Structural Determinants of the Ligand-Binding Site of the Human Retinoic Acid Receptor α†

Bruno Lefebvre, Christophe Rachez, Pierre Formstecher, and Philippe Lefebvre*

CJF-INSERM 92-03, Laboratoire de Biochimie Structurale, Faculté de Médecine de Lille, 1 Place de Verdun, 59045 Lille Cédex, France

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ABSTRACT: The ligand-dependent transactivating properties of retinoic acid receptors are controlled through a complex structure at the C-terminus of these proteins, commonly referred to as the hormone binding domain. This domain is involved not only in ligand recognition but also in protein−protein interactions such as homo- and heterodimerization processes. To identify more precisely regions of the human *all-trans*-retinoic acid receptor α (hRARα) that are involved in ligand binding, we constructed a series of deletion mutants of this molecule and overexpressed them in bacteria. We found that the C-terminal part of the D domain (amino acids 186−198) was necessary for ligand binding. The F domain and the 10 C-terminal amino acids of the E domain were dispensable for high-affinity binding of various natural and synthetic retinoids. A further deletion to position 403 resulted in a moderate decrease in affinity for *all-trans*-(ATRA) and 9-*cis*-retinoic acids, whereas the binding of two RARα-specific ligands (Am80 and Am580) was abolished. In addition, hRARα and the minimal hormone binding domain (amino acids 186−410) bound ATRA with a positive, cooperative mechanism. This behavior was not observed with CD367, a conformationally restricted synthetic retinoid. The positive cooperativity could be correlated with stable ATRA binding to RAR homodimers, whose formation was triggered by ligand. In the same conditions, only monomeric CD367−RARα complexes were detected. These data indicate that ligand binding to hRARα requires the presence of part of the D domain, whereas the C-terminal end of the E domain is involved in more subtle ligand recognition processes. They also clearly suggest that structurally distinct retinoids interact differently with the ligand-binding site of this receptor.

Retinoic acid receptors mediate the pleiotropic effects of retinoic acids, which exert strong effects on vertebrate development, cellular proliferation, and differentiation. On the basis of their sequence homologies with other nuclear receptors (steroid hormones, thyroid and vitamin D receptors), six functional domains have been defined for these proteins [reviewed in Leid et al. (1992)]. Among them, the hormone binding domain (HBD)[1] appeared as bearing multiple functions besides ligand binding [reviewed in Forman and Samuels (1990)]. Discrete regions of the HBD are involved in the ligand-dependent activation of transcription by steroid hormone receptors (Danielian et al., 1992), retinoic acids (Nagpal et al., 1993), and thyroid hormone receptors (Barettino et al., 1994), or repression of transcription (Baniahmad et al., 1993), and this domain is also involved in protein/protein interactions. Steroid receptors interact most noticeably with heat-shock proteins, and this association is necessary to maintain the glucocorticoid receptor under a conformation compatible with hormone binding [reviewed in Pratt (1993) and Smith and Toft (1993)]. Thyroid, retinoic acid, and vitamin D_3 receptors homo- or heterodimerize with each other, and this interaction involves discrete regions located in the DNA binding domain (noted C domain), the hinge region (D domain), and the HBD (E domain). This dimerization process modulates their DNA binding affinity for their cognate response elements and ultimately their transcriptional properties [reviewed in Zhang and Pfahl (1993)].

Agonist binding to the receptor increases the *in vitro* binding affinity of the androgen receptor (Wong et al., 1993), progesterone receptor (Elliston et al., 1992; Allan et al., 1992), estrogen receptor (Beekman et al., 1993), and RXR (Zhang et al., 1992) for their cognate response elements to which they bind as homodimers. *In vivo* studies showed that agonists, but not antagonists, promote binding to DNA in intact cells of the GR (Becker et al., 1986) and of RARα (Minucci et al., 1994). Moreover, agonist binding to the progesterone receptor and retinoic acid receptor α induces conformational changes that are clearly different from the one observed in the presence of antagonists. A correlation between the biological activity of the ligand and an altered conformation of the C-terminal end of these receptors has been established (Vegeto et al., 1992; Keidel et al., 1994). Very interestingly, the C-terminal end of the HBD of nuclear receptors contains the ligand-dependent activating function region 2 (AF-2) which is thought to interact with component(s) of the transcription complex (Halachmi et al., 1994).

The functional significance of the HBD subregions has been established by mutational and biochemical studies. In the case of the glucocorticoid receptor (GR), a minimal

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* Corresponding author [telephone (33) 20-62-67; fax (33) 20-62-68-68].

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[1] Abbreviations: HBD, hormone binding domain; ATRA, *all-trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; RAR, *all-trans*-retinoic acid receptor; RXR, 9-*cis*-retinoic acid receptor; hsp90, heat-shock protein of 90 kDa; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; NiTA, nitrilotriacetic acid; Rs, Stokes radius.

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domain of 16 kDa displays a fair affinity for hormone as well as its association with hsp90 (Chakraborti & Simons, 1991). However, it is not clear whether this fragment is the minimal ligand-binding site since further truncations resulted in the loss of the interaction of hsp90 and therefore of the ligand-binding activity. Potentially important residues have also been identified by point mutations or affinity labeling of GR or estrogen receptors. These results have yielded a three-dimensional model based on a motif-oriented structural search that identified the subtilisin-like proteases as the proteins having the closest structure to the HBD of the GR (Goldstein et al., 1993). The GR HBD is likely to belong to the mixed α/β class of proteins, and this conclusion extended to the steroid receptor family a similar prediction made for the thyroid hormone receptor (T_3R) (McPhie et al., 1993). Circular dichroism study of several deletion mutants of the $\beta 1$ isoform of this receptor revealed that the T_3R HBD contains extensive regions of α-helix and β-sheet. The cooperative nature of the structure is lost upon deletion of eight amino acids from the C-terminal region of the receptor, which correlates with the loss of hormone binding activity. These results have been interpreted as compatible with an α/β barrel structure, in which only the first helix, located in the D domain, is dispensable (McPhie et al., 1993). In enzymes displaying such a structure, the substrate specificity is dictated by residues located in loops. Finally, the crystal structure of the retinol binding protein and the epididymal retinoic acid binding protein also revealed a β-barrel structure that, in the latter case, must undergo a conformational change to accommodate *all-trans*-retinoic acid (Zanotti et al., 1993; Newcomer et al., 1993).

The retinoic acid receptor family comprises two major types of receptor: the *all-trans*-retinoic acid (ATRA) receptors (RARs), which bind ATRA and 9-*cis*-retinoic acid (9-*cis*-RA), and the 9-*cis*-retinoic acid receptors (RXRs), which bind exclusively 9-*cis*-RA. Each of these receptor types is subdivided in α, β, and γ subtypes, with each gene encoding a variable number of isoforms (Leid et al., 1992). A major challenge is to decipher specific biological effects for each receptor, and conceiving specific ligands for each of them is one way to achieve this goal as well as to identify therapeutically active retinoids (Bernard et al., 1992; Crettaz et al., 1990; Delescluse et al., 1991; Graupner et al., 1991; Lehman et al., 1991; Martin et al., 1992) and anti-retinoids (Apfel et al., 1992; Eyrolles et al., 1994).

As a first step to identify structural determinants of the HBD of the human RARα responsible for the interaction with the ligand, we made a series of deletion mutants and assessed their hormone binding affinity. This allowed us to define two regions critical for *all-trans*-retinoic acid binding. Competition studies revealed the C-terminal end of the ligand-binding domain as bearing a sequence important for ligand recognition. Moreover, we detected a cooperative binding of ATRA to the receptor or the isolated HBD, which was not observed with CD367, a synthetic retinoid (Cavey et al., 1990). This particular behavior was correlated with the ability of ATRA to bind stably to RARα homodimers, as opposed to CD367 which bound exclusively to RARα monomers.

MATERIALS AND METHODS

Materials. [11,12-^{3}H]-*all-trans*-Retinoic acid (55.6 Ci/mmol) was purchased from NEN-Dupont de Nemours (Les Ulis, France). Tritiated and unlabeled CD367 (Cavey et al., 1990) was a gift from B. Shroot, CIRD Galderma, Valbonne, France. The purity of retinoid solutions was routinely assessed by reverse-phase chromatography essentially as described by Dawson et al., (1993). Radioinert *all-trans*-retinoic acid was purchased from Sigma (St. Louis, MO), as well as antiproteases. Taq DNA polymerase, isopropyl thio-β-thiogalactopyranoside (IPTG), ampicillin, and kanamycin were from Appligene (Strasbourg, France). Acrylamide and bis(acrylamide) mix (Protogel) was from National Diagnostics (Atlanta, GA). Dextran T-70 and charcoal (Norit-A) were from Prolabo (Paris, France). Restriction enzymes were from Promega (Madison, WI), and oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium).

Plasmids and Bacterial Strains. Plasmid pHK1, containing the cDNA of hRARα (Giguere et al., 1987), was obtained from V. Giguere and R. M. Evans (The Salk Institute, HHMI, La Jolla, CA). The pQE-9 vector was obtained from Diagen Gmbh (Dusseldorf, Germany). DNAs were obtained either by PCR amplification or by using appropriate restriction sites (see Figure 2) and inserted into the pQE-9 vector as a *Bam*HI-*Hin*dIII fragment, in order to generate an in-frame fusion protein made of a histidine tag followed by sequences coding for the receptor. Histidine residues were shown not to interfere with ligand and DNA binding properties of the receptor (C. Rachez, data not shown). DH5α (Gibco-BRL, Gaithesburg, MD) cells were used for routine subcloning procedures; M15 or SG 10039 (Diagen) bacterial strains containing the Rep4 plasmid coding for the lac repressor were the host cells for overexpression of the receptor and its various deletion mutants.

Expression of Full-Length and Deletion Mutants of RARα. Transformed bacteria were grown overnight in LB broth supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin. These precultures were grown in 1 L of LB broth to an $OD_{600} = 0.7-0.9$, and 1 mM IPTG was added. Derepression proceeded for 3 h after which time cells were pelleted and resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mM DTT, 10 mM PMSF, and aprotinin, leupeptin, and pepstatin at 20 μg/mL) supplemented with 50 mM glucose. Lysozyme was added to a final concentration of 0.5 mg/mL, and the cell suspension was incubated for 45 min on ice. Cells were lyzed by five successive freeze−thawing cycles, and the lysate was brought to 0.4 M NaCl. The homogenate was centrifuged for 1 h at 100000*g* at 4 °C. The supernatant was submitted to a poly(ethylenimine) precipitation step (0.2% final) to remove most of the DNA (Burgess, 1991). Similar results were obtained with DNase I or poly(ethylene glycol)/NaCl. DNA removal from the extracts yielded receptor preparations with a strongly decreased nonspecific binding, which dropped from 20% of the total binding activity in untreated extracts to less than 5% (B. Lefebvre, data not shown). The bacterial extract was then adjusted to 10% glycerol and stored at −80 °C.

Retinoid Binding Assays. Bacterial extracts (15−20 mg/mL proteins) were adjusted to ca. 1−2 mg/mL by dilution in buffer A supplemented with 150 mM NaCl. Retinoids were added to the tubes in solution in either ethanol or DMSO, and solvents were evaporated to dryness in the dark. Then 200 μL of extract was added to these tubes, in which the ligand concentration usually varied from 0.1 to 10 nM (labeled ligand), and the mixture was incubated for 16 h at

4 °C in the dark. Each assay was performed in triplicate as follows: 10 μL of the extract was removed to assess the actual concentration of the ligand, and the remaining sample was incubated for 10 min at 4 °C with 100 μL of a charcoal−dextran suspension (3% charcoal, 0.3% dextran in buffer A). Samples were then centrifuged for 10 min at 4 °C at 6000*g*, and the radioactivity content of the supernatant was assayed by scintillation counting. Nonspecific binding was assayed similarly, except that a 200-fold excess of radioinert ligand was added to the incubation mix. Scatchard analysis was performed using the Rack-Beta Receptor program from LKB-Pharmacia.

Other Techniques. SDS−polyacrylamide gel electrophoresis and western blotting were performed as described previously (Tahayato et al., 1993). The anti-RARα polyclonal antibody is directed against the 425−443 region of this receptor. Alternatively, the monoclonal antibody Rα10 directed against the 444−462 region of the same receptor was used (Ali et al., 1992.) Purification of the denatured proteins was performed exactly as suggested by the manufacturer, except that a batch adsorption procedure was substituted to the column protocol described in the manual (Diagen). High-performance size exclusion chromatography was performed according to Dallery et al. (1993).

RESULTS

Expression of Histidine-Tagged Full-Length and Truncated hRARα Polypeptides. The intact cDNA and several deletion mutants of hRARα were cloned into the pQE9 expression vector (Figure 1). Deletions were introduced at either the N-terminal or the C-terminal end of the cDNA domain to remove successively the A, B, C, and D domains or the F domain, respectively. Smaller deletions were also made to remove smaller pieces from the N- and C-terminal extremities of the E domain of hRARα. Numbers represent the first and the last amino acid from hRARα present in the molecule, according to the sequence published by Giguere et al. (1987). ΔN indicates that the molecule is deleted from its N-terminal end, whereas ΔC designates a C-terminally truncated receptor.

Each of these mutants is bearing a histidine tag, allowing for a rapid purification and characterization of the protein. This property is exemplified for the mutant ΔN186/462, made of the C-terminal part of the D domain and the intact E and F domains of hRARα, which was expressed in *Escherichia coli* M15 strain under the control of an IPTG-inducible promoter (Figure 2A). Proteins were substantially overexpressed in this system (Figure 2B, left panel; compare lane Ind to lane NI) to reach levels of 60−100 nM (3−5 mg/L of culture). Although we noted dramatic differences in recovery between the full-length receptor (less than 1 mg/L of culture) and truncated versions of the same protein (more than 10 mg/L in some cases), soluble (His_6) fusion proteins were easily purified under denaturing (Figure 2B, lane E) or under nondenaturing conditions (C. Rachez, in preparation). Histidine-tagged hRARα displayed an affinity for the ligand (see below) or retinoic acid response elements (C. Rachez, in preparation) comparable to that of the wild-type molecule.

Mutants were characterized (Figure 3) for the expression of the protein by NiTA affinity chromatography (Figure 3A) and western blot analysis (Figure 3B). The expression

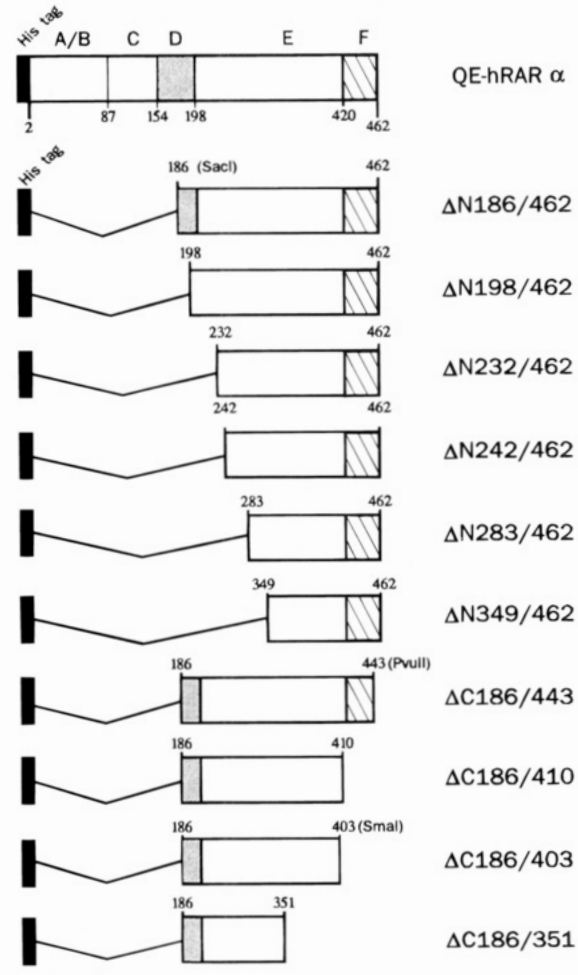


FIGURE 1: Construction of the full-length and truncated hRARα expression vectors. The 5′ and 3′ ends of the hRARα cDNA were modified by PCR amplification to create *Bam*HI and *Hin*dIII sites, respectively. The amplified cDNA was inserted into the pQE9 vector as a *Bam*HI/*Hin*dIII fragment. A similar procedure was used to generate other mutants, except when convenient restriction sites could be used (ΔC186/351, *Pst*I; ΔC186/403, *Sma*I; ΔC186/443, *Pvu*II). In that case, the initial vector ΔN186/462 was cut at the indicated position and a linker was ligated to generate a stop codon after the indicated amino acid. The conventional letter code (A−F) was used to designate the different receptor domains.

vectors encoded proteins with the expected molecular masses ranging from 17 to 31 kDa (Figure 3A) and were recognized, when the F domain was left intact, by a polyclonal antibody directed against this domain (Figure 3B). Thus this overexpression system allows for the production of a large amount of functional hRARα and truncated receptors that can be tested for ligand binding activity.

Ligand-Binding Properties of the Full-Length and Truncated hRARα Polypeptides. In order to define the structural determinant(s) of hRARα required for hormone binding, we assessed the ligand binding activity of each mutant described above by the charcoal adsorption assay (Table 1). Each protein encoded by the vectors described above was overexpressed in M15 cells and extracted from the bacterial lysate as described in Materials and Methods. The full-length receptor displayed a dissociation constant (K_d) for ATRA of 2.8 ± 1.3 nM ($n = 15$) and of 4.8 ± 1.2 nM ($n = 5$) for CD367, a synthetic retinoid binding to RARα, RARβ, and RARγ (Table 1). These values are in agreement with already published values, obtained by comparable assays, for the two ligands by our laboratory (Dallery et al., 1992) and others

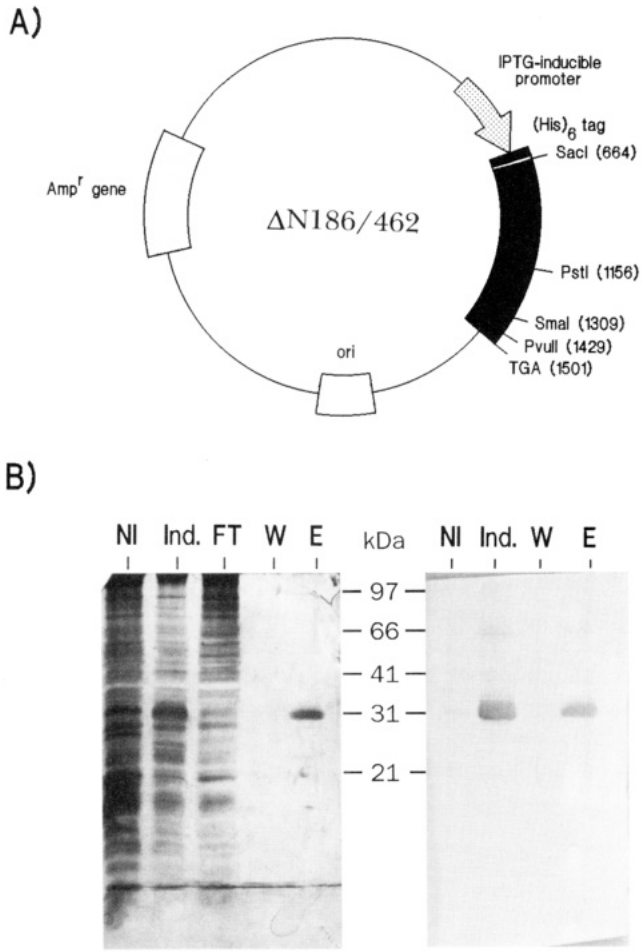


FIGURE 2: Description of the overexpression system. (A) Schematic diagram of the pQE9 expression vector. This vector contains a synthetic IPTG-inducible promoter driving the histidine-tagged protein (here the ΔN186/462 mutant containing the C-terminal end of the D domain and the intact E and D domains). (B) Characterization of the isolated HBD of hRARα following *E. coli* overexpression and purification under denaturing conditions over a NiTA column. Crude extracts from noninduced (NI) and IPTG-induced bacteria (Ind.) were resolved on an 8% SDS−PAGE that was either silver stained (left) or transferred on a nitrocellulose membrane and immunodetected using an anti-RARα polyclonal antiserum (right). A similar analysis was performed for the NiTA column flow-through (FT), the last wash (W), and the eluate (E).

(Bernard et al., 1992; Keidel et al., 1992; Fukasawa et al., 1993). Deletion of the A, B, and C domains and the N-terminal half of the D domain (mutant ΔN186/462; see Figure 1) did not induce major changes in the affinity for ATRA or CD367. However, these domains may stabilize the tertiary structure of the HBD since we noted that ΔN186/462 has a 2−3-fold lower affinity for both ligands when compared to the full-length receptor (hRARα). Further deletions at either the N-terminal or the C-terminal end from the ΔN186/462 molecule affected more dramatically the retinoid binding capacity of this protein. Indeed, deleting the last 12 amino acids from the C-terminal part of the D domain (186−198) abolished the receptor ability to bind ATRA and CD367 (Table 1, mutant ΔN198/462). Further deletions (ΔN232, 242, and 283 and 349/462) had no effect, indicating that the initial deletions did not unmask any structure that could have an inhibitory effect on hormone binding. Thus the highly charged sequence (186) ELIEKVRKAHQE (198) of the D domain is required for ligand binding.

Removal of 29 amino acids from the F domain (ΔC186/443) yielded a mutant having ligand-binding affinities identical with that of the HBD (ΔN186/462), demonstrating that this part of the F domain is dispensable for ligand recognition. The next mutant (ΔC186/410) was built to remove the entire F domain plus 11 amino acids from the E domain, a sequence encompassing most of the AF-2 region. This mutant turned out to bind ATRA and CD367 as efficiently as the HBD, with a calculated K_d for ATRA of 5.2 nM and 7.0 nM for CD367. Seven amino acids were therefore removed to yield the ΔC186/403 mutant. This deletion removed totally the AF-2 region and has been shown to yield a dominant negative mutant still able to heterodimerize with RXR (Pratt et al., 1990; Tsai et al., 1992; Damm et al., 1993), suggesting that the tertiary structure of the polypeptide is not strongly altered. The overexpressed protein displayed a 3-fold lower affinity for ATRA and CD367 than the isolated HBD and a 10-fold lower affinity for these ligands than the intact receptor, and we consistently observed a strong decrease in the number of binding sites in our extracts by at least 85−90%, when compared to other mutants, despite a similar rate of expression (data not shown). This is suggestive of a much lower stability of the interaction of the ligand with this truncated form of the HBD, indicative of an important role of the 403/410 region in ligand binding.

Ligand-Binding Selectivity of hRARα Mutants. The ability of RARα and ΔN186/462, ΔN186/410, and ΔN186/403 mutants to bind other retinoids was investigated by competition experiments. The receptor or its derivatives were incubated with [^{3}H]ATRA concentrations yielding an 80% saturation, in the presence of increasing concentrations of the radioinert competitor. Retinoids used as such were ATRA itself, 9-*cis*-RA, and retinobenzoic acids Ch55, Am580, and Am 80 (Figure 4A). The order of potency of the compounds to inhibit ATRA binding was evaluated and expressed as K_i values in Figure 4B. ATRA, 9-*cis*-RA. and Am580 inhibited ATRA binding to the full-length receptor, ΔN186/462, and ΔN186/410 with a similar efficiency, giving K_i values in a range from 8 to 20 nM. Ch55 and Am80 were less efficient and yielded K_i values around 30−50 nM. Thus the order of potency for binding to the full-length receptor, ΔN186/462, and ΔN186/410 was identical with ATRA ≈ 9-*cis*-RA ≈ Am580 > Am80 > Ch55. This ranking is in good agreement with values reported for hRARα (Keidel et al., 1992) or the isolated HBD (Crettaz et al., 1990) expressed in *E. coli.* More surprisingly, we observed that the deletion to amino acid 403 caused the loss of the ability of Am80, and to a lesser extent of Am580, to inhibit ATRA binding to the receptor, whereas the affinity of Ch55 was not affected. The relative affinities of ATRA and its metabolic derivative 9-*cis*-RA decreased by 2−3. Thus the region 403/410 contains residues involved in the specific recognition of synthetic retinobenzoic derivatives and is also involved in maintaining a high affinity of the receptor for natural retinoids.

hRARα Binds Cooperatively all-trans-Retinoic Acid but Not CD367. While investigating the ligand-binding properties of hRARα, we noted that the full-length receptor had a peculiar behavior at low ATRA concentrations (0−1 nM). We were able to detect reproducibly this phenomenon when contaminating bacterial DNA was carefully removed from high salt extracts. This was achieved by either poly(ethylenimine) (Burgess, 1991), poly(ethylene glycol) pre-

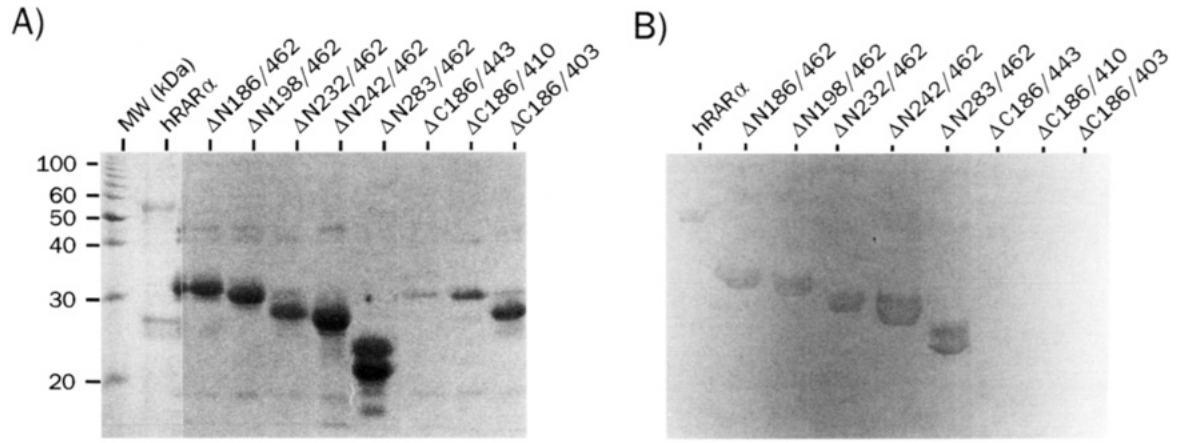


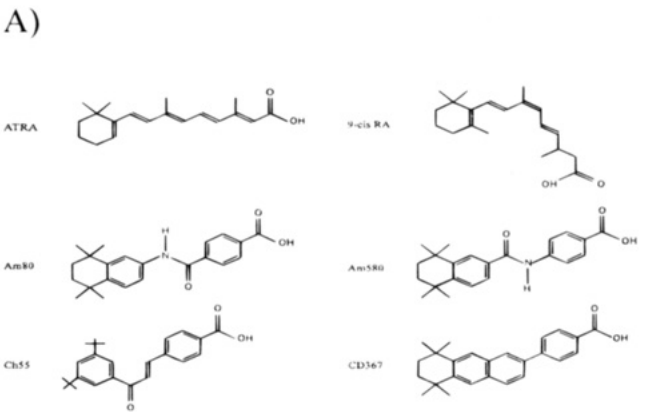
FIGURE 3: Characterization of overexpressed receptors. (A) Following overexpression in *E. coli*, His-tagged receptors were purified under denaturing conditions and resolved on a 15% SDS−polyacrylamide gel. After transfer on a PDVF membrane, proteins were stained with Ponceau Red (panel A). After destaining, the membrane was immunoprobed using an anti-RARα monoclonal antibody directed against the 425−443 region of RARα (panel B). Molecular masses are indicated on the left of the panel A.

Table 1: Dissociation Constants for ATRA and CD367 of RARα and Deletion Mutants Expressed in *E. coli*[a]

mutant	K_d (nM)	
	ATRA	CD367
RARα	2.8 ± 1.3	3 ± 2
ΔN186/462	6 ± 2	5 ±2
ΔN198/462	NB[b]	NB
ΔN232/462	NB	NB
ΔN242/462	NB	NB
ΔN283/462	NB	NB
ΔN349/462	NB	NB
ΔC186/443	8 ± 2	7 ± 1
ΔC186/410	5 ± 2	6 ± 1
ΔC186/403	17 ± 3	16 ± 4
ΔC186/351	NB	NB

[a] Intact or truncated RARα were expressed in *E. coli*, and their affinities for tritiated ATRA or CD367 were assayed as described in Materials and Methods. K_d values were deduced from Scatchard plots and are the mean of at least four independent assays. [b] NB: no binding detectable.

cipitation, or DNase I treatment. Reproducible binding data could be obtained after optimization of the DNA removal procedure which reduced the nonspecific binding in a dramatic fashion and allowed for the accurate determination of ligand binding at low ligand concentrations (between 0.10 and 0.5 nM). As shown by saturation curves (Figure 5C), the amount of specifically bound ATRA increased in a quasilinear fashion at these low concentrations and then increased exponentially between 1 and 5 nM ATRA, to reach saturation at 8−10 nM ATRA. This triphasic curve, when plotted according to Scatchard (1949), yielded a convex curve typical of a cooperative binding of the ligand to the receptor (Figure 5D). The Hill coefficient n_H had a value of 2, suggesting that two receptor subunits interact with each other. On the contrary, CD367 saturation binding experiments (Figure 5A) yielded a linear Scatchard plot, with an estimated K_d of 5.0 nM and a Hill coefficient of 0.95 (Figure 5B), characteristic of a noncooperative binding mechanism. The receptor concentration in these experiments was typically around 1 nM (60 μg/mL), and it should be noted that results shown here were obtained with different extracts. In comparative experiments, the number of binding sites for ATRA and CD367 was always equal. Interestingly, we observed a similar behavior for the mutants ΔN186/462, ΔN186/443, and ΔN186/410 (data not shown).



Ligand	RARα	ΔN186/462	ΔN186/410	ΔN186/403
ATRA	7.9±1.9	13.2±1.0	14.2±1.6	33.1±0.2
9-cis-RA	12.1±1.7	17.9±1.2	16.9±0.7	39.5±2.3
Ch55	32.9±4.4	53.1±1.0	44.4±1.2	35.3±0.1
Am80	18.5±1.6	34.9±5.4	47.6±1.8	NB
Am580	12.1±1.2	16.5±3.2	17.6±3.0	)200

FIGURE 4: Ligand specificities of hRARα and mutants ΔN186/462, ΔN186/410, and ΔN186/403. (A) Structures of the ligands used in competition experiments. ATRA and 9-*cis*-RA are natural derivatives of vitamin A, whereas Ch55, Am580, and Am80 are retinobenzoic acid derivatives. Am80 and Am580 are RARα-specific ligands. (B) K_i values of retinoids for hRARα and mutants ΔN186/462, ΔN186/410, and ΔN186/403. Bacterial extracts were incubated in the presence of a concentration of [^{3}H] ATRA yielding an 80% saturation of the binding sites (6 nM for all receptors but ΔN186/403, for which 30 nM was used) and with increasing concentrations of the unlabeled competitor, ranging from 1 nM to 1 μM. Incubations were for 16 h at 4 °C in the dark. The K_i value for each compound is indicated in nanomolar and was calculated from the formula $K_i = IC_{50}[(1 + [[^3H\ ATRA])/K_d]^{-1}$. IC_{50} is the concentration in the competitor required to inhibit 50% of ATRA binding, [[^{3}H] ATRA] is the concentration of ATRA used (6 or 30 nM), and K_d is the K_d of each receptor mutant for ATRA. Data are the average of at least four independent experiments.

Several parameters that could affect the dimerization state of the receptor were tested for their effects on the cooperative binding of ATRA to hRARα. The presence of either RXRβ (Hamada et al., 1989) or a retinoic acid response element from the promoter of the RARβ gene (de The et al., 1990),

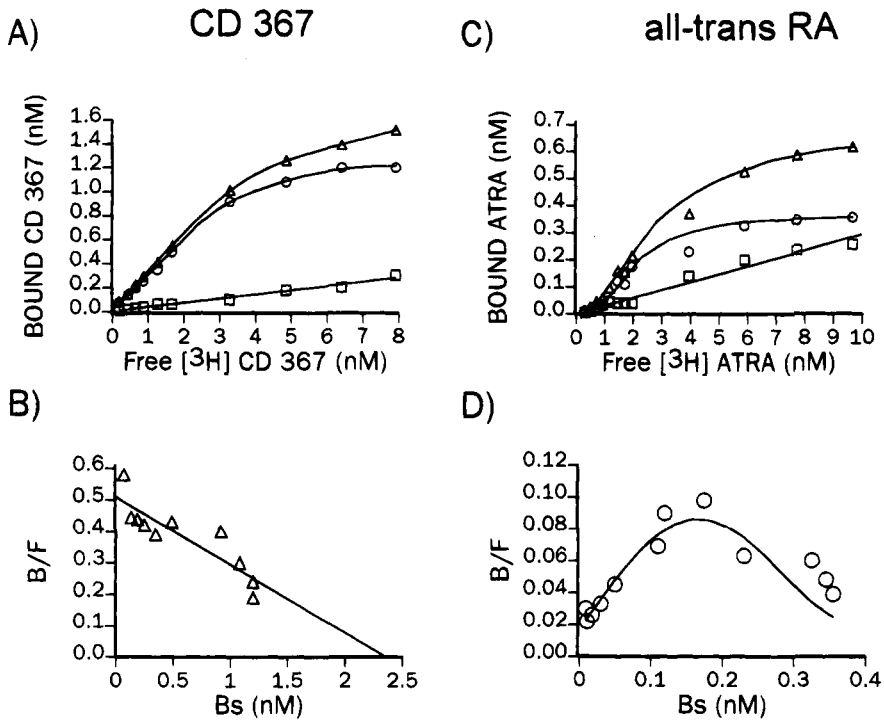


FIGURE 5: Saturation curves and Scatchard analysis of CD367 and ATRA binding to hRARα. (A) CD367 binding assayed by the charcoal adsorption assay. Bacterial extracts were incubated with increasing concentrations of tritiated CD367 for 16 h in the dark at 4 °C. Nonspecific binding was determined by adding a 200-fold excess of unlabeled CD367. Each point was assayed in triplicate. Free radioactive ligand concentrations were calculated by substracting the concentration of labeled ligand from that of the receptor-bound labeled ligand. (B) Scatchard analysis of CD367 binding to hRARα. Data points were best fitted using a nonlinear regression analysis of the data by a Stanford Graphics program. (C) ATRA saturation curve. A procedure similar to that described in (A) was followed to determine ATRA binding properties of hRARα. (D) Scatchard analysis of the ATRA saturation curve shown in (C). Similar results were obtained with ΔN186/462 and ΔN186/410 (data not shown). Note that representative experiments are shown here and were not performed with the same extract. Symbols: △, total binding of the tritiated ligand; ○, specific binding of the same ligand; □, nonspecific binding of the labeled ligand detected in the presence of a 200-fold molar excess of the same radioinert ligand.

in proportions compatible with an optimal DNA binding activity, did not change the positive cooperativity. Similarly, physical parameters like elevated temperature (22 °C) or concentration (from 0.5 to 50 nM receptor) did not alter the cooperative nature of ATRA binding to hRARα, which was equally detected with other expression vectors in different bacterial strains (data not shown).

We thus investigated a possible relationship between the dimerization state of the receptor and the observed cooperative binding mechanism. Identical samples were incubated with vehicle (DMSO), 30 nM [3H]ATRA, or [3H]CD367 and fractionated over a size exclusion column (panels A−C of Figure 6, respectively). [35S]Methionine-labeled hRARa was also used as a tracer for the unliganded form of the receptor (ca. 50 fmol, Figure 6A). In the latter case, the receptor was eluted mostly as a monomeric species (60−70% of total receptor), with a Stokes radius of 3.0 nm (Figure 6A). Western blotting of fractions eluting at positions corresponding to monomeric (Rs = 3 nm, denoted from f to j) or dimeric species (Rs = 6 nm, denoted from a to e) also showed that the RAR polypeptide was present essentially as a monomeric species under these conditions and appeared to be more sensitive to proteolysis than the liganded receptor (upper panel). Since two bands of apparent molecular masses 52 and 48 kDa were detected, it is likely that the 48 kDa polypeptide results from partial proteolysis of the A/B domain. The CD367-bound receptor was eluted as a monomeric species (Figure 6C), whereas, in sharp constrast, the ATRA-bound receptor was predominantly eluted (90−100% of total receptor) in fractions corresponding to a Stokes radius of 6.0 nm, compatible with a dimeric state of the receptor (Figure 6B). More surprisingly, the RARα polypeptide was eluted, as shown by western blot, as a dimeric species, whatever ligand was used (upper panels of Figure 6B,C). Thus ATRA appeared to induce the formation of a stable homodimeric form of the receptor and bind to it, whereas CD367, although equally able to promote homodimer formation, did not bind to these homodimers. This observation establishes a direct correlation between the observed cooperative ligand-binding mechanism of ATRA to RARα and the quaternary structure of the protein.

DISCUSSION

The study of the major function of the hormone binding domain of nuclear receptors, namely, its ligand binding activity, has been hampered by the structural and functional complexity of this domain. Indeed, the hormone binding domain not only has a structure conferring its ability to recognize specifically a subset of small molecules but is also involved in protein/protein interactions that dictate the quaternary structure and regulate the transcriptional activity of the receptor. To determine what parts of the hRARα contribute to the hormone binding activity, we deleted systematically domains from the wild-type molecule. These truncated receptors were expressed in bacteria in which the receptor does not form heterodimers nor is involved in transcriptional regulation, and thus this system allowed the study of the hormone binding function per se. Deletion of the A and B domains, as well as the C- and the N-terminal part of the D domain, did not result in a major alteration of

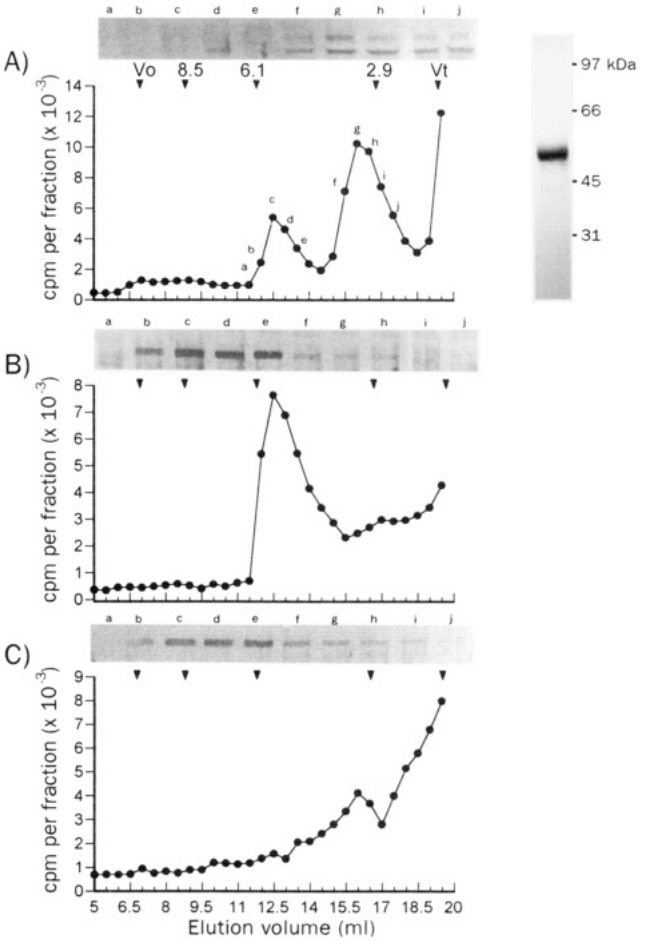


FIGURE 6: Influence of ligand on the dimerization state of hRARα. (A) Unliganded hRARα (100 μL of extract) was fractionated over a Superdex 200 HR10/30 equilibrated in 20 mM HCl, pH 7.4, 150 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM PMSF. ^{35}S-labeled hRARα was used as a tracer in this particular experiment (~100 000 cpm), and an autoradiogram shows a fraction of the labeled receptor. Note that this amount of labeled receptor is not detectable by western blot. hRARα was detected by quantitating ^{35}S radioactivity and by western blotting (upper panel). Five fractions, denoted from a to e, corresponding to the elution volume of the dimer (11.50−13.50 mL), and five fractions, denoted from f to j, corresponding to the elution position of the monomer (16.00−17.50 mL) were resolved by SDS−PAGE, blotted, and revealed by an anti-RARα antibody. Arrowheads at the top of the elution profile show the elution volume of Blue dextran (V_0), thyroglobulin (Rs = 8.5 nM), ferritin (6.1 nM), and ovalbumin (2.9 nM). V_t is determined by injection of free radiolabeled ATRA. Blotted fractions are indicated by arrowheads located along the curve shown. (B) ATRA-bound hRARα exists as a dimer. Bacterial extracts were incubated for 16 h at 4 °C in the dark with 30 nM [^{3}H]ATRA, fractionated, and analyzed as described in (A). Arrowheads indicate the elution volume of standard proteins. (C) CD367-bound hRARα exists as a monomer. Bacterial extracts were processed exactly as described above, except that tritiated CD367 was used in place of ATRA.

the K_d of hRARα for its natural ligand, *all-trans*-retinoic acid, or for a synthetic retinoid, CD367, which binds equally well to the RARβ and RARγ (Martin et al., 1992). However, deletion of the remaining part of the D domain (mutant ΔN198/462) yielded a receptor that did not bind either ATRA or CD367. The critical importance of the C-terminal end of the D domain has also been noticed for T3 binding to the human thyroid hormone receptors α1 and β1 (Lin et al., 1991 and references therein). Interestingly, agonist-induced conformational changes of RARα (Keidel et al., 1994) and of RXRα (Keidel et al., 1994; Leid, 1994) yielded a 30-kDa fragment resistant to proteolysis. In the case of RXRα, the N-terminal end of this protease-resistant fragment mapped to Ser 229, which is located in the middle of the D domain (Leid, 1994). Taken together, these results therefore point to a critical role of this region in ligand recognition.

Deletions removing the F domain did not prevent the receptor from binding ATRA and CD367, whereas further deletions introduced at the C-terminal end of the E domain led to the loss of the hormone binding capacity, identifying a region (403-PGSMPPLI-410) required to confer a high affinity for ATRA and CD367 (see Figure 7). Our competition studies showed that this region is also responsible for the specific recognition of retinobenzoic derivatives Am80 and Am580, whereas ATRA, 9-*cis*-RA, and Ch55 still bound to the receptor lacking this region, albeit with a 10-fold decreased affinity when compared to the full-length receptor. This observation is intriguing since these two compounds, in opposition to ATRA, 9-*cis*-RA, and Ch55, have been shown to display a strong selectivity for RARα (Bernard et al., 1992; Delescluse et al., 1991; Martin et al., 1992). Tate et al. (1994) reported that hRARα truncated to amino acid 404 bound ATRA and Am580 with an affinity similar to that of the wild-type receptor, which would identify Gly 404 as the amino acid involved in Am80 and Am580 recognition. The role of Gly 404 is also emphasized by the fact that its deletion yielded a receptor with a 10-fold lower affinity for ATRA when compared to the full-length receptor (Tate et al., 1994, and our results). However, Gly 404 is conserved in the three RAR isoforms (see Figure 7), thereby excluding its involvment in the specific recognition of these compounds. In the same report, the authors reported that hRARα truncated at position 404 was unable to bind 9-*cis*-RA, whereas our ΔN186/403 mutant still binds this compound. The dramatically different experimental conditions for receptor overexpression and ligand-binding assays may be an explanation for this discrepancy, although the authors reported that the Δ404 mutant displayed an abnormal electrophoretic mobility in denaturing conditions, which could reflect an altered charge distribution of this particular receptor mutant. On the contrary, the Δ186/403 mutant did not display such an abnormal property (see Figure 3).

FIGURE 7: Sequence alignment of human *all-trans*-retinoic acid receptors α, β, and γ and of hRXRα and the *Drosophila* usp gene product. Regions conserved among ATRA-binding receptors are indicated by squares, and residues conserved among the five nuclear receptors are indicated by oval boxes. The 403−410 region is boxed in gray for hRARα. Dots at the top of the sequences indicate conservative mutations.

These results demonstrate that a critical region located between amino acids 403 and 410 is involved in ligand recognition. Very interestingly, other mutants of retinoic acid receptors have been described, for which the ligand binding activity has been studied. A subclone of HL60 cells is partially resistant to ATRA-induced differentiation due to a truncation of RARα to position 410 (Robertson et al., 1992). RAC 65 cells contain a truncated hRARα at the amino acid 395, which acts as a dominant negative receptor devoid of any ATRA binding activity (Pratt et al., 1990). A dominant negative activity was also evidenced for Δ403 RARα, Δ397 RARβ, and Δ406 RARγ (Damm et al., 1993). Other mutations were also described: the RARβ deleted from the F domain binds ATRA with an affinity equal to that of the wild-type receptor (Shen et al., 1993). Taken together, our data and others show that the C-terminal end of the E domain has a critical role in hormone binding. The domain F is dispensable, whereas the 403−410 region contains critical residues or has a structure required for high-affinity ATRA binding. Importantly, this region contains the AF-2 region, a ligand-dependent transactivating sequence that can potentially form an α-helix (see Figure 7). This sequence is highly conserved in ATRA binding receptors, whereas it displays strong divergence in RXRs, which do not bind ATRA. Whether specific residues are directly involved in a direct interaction with the ligand will be tested by site-directed mutagenesis. Moreover, RARs display a differential affinity for ATRA and 9-*cis*-RA (Allenby et al., 1993), and again, assessing the contribution of individual amino acids to this differential binding may prove useful to the conception of specific ligands.

ATRA binding to the intact receptor or the isolated HBD was characterized by a Hill coefficient of 2, indicative of positive cooperativity. In contrast, CD367 displayed no cooperative binding in identical conditions. This cooperative binding was observed at receptor concentrations ranging from 0.5 to 50 nM, which are close to that observed in nuclei (100−5000 sites/nucleus). At lower receptor concentrations, this cooperativity was hardly detected, due to the lack of sensitivity of the hormone binding assay. Indeed, ATRA binding has so far been reported to be noncooperative in various overexpression systems. The failure to observe such a mechanism can be explained by the lack of data points at low ligand concentration (Allegretto et al., 1993; Crettaz et al., 1990; Dallery et al., 1993) or to lower receptor concentrations (Fukasawa et al., 1993; Keidel et al., 1992; Yang et al., 1991). Such pitfalls have already been evoked to explain apparent discrepancies in the observed ligand-binding mechanism of the estrogen receptor [see Schwartz and Skafar (1993) and references therein]. Positive cooperativity has also been reported for the estrogen and progesterone receptors (Schwartz & Skafar, 1993; Skafar, 1991, and references therein). Like those for enzymatic complexes, this ligand-binding mechanism has been interpreted as a means to regulate closely the activity of steroid hormone receptors which bind to their cognate hormone response elements as homodimers. In contrast to these receptors, other nuclear receptors like RARs, T_3R, or vitamin D receptors (VDR) are known to bind more efficiently to DNA when forming heterodimers with RXR than when forming homodimers (Mader et al., 1993a; Mader et al., 1993b). However, the exact role of the ligand remains unclear for these receptors since 9-*cis*-RA induces RXR homodimerization (Zhang et al., 1992), whereas the cognate ligands for T_3R and VDR favor homodimer dissociation and in turn facilitate heterodimer formation (Miyamoto et al., 1993; Cheskis & Freedman, 1994). Whether such differences can be evidenced for hRARα bound to CD367 or ATRA remains a hypothesis, as well as the relation between CD367 particular behavior (i.e., binding to RARs with an affinity similar to that of ATRA but transient association with RAR homodimers) and its higher efficiency in transactivation assays (Martin et al., 1992). It is interesting to note that CD367 is a rigid polycyclic compound which probably cannot undergo conformational changes imposed by receptor structure alteration observed upon ligand binding, in opposition to the more flexible ATRA molecule which could still bind to the structurally reshaped ligand-binding site. Such conformational changes, already proposed to be related to the transcriptional efficiency of RARβ induced by flexible or rigid ligands (Lombardo et al., 1994), could also play a role in the cooperative binding mechanism observed here.

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