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Dimerization of v-erbA on inverted repeats☆

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Abstract

Thyroid hormone receptors (TRs) and the oncoprotein v-erbA can heterodimerize with retinoid X receptor (RXR) on core motifs arranged as inverted repeats (IR0) which contain the consensus sequence AGGTCA. On this core motif, v-erbA can also form homodimers whereas TRs homodimerize very poorly. Therefore to obtain a better understanding of distinct homodimerization properties of TRα1 as compared to those of v-erbA, we created chimeras between these two receptors and tested their abilities to homodimerize on IR0. We found that the enhanced homodimerization properties of v-erbA compared to those of TRα1 on IR0 map to amino acids 107–156 in v-erbA/121–170 in TRα1 (VT-2 chimera). Furthermore, functional studies on transient transfections showed that v-erbA–RXR heterodimers do not mediate the dominant negative activity of v-erbA on an inverted repeat response element. These data, in conjunction with our previous studies, indicate that v-erbA homodimers mediate the repressor activity of v-erbA on IR0.

Keywords: v-erbA; Thyroid hormone receptor; Oncoprotein; Repressor; Inverted repeat; Nuclear receptor; Homodimer; DNA binding

The v-erbA oncoprotein is a mutated form of the thyroid hormone receptor α1 (TRα1) and belongs to the large family of zinc finger transcription factors which includes the receptor for steroids, retinoic acid (RAR), and vitamin D [1].

As a consequence of a nine amino acid deletion in the carboxy-terminal domain, v-erbA expressed in mammalian cells is unable to bind thyroid hormone [2]. In mammalian and avian cells, v-erbA acts as a constitutive dominant repressor of transcription regulated by TR and RAR, suggesting that these activities may be central to its oncogenic activity [3,4].

At least two regions within the nuclear receptors are involved in dimerization. One region is located within the DNA-binding domain (DBD); this region confers a weak dimerization interface. In contrast to this weak dimerization interface of the DBD, a second region located in the C-terminal domain confers a strong dimerization interface [5–10]. v-erbA can form homodimers on DNA containing the consensus sequence AGGTCA arranged as direct, everted or inverted repeats (DRs, ERs, IRs) and this homodimerization may be critical for the oncogenic activity of v-erbA. Recently, we have shown that homodimerization process of v-erbA on IRs may differ from those on ERs or DRs [11]. Therefore current studies were aimed at obtaining a better understanding of the formation of v-erbA homodimers on IRs. It has been proposed that the amino-terminal region of TRα inhibits homodimer formation [12,13]; specifically it is believed that the five basic amino acids 23 KRKRK 27 in the amino-terminal domain of TRα are responsible for the inability of this TR isoform to homodimerize on IR0 [13]. However, v-erbA, which binds DNA primarily as homodimers rather than monomers, also has five basic amino acids (KHKRK) at the corresponding positions. This suggests that sequences other than those five basic amino acids are responsible for the difference in the abilities of v-erbA and TRα1 to homodimerize on the inverted repeat IR0. Since TRs bind to this core motif as

☆ *Abbreviations*: bp, base pair; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; DR, direct repeat; EMSA, electromobility shift assay; ER, everted repeat; GH, growth hormone; IR, inverted repeat; PAGE, polyacrylamide gel electrophoresis; RAR, retinoic acid receptor; RXR, retinoid X receptor; T3, thyroid hormone (3,5,3′-triiodothyronine); TR, thyroid hormone receptor.

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monomers rather than as homodimers, we addressed this question by making chimeras between TRα1 and v-erbA. Specifically, we swapped different domains between TRα1 and v-erbA receptors, to define the region(s) responsible for the difference in homodimerization between v-erbA and TRα1 bound to consensus DNA sequences arranged as IRs. Furthermore, we performed transient transfections to investigate the dominant negative activity on an inverted repeat response element of the v-erbA mutant created which was unable to bind as homodimers to this core motif.

Materials and methods

Chimeric constructs. pBS-tr(1–33)–v-erbA was created by removing the *Eco*RI/*Pfl*mI fragment that encodes for amino acids 1–19 of v-erbA and replacing these sequences with a PCR-generated DNA fragment encoding amino acids 1–33 of mouse TRα1. The constructs VT and TV were created by digesting pBS–v-erbA and pBS–mTRα1 with *Bst*XI. In this way, the DNA fragment that encodes for amino acids 1–109 of v-erbA was replaced with the corresponding DNA fragment that encodes amino acids 1–123 of mTRα1 (TV); also, the DNA fragment that encodes amino acids 110–387 in v-erbA was replaced by the corresponding DNA fragment that encodes amino acids 124–410 in mTRα1 (VT). VT-1 construct was formed by digesting pBS–v-erbA and pBS–TRα1 with *Pst*I and replacing the DNA fragment that encodes amino acids 141–306 in v-erbA with the corresponding DNA fragment that encodes amino acids 155–320 in TRα1. VT-2 construct was formed by removing the *Msc*I/*Bst*EII fragment from pBS–v-erbA that encodes amino acids 107–156 in v-erbA and replacing these sequences with a PCR-generated DNA fragment encoding for the corresponding amino acids 121–170 in TRα1. VT-3 construct was created by removing the *Blp*I/*Nar*I fragment from pBS–v-erbA that encodes amino acids 262–364 in v-erbA and replacing these sequences with a PCR-generated DNA that encodes the corresponding amino acids 276–378 in TRα1. v-erbA–H12 and VT-2–H12 were created by removing the *Sac*I fragment from pBS–v-erbA and pBS–VT-2, respectively, that encodes amino acids 383–387 in v-erbA and replacing these sequences with a PCR-generated DNA fragment encoding amino acids in helix 12 in TRα1.

Site directed mutagenesis. The Stratagene QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA) was used to create point mutations in v-erbA. Mutant products were sequenced to confirm the mutations and to exclude errors.

Production and purification of proteins. CDNAs for wild type v-erbA, wild type mouse TRα1, as well as the corresponding chimeric and mutant constructs, and wild type mouse RXRα, were transcribed from pBluescript plasmids and then translated using the rabbit reticulocyte lysate system (Promega) in the presence of [^{3}H]leucine [14–16]. Trichloroacetic acid precipitable-counts per minute were determined. SDS–PAGE and fluorography were performed to demonstrate that all proteins were of the appropriate size [17].

Electrophoretic mobility shift assays (EMSA). Protein–DNA-binding reactions were performed in 35 μl of 20 mM HEPES, pH 7.8, 20% glycerol, 1.4 μg polydI·dC, 1 mM dithiothreitol, 50 mM KCl, 0.1% Nonidet P40 (NP-40), ^{32}P-labeled DNA, and the protein(s) of interest. The double stranded DNAs were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. An EMSA was performed with 40,000 cpm of ^{32}P-labeled DNA per sample. For each core motif, the amounts of in vitro translated wild type and mutant v-erbA and TRα1 proteins used were equal as assessed by [^{3}H]leucine incorporation, taking into consideration the number of leucines in the specific proteins. Reactions were incubated at room temperature for 45 min prior to electrophoresis. Electrophoresis was carried out on 0.25X TBE (22 mM Tris base, 22 mM boric acid, 0.5 mM EDTA) 6% polyacrylamide gels (29:1 acrylamide:bisacrylamide) at room temperature. Gels were fixed in 30% methanol, 10% acetic acid; dried and exposed to film with an intensifying screen for 6–24 h at −70 °C.

The DNA–protein complexes were quantified on a Molecular Dynamics PhosphorImager. Experiments were performed at least twice using two different batches of rabbit reticulocyte lysates.

The sequences of the oligonucleotides used in EMSA are shown below (the hexameric half sites are underlined):

M1: GATCCGGGCGATGAAATAATTGAGGTCACGTGCA
IR0: GATCCTAAGGTCATGACCTTAGGATC
DR4: GATCCGGGCGATGGGGTCATATGAGGTCACGTGCA
ER6: GATCCGGGCGATGACCTAACTTGAGGTCACGTGCA

Transient transfections. JEG-3 cells were grown in 90% Eagle's minimum essential medium plus 10% fetal bovine serum and transfected using standard calcium phosphate precipitation [18]. The oligonucleotides IR0 and ER6 were used as TR/v-erbA response elements. These oligonucleotides were ligated as single inserts into pUTKAT3 at a *Bam*HI site 5′ to the basal herpes simplex virus thymidine kinase promoter driving expression of CAT [19]. Reporter plasmid was transfected at a dose of 4 μg per 60 mm petri dish. Mouse TRα1 was expressed from the vector pCDM [20]; wild type and mutant v-erbAs were expressed from the vector pRSV [21]. Transfections included 100 ng pCDMTRα1, which represents a non-saturating dose of this expression plasmid; and between 6 and 12 μg pRSV–v-erbAs (or vector) for IR0 and 450 ng pRSV–v-erbAs for ER6. Vector pRSV was added to achieve a total of 12 μg pRSV-based plasmid per transfection.

Co-transfections included 1 μg of a human growth hormone (GH) expressing vector (pTKGH) per 60 mm petri dish to control for transfection efficiency. Cells were transfected in the presence of 10% charcoal stripped fetal bovine serum and 100 nM dexamethasone. Cells were cultured ± 10 nM T3 for two days prior to harvest. CAT and hGH assays were performed as described previously [18]. Ligand responsiveness is defined as CAT/hGH for cells cultured with ligand divided by CAT/hGH for cells cultured without ligand. v-erbA suppression of CAT reporter gene expression was calculated as CAT/hGH for cells cultured with v-erbA divided by CAT/hGH for cells cultured without v-erbA in the presence or absence of ligand. Results are presented as means ± SE for four to six independent transfections per assay condition.

Results

The five basic amino acids in the amino-terminal region are not responsible for the difference in homodimerization between v-erbA and TRα1

First, we compared the abilities between v-erbA and TRα1 to form homodimers on the inverted repeat core motif IR0. As shown in Fig. 1, v-erbA binds predominantly as homodimers on this core motif. On the contrary, TRα1 binds predominantly as monomers on IR0 with the formation of a weak "homodimer"–DNA complex at higher doses.

A previous study has suggested that five basic amino acids in the amino-terminal region of TRα1 (23 KRKRK27) inhibit homodimerization on IR0 [13]; however, v-erbA, which binds to DNA predominantly as homodimers also has five basic amino acids at the corresponding positions (KHKRK). Thus, four of those five

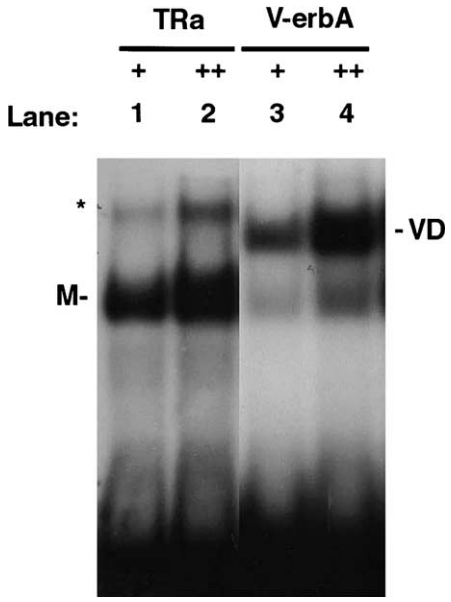

Fig. 1. EMSA analysis of v-erbA and TR α1 binding on the inverted repeat IR0. Increasing doses of vitro translated v-erbA, or TRα1, were incubated with ^{32}P-labeled IR0. M represents TRα1 or v-erbA monomer–DNA complexes whereas VD represents v-erbA homodimer–DNA complexes. (∗) A faint slower migrating band is seen with increasing doses of TRα1, which has been shown to represent two monomer–DNA complexes rather than true homodimer formation.

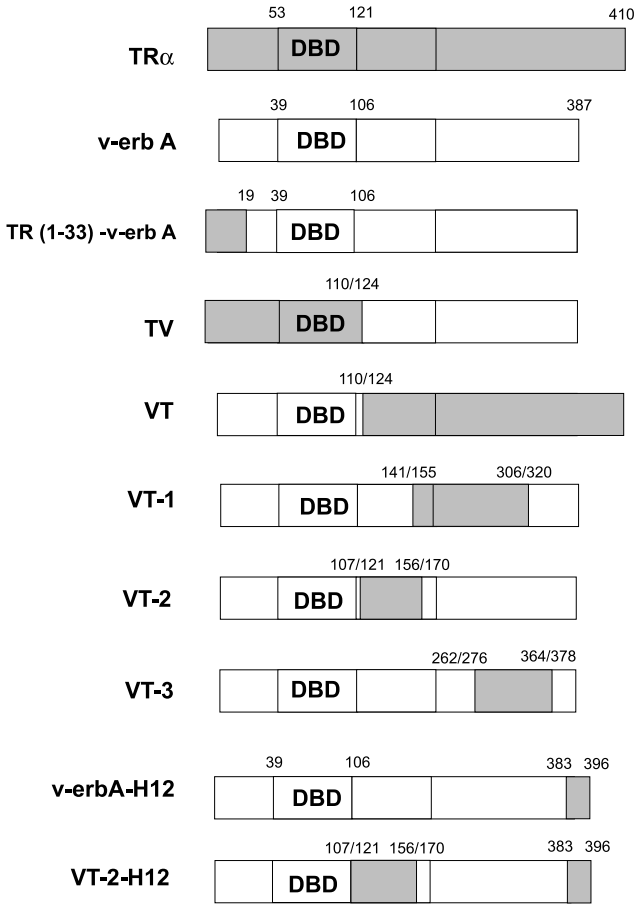

Fig. 2. Structure of TRα1 and v-erbA chimeras and mutants. A number of v-erbA/TRα1 chimeras and mutants were created using restriction enzyme digestion and polymerase chain reaction. The numbering indicates the amino acid number in each protein.

amino acids are identical between TRα1 and v-erbA. Since it is possible that the histidine in v-erbA instead of arginine at the corresponding position in TRα1 was responsible for the difference in homodimerization between these two proteins, a v-erbA mutant containing an arginine for histidine at this position (tr(1–33)–v-erbA mutant) was made. The schematic illustration of this and other chimeras used in these studies is depicted in Fig. 2. Our results show that the mutant tr(1–33)–v-erbA homodimerizes as well as the wild type (Fig. 3). Taken together, the above information indicates that sequences other than those five basic amino acids in the amino-terminal region of TRα1 are responsible for the difference in homodimerization between v-erbA and TRα1.

The difference in homodimerization between v-erbA and TRα1 maps to sequences distal to the DBD

Next, we created chimeras between v-erbA and TRα1 by exchanging the amino-terminal region and the DBD (TV) or the carboxy-terminal region (VT) and investigated the ability of these chimeras to form homodimers on IR0 by EMSA. As shown in Fig. 4A, TV binds predominantly as homodimers to IR0, indicating that neither the amino-terminal region or the DBD is responsible for the difference in homodimerization between these two receptors. Interestingly, TV binds to DNA as monomers and homodimers with ~2-fold higher relative binding affinity than the wild type v-erbA (for example, compare lanes 1 and 2 with lanes 3 and 4 in Fig. 4A). This increase in overall DNA binding by TV is reproduced by the mutant C32Y (lanes 5 and 6 in Fig. 4A), confirming previous studies that this particular amino acid in the amino-terminal region of TRα1 and v-erbA plays a role in DNA binding [22].

Homodimer binding was disrupted on IR0 with the VT chimera (Fig. 4B). These results indicate that sequences distal to the DBD are responsible for the difference in homodimerization between TRα1 and v-erbA on this core motif.

The difference in homodimerization between v-erbA and TRα1 maps to amino acids 107–156 in v-erbA (121–170 in TRα1)

To define the region responsible for the distinct homodimerization properties between TRα1 and v-erbA bound to IRs, chimeras were made within sequences distal to the DBD of these two receptors. In VT-1, amino acids 141–306 in v-erbA were exchanged for the

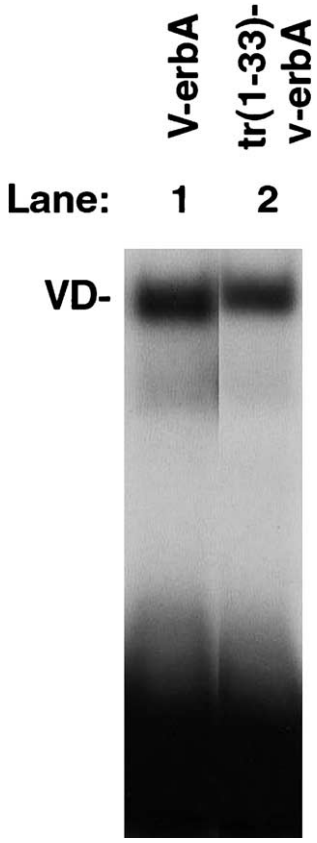

Fig. 3. EMSA analysis of wild type v-erbA and the chimera tr(1–33)–v-erbA on IR0. In vitro translated wild type v-erbA or chimera tr(1–33)–v-erbA were incubated with ^{32}P-labeled IR0. VD represents homodimer–DNA complexes. Equal amounts of wild type and mutant v-erbAs were used as assessed by [^{3}H]leucine incorporation, taking into account the number of leucines in the specific proteins.

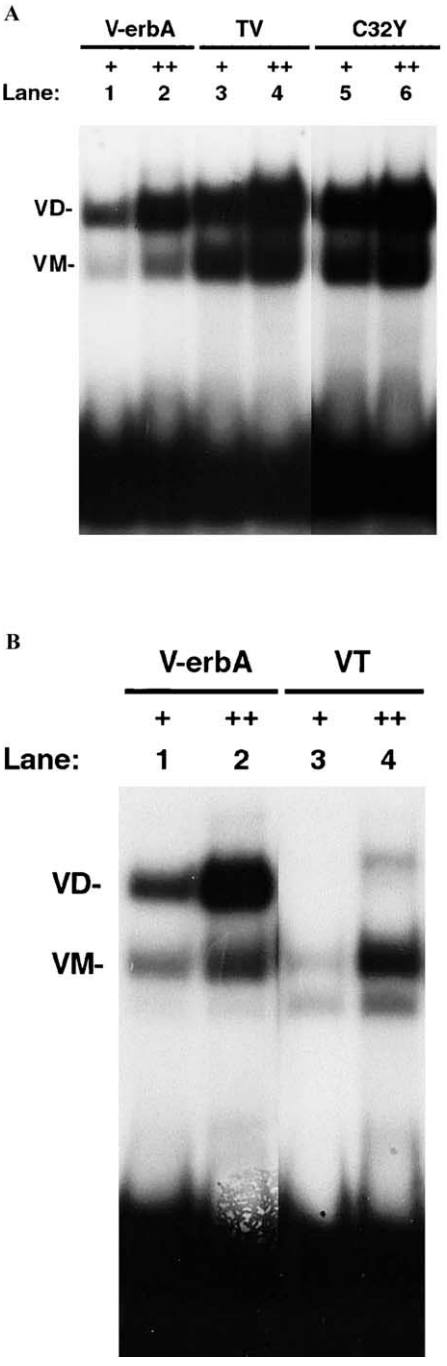

Fig. 4. EMSA analysis of wild type or mutant v-erbAs on IR0. (A) Increasing doses of in vitro translated wild type v-erbA, chimera TV or a v-erbA containing the point mutation C32Y were incubated with ^{32}P-labeled IR0. (B) Similar to (A), except the incubations were performed with in vitro translated wild type v-erbA and chimera VT. VM and VD represent monomer and homodimer complexes. Equal amounts of wild type and mutant v-erbAs were used as assessed by [^{3}H]leucine incorporation, taking into account the number of leucines in the specific proteins.

corresponding amino acids in TRα1 (155–320); in VT-2, amino acids 107–156 in v-erbA were exchanged for the homologous amino acids in TRα1 (121–170); in VT-3, amino acids 262–364 in v-erbA were exchanged for the corresponding amino acids in TRα1 (276–378).

As shown in Fig. 5, VT-1 binds predominantly as homodimers on IR0, albeit with somewhat decreased relative affinity compared to wild type v-erbA. VT-3 also binds predominantly to homodimers on IR0, with no difference compared to wild type v-erbA. However, the difference between VT-2 and wild type v-erbA is dramatic. Specifically, homodimerization on IR0 is disrupted with VT-2 (lane 3 in Fig. 5) whereas the ability of this chimera to bind to DR4 (lane 6 in Fig. 5) or ERs is unaffected.

Homodimerization of v-erbA on inverted repeats is not affected by helix 12 from TRα1

The above results have shown that amino acids 121–170 of TRα1 inhibit v-erbA homodimerization on IR0 (VT-2 chimera). However, helix 12, which is present in TRs but not in v-erbA, is present in the chimera VT but not in VT-1, VT-2, or VT-3. Therefore it was possible that helix 12, in addition to the region of amino acids

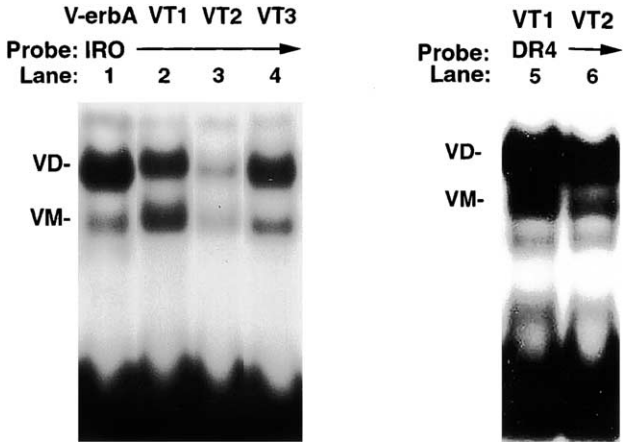

Fig. 5. EMSA analysis of wild type v-erbA or chimeras VT-1, VT-2, and VT-3 on core motifs arranged as IR0 or DR4. In vitro translated wild type v-erbA, or chimeras VT-1, VT-2, or VT-3 were incubated with ^{32}P-labeled IR0 (lanes 1–4). Also, in vitro translated VT-1 and VT-2 chimeras were incubated with ^{32}P-labeled DR4 (lanes 5 and 6). VM and VD represent monomer and homodimer complexes. Equal amounts of wild type and mutant v-erbAs were used as assessed by [^{3}H]leucine incorporation, taking into account the number of leucines in the specific proteins.

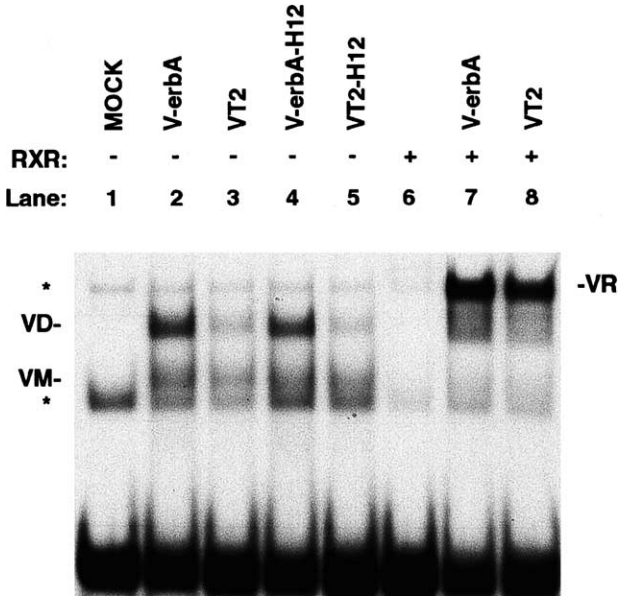

Fig. 6. EMSA analysis of wild type v-erbA or mutants VT-2, v-erbA–H12, or VT-2–H12 on IR0 in the absence or presence of RXRα. (A) In vitro translated wild type v-erbA or v-erbA mutants (or mock control) were incubated with ^{32}P-labeled IR0. Also, in vitro translated wild type or chimera VT-2 (or control) were incubated with ^{32}P-labeled IR0 in the presence of RXRα (lanes 6–8). VM, VD, and VR represent monomer, homodimer, and heterodimer–DNA complexes. The symbol $*$ represents faint non-specific bands. Equal amounts of wild type and mutant v-erbAs were used as assessed by [^{3}H]leucine incorporation, taking into account the number of leucines in the specific proteins.

107–156 (v-erbA)/121–170 (TRα1), may play a role in the difference in homodimerization between TRs and v-erbA on IRs. Therefore chimeras were made by transferring helix 12 from TRα1 into v-erbA (v-erbA–H12) and into the construct VT-2 (VT-2–H12) to address whether or not helix 12 from TR inhibits v-erbA homodimerization on IR0 by EMSA. As depicted in Fig. 6, v-erbA–H12 binds with similar relative affinity as wild type v-erbA whereas the chimera VT-2 disrupts homodimerization on this site. Furthermore, H12 does not have any additional effect on the impaired homodimerization shown by VT-2. Taken together, our data indicate that amino acids 121–170 in TRα (VT-2) inhibit homodimerization of v-erbA on IRs. Furthermore, these sequences did not inhibit heterodimerization with RXR on this core motif (Fig. 6).

Dominant negative activity of wild type v-erbA and VT-2 chimera on inverted repeats

Experiments were conducted to examine the ability of VT-2, a v-erbA mutant unable to homodimerize but able to heterodimerize with RXR to effect dominant negative activity on the inverted repeat IR0. For this purpose, 6 and 12 μg wild type or mutant VT-2 v-erbAs were transfected. As shown in Fig. 7, the co-transfection of 6 and 12 μg of the wild type v-erbA suppressed T3-mediated CAT activity to $72 \pm 3\%$ and $47 \pm 2\%$, respectively, of that observed in the absence of v-erbA. On the contrary, the co-transfection of 6 and 12 μg mutant VT-2 did not suppress T3-mediated CAT activity ($138 \pm 5\%$, $138 \pm 3\%$, respectively, of

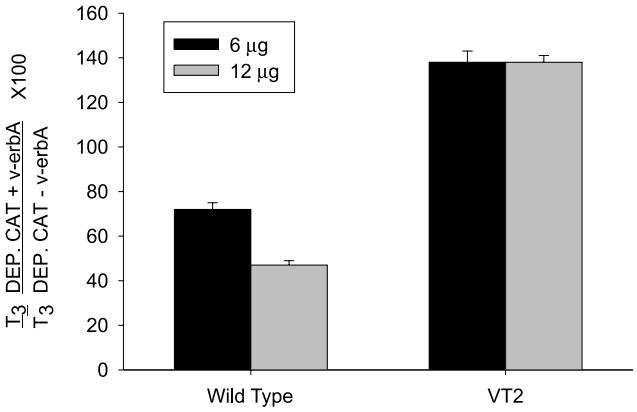

Fig. 7. Dominant negative effect of wild type v-erbA and the mutant VT-2 on TRα1 action on an inverted repeat core motif. JEG-3 cells were transfected with the reporter plasmid pUTKAT3 containing a single copy of IR0, along with the internal control plasmid pTKGH. All cells also received expression vectors for TRα1 ± wild type or mutant v-erbA (or empty vector). Cells were cultured for two days in the presence of 10 nM T3 and then cell lysates were analyzed for CAT and media for hGH.

that observed in the absence of v-erbA). Since the mutant VT-2 is able to heterodimerize with RXR but unable to homodimerize on IR0, these results indicate

that v-erbA–RXR heterodimers are not important for the repressor activity of v-erbA on inverted repeats.

To confirm that VT-2 was functional in transient transfections, experiments were also performed testing the dominant negative activity of 450 ng co-transfected wild type or the mutant VT-2 vectors on a response element arranged as ER6. The wild type and mutant VT-2 v-erbAs suppressed T3-mediated CAT activity similarly (~25% of that observed in the absence of v-erbA).

Discussion

v-erbA can form homodimers either on DRs, ERs, or IRs. In contrast, TRα1 forms homodimers poorly overall. At least two regions within the nuclear receptors are involved in the heterodimerization with RXR. One region is located within DBD. This region confers a weak dimerization interface; however, it dictates the spacing preference of direct repeat response elements [5–9]. In contrast to this weak dimerization interface of the DBD, a second region located in the carboxy terminal domain confers a strong dimerization interface. This region encompasses helices 10 and 11 in TR and v-erbA [10].

v-erbA can form homodimers on IRs, albeit with lower affinity than on ERs or DRs [11]. The possibility of the independent occupancy of two half sites by two v-erbA protein molecules rather than protein–protein interactions on IR0 is unlikely because the predominant complex observed bound to DNA is homodimer (with minimal evidence of a monomeric intermediate). Furthermore, our previous studies have shown that v-erbA binding was not observed on "spacing mutants" of the IR with a 6 bp spacer or with a 9 bp spacer [11]. Recently, we have shown that helices 10 and 11 in v-erbA do not appear to be involved in v-erbA homodimerization on IRs. In fact, deletion of these helices enhanced the ability of v-erbA to homodimerize on this core motif [11]. These data indicated that other region(s), perhaps the DBD or specific sequences in the C-terminal domain, are involved in v-erbAs homodimerization on IRs.

In a recent study, results obtained by performing X-ray crystal structure-guided mutation analysis of the TR ligand-binding domain suggested that TR "dimers" on IRs consist of independent binding of two monomers [23]. The observation that "homodimers" on IRs are formed very weakly as compared to those on DRs or ERs and the fact that T3 increases TR binding as monomers with several TREs and as "homodimers" on IR0, whereas T3 decreases TR binding as homodimers with DRs or ERs, present additional evidence for this theory.

Our data indicate that the chimera VT-2, in which amino acids 107–156 in v-erbA have been replaced with the corresponding amino acids 121–170 in TRα1, disrupts homodimerization of v-erbA on IR0. Interestingly, this chimera is still able to homodimerize on DRs or ERs. Furthermore, the ability of this chimera to heterodimerize on IR0 remains unaffected. This region contains the T and A boxes, suggesting that T and/or A boxes of v-erbA may play an important role in v-erbA's homodimerization on IRs. It has been previously shown that the A box of TR plays an important role in mediating DNA binding of TR homodimers [24].

Previously, we have shown that the co-transfection of RXR did not enhance the repressor activity of v-erbA on IR0 [14]. Furthermore, we have recently described v-erbA mutants that retain their ability to homodimerize but were not able to heterodimerize with RXR on IR0. These mutants were as potent repressors as the wild type v-erbA on this response element [11]. The current studies have identified a v-erbA mutant unable to homodimerize but is still fully able to heterodimerize with RXR on IR0 (VT-2) and which is unable to effect dominant negative activity in a transient transfection assay. Taken together, previous and current studies would indicate that v-erbA homodimers rather than v-erbA–RXR heterodimers mediate the dominant negative activity of v-erbA on IRs.

Most of the natural response elements for thyroid hormone or retinoic acid consist of core motifs arranged as DRs. Until recently, inverted repeat response elements for thyroid hormone or retinoic acid had only been demonstrated by using a synthetic DNA fragment. However, the first natural IR0 has now been characterized. This IR0 is found in the proximal promoter region of the mouse testicular receptor TR2-11 gene, and characterized as a functional retinoic acid-response element [25,26]. Therefore it is possible that v-erbA's oncogenic activity could be mediated by effecting dominant negative activity of retinoic acid or thyroid hormone action on genes containing a response element arranged as IR0.

In current and previous studies, we have identified a series of v-erbA mutants that selectively disrupt either homodimerization or heterodimerization with RXR on differently oriented core motifs [11]. The study of these mutants indicates that v-erbA homodimers rather than v-erbA–RXR heterodimers mediate the dominant negative activity of v-erbA on IRs. The study of these mutants in vivo may clarify the role of RXR in the dominant negative activity and oncogenic activity of v-erbA. Moreover, since these v-erbA mutants affect dimerization on a subset of core motifs (DR, ER, or IR), their study may help to predict the nature of the thyroid hormone or retinoic acid-response elements found in genes targeted by v-erbA. This is particularly

important, since thyroid hormone or retinoic acid-response elements have been characterized in only a few of the thyroid hormone or retinoic acid responsive genes.

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