H11-H12 Loop Retinoic Acid Receptor Mutants Exhibit Distinct trans-Activating and trans-Repressing Activities in the Presence of Natural or Synthetic Retinoids†

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ABSTRACT: Retinoids, such as the naturally occurring *all-trans*-retinoic acid (atRA) and synthetic ligand CD367 modulate ligand-dependent transcription through retinoic acid receptors (RARs). Retinoid binding to RAR is believed to trigger structural transitions in the ligand-binding domain (LBD), leading to helix H1 and helix H12 repositioning and coactivator recruitment and corepressor release. Here, we carried out a detailed mutagenesis analysis of the H11−H12 loop (designated the L box) to study its contribution to hRARα activation process. Point mutations that reduced transactivation by atRA also reduced atRA-induced transrepression of AP1 transcription, correlating ligand-induced activation and repression. However, a correlation was not observed with these mutations when tested with another ligand CD367, a synthetic agonist with binding properties identical to those of atRA. Transcription was strongly inhibited in the presence of CD367 for some mutants, thus leading to an inverse agonist activity of this ligand. None of these mutations significantly altered binding affinity for either ligand, indicating that altered transcription was not caused by altered ligand binding by these mutations. Although simple correlations with transcriptional activities were not found, these mutations were also characterized by altered ligand-induced structural transitions, which were distinct for the atRA−hRARα or CD367−hRARα complexes. These results indicate that amino acids in the L box are involved in specifying trans-repressive and trans-activating properties of the hRARα, and support the notion that different agonists induce distinct conformations in the LBD of the receptor.

Molecular evolution has shaped nuclear receptors (NRs)[1] so that members of this superfamily, which have a modular structure and a highly conserved DNA-binding domain (domain C) interacting with closely related hormone response elements (*1*), act as transcription factors activatable by distinct lipophilic hormones. Although it was thought to be limited to the C-terminal domain E, essentially by virtue of its sequence homology with other members of the steroid/thyroid receptor family, the ligand-binding domain of hRARα turned out to be an α-helical structure encompassing also the C-terminal part of domain D (*2*, *3*). Biochemical and crystallographic studies further documented striking structural analogies with other members of the nuclear receptor superfamily such as hRXRα (*4*, *5*), the thyroid hormone receptor β (T3R-β1) (*6*, *7*), and the estrogen receptor (*8*). This three-layered antiparallel α-helical sandwich undergoes structural transitions upon ligand binding which generate or disrupt protein:protein interaction interfaces that govern the transcriptional activity of the receptor. Structural remodeling of the holo-LBD was first identified by biochemical techniques (*9*) and detailed further by three-dimensional structures comparison (*2*). The hRARα LBD is therefore a multifunctional domain bearing structures regulating corepressor [NCoR (*10*, *11*)], coactivator (NCoA) binding (*12*, *13*), and dimerization with RXR (*14*). Although the minimal structural requirements for binding of the natural ligand *all-trans*-retinoic acid (atRA) have been defined, recent reports suggested that the core ligand-binding site could vary according to the ligand structure. We indeed initially reported that a region, located at the C-terminus of the domain E (residues 403−410) is selectively required for the binding of two RARα-specific ligands (*3*). In addition, Apfel and co-workers recently showed that three distinct regions of hRARα LBD are required for the binding of atRA, 9-*cis*-RA and Ro41−5152, a RARα-specific antagonist (*15*). Since molecular basis underlying the specific recognition of structurally distinct ligands and the subsequent transcriptional activation of retinoic acid receptors are still poorly understood, we wished to define domain(s) of hRARα regulating these molecular events.

As part of a preliminary study designed to define the core ligand-binding site of hRARα, we identified a 7 amino acid

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[1] Abbreviations: atRA, *all-trans*-retinoic acid; LBD, ligand-binding domain; hRARα, *all-trans*-retinoic acid receptor; RXR, 9-*cis*-retinoic acid receptor; T3R, thyroid hormone receptor; ER, estrogen receptor; RARE, retinoic acid response element; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NCoA: nuclear receptor coactivator; NCoR: nuclear receptor corepressor; CBP, CREB binding protein.

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region involved in a specific interaction with ligands able to bind selectively to RARα (*3*). This region, which forms a loop, is located at the C-terminus of the LBD, between helix H11 and the AF2-AD region. Given its location within the three-dimensional structure of the hRARα LBD, which could potentially confer to this region a key regulatory role in ligand recognition and protein:protein interactions, we carried out a mutagenesis study of this domain that we refer to below as the L box. Mutations that were initially designed to alter ligand:receptor interactions identified amino acids involved in specifying structural transitions occurring in vitro at the N- and C-terminus of the receptor LBD upon ligand binding. Mutagenesis of these residues had, however, distinct consequences depending on the ligand tested. In addition, some of these mutations dissociated ligand-dependent activation from trans-repressive (anti-AP1) activity of hRARα. They also played a key role in the ligand-dependent trans-activation of hRARα, since mutating some of these amino acids converted a synthetic agonist into an inverse agonist. Very importantly, phenotypic changes brougt by mutations were again clearly distinct in the presence of atRA or CD367, a synthetic retinoid with binding properties similar to that of atRA. Taken together, these data identify the L box as a determinant of the biological activities of hRARα sensitive to ligand structure.

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 were a gift from B. Shroot, CIRD-Galderma, Valbonne, France. Radioinert *all-trans*-retinoic acid was purchased from Sigma (St. Louis, MO), as well as antiproteases. Acrylamide and bis(acrylamide) mix (Protogel) was from National Diagnostics (Atlanta, GA). Dextran T-70 and charcoal (Norit-A) were from Prolabo (Paris, France). Ampicillin and kanamycin were from Appligene (Strasbourg, France). Taq DNA polymerase was from Gibco-BRL (Cergy-Pontoise, France), and restriction enzymes were from Promega (Madison, WI). Oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium).

Plasmids and Bacterial Strains. Plasmids pHK1, containing the cDNA of hRARα, pSG5-hRARα, pSG5-hRXRα, pGST-RIP 140, pGST-CBP, and pGST-SMRT have been described elsewhere (*11*, *16*−*18*).

Point mutations were introduced in the hRARα sequence using oligonucleotides that were inserted into ΔC186/403 (*3*) as *Sma*I−*Cla*I fragments, generating a first set of mutated receptors deleted at position I410. This set of pQE9/Δ410 hRARα mutants was used to produce the pQE9/Δ418 (termed pQE9[AF2] further in the text) expression vectors, by inserting the oligonucleotidic sequence coding for the AF2-AD region, from residues 411−418, as a *Cla*I−*Hin*dIII fragment. These plasmids are described elsewhere (*19*).

hRARα eukaryotic expression vectors used in transfection experiments were constructed starting from pSG5−hRARα (*16*), from which the *Sac*I−*Bgl*2 fragment was removed and replaced by the *Sac*I−*Bam*HI fragment excised from the corresponding pQE9[AF2] mutant. The reporter gene p(TRE-$_{pal}$)$_3$ Luc was constructed by inserting three repeats of the synthetic thyroid response element **AGGTCA**TGACCT upstream of the AdMLP TATA box present in the pTATA Luc vector (*20*). The phorbol ester-inducible pTPA-RE Luc vector has been described elsewhere (*20*). All constructs were checked by restriction enzyme analysis and automatic sequencing.

JM109 (Promega, Madison, WI) cells were used for routine subcloning procedures; M15 (Diagen) bacterial strains containing the Rep4 plasmid coding for the lac repressor were the host cells for overexpression of hRARα mutants (*14*), GST fusion proteins were overexpressed in JM109 cells.

Expression of Mutant Receptors, Bacterial Extracts Preparation, and Ligand-Binding Assays. Receptor polypeptides and bacterial extracts were processed as described (*14*). Ligand-binding assays were performed as previously described (*3*). Briefly, bacterial extracts were incubated in the presence of a concentration of [^{3}H]CD367, yielding a 60% saturation of the binding sites ($K_d \times 1.5$) and with increasing concentrations of the unlabeled competitor, ranging from 1 nM to 10 μM. Incubations were for 2 h at 4 °C in the dark. The K_i value for each compound is indicated in nanomolarity and was calculated from the formula $K_i = IC_{50}[(1 + [^3HCD367]/K_d]^{-1}$. IC_{50} is the concentration in competitor required to inhibit 50% of CD367 binding, [[^{3}H]CD367] is the concentration of CD367 used and K_d is the K_d of each receptor mutant for CD367. Data are the average of at least five independent experiments performed with at least three different receptor preparations. Scatchard analysis and K_i values were calculated using the Prism program (GraphPad Software Inc., San Diego, Ca.)

In Vitro Coupled Transcription/Translation and Protease Mapping. [^{35}S]Methionine wild-type and Δ418 labeled receptors were generated using the TnT system (Promega) according to the manufacturer instructions. Limited proteolysis reactions and products analysis were carried out as described (*21*).

Transient Transfection Assays. Transfections were carried out using the polyethyleneimine (PEI) coprecipitation method. COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum and split in 6 wells clusters (10^5 cells/well). One milliliter of fresh medium was added to the monolayer the next day, and 200 μL of the PEI:DNA complex mixture was added to the culture medium. The PEI:DNA mix (for 1 well) was prepared as follows: 1 μg of the appropriate reporter gene was mixed with 200 ng of hRARα and hRXRα expression vectors in 100 μL of sterile 150 mM NaCl. A total of 7 μL of ExGen 500 (Euromedex, Strasbourg) was added to 100 μL of sterile 150 mM NaCl and mixed to the DNA mix. After a 10 min incubation at room temperature, the mix was added to the medium and incubated overnight. Medium was then replaced and cells stimulated 8 h later with retinoids and/or TPA for 18 h. The luciferase assay was performed as described (*16*).

GST Pulldown Procedure. GST fusion proteins were prepared as a crude bacterial extract and immobilized on glutathione-Sepharose beads. Binding of labeled mutant or wild-type receptors was analyzed as described previously (*11*, *22*) with minor modifications.

Statistical Analysis. All incubations or assays were performed in triplicate. Measured values were used to calculate a mean ± SEM and groups of data were compared using an unilateral ANOVA followed by a Student's paired *t*-test. Significance was defined as $p < 0.05$.

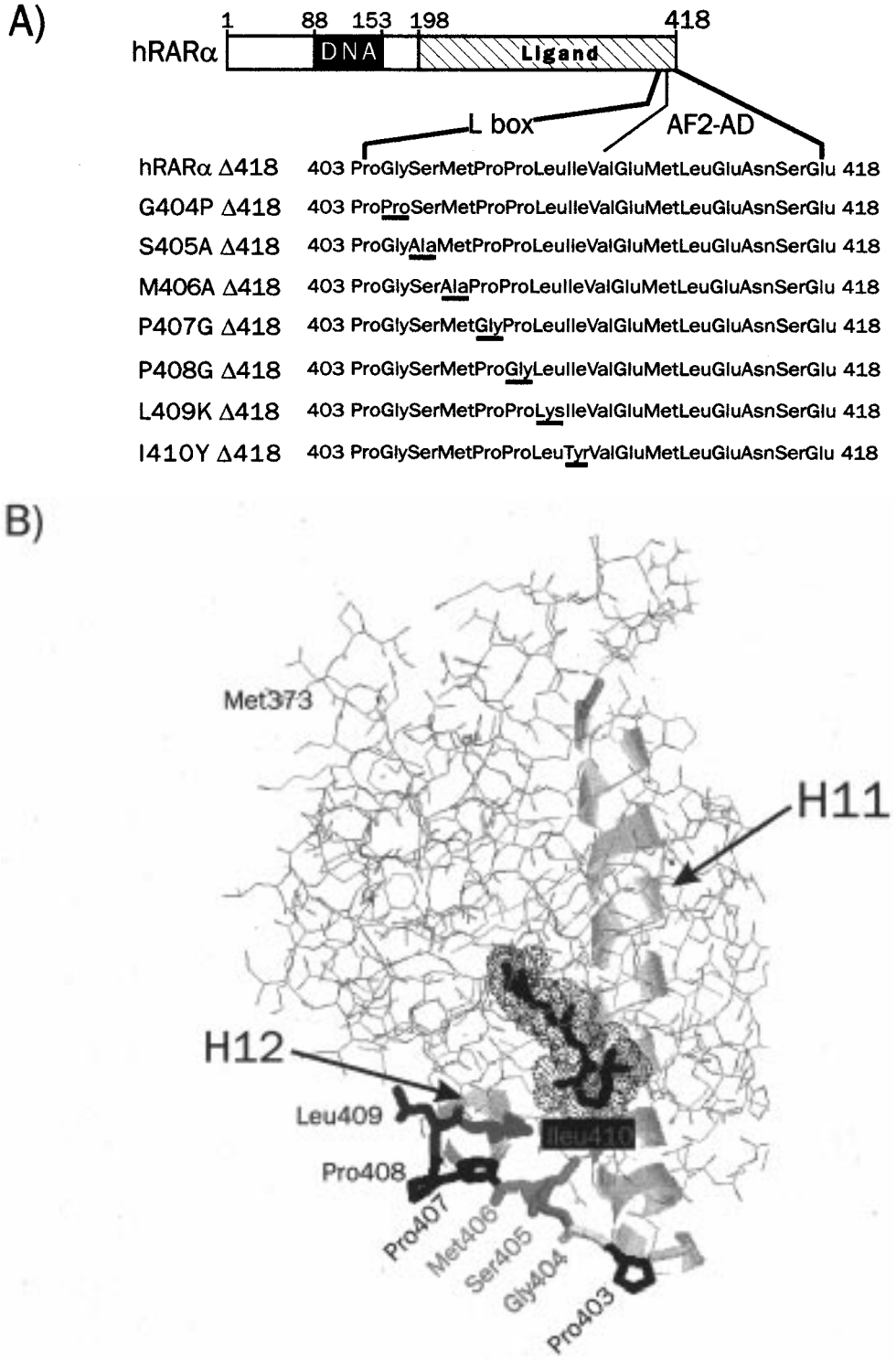


FIGURE 1: Mutations in the core LBD of hRARα. (A) Primary sequence of the wild-type and mutants in the L box of hRARα: the sequence of each construct is shown and mutated residues are underlined. Note that an additional mutation at position 411 was introduced, converting Gln411 into a valine residue. This amino acid, which is highly variable among nuclear receptors, has no detectable influence on the ligand binding, DNA-binding and transactivation properties of the receptor (this paper and unpublished data). (B) Three-dimensional structure of the L box. The two C terminal α helices H11 and H12 are represented here. The receptor is truncated here at position 418. Side-chain orientation is shown for amino acids from 404 to 410, and atRA is positioned in the ligand-binding pocket and indicated in black. Additional three-dimensional views that are virtually identical to this model can be found in ref *2*.

RESULTS

Transactivation, Transrepression, and Ligand Interpretation Are Controlled through the L Box. Figure 1A illustrates the position and the nature of point mutations introduced in the L box of hRARα. Nonconservative mutations were used to introduce either strong structural constraint on this region or to modify the charge or the polarity of the targeted amino acid. These mutations were introduced in the context of hRARα deleted from the domain F (Δ418), since we showed that this truncated receptor displays ligand-binding activities (*3*, *19*) and transcriptional properties (see below) similar to those of the wild-type receptor. First, since secondary

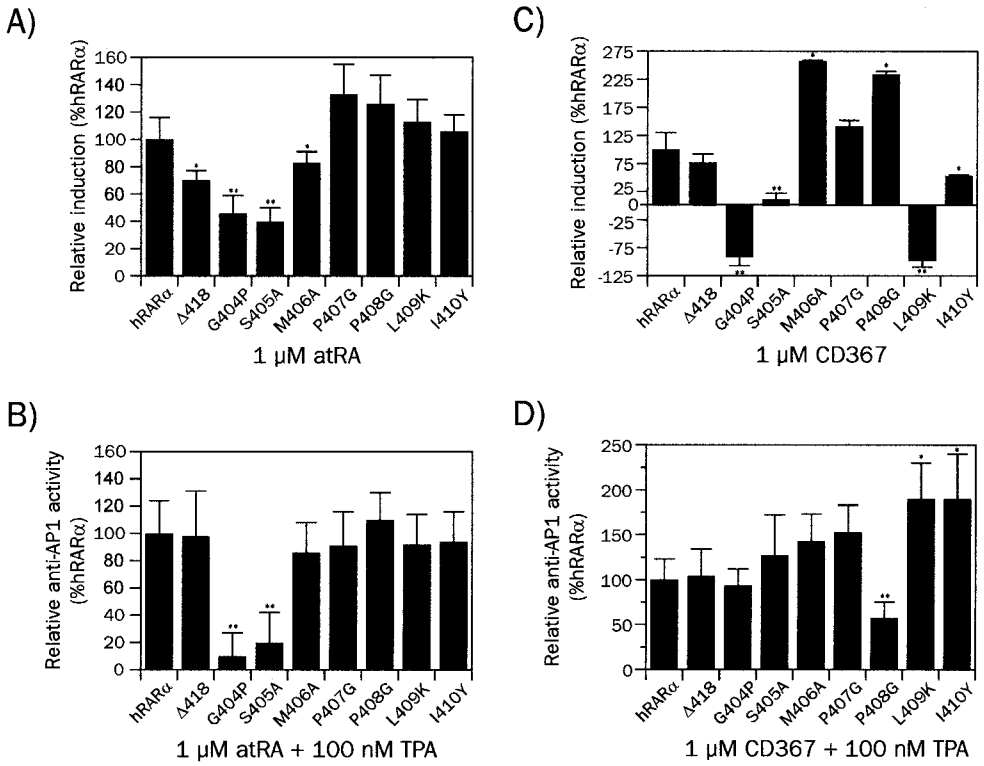


FIGURE 2: atRA- and CD367-induced transactivating and transrepressing activities of hRARα mutants. (A) Transactivation in the presence of atRA: COS-7 cells were transfected with expression vectors for wild-type hRXRα and hRARα or mutated hRARα. Their transactivating activity was assayed by cotransfecting the p(TRE_{pal})$_3$ Luc reporter plasmid. Results are expressed as the percentage of maximal wild-type activity in the presence of 1 μM atRA and are the mean ± SEM of at least eight independent determinations of the luciferase activity. An 8−10-fold induction was typically observed with the wild-type receptor. (B) Transrepression in the presence of atRA: COS-7 cells were transfected with expression vectors for wild-type hRARα or mutated hRARα and the TPA-inducible reporter gene pTPA-RE Luc. Cells were treated with 100 nM TPA and 1 μM atRA for 18 h. The anti-AP1 activity of the wild-type hRARα was set at 100%, the observed percentage of inhibition of the AP1 activity was of 70−85%. Results are representative of at least 15 independent assays, with the standard deviation shown in the diagram. (C) Transactivation in the presence of CD367: the activity of the p(TRE_{pal})$_3$ Luc reporter gene was assayed as described in legend to panel A after treatment of cells with 1 μM CD367. Results are expressed as the percentage of maximal wild-type activity in the presence of 1 μM CD367, and are the mean ± SEM of at least eight independent determinations of the luciferase activity. A 3−4-fold induction was typically observed with the wild-type receptor. (D) Anti-AP1 activity of hRARα mutants challenged with CD367: the anti-AP1 activity of each mutant was assayed as described in legend to panel B in the presence of 100 nM TPA and 1 μM CD367. Results are representative of at least 15 independent assays and are expressed as the percentage of inhibition observed with 1 μM CD367. A 65−80% repression of the AP1 activity was typically observed with the wild-type receptor. (*) Significant differences vs control ($p < 0.05$); (**) Significant differences vs control ($p < 0.01$).

structure predictions and crystallographic data (Figure 1B) positioned the L box in a turn, we tested the importance of this structure by replacing glycine 404 by a proline residue. Serine at position 405 was replaced by an alanine residue to test whether the polarity conveyed by this residue is important for the ligand-binding function. A methionyl residue occurs at position 406, which was replaced by an alanine to investigate the importance of the sulfur group. The two following amino acids are proline residues. Given their impact on the global folding of proteins, these residues were mutated to glycine, known to confer a maximal conformational freedom. Leucine at position 409 was replaced by a lysine to assess the importance of the hydrophobic contribution of its side chain to the ligand−receptor interaction. Finally, we replaced isoleucine 410 by a tyrosyl residue. Only M406 and I410 are in the vicinity of the β-ionone ring of atRA (ref *2* and Figure 1B), whereas the contribution, if any, of other residues from the L box to the overall structure of the LBD is not clearly established.

The ability of these hRARα mutants to stimulate transcription as hRARα/hRXRα heterodimers from a retinoic response element, as well as to repress the TPA-induced activity of an AP-1 response element, was thus examined in COS-7 cells (Figure 2). This cell line expresses only a low level of endogenous retinoid receptors and thus is unable to activate a reporter gene under the control of the adenovirus major late promoter TATA box flanked in 5′ of three repeats of a synthetic, palindromic thyroid hormone element (TRE_{pal}-TATA Luc). In the presence of overexpressed wild-type hRARα and hRXRα, this reporter gene is activated by natural retinoids (atRA, Figure 2A and 9-*cis*-RA, data not shown) and synthetic agonists such as CD 367 (Figure 2C), Am580, and TTNPB (data not shown). Removal of domain F yielded a receptor (hRARαΔ418) still able to activate transcription in response to atRA and CD 367 with wild-type efficiency (Figure 2, panels A and C). Since this response could be inhibited by the RARα antagonist (*23*) Ro41−5253 (data not shown), we conclude that this truncated receptor recapitulates most, if not all, functions of the full-length receptor and is therefore a valid model system to assess the contribution of the L box to the regulation of hRARα functions.

Point mutations at positions 404 and 405 reduced the atRA-induced transactivation by more than 60% (Figure 2A).

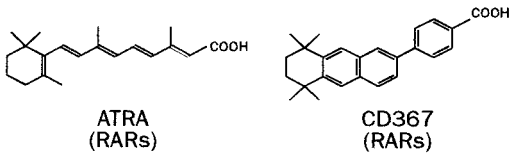
While mutation at position 406 left the level of atRA-induced transactivation unchanged, other mutations yielded receptors showing a sligthly increased response to this ligand. The molecular mechanism governing the anti-AP-1 activity of retinoids is clearly distinct from that leading to transcriptional activation, since ligands dissociating AP-1 transrepression and transactivation have been described (*24*−*26*). We therefore evaluated the anti-AP-1 activity of atRA mediated through mutated receptors by monitoring the transcription level of a TPA-inducible reporter gene in the presence of overexpressed wild-type or mutated hRARα (Figure 2C). A striking parallel was observed between the anti-AP-1 response and the transactivation in the presence of atRA. The transrepressing activities of mutants G404P and S405A were reduced by about 80%, in contrast to other mutations which had no effect.

CD367 is a synthetic retinoid that binds to RARs with high affinity (*3*). However, comparison of CD367 and atRA for the ability to stimulate transcription from the TRE_{pal}-TATA promoter showed that CD367 is a poor activator (2−3-fold induction vs 7−10-fold for atRA, data not shown). This suggests that CD367 induces the formation of a less active complex in our experimental model. The ability of receptor mutants to convey CD367 responsiveness was thus evaluated as described above for atRA. hRARαΔ418 and I410YΔ418 mutants showed reporter inducibilities similar to that of the wild-type receptor (Figure 2C). Surprisingly, the other mutations exhibited differences from the wild-type receptor in CD367-induced transactivation. While mutations at positions 406, 407, and 408 increased the transactivating potential of CD367, mutants G404PΔ418 and L409KΔ418 showed reduction in reporter activity after CD367 addition. Ligand-binding assays demonstrated that CD367 is competing with atRA for binding to hRARα, suggesting the same overlapping binding site for these ligands (Figure 3 and ref *3*). Thus, the RAR agonist CD367 behaved as an inverse agonist when hRARα was mutated at positions G404 and L409, since it was able to reduce the constitutive activity of this receptor by 40−55%. However, Ro41−5253 still acted as a potent antagonist for these mutants (data not shown), showing that the alteration of the transcriptional activities of both receptor mutants was ligand specific.

The anti-AP1 activity of CD367 mediated through L box mutants was tested (Figure 2D). In contrast to responses observed in the presence of atRA, no correlation between transactivating and transrepressive activities was observed. While G404PΔ418 and L409KΔ418 displayed strongly reduced transactivating activities in the presence of CD367, none of these mutants exhibited reduced transrepressive activities (Figure 2C). More surprisingly, P408GΔ418 showed a strongly reduced anti-AP1 activity, while being a strong transactivator (Figure 2C). This pattern of activity is opposite to that observed with S405AΔ418, which, while being inactive in transactivation assays, showed a wild-type anti-AP1 activity. Thus, CD367 exhibited dissociated anti-AP1 and transactivating activities when tested with several L box mutants, in contrast to atRA which elicited analogous response profiles.

Ligand-Binding Properties of the L Box Mutants. To elucidate which type of interference these mutations could generate with hRARα function(s), we first assayed the affinity of each mutant for both ligands by competition

A)



B)

Ligand / Mutant	CD367[a]	ATRA
hRARα	2.0	6.15
G404P[AF2]	4.6* (÷2.3)	28.8** (÷ 4.7)
S405A[AF2]	0.2** (x10)	1.4* (x 4.4)
M406A[AF2]	4.8* (÷2.4)	7.5
P407G[AF2]	0.4* (x 5)	6.6
P408G[AF2]	0.8	4.4
L409K[AF2]	4.0* (÷2)	8.8
I410Y[AF2]	1.2	16.5* (÷2.7)

FIGURE 3: Ligand binding activities of hRARα L box mutants. (A) Ligands structure: atRA is a natural derivative of vitamin A. CD367 is a synthetic retinoid binding to and activating RARα, *β*, and *γ*. This synthetic compound is characterized, when compared to natural retinoids, by the aromatization of the polyenic chain, restricting its flexibility. Biological activities of these retinoids and derivatives have been described in detail in ref *41*−*44*. (B) Receptor binding activity: K_d values for CD367 and K_i values for atRA were assayed as described in the Materials and Methods and are expressed in nanomolarity. (*) Significant differences vs control ($p < 0.05$). (**) Significant differences vs control ($p < 0.01$). (a) K_d values.

experiments. K_i values are shown in Figure 3B and revealed that, while being detectable, differences in affinity caused by these mutations varied in most cases in the 2−5-fold range. We noted that the impact of some mutations was different on atRA or CD367 affinities. Indeed, mutation of P407 and P408 increased the affinity of CD367 for the receptor by a factor of 5, whereas virtually no effect was seen on the atRA-binding activity. On the contrary, mutation at position 410 decreased the affinity for atRA (3-fold) but was ineffective at altering CD367 binding. Finally, we note that, although having been reported as a discriminant between atRA and 9-*cis*-RA (*27*), M406 has no such contribution to CD367 binding. Thus, while suggesting that subtle differences in the binding mechanism of these ligands may occur, these variations in affinity cannot account for the strikingly different transcriptional properties reported above (Figure 2), since they were observed when using ligand concentration of 1 μM, for which hRARα is saturated. These results suggest also that the overall structure of hRARα is not affected by mutations introduced in the L box.

Mutations in the L Box Alter Structural Transitions Occurring in the N-Terminus of the hRARα LBD. Limited proteolysis of hRARα showed that ligand binding induces conformational changes presumably required for NCoA recruitment and/or NCoR displacement (*9*). The effect of C-terminal deletions and point mutations described above on the three-dimensional structure of the full-length receptor was therefore assessed using trypsin as the cleaving agent (Figure 4). Truncation of the C-terminus of hRARα at

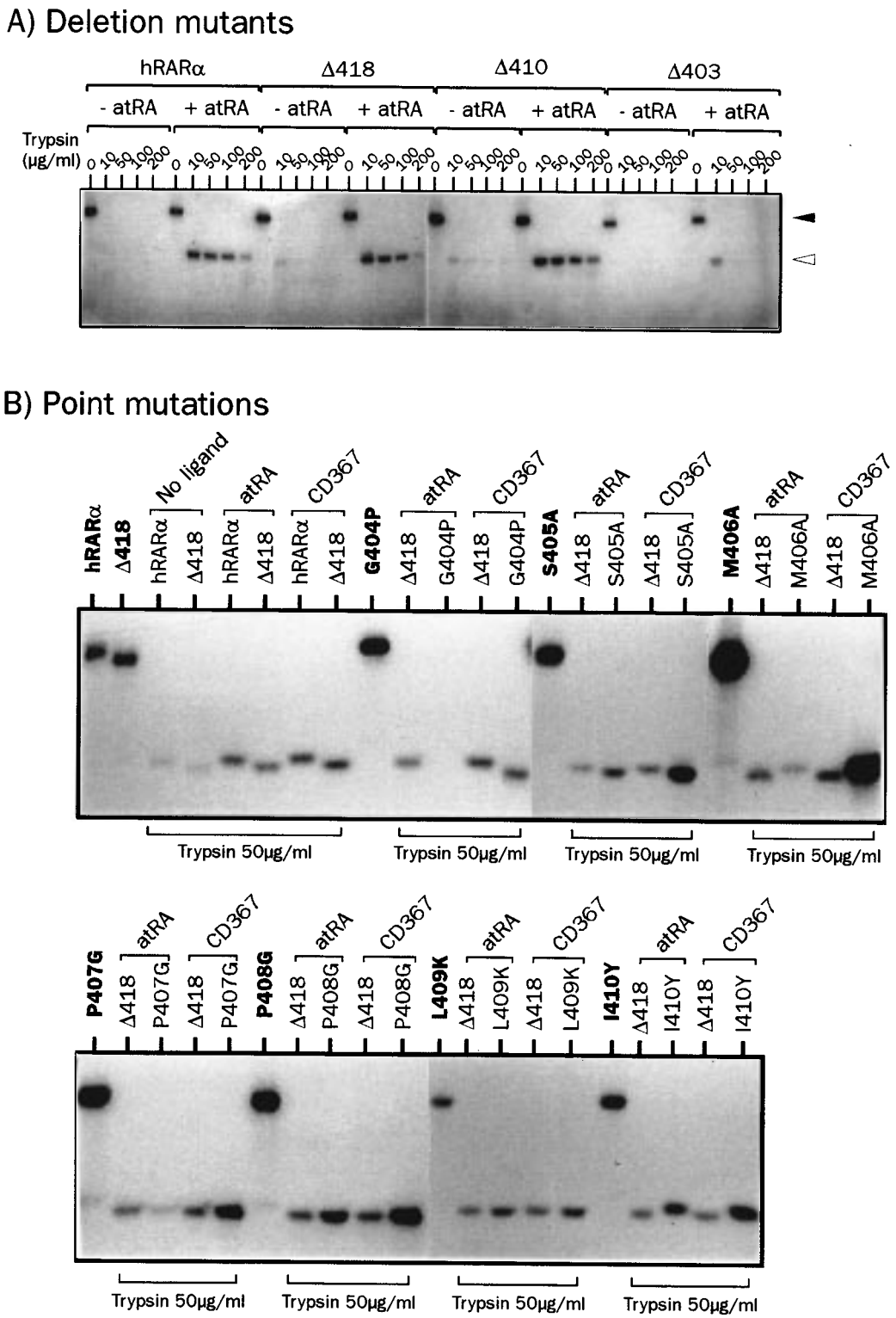


FIGURE 4: Limited proteolysis of hRARα mutants reveals an altered structure of the N-terminus of the LBD. Receptors were obtained by in vitro translation from pSG5-based plasmids encoding either the full length receptor (1−462), C-terminally truncated polypeptides (1−418, Δ418; 1−410, Δ410; 1−403, Δ403), or the Δ418 constructs bearing point mutations described in Figure 1. (A) Tryptic digestion profiles of hRARα deletion mutants. ^{35}S-labeled receptors were incubated in the presence of 1 μM at RA or vehicle (2% DMSO) as described in the Materials and Methods and digested for 30 min at room temperature with increasing amounts of trypsin (0 to 200 μg/mL final concentration). Tryptic products were resolved by 10% SDS−PAGE and visualized by autoradiography. (B) Tryptic digestion profiles of Δ418 L box mutants. Receptors were generated as described above and incubated with either 1 μM atRA or 1 μM CD367. Trypsin digestion were run as above using a concentration of 50 μg/mL. Bold characters indicate native receptors, which all migrated at 50−52 kDa (see text).

residue 418 generated in a ligand concentration-dependent manner (data not shown) a trypsin-resistant polypeptide 1.5 kDa smaller than that obtained from the full-length receptor (compare hRARα lanes to Δ418 lanes in Figure 4B). Since trypsin cleavage sites in the wild-type, native hRARα have been mapped to R432 and within or in the vicinity of the

CoR box (R192, K193, and K207) (*9*), this observation implies that the appearance of the trypsin-resistant fragment reflects conformational changes occurring in the N-terminal part of the LBD of the receptor point mutants, deleted at position 418. Removal of the L box (Δ403) led to the loss of the ligand-dependent resistance to trypsin cleavage (Figure 4A), although this receptor bound atRA with a high affinity, as previously reported (*3*). In contrast, the Δ410 mutant generated a trypsin-resistant fragment, therefore identifying L box residues as critical determinants for this structural transition (Figure 4A).

The influence of point mutations on the ligand-dependent remodeling of the N-terminus of the LBD was assayed by limited digestion of CD367- or atRA-bound receptors. These experiments evidenced both qualitative and quantitative differences in the proteolysis profile of each mutant (Figure 4B). Mutants G404P and S405A migrated consistently faster than the reference receptor Δ418, whereas mutants M406A and I410Y migrated more slowly. These specific electrophoretic mobilities, which are observed in the presence of atRA and CD367 (except for G404P, see below) indicate an increased accessibility of otherwise masked tryptic sites. S405A generated a fragment smaller of about 0.8−1.0 kDa when compared to Δ418. On the basis of the hypothesis that R192 and K193 are major tryptic sites, this suggests that trypsin is now cutting preferentially at K207. Alternatively, this might reflect a higher accessibility of K217. Molecular masses differences could be estimated at 1.6−2.0 kDa for mutants M406A and I410Y, mapping putative tryptic sites to the highly basic nuclear localization signal, located 20 amino acids upstream of R192. Although these distinct proteolysis patterns could also be attributed in principle to a modified accessibility of K and R residues at the C-terminus of hRARα, this seems unlikely since expected molecular masses differences are of at least 2.1 kDa in this region. We also note that introducing a new potential trypsin cleavage site at position 409 (L409K) did not cause detectable change in the electrophoretic mobility of the trypsin-resistant polypeptide, suggesting that this amino acid is poorly accessible in the holo receptor and that this mutation has virtually no effect on receptor conformation.

While our data do not rule out a possibly altered electrophoretic of mutated fragments when compared to the wild-type counterpart, we however do not favor this hypothesis for the two following reasons: (i) high-resolution SDS−PAGE analysis of native receptor mutants did not reveal alteration in their electrophoretic mobilities when compared to the wild-type receptor (data not shown), and (ii) converting S405 or M406 into an alanine yielded proteolytic fragments with opposite electrophoretic properties, with either an increased (S405A) or decreased mobility (M406A).

In the presence of atRA, G404P did not exhibit an increased resistance to proteolysis, although this mutant binds atRA with a high affinity (Figure 3). This property is reminiscent of that of the Δ403 deletion mutant and suggests that this mutant is unable, when bound to atRA, to undergo appropriate structural transitions. This was also observed to a lesser extent for mutant P407G. An increased resistance to proteolysis in the presence of CD367 was noticed for M406A, P407G, and P408G, suggesting that this ligand binds more stably to these particular hRARα mutants or that CD367 binding is less sensitive to proteolytic degradation of hRARα mutants. Taken together, these data show that mutations in the L box are very likely to alter the remodeling of the N-terminus of the LBD and that ligands have varying efficiencies at promoting these structural changes. However, we note that trypsin sensitivity itself did not correlate with transrepression and transactivation properties of hRARα mutants.

Differential Interaction of hRARα Mutants with RIP140 and SMRT. Conformational changes observed upon ligand binding induce AF2-AD (helix 12) and helix1 repositioning. Concomitantly, a functional binding interface for NCoA is generated, and the NCoR-binding domain is disrupted. NCoAs couple ligand-activated NRs to the basal transcription machinery. RIP140, TIF1, and TRIP1/Sug1 interact with NRs in an agonist- and AF2-AD-dependent manner, as well as SRC-1 (*28*), TIF2/GRIP1 (*29*, *30*), and CBP/p300 (*31*), which fulfill criteria defining a NCoA. NCoRs binding to unliganded retinoid receptors is dependent upon the integrity of the CoR box, which is located at the N-terminus of the LBD (*32*) and required for ligand binding (*3*). A protein:protein interaction assay was thus used to test further whether mutations could influence these structural alterations, as suggested by limited proteolysis experiments: GST-SMRT and RIP140 fusion proteins were immobilized on glutathione-agarose beads and incubated with in vitro labeled receptors, in the presence of atRA or CD367 (Figure 5).

The observation that point mutations in the L box altered the LBD N-terminus structure (Figure 4) was of interest because it identified this region as a critical determinant of the holo-LBD structure. Protein:protein interactions in this region have been shown to be highly sensitive to structural alterations, and mutations that affect neither ligand binding, DNA binding, nor transactivation inhibited N−CoR binding to hRARα (*33*) and references therein]. The ligand-induced release of point-mutated hRARα from a GST-SMRT fusion protein was thus monitored when challenged with atRA or CD367. As expected, the full-length, wild-type receptor released SMRT very efficiently when complexed to CD367 or atRA, as well as CD367- or atRA-bound Δ418 deletion mutant, albeit, it exhibited a somewhat decreased efficiency (Figure 5). CD367 appeared to be generally more efficient at promoting SMRT release than atRA: this differential efficiency was clearly emphasized for M406A, L409K, and I410Y mutations. The M406AΔ418 and I410YΔ418 mutants were unable to release SMRT when bound to atRA, but not to CD367, and we noted also that the level of interaction of SMRT with G404PΔ418 was very weak. A clear ligand-dependent interaction of the full-length hRARα with RIP140 was observed with both agonists, whereas the reference receptor Δ418 displayed a strongly diminished affinity for this protein (Figure 5). This suggests that RIP140 binding is, in these experimental conditions, conditionned by the integrity of the C-terminus of hRARα. Most importantly, point mutations had a differential impact on RIP140 recruitment by CD367- or atRA-bound receptors. The G404P mutation abolished RIP140 recruitment, whereas mutating residues 406−408 led to a stronger interaction in the presence of CD367, albeit with varying efficiencies. In this respect, we noted that RIP140 bound very efficiently to mutant P408GΔ418. In opposition, I410Y and, to a lesser extent, S405A favored RIP140 binding in the presence of atRA. The affinity of liganded receptors for CBP varied in

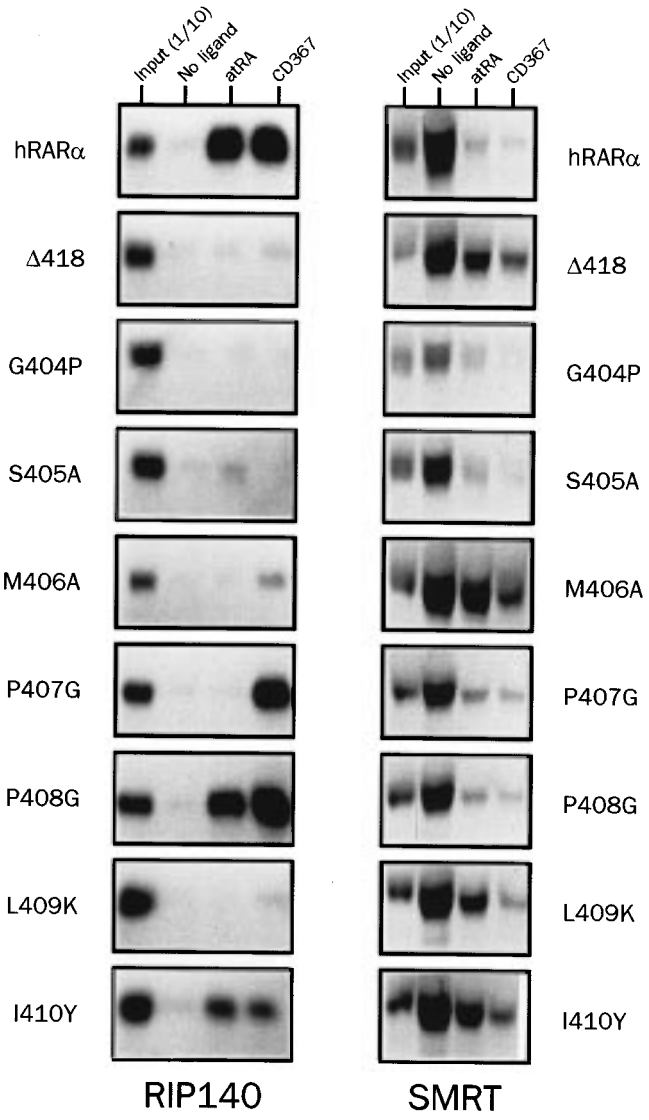


FIGURE 5: Ligand structure modulates SMRT and RIP140 binding to hRARα through the L box. (Right panel) Interaction between hRARα mutants and the NCoR SMRT. Complexes were formed between a GST fusion protein with AAs 982−1495 from SMRT and ^{35}S-methionine labeled receptors. The amount of receptor bound to SMRT in the presence of 1 μM atRA, 1 μM CD367, or vehicle (ethanol, lanes "no ligand") was assayed by 8% SDS−PAGE followed by autoradiography. (Left panel) Interaction between hRARα mutants and the NCoA RIP140. The ability of RIP140 to associate with receptors in the presence of 1 μM atRA or CD367 was monitored as described in Materials and Methods. Complexes were formed between a GST fusion protein with site II of RIP140 (AAs 683−1158) and ^{35}S-methionine labeled receptors.

a strictly similar manner, indicating that these two coactivators interacts with the same interface (data not shown). These results demonstrate that the structure of the ligand affects directly AF2-AD positioning, which in turn modulates NCoA recruitment.

Taken together, the results described above demonstrate that mutations in the L box not only affect AF2-AD positioning, but also modulate the structure of the N-terminal NCoR-binding interface of hRARα LBD, all of these effects being modulated by ligand structure. However, as discussed below, no correlation between these in vitro protein:protein interaction assays and transcriptional activities is observed, as suggested by the nearly wild-type transrepressing and transactivating properties of the Δ418 mutant, which is, however, unable to bind to CBP and RIP140 in these conditions.

DISCUSSION

This study aimed at deciphering intramolecular processes leading to hRARα activation in response to natural or synthetic retinoids and to provide evidence for distinct modes of activation by structurally different retinoids. Ours and other reports (*3*, *27*, *34*) suggested that the H11−H12 loop (L box) of hRARα could be involved in the differential activation of the retinoid-signaling pathway upon binding of natural or synthetic retinoids to overlapping, but not identical ligand-binding pockets. For this purpose, we have examined by site-directed mutagenesis the role of the L box structure in (i) transcriptional activities, (ii) ligand binding, and (iii) ligand-induced structural transitions of hRARα as well as interactions with cofactors. We compared systematically the effect of mutations in the presence of the natural ligand, atRA, to that observed in the presence of a synthetic retinoid, CD367. Despite similar receptor-binding properties, these two ligands elicited distinct biological responses when used to challenge L box receptor mutants.

The trans-repression of AP1 transcription by retinoids is thought to be caused, at least in part, by competition for CBP/p300 by liganded receptors with AP1 (*35*). To investigate the role of the L box in CBP/p300 recruitment in vivo, we compared the anti-AP1 activity of each L box mutant in the presence of atRA and CD367. We observed a striking parallel between atRA-induced transrepression and transactivation, thereby strengthening the hypothesis of CBP/p300 as a relevant molecular relay of atRA-activated hRARα. If transactivation reflects CBP/p300 recruitement, then CD367-induced transactivating and transrepressive activities of each mutant should fluctuate in parrallel, as observed with atRA. An unexpected uncoupling of transrepressive and transactivating properties of mutants challenged with CD367 was observed: while mutant P408GΔ418 appeared to be fully active in transactivation assays, it displayed a reduced anti-AP1 activity. Furthermore, mutation at position 405 generated a receptor with features opposite to those of P408GΔ418: while displaying a wild-type anti-AP1 activity, this mutant was inactive in transactivation assays, therefore converting CD367 into a retinoid with only anti-AP1 activity. A RAR antagonist was unable to promote CBP binding to hRARα and to induce transcriptional activation (*35*), leading to the prediction that the transcriptionally inactive G404PΔ418 and L409KΔ418 mutants should display, when challenged with CD367, a strongly reduced transrepressive activity in the presence of CD367. However, both exhibited wild-type transrepressive activities. There are several possibilities to account for observed effects: CD367-mediated transactivation can either proceed through an alternative, CBP/p300-independent pathway, or CD367 can interfere with AP1 activity in a CBP/p300-independent manner. This latter hypothesis has been recently documented and attributed to the inhibition of the jun kinase (JNK) pathway (*36*). Alternatively, free and DNA-complexed receptors adopt distinct conformations when bound to this ligand, yielding a receptor able to recruit CBP/p300, albeit in a configuration not favorable for transcription. The L box also appeared to be critical in determining how a ligand functions in transcrip-

tion: indeed, CD367 displayed inverse agonist properties when bound to mutant G404PΔ418 and L409KΔ418. Interestingly, point mutations in ER at L540, analogous to hRARα I410, led to a similar alteration in ligand functionality of this receptor (*37*).

Distinct structural transitions of the holo-receptor were also evidenced in the presence of structurally different ligands. The observation that several point mutations in the L box led to a nontypical endopeptidase digestion pattern indicated that alterations of the structure in this region modulate the LBD N-terminus structure. This hypothesis was directly tested by a protein−protein interaction assay using SMRT, a transcriptional NCoR binding to the CoR box of unliganded RARs and T_3R (*11*). Our current study showed that most of the L box mutants were affected in their ability to release, but not to bind to, the corepressor SMRT. However, we note that the unliganded G404 mutant associates less efficiently to SMRT (Figure 5), and that hRARαΔ403 is unable to release SMRT (*11*). Thus, we infer that the structural integrity of the L box is required for both binding and ligand-induced release of this corepressor in vitro. The ligand-dependent structural transition of H1 thus appeared to be controlled by key residues of the L box. In this respect, we note that mutations at M406 and I410, which have their side chains pointing toward atRA in the holo-receptor, severely impeded atRA-induced release of SMRT. On the contrary, CD367 promoted the release of this corepressor with a similar efficiency.

CBP and RIP140 are two transcriptional NCoAs that associate with ER and RARs in an AF2-AD and agonist-dependent manner (*22*, *30*). We found that RIP140 and CBP binding to hRARα in vitro was strongly affected by truncation at position 418, suggesting that domain F is an essential component of the binding interface in this configuration. We also found that the Δ418 wt receptor exhibited functional properties of the full-length receptor, yet it did not bind these two NCoAs in the same manner in vitro. While it may suggest that these factors are unlikely to play a significant role in the transactivation and transrepression processes reported here, it seems more likely that assessing protein:protein interactions in solution does not reflect the actual architecture of heterodimers complexed to DNA and to the transcriptional machinery. In addition, the proteins used in our protein:protein interaction assays are reduced to the receptor-interacting domain, thereby obliterating the contribution of other domains of the coactivator to the overall stability of the complex. Thus, these assays only reflect alteration of the three-dimensional structure of the receptor and not their transcriptional properties. Some of the mutants were able to compensate the loss of function observed with the Δ418 wt receptor (see M406A, P407G, P408G, and I410Y). Very interestingly, this compensation appeared to be ligand specific, except for I410Y, demonstrating that the L box is involved in specifying the formation of NCoAs binding interface, which is dependent on ligand structure.

The structural transition leading to NCoAs recruitment has been proposed to be coincidental with the structural inactivation of the NCoRs interaction surface, which is a consequence of helix H1 dislodging from the HBD core (*38*). The folding back of the AF2-AD could thus play a direct role in NCoR release, since truncation at position 403 abolished the ligand-dependent release of SMRT (*39*). However, our data show that there is no correlation between the formation of a given NCoA-binding interface and SMRT release in this configuration. Indeed, while atRA was unable to promote RIP140 binding to P407G Δ418, we found that this ligand promoted very efficiently SMRT release from the same polypeptide. This decoupling was also observed for S405AΔ418 and L409KΔ418, and we noted that there was no correlation between the capacity of a given ligand to promote RIP140 recruitment and SMRT release. This suggests that allosteric changes are propagated through the polypeptidic chain and dictated by the ligand structure. Interestingly, Privalski and co-workers (*40*) showed that T3R P453, equivalent to hRARα P408, is a key residue controlling SMRT release from T3R, consistent with the hypothesis of a function for this hinge region shared by several nuclear receptors. Our in vitro data thus established clearly that these two structural transitions (helices H1 and H12 repositioning) are independent and conditionned by ligand structure, although they are not representative of protein:protein interactions occurring in the assembled transcriptional complex. The lack of quantitative correlation between NCoA binding and transcriptional activities of mutant receptors underscores the complexity of the regulation of hRARα structure and indicate that these in vitro studies must be viewed only as a means to investigate structure−function relationships of the hRARα activation domain 2.

Our present results therefore suggest that the LBD of hRARα can adopt distinct conformations which are controlled through the L box, and that ligand structure is a critical parameter for controlling structural transitions occurring in the hRARα LBD. They also raise an intriguing question about the mechanism by which synthetic ligands may induce retinoid-dependent transcription and cellular processes such as apoptosis and differentiation, an issue currently under investigation in our laboratory.

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