction with 0.25 and 1.25 mM isopropyl β -D-thiogalactopyranoside respectively for 3 h at 37 °C, providing a final protein concentration of approx. 4 $\mu\text{g}/\mu\text{l}$ (average 90% purity).

Gel-shift, supershift and gel-shift clipping assays

Equal amounts of *in vitro*-translated VDR and RXR proteins (approx. 10 ng each) were mixed and incubated in the presence of a saturating concentration (10 μM) or graded concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ or CD2425 (or solvent as control) for 15 min at room temperature in a total volume of 20 μl of binding buffer [10 mM Hepes (pH 7.9), 1 mM dithiothreitol, 0.2 $\mu\text{g}/\mu\text{l}$ poly(dI-dC) and 5% glycerol]. The buffer was adjusted to 100 mM of univalent cations by the addition of KCl. For supershift assays, 3 μl of GST–SRC-1-(596–790) or GST–NCoR-(1679–2453) fusion protein was included in the incubation for a further 10 min. Double-stranded oligonucleotides carrying the different VDRE core sequences (Table 1) were labelled by fill-in reactions using [³²P]dCTP and the Klenow fragment of DNA polymerase I (Promega). Approx. 1 ng of labelled VDRE probe (50000 c.p.m.) was added to the receptor/ligand mixture and incubation was continued for 20 min. For gel-shift clipping assays, trypsin (final concentration 8.3 $\mu\text{g}/\text{ml}$) or chymotrypsin (final concentration 33.3 $\mu\text{g}/\text{ml}$) was added and the incubation was continued for a further 10 min. Protein–DNA complexes were resolved through non-denaturing 8% (w/v) polyacrylamide gels (at room temperature) in 0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.3) and exposed to a Fuji MP2040S imager screen. The ratio of protein-complexed probe to free probe was quantified with the use of a Fuji FLA2000 reader (Fuji, Tokyo, Japan) using Image Gauge software (Raytest, Sprockhövel, Germany).

RESULTS

Ligand-dependent gel-shift assays were performed using *in vitro*-translated VDR and RXR proteins and all presently known natural DR3-type VDREs (for core sequences, see Table 1) in the presence and absence of a saturating concentration of $1\alpha,25(\text{OH})_2\text{D}_3$. The rat Pit-1 DR4-type VDRE [23] was included as a reference in all experimental series. The amount of VDR–RXR complex-formation (in relation to the individual free probe) was expressed for each VDRE in comparison with the amount of unliganded VDR–RXR heterodimers bound to the Pit-1 VDRE (Figure 1). The strongest VDR–RXR heterodimer-binding DR3-type VDREs appear to be from the rat atrial natriuretic factor (ANF) promoter [24], the mouse and pig osteopontin (OPN) promoters [25,26] and the chicken carbonic anhydrase II promoter [27]. These four elements were categorized into class I (see Table 1), but their binding affinity for VDR–RXR heterodimers was found to be only 10–30% of that of the rat Pit-1 DR4-type VDRE. The DR3-type VDREs of the human and rat 24-hydroxylase (CYP24) promoter [28,29], the human Na⁺-dependent P_i transporter type II promoter [30], the rat osteocalcin promoter [31], the human parathyroid hormone (PTH) promoter [32] and the first VDRE of the rat PTH-related peptide (PTHrP) promoter [33] demonstrated only 2–10% of the binding strength of the Pit-1 element, and were grouped into class II. The binding of VDR–RXR heterodimers to the ten DR3-type VDREs in classes I and II, as well as to the Pit-1 element, was found to be enhanced by $1\alpha,25(\text{OH})_2\text{D}_3$ by a factor of 2–5. In contrast, the

Table 1 Natural DR3-type VDREs

The core sequences of the 17 examined DR3-type VDREs and of the reference rat Pit-1 DR4-type VDRE in relation to the transcription start site are indicated. Hexameric core binding motifs are in bold, and deviations from the consensus sequence RGKTSA are underlined. The 5'- and 3'-flanking sequences of all core sequences were ATTCTAGA and TCTAGACC respectively.

VDRE	Sequence	Position	Species	Gene product	Reference
Reference DR4-type VDRE					
rPit-1	GA AGTTCA TGAG AGTTCA	— 683	Rat	Pit-1	[23]
Class I DR3-type VDREs					
rANF	AG AGTCATGAAGGACA	— 907	Rat	Atrial natriuretic factor	[24]
mOPN	A AGGTTCA CG AGGTTCA	— 759	Mouse	Osteopontin	[26]
pOPN	AT GGGTCATATGGTTCA	— 2261	Pig	Osteopontin	[25]
cCAII	GA AGGCATGGAGTTCG	— 62	Chicken	Carbonic anhydrase II	[27]
Class II DR3-type VDREs					
hCYP24	GG AGTTCA CCGGGTGTG	— 291	Human	24-Hydroxylase	[28]
hNaP _i	CAGGGGCAGCA AGGGCA	— 1977	Human	Na ⁺ -dependent P _i transporter type II	[30]
rOC	CTGGGTGAATG AGGACA	— 457	Rat	Osteocalcin	[31]
rCYP24	AGGGTTCA AGCGGGTGGC	— 259	Rat	24-Hydroxylase	[29]
hPTH	ATGGTTCA AGCAGACA	— 122	Human	Parathyroid hormone	[32]
rPTHrP ₁	TA AGGTTACTCAGTGAA	— 805	Rat	PTH-related peptide	[33]
Class III DR3-type VDREs					
rPTHrP ₂	AGGGTGGAGAGGGGTGA	— 1107	Rat	PTH-related peptide	[34]
rD9k	GAGGGTGT CGGAAGCCC	— 490	Rat	Calbindin D _{9k}	[35]
aMyHC	GA AGGACA AAGAGGGGA	— 801	Quail	Slow myosin heavy chain	[36]
hGH	TGGGGTCAACAGTGGGA	— 59	Human	Growth hormone	[37]
clntβ	GC AGGCAGAAAGGGAGA	— 772	Chicken	Integrin β ₃	[38]
cPTH	GAGGGTCAGGAGGGTGT	— 76	Chicken	Parathyroid hormone	[39]
hp21	GT AGGGAGATTGGTTCA	— 779	Human	p21 ^{WAF1/CIP1}	[40]

Figure 1 Classification of DR3-type VDREs

Ligand-dependent gel-shift assays were performed using *in vitro*-translated VDR–RXR heterodimers, 10 μ M 1 α ,25(OH)₂D₃ (or solvent as a control) and 17 ³²P-labelled DR3-type VDREs. Protein–DNA complexes were separated from free probe on non-denaturing 8% (w/v) polyacrylamide gels. Representative experiments are shown. The amount of VDR–RXR–VDRE complexes was quantified in relation to free probe by phosphorimaging. Relative complex-formation is presented by reference to the VDR–RXR heterodimer complex on the rat Pit-1 DR4-type VDRE in the absence of 1 α ,25(OH)₂D₃. Values are means $\pm$ S.D. of triplicate determinations. See Table 1 for definitions of abbreviations.

second VDRE of the rat PTHrP promoter [34], the rat calbindin D_{9k} promoter [35] and the putative DR3-type VDREs of the quail slow myosin heavy chain (MyHC) promoter [36], the

human growth hormone promoter [37], the chicken integrin β_3 promoter [38], the chicken PTH promoter [39] and the human p21^{WAF1/CIP1} promoter [40] were grouped together into class III,

Figure 2 Conformations of VDR-RXR heterodimers bound to DR3-type VDREs

Gel-shift clipping assays were performed using *in vitro*-translated VDR-RXR heterodimers, 10 μM $1\alpha,25(\text{OH})_2\text{D}_3$ (or solvent as a control) and ^{32}P -labelled DR3-type VDREs of classes I and II. Trypsin was added (final concentration 8.3 $\mu\text{g}/\text{ml}$) to the protein-DNA complexes and incubated for 10 min. Digested and non-digested VDR-RXR-VDRE complexes were separated from free probe on non-denaturing 8% (w/v) polyacrylamide gels. Representative experiments are shown. The amounts of trypsin-digested VDR-RXR-VDRE complexes 1 and 2 (designated as VDR-RXR heterodimer conformations c1_{GSC} and c2_{GSC}) were quantified in relation to the respective amounts of ligand-induced, non-digested VDR-RXR heterodimers by phosphorimaging. Values are means $\pm$ S.D. of triplicate determinations. See Table 1 for definitions of abbreviations.

Figure 3 Ligand sensitivity of VDR-RXR heterodimers and of VDR-RXR heterodimer conformations on class I and class II DR3-type VDREs

Gel-shift (A) and gel-shift clipping (B) assays were performed using *in vitro*-translated VDR-RXR heterodimers, graded concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ and selected ^{32}P -labelled DR3-type VDREs from classes I and II. Trypsin was added (final concentration 8.3 $\mu\text{g}/\text{ml}$) to the protein-DNA complexes for gel-shift clipping assays and incubated for 10 min. Digested and non-digested VDR-RXR-VDRE complexes were separated from free probe through non-denaturing 8% (w/v) polyacrylamide gels. Representative experiments are shown. The amounts of VDR-RXR heterodimers (A) or of VDR-RXR heterodimer conformations c1_{GSC} and c2_{GSC} in relation to respective ligand-induced, non-digested VDR-RXR heterodimers (B) were quantified by phosphorimaging. Values are means $\pm$ S.D. of triplicate determinations. EC_{50} values were calculated from the dose-response curves.

as they showed less than 2% affinity for VDR-RXR heterodimers compared with the Pit-1 element and no significant ligand-inducibility.

For the analysis of ligand-induced VDR-RXR conformations,

gel-shift clipping assays were performed with VDR-RXR-VDRE complexes that showed ligand inducibility, i.e. with all members of classes I and II and the Pit-1 element as a reference (Figure 2). In this assay, VDR-RXR heterodimer complexes

Figure 4 Effects of VDR and RXR ligands on VDR-RXR heterodimer conformations on class I and class II DR3-type VDREs

Gel-shift clipping assays were performed using *in vitro*-translated VDR-RXR heterodimers, 10 μM 1α,25(OH)₂D₃ (VD) and/or 10 μM CD2425 (or solvent as a control) and selected ³²P-labelled DR3-type VDREs from classes I and II. Chymotrypsin was added (final concentration 33.3 μg/ml) to the protein-DNA complexes and incubated for 10 min (hPTH VDRE), 20 min (hCYP24 VDRE) or 30 min (rat Pit-1, rANF and mOPN VDREs). Digested and non-digested VDR-RXR-VDRE complexes were separated from free probe through non-denaturing 8% (w/v) polyacrylamide gels. Representative experiments are shown. The amount of the VDR-RXR heterodimer conformation c_{GSC} was quantified in relation to respective ligand-induced, non-digested VDR-RXR heterodimers by phosphorimaging. Values are means ± S.D. of triplicate determinations. NS indicates unspecific complexes.

were first formed on the different VDREs in the absence or presence of saturating concentrations of 1α,25(OH)₂D₃ as in the gel-shift assay, and then digested with a limited concentration of the endoprotease trypsin [10]. This resulted in the same two protein-DNA complexes (c_{1GSC} and c_{2GSC}) which migrated faster for all tested VDREs, i.e. which appeared to be of lower molecular mass than non-digested VDR-RXR heterodimers (Figure 2). These complexes have recently been described as being representatives of different conformations of DNA-bound VDR-RXR heterodimers [41]. The complexes c_{1GSC} and c_{2GSC} were quantified in relation to the respective ligand-induced, non-digested VDR-RXR heterodimers for each VDRE. Interestingly, the ligand-stabilized conformations c_{1GSC} and c_{2GSC} provided a very similar pattern for all VDREs, although the absolute amount of ligand-triggered VDR-RXR complex-formation differed between the VDREs tested (compare with Figure 1). In the presence of 1α,25(OH)₂D₃, 37–72% of DNA-bound VDR-RXR heterodimers were found to be stabilized in c_{1GSC}, whereas 17–32% were stabilized in c_{2GSC}.

The ligand sensitivity of VDR-RXR heterodimer complex-formation on DNA and of VDR-RXR heterodimer conformations c_{1GSC} and c_{2GSC} was analysed on representative DR3-type VDREs from class I (rat ANF and mouse OPN) and class II (human CYP24 and human PTH) with reference to the rat Pit-1 DR4-type VDRE. Therefore ligand-dependent gel-shift assays (Figure 3A) and gel-shift clipping assays (Figure 3B) were performed in the presence of graded concentrations of 1α,25(OH)₂D₃. Interestingly, the dose-response curves provided

EC₅₀ values (i.e. an indication of ligand sensitivity) for VDR-RXR complex-formation of the order of 0.1 nM (0.09–0.12 nM) for all five VDREs tested (Figure 3A). Consistent with this observation, the ligand sensitivity of both VDR-RXR heterodimer conformations c_{1GSC} and c_{2GSC} was also found to be approx. 0.1 nM (0.05–0.12 nM) (Figure 3B). Moreover, the dose-response curves indicate that the results from Figures 1 and 2 that were obtained at the pharmacological 1α,25(OH)₂D₃ concentration of 10 μM should be comparable with those from assays performed at physiological concentrations of 1 nM or lower. Taken together, the results from the representative VDREs suggest that DR3-type VDREs cannot be differentiated by the ligand sensitivities of the complexed VDR-RXR heterodimers.

Potentially differential effects of the RXR-selective ligand CD2425 on conformations of VDR-RXR bound to DR3-type VDREs were assessed by gel-shift clipping assays (Figure 4). In this experimental series the same set of representative VDREs from classes I and II (and the rat Pit-1 element as a reference) was used as in the previous series (Figure 3), but the endoprotease chymotrypsin was used. Consistent with previous findings [11], under these conditions only one specific 1α,25(OH)₂D₃-stabilized VDR-RXR conformation (c_{GSC}) could be observed (representing 18–30% of all DNA-bound VDR-RXR heterodimers). Interestingly, a saturating concentration of CD2425 had no significant stabilizing effect on the VDR-RXR-VDRE complex, whereas in the case of the rat ANF DR3-type VDRE only an additional effect of CD2425 on 1α,25(OH)₂D₃-stabilized VDR-RXR-VDRE complexes could be observed.

Figure 5 Co-activator and co-repressor interactions of VDR-RXR heterodimers on class I and class II DR3-type VDREs

Gel-shift experiments were performed with *in vitro*-translated VDR-RXR heterodimers, bacterially expressed GST-SRC-1-(596-790) (**A**) or GST-NCOR-(1679-2453) (**B**), 10 μM $1\alpha,25(\text{OH})_2\text{D}_3$ (or solvent as a control) and selected ^{32}P -labelled DR3-type VDREs from classes I and II. Protein-DNA complexes were separated from free probe through non-denaturing 8% (w/v) polyacrylamide gels. Representative experiments are shown. The amount of VDR-RXR-VDR and VDR-RXR-VDR-SRC-1 complex-formation in the presence of SRC-1 was quantified in relation to VDR-RXR-VDR complex-formation in the absence of SRC-1 in (**A**), whereas the ratio of VDR-RXR-VDR-NCOR to VDR-RXR-VDR complex-formation was quantified in the absence and in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ in (**B**). Values are means $\pm$ S.D. of triplicate determinations. NS indicates unspecific complexes.

Finally, potentially different interactions of cofactors with DR3-type VDRE-bound VDR-RXR heterodimers were tested in supershift experiments with the representative co-activator SRC-1 and the representative co-repressor NCoR (Figure 5). For this purpose ligand-dependent supershift assays were performed with VDR-RXR heterodimers using the same set of representative VDREs from classes I and II (and the rat Pit-1 element as a

reference) and saturating concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ in the presence of bacterially expressed GST-SRC-1-(596-790) (Figure 5A) or GST-NCOR-(1679-2453) (Figure 5B). GST protein alone provided no supershift (results not shown). Interestingly, despite the different individual affinities of VDR-RXR heterodimers for the five selected VDREs, in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ 46-82% of all VDRE-bound VDR-RXR heterodimers formed

complexes with SRC-1 on each of the five VDREs (Figure 5A). Complex-formation with NCoR was also observed on all five representative VDREs (Figure 5B), but analysis of the ligand-dependent dissociation of VDR–RXR–VDRE–NCoR complexes is complicated by the fact that ligand enhances VDR–RXR–VDRE complex-formation in parallel. Therefore the individual ratio of VDR–RXR–VDRE–NCoR complexes to VDR–RXR–VDRE complexes was calculated for each VDRE, and it was demonstrated that this ratio generally decreased from a value of 0.34–0.76 in the absence of ligand to a level of 0.23–0.39 in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$. Taken together, these cofactor-dependent assays did not demonstrate significant differences in the *in vitro* functionality of VDR–RXR heterodimers that are bound to DR3-type VDREs.

DISCUSSION

Probably the most important step in $1\alpha,25(\text{OH})_2\text{D}_3$ signalling is the complex-formation of VDR–RXR heterodimers with VDREs in the promoter of primary $1\alpha,25(\text{OH})_2\text{D}_3$ -regulated genes. The present study tried to answer the question of whether the predominant VDRE type, i.e. the family of DR3-type elements, can be grouped into different classes with individual functionalities. The affinity of the 17 presently known DR3-type VDREs for VDR–RXR heterodimers (at a fixed protein/DNA ratio) was found to be their major discriminating parameter, which allowed us to group the VDREs into three classes (Table 1). Class I contains the very potent VDREs of the rat ANF, the mouse and pig OPN and the chicken carbonic anhydrase II genes. The affinity for VDR–RXR heterodimers of the six VDREs that form class II (from the human genes for CYP24, the Na^+ -dependent P_i transporter type II and PTH and the rat genes for CYP24, osteocalcin and PTHrP) is lower than in class I, but they are also ligand sensitive. In contrast, the seven VDREs of class III show very weak binding of VDR–RXR heterodimers and no response to $1\alpha,25(\text{OH})_2\text{D}_3$. According to these stringent *in vitro* criteria, the core sequences of the class III members cannot be considered as functional VDREs. The *in vitro* binding affinity of VDR–RXR heterodimers for VDREs was shown to be proportional to their *in vivo* functionality in transiently transfected cells, i.e. to mediate, in a heterologous promoter context, induction of reporter gene activity [42,43]. In this assay system class III VDREs did not show any *in vivo* functionality (results not shown). However, these findings do not exclude the possibility that class III VDREs may gain responsiveness to $1\alpha,25(\text{OH})_2\text{D}_3$ in their natural promoter context through the help of flanking partner proteins. Moreover, the functionality of a $1\alpha,25(\text{OH})_2\text{D}_3$ -responsive gene will also depend on a potential co-operative action of two or more VDREs, such as in the case of the CYP24 gene [44].

A comparison of the individual VDRE core sequences (Table 1) with their classification according to their affinity for VDR–RXR heterodimers suggests that the degree of deviation from the core binding motif consensus sequence RGKTSA (R = A or G, K = G or T and S = C or G) [3] is proportional to the loss of *in vitro* functionality. Interestingly, the DR4-type VDRE of the rat Pit-1 gene, which contains perfect core-binding motifs, was shown to bind more strongly than any of the DR3-type VDREs. However, *in vivo*, VDR–RXR heterodimers have to compete with heterodimeric complexes of the thyroid hormone receptor or different orphan nuclear receptors with RXR for binding to DR4-type response element [45,46]. This may be the reason that DR4-type response elements are rarely used as natural VDREs.

VDR–RXR heterodimers appear to form identical complexes on the ten VDREs of classes I and II, since indistinguishable VDR–RXR heterodimer conformations were observed on these

VDREs. This is in contrast with a report that VDR–RXR heterodimers take different conformations on the mouse OPN and the rat osteocalcin DR3-type VDRE [9]. In that study nuclear extracts from bone cells provided protein complexes on the two VDREs that were differentially recognized by antibodies. One could assume that the VDR–RXR heterodimers in these complexes interact with different types of cofactors, as shown for oestrogen response elements [47]. However, the present study indicates that VDR–RXR heterodimers that are formed on the different DR3-type VDREs show no significant differences in their interactions with a given co-activator or co-repressor protein. Interestingly, VDREs from genes that are negatively regulated by $1\alpha,25(\text{OH})_2\text{D}_3$, such as those from the rat ANF and the human PTH gene, show the same interactions with cofactors as VDREs from positively regulated genes. This suggests that positive and negative regulation by $1\alpha,25(\text{OH})_2\text{D}_3$ is not related to the VDRE sequence.

The ligand sensitivity of VDR–RXR heterodimers bound to the different DR3-type VDREs showed no significant deviation from the average value of 0.1 nM, as confirmed independently on the level of VDR–RXR–VDRE complex-formation and of stabilization of VDR–RXR heterodimer conformations. Moreover, VDR–RXR heterodimers that were formed on the DR3-type VDREs from classes I and II showed no significant differences in their insensitivity to an RXR ligand and in $1\alpha,25(\text{OH})_2\text{D}_3$ -triggered interactions with co-activator and co-repressor proteins. The only exception might be the rat ANF VDRE, on which the RXR ligand CD2425 showed an additional effect to the $1\alpha,25(\text{OH})_2\text{D}_3$ -induced stabilization of the VDR–RXR heterodimer conformation. In addition, the interaction with NCoR in the absence of $1\alpha,25(\text{OH})_2\text{D}_3$ was higher than average and the $1\alpha,25(\text{OH})_2\text{D}_3$ -triggered interaction with SRC-1 was lower than average. Taken together, the results of the present study suggest that VDR–RXR heterodimers are able to differentiate between the individual DR3-type VDREs on the basis of the different protein–DNA affinity, but when the complexes are formed, i.e. the molecular switches of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling are built up, they appear to react in exactly the same fashion.

This means that, *in vitro*, there are no indications for multiple DR3-type VDRE-mediated $1\alpha,25(\text{OH})_2\text{D}_3$ signalling pathways. In the recent past, *in vitro* investigations of VDR–RXR heterodimers (and their conformations) have provided many new insights into the functionality of these molecular switches that also explain their function in living cells [48,49]. This makes it likely that the lack of variation of the *in vitro* functionality of DR3-type VDREs (from positively as well as negatively regulated genes) can be transferred to the *in vivo* situation. This has the advantage that an observation that has been made with a specific DR3-type VDRE can be generalized to the whole family of DR3-type VDREs. However, expectations were dashed that a variety of DR3-type VDREs may explain the pleiotropic physiological actions of $1\alpha,25(\text{OH})_2\text{D}_3$ and may allow dissection of these effects through selective activation of a subset of DR3-type VDREs by $1\alpha,25(\text{OH})_2\text{D}_3$ analogues. Therefore alternative VDRE structures, such as DR4-, DR6- and IP9-type elements, have to be considered as mediators of selective actions of $1\alpha,25(\text{OH})_2\text{D}_3$ analogues. The selective anti-proliferative effect of the $1\alpha,25(\text{OH})_2\text{D}_3$ analogue EB1089, for example, has been associated with selective activation of IP9-type-VDRE-driven reporter genes [50].

In conclusion, natural DR3-type VDREs were found to vary in their affinity for VDR–RXR heterodimers, but the heterodimers appear to take identical conformations on all of them and also to show similar interactions with cofactors. This facilitates

further investigation of the principles of DR3-type-VDRE-mediated gene regulation, but also strongly suggests that DR3-type VDREs alone cannot explain the pleiotropic genomic actions of $1\alpha,25(\text{OH})_2\text{D}_3$.

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