Engineering novel specificities for ligand-activated transcription in the nuclear hormone receptor RXR

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Background: The retinoid X receptor (RXR) activates transcription of target genes in response to its natural ligand, 9-*cis* retinoic acid (9cRA), and a number of RXR-specific synthetic ligands. To discover the potential for engineering nuclear receptors for activation of transcription by novel ligands, we used structure-based mutagenesis to change the ligand specificity of RXR.

Results: By making substitutions at only two positions (Phe313 and Leu436) we engineered two new classes of RXR proteins that had altered ligand specificities. The first class exhibits decreased activation by 9cRA and increased activation by synthetic ligands. The second class continues to be activated by 9cRA but no longer responds to synthetic ligands. The magnitude of the change in specificity that can be accomplished is greater than 280-fold.

Conclusions: These results confirm that Phe313 and Leu436 are crucial determinants of ligand specificity for RXR and demonstrate that nuclear receptors are exceptionally promising protein scaffolds for the introduction of novel ligand specificities through structure-based protein engineering.

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Introduction

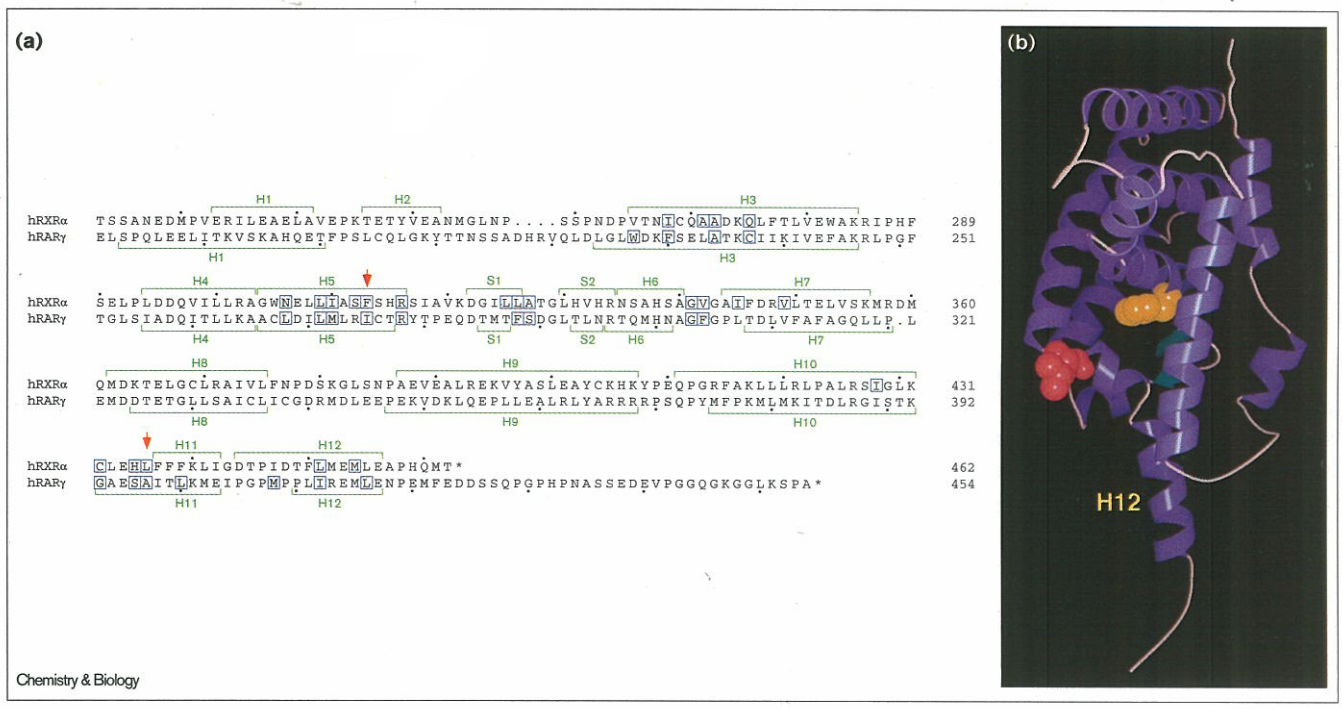
Nuclear receptors are a superfamily of ligand-inducible transcription factors that control a broad range of physiological processes [1,2]. These modular proteins contain an evolutionarily conserved DNA-binding domain composed of two zinc fingers and a ligand-binding domain (LBD) containing a common fold that creates a conserved ligand-binding pocket [3]. The known ligands for these receptors are chemically diverse, including steroid and thyroid hormones, vitamin D, prostaglandins, fatty acids, eicosanoids and retinoids. Evolutionarily closely related receptors (e.g., thyroid hormone receptor and retinoic acid receptor, RAR) bind different ligands, whereas some members of distant subfamilies (e.g., retinoid X receptor, RXR, and RAR) bind the same ligand [4]. This diversity of high-affinity ligand–receptor interactions demonstrates the versatility of the fold for ligand binding and suggests that it should be possible to engineer LBDs with a wide range of novel specificities.

Among nuclear hormone receptors, altered activation in response to ligand occurs in natural and directed mutants. Naturally occurring point mutants of nuclear receptors that alter responses to natural ligands and result in diseased states have been well characterized. For example, the Thr877→Ala mutation in the androgen receptor, associated with pharmaco-resistant prostate cancer, results in a variant that is able to bind and activate in response to progesterone, estradiol and anti-androgens [5]. Site-specific mutants made in attempts to understand receptor function have also shown changes in specificity. Examples include receptors for glucocorticoids [6,7], estrogen [8–10], thyroid hormone [11], retinoic acid [12,13], androgen [14] and progesterone [15].

Altered specificities for ligand-dependent activation not only reveal insight into the structural basis of specificity and the origin of disease states, they may also allow development of new tools for controlling genetic and metabolic processes. For example, the ecdysone receptor from *Drosophila* has been introduced into mice for targeted gene disruption in response to ecdysone, a hormone not found in mammals [16]. Similarly, a mutant estrogen receptor that is not activated by estrogen but is activated by tamoxifen, an anti-estrogen, has been used to selectively control expression of target genes in mammalian cells [17]. The ability to generate receptors activated by selected ligands would expand the applications for metabolic and genetic control.

Development of such receptors is facilitated by the recent availability of high resolution crystal structures of nuclear receptor LBDs [18–20]. We chose the nuclear hormone receptor RXR as a target for structure-based engineering to alter the ligand dependence of transcriptional activation. RXR is able to function as a silent, non-ligand-binding partner in a heterodimer with other nuclear receptors, and can also function as a ligand-binding homodimer [21,22]. The functional homodimer

Figure 1



The structure of hRXRα. **(a)** The primary structures of the ligand-binding domains of hRXRα and hRARγ. Sequences were aligned on the basis of observed secondary structure in the crystal structures [3]. Residue numbers are indicated on the right. Red arrows indicate the residues F313 and L436 in hRXRα substituted in this study. Boxed residues in blue denote residues predicted to contact 9cRA [3,18]. Helices 1 to 12 (H1–H12) and β strands (S1–S2) are indicated in green. Asterisks denote the carboxyl termini. **(b)** A ribbon diagram showing apo-hRXRα secondary structure. The coordinates are from Bourguet *et al.* [18] (Brookhaven Protein Database entry '1LBD'). Residue F313 (yellow) is deep within the structure and forms part of the ligand-binding pocket. Residue L436 (orange/pink) is located on the surface and exposed to solvent. Helix 12 is predicted to be repositioned over the binding pocket in the active form putting L436 in contact with the ligand [3].

binds to RXR response elements and activates transcription in response to its natural ligand 9-*cis* retinoic acid (9cRA). RXR's ability to function as a homodimer, its nuclear localization, the availability of a high-resolution crystal structure [18], and a range of synthetic agonists and antagonists make it an ideal candidate for the introduction of novel specificities by structure-guided protein engineering.

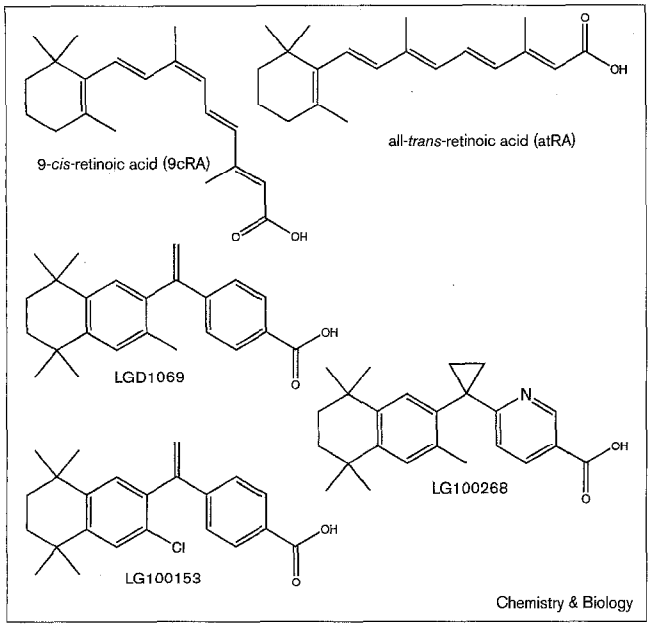
From a comparison of the X-ray crystal structures of apo-hRXRα LBD and holo-all-*trans* retinoic acid (atRA)-hRARγ LBD, Moras and colleagues suggested that the residues Phe313 (F313) and Leu436 (L436) in hRXRα (Figure 1a,b) are important determinants of ligand specificity [3,18,19]. We show here that by substituting a range of amino acids for F313 and L436, both singly and in combination, two classes of receptors with novel ligand specificities are generated. The first class displays a shift in specificity for transcriptional activation of up to 40-fold away from the natural hormone 9cRA and towards the synthetic ligands. The second class displays a change in specificity greater than 280-fold away from the synthetic ligands. These results illustrate the exceptional plasticity of nuclear hormone receptors for engineering altered ligand specificities.

Results

Mutagenesis, expression and analysis of variant hRXRα proteins

Site-directed mutagenesis was used to substitute various uncharged amino acids for residues F313 and L436 (Figure 1a), either singly or in combination. The variant proteins were analyzed in mammalian cells for the ability to activate transcription of an RXR response element linked to a luciferase reporter gene in response to the natural hormone 9cRA and the RXR-specific synthetic agonists LGD1069, LG100153 and LG100268 [23,24]. The structures of these compounds are shown in Figure 2. Western analysis of transfected mammalian cells indicated that all variants were expressed at similar levels (data not shown). We also analyzed the variant proteins for their ability to bind 9cRA and LGD1069 *in vitro* using bacterially expressed protein. All wild-type EC_{50} and K_d values (Tables 1 and 2) are similar to those previously reported for hRXRα [25,26].

Figure 2



Structures of natural and synthetic RXR ligands.

Effect of Phe313 substitutions on specificity

We first altered residue F313 in isolation to examine its role in modulating ligand specificity. Figure 3 shows representative dose–response curves used to determine the ligand concentration required for half-maximal luciferase activity (EC_{50}) and the relative efficacy of each ligand (Table 1). The most conservative hRXRα substitution, Phe313→Ile (F313I), produces one of the most dramatic shifts in ligand specificity (Figure 4). Compared to the wild-type receptor, the F313I variant exhibits a significant increase in EC_{50} for 9cRA (greater than fivefold, from 100 nM to ≥500 nM) and a decrease in EC_{50} for LGD1069 (eightfold, from 20 nM to 2.5 nM), resulting in at least a 40-fold change in specificity in favor of the synthetic ligand. The change in specificity between 9cRA and LG100153 or LG100268 is 25-fold and 5-fold, respectively, toward the synthetic ligands. Although less dramatic, the Phe313→Ser (F313S) variant displays a similar change in specificity towards the synthetic ligands and away from 9cRA. The Phe313→Val (F313V) and Phe313→Ala (F313A) variants activate poorly with all of the ligands tested (Table 1).

The F313I variant displays increased efficacies with all of the ligands and decreased EC_{50} values for LGD1069 and LG100153. In particular, with LGD1069 the maximal activation for this variant is almost twice that achieved with wild-type (119% compared to 65%, Table 1, Figure 3b). With 9cRA, the F313I variant has a greater EC_{50} and efficacy than wild-type. The result is a steeper slope in the dose–response curve (Figure 3a), possibly due to enhanced cooperativity in ligand binding, DNA binding, multimerization, or the assembly of transcription machinery. The other substitutions for F313 (valine, serine and alanine) result in basal transcription at least twice that of wild-type (Figure 3c). Ligand-dependent increases in transcription are generally preserved in F313 variants (except for F313V with LG100268 and F313A with LGD1069 and LG100268), although efficacy is reduced. The high basal activity is suppressible to wild-type basal level upon addition of 10^{-5} M LG100754, an antagonist of RXR homodimers (Figure 3c) [27].

Table 1

Activation of transcription by wild-type and variant hRXRα proteins.

	9cRA		LGD1069		LG100153		LG100268	
Variant	EC_{50}* (nM)	Eff† (%)	EC_{50} (nM)	Eff (%)	EC_{50} (nM)	Eff (%)	EC_{50} (nM)	Eff (%)
WT	100	100	20	65	20	77	4	61
F313I	≥500	123	2.5	119	4	106	4	70
F313V‡	≥1000	46	>10,000	12	>10,000	22	>10,000	
F313S‡	≥800	65	9	55	10	94	100	46
F313A‡	≥1000	37	>10,000		>10,000	19	>10,000	
L436F	≥900	64	>10,000		>10,000		>10,000	
L436V	≥1000	48	200	33	>500	39	100	21
L436S	>10,000		>10,000		>10,000		>10,000	
L436A	>10,000		>10,000		>10,000		>10,000	
F313I/L436F	3000	101	>10,000		>10,000		>10,000	
F313I/L436V	≥2000	36	30	68	50	75	150	39
F313I/L436S	>10,000		>10,000		>10,000		>10,000	
F313S/L436S	>10,000		>10,000		>10,000		>10,000	
F313S/L436V	ND	53	>10,000		>10,000		>10,000	

*EC_{50}, concentration of ligand producing half maximal activity, ≥ indicates that a saturable maximum response is not reached (for wt with 9cRA, 10^{-5} M is assumed to produce maximal activation). Values represent averages of at least two measurements in triplicate. †Eff, efficacy, maximum increase in activation relative to the increase in activation of wild type with 9cRA. Values represent averages of at least two measurements in triplicate. ‡These variants have a basal activity >twofold above wild type; ND, not determined.

Table 2

Equilibrium binding constants of wild-type and variant hRXRα proteins.

Variant	9cRA K_d (nM)*	LGD1069 K_d (nM)
WT	13 ± 3	36 ± 12
F313I	NB	7 ± 1
F313V	W	ND
F313S	W	26
F313A	W	42
L436F	40 ± 15	W
L436V	30 ± 5	51 ± 42
L436S	NB	NB
L436A	NB	ND
F313I/L436F	NB	28 ± 9
F313I/L436V	NB	18 ± 5
F313I/L436S	NB	NB
F313S/L436S	NB	ND
F313S/L436V	W	ND

*K_d values determined from Scatchard analysis, values represent averages of at least two measurements in triplicate ± standard error. Where no uncertainties are indicated, values are the K_d of a single experiment with triplicate determinations. NB, no specific binding detected at ligand concentrations up to 40 nM; W, weak, unsaturable specific binding observed at ligand concentrations ≤ 40 nM; ND, not determined.

The specificity changes for the F313 variants correlate with changes in ligand binding affinity *in vitro*. Figure 5 shows representative binding curves used to determine equilibrium dissociation constants (K_d). The results are summarized in Table 2. The F313I and F313S variants display much lower affinity for 9cRA (Table 2) in agreement with their increased EC_{50} values for transcription upon activation by 9cRA (Figure 3a, Table 1). Conversely, these variants exhibit decreased K_d values for LGD1069 (Table 2) that correspond to decreased EC_{50} values for activation by LGD1069. Although the F313I variant is activated by 9cRA, no binding of 9cRA *in vitro* is detected at concentrations up to 40 nM. This observation is not surprising, because at 40 nM 9cRA concentration in the dose response curve (Figure 3a) activation is

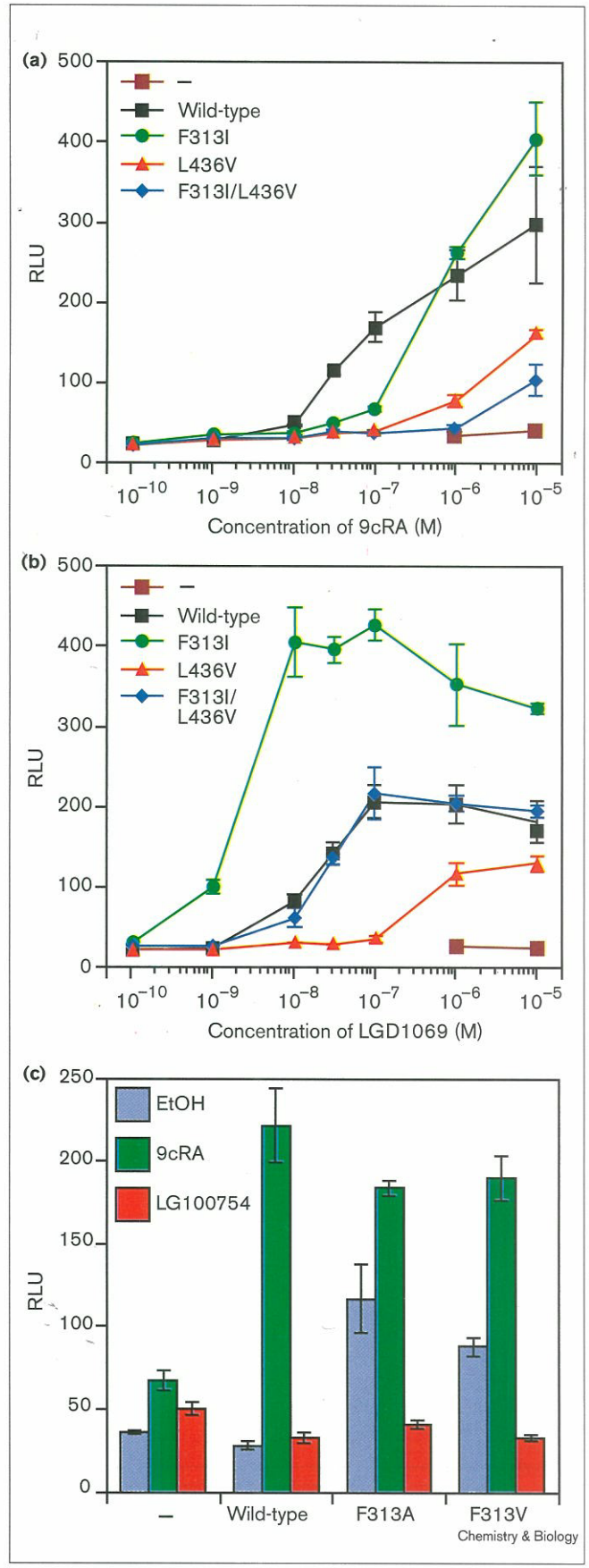
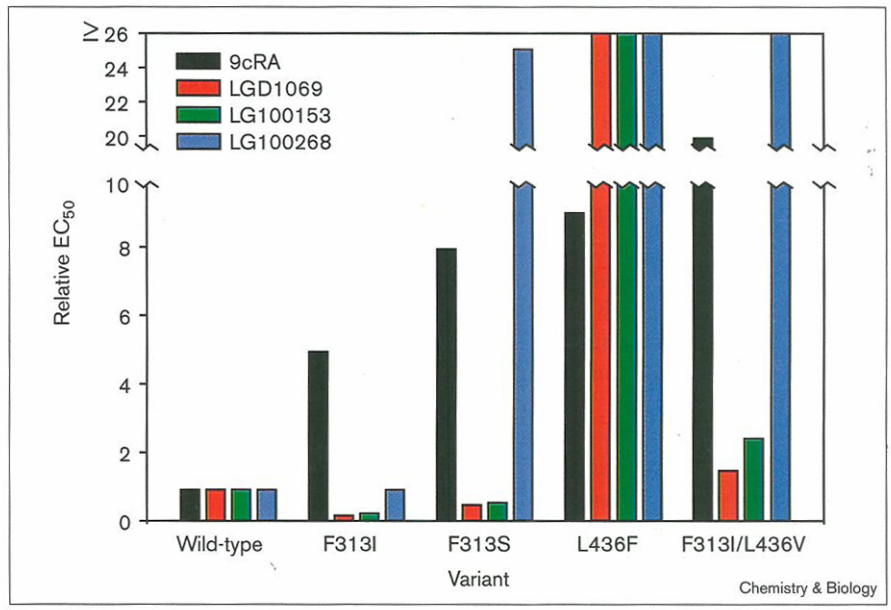


Figure 3

Activation of transcription of wild-type hRXRα and variants. CV-1 cells were cotransfected with either a control plasmid (–), or an hRXRα expression plasmid together with the pTK-CRBPII-LUC reporter plasmid, and then incubated with various concentrations of ligand. Activation of the luciferase reporter gene is measured in relative light units (RLU) and plotted against concentration of ligand. Symbols and bars represent the mean of triplicate determinations and error bars indicate the standard deviation. Representative dose–response curves show changes in ligand specificity between **(a)** 9cRA and **(b)** LGD1069. **(c)** Variants displaying high basal levels of transcription. Concentrations of 9cRA and LG100754 are 10^{-5} M.

Figure 4

Relative EC_{50} for wild-type hRXRα and variants showing changes in specificity. Plotted values are relative to the EC_{50} of wild-type for each ligand. Relative EC_{50} = variant EC_{50}/wild-type EC_{50}.



only 5% of maximal activation compared to 40% for the wild-type receptor.

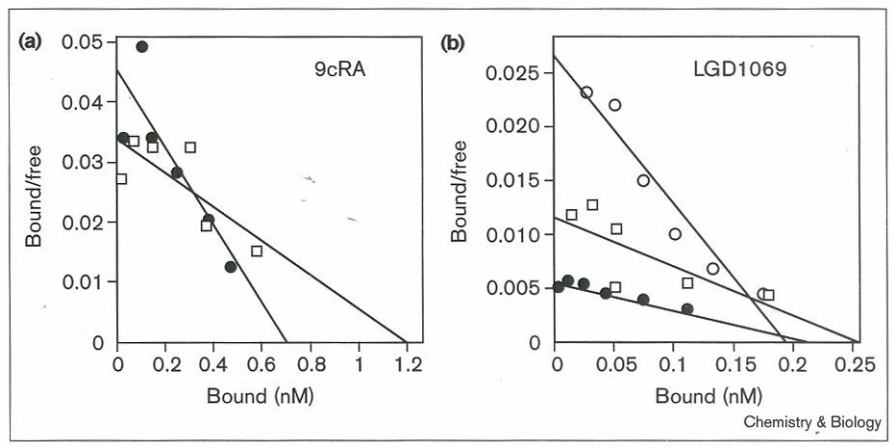
Effect of Leu436 substitutions on specificity

We next analyzed the hRXRα residue Leu436 (L436) in isolation to examine its role in modulating ligand specificity. The only L436 substitution to show a change in specificity is L436F (Table 1). In contrast to the F313 variants, this alteration adjusts specificity away from the synthetic ligands. Compared to the wild-type receptor, the L436F variant exhibits a ninefold increase in EC_{50} for 9cRA and abolishes activation by LG100268 (EC_{50} > 10,000 nM compared to 4 nM for the wild-type receptor, corresponding to a relative increase > 2,500-fold), resulting in a > 280-fold change in specificity in favor of the natural ligand. The most conservative substitution, L436V, resulted in reduced activation with all ligands but no major change in specificity, whereas the more radical substitutions (L436A and L436S) abolished activation with all of the ligands.

Two substitutions for L436, phenylalanine and valine, cause a moderate (twofold to threefold) increase in K_d for binding 9cRA (Table 2) which is in contrast to the large decreases in their activation of transcription (Table 1). This result suggests that for these variants ligand binding has been partially decoupled from efficient transcriptional activation. The two smaller substitutions for L436 (alanine

Figure 5

Representative Scatchard plots used to determine K_d. **(a)** ^{3}H-9cRA with wild-type hRXRα (●, K_d = 15.4 nM, r = 0.86) and L436V (□, K_d = 34.3 nM, r = 0.79). **(b)** ^{3}H-LGD1069 with wild-type hRXRα (●, K_d = 37.3 nM, r = 0.997), L436V (□, K_d = 21.3 nM, r = 0.82) and F313I (○, K_d = 7.2 nM, r = 0.97). Symbols represent the mean of triplicate determinations.



and serine) are unable to bind 9cRA or LGD1069 at concentrations up to 40 nM.

Effect of simultaneous Phe313 and Leu436 substitutions

Finally, we combined F313 and L436 substitutions to examine their cumulative effects on RXR ligand specificity. The hRXRα double variants F313I/L436F and F313S/L436V show a change in specificity in favor of 9cRA, similar to the L436F variant (Table 1, Figure 4). F313I/L436F is unable to activate in response to any of the synthetic ligands, but activates transcription with wild-type efficacy at high concentrations of 9cRA. F313S/L436V exhibits a similar preference for activation by 9cRA relative to the synthetic ligands, although maximal activation by 9cRA is lower than for F313I/L436F. In direct contrast, the F313I/L436V variant shows a change in specificity toward the synthetic ligands, similar to the F313I variant (Table 1, Figure 4). The F313I/L436V variant exhibits a significant increase in EC_{50} for 9cRA (at least 20-fold, from 100 nM to >2000 nM) while maintaining a similar EC_{50} for LGD1069 (from 20 nM to 30 nM), resulting in at least a 13-fold change in specificity toward the synthetic ligand.

The effects of the individual substitutions on ligand binding combine to produce intermediate effects in the double variants. For example, the variants F313I and L436V have K_d values of 7nM and 51 nM with LGD1069, and for F313I/L436V the K_d is 18 nM (Figure 5, Table 2). For transcription, in all cases where a single substitution abolishes activation by a particular ligand, double variants containing this substitution also do not activate (Table 1). Activation of F313I/L436V by LGD1069 occurs with an EC_{50} of 30 nM, intermediate between the 2.5 nM EC_{50} of the F313I variant and the 200 nM EC_{50} of the L436V variant. The same substitutions do not yield an intermediate dose–response curve for 9cRA activation (Figure 3a,b, Table 1).

The F313I/L436V variant contains residues identical to those in equivalent positions of hRARα. Examination of the crystal structures suggested that these two residues would be important determinants for the difference in ligand specificities of RXR and RAR, in particular, the ability of RAR to activate in response to atRA [3]. To investigate whether activation by ligands of RAR had been conferred upon this variant, we analyzed activation by the RAR-specific ligands atRA, ALRT1550 [28], and TTNPB [29]. No activation was observed for F313I/L436V or any of the other variants (data not shown), nor was any specific binding of [^{3}H] atRA detected *in vitro* by F313I, L436A, F313I/L436F or F313I/L436V. Therefore, while F313 and L436 are important determinants of RXR specificity, they are not the sole determinants for discrimination between RAR and RXR ligands.

Discussion

Nuclear receptor specificity is highly adaptable

Attempts to engineer changes in protein function by site-directed mutagenesis have often yielded limited success. Trypsin, for example, an enzyme that has been used as a paradigm for engineering specificity [30], requires 10 to 12 substitutions to combine acquisition of chymotrypsin-like specificity with retention of significant levels of catalytic activity [31]. Changing the substrate specificity of chymotrypsin has been even more difficult to achieve [32]. The specificities of other serine proteases, including subtilisin and alpha lytic protease, are more amenable to engineering [33,34]. The greater ability to alter substrate recognition in the latter cases has been attributed to enhanced structural plasticity caused by the greater involvement of amino acid sidechains, as opposed to backbone contacts, in substrate recognition.

Examination of recently solved X-ray crystal structures of apo-hRXRα, holo-hRARγ and holo-rat thyroid hormone receptor α_1 ligand-binding domains suggested that nuclear hormone receptors are dependent on numerous sidechain–ligand contacts and might be readily adapted for novel ligand-binding specificities [3,18–20]. Our finding that specificity can be readily altered by substitutions at positions 313 and 436 confirms that structure-based mutagenesis can lead to novel specificity profiles for nuclear hormone receptors. It further suggests that engineering specificity will be more similar to engineering subtilisin and alpha lytic protease than to trypsin and chymotrypsin. Additionally, these changes were achieved by substituting only 2 of 22 amino acids predicted to make contact with the ligand (Figure 1a), suggesting that an even wider range of changes in specificity are possible.

Structural origins for specificities of Phe313 variants

From the X-ray crystal structures of apo-hRXRα [18], holo-hRARγ [19] and holo-thyroid hormone receptor [20], it has been predicted that, upon binding of an agonist, a conformational change in the LBD to the active form occurs [3]. The mechanism of this change implies that some residues are essential for initial ligand recognition and binding affinity, while others interact with bound ligand to produce the conformational change necessary for activation.

We find that substitutions at position 313 predominantly affect ligand binding (and therefore necessarily affect activation). In the crystal structure of apo-hRXRα residue F313 is buried within the hydrophobic ligand-binding pocket and projects into the cavity (Figure 1b). On the basis of the model for 9cRA-bound hRXRα generated using the holo-hRARγ as a guide, F313 is believed to interact directly with 9cRA [3]. Its position in helix 5 suggests that it is unaffected by the conformational changes that occur upon ligand binding, and is more important for ligand-binding affinity rather than for the structural transition necessary for

activation. This suggestion is supported by our data which demonstrate that any substitution for residue F313 results in a large reduction in binding affinity for 9cRA (Figure 5, Table 2) and an increased EC_{50} (Figure 4, Table 1). However, the variants F313I and F313S retain the ability to bind the synthetic ligands with a high affinity and activate transcription with EC_{50} values similar to the wild-type receptor. Furthermore, the variant F313I has at least fivefold lower EC_{50} values than wild-type in response to LGD1069 and LG100153. Therefore, F313 has a crucial role in activation by influencing binding affinity, and interacts differently with different ligands to determine specificity.

The elevated levels of constitutive activation observed with F313V, F313S and F313A suggest that these substitutions mimic the binding of agonists by stabilizing the active conformation of the receptor. This mechanism is supported by the ability of LG100754, an antagonist of RXR homodimers [27], to repress the elevated constitutive activity (Figure 3c). Mutations in the estrogen receptor, located immediately amino-terminal to the putative helix 12, also display similar elevated levels of constitutive activity [35,36]. Interestingly, this constitutive activity could also be repressed by estrogen receptor antagonists. The close proximity of these residues in the estrogen receptor to helix 12, crucial for ligand-dependent activation, were thought to directly affect its conformation and hence the activity of the receptor. However, F313 in hRXRα is located in helix 5 (Figure 1a,b), a region believed to undergo no major conformational change in response to ligand binding [3,19].

Structural origins for altered specificities by Leu436 variants

Substitutions at position 436 affect either activation alone or, for the smaller substitutions, both binding and activation. The residue L436 in hRXRα immediately precedes the amino terminus of helix 11 (Figure 1a). In the apo-hRXRα crystal structure, L436 projects away from the ligand-binding cavity (Figure 1b). Upon ligand binding, it is thought to be repositioned so it can make direct contact with 9cRA, equivalent to A397 in hRARγ. The variants L436F and L436V have a minor effect on the binding affinity of the receptor for 9cRA (Table 2, Figure 5), yet have greatly decreased transcriptional activation (increased EC_{50} values and decreased efficacies) with all of the ligands (Table 1). This suggests that L436 is more important for stabilizing the conformationally active form of the receptor rather than for high-affinity ligand binding and specificity. However, when L436 is substituted for smaller residues such as serine or alanine, high-affinity ligand binding with 9cRA and LGD1069 and activation with all ligands are abolished. Although it is possible that these variants are inactive because they are unable to fold correctly, this result may indicate that L436 still has a role in determining binding affinity. Substitutions of the equivalent residues in the glucocorticoid and androgen receptors also result in altered specificities [5,37].

Significance

We have used structure-based protein engineering to generate two classes of retinoid X receptors (RXRs) that differ in their specificities for transcriptional activation by varied ligands, and we have confirmed that residues Phe313 and Leu436 have important roles in defining RXR specificity. The first class of RXR variants shows a shift in specificity up to 40-fold away from the natural hormone 9-*cis* retinoic acid (9cRA) and towards the synthetic ligands. The second class has a change in specificity greater than 280-fold in favor of 9cRA. The changes in the specificity for ligand-dependent activation have been achieved through altering EC_{50} values, efficacies, and binding affinity. Each of the individual ways that the transactivation profiles have been altered can be exploited in engineering regulation of transcription. Increased binding affinity for synthetic ligands can be used to enhance activation at low ligand concentrations, while altered specificity, both towards and away from the natural hormone, allows the tailoring of activation among multiple ligands. The ability to activate or repress transcription of specific genes in response to selected compounds will expand the options for control of gene therapy, introduced communication between unrelated metabolic pathways, imposed viability requirements for experimental organisms, controlled onset of disease in animal models, controlled expression of defense proteins in crops and conditional antisense RNA expression.

These data demonstrate that high-resolution structural data can be used to identify residues important for modulating ligand specificity for nuclear hormone receptors and that these residues can be mutated to produce substantial functional changes. The success of this approach demonstrates that, in contrast to trypsin, nuclear hormone receptors possess a high degree of structural plasticity that allows variants to bind different ligands and activate transcription. As additional high-resolution structural data become available, it should be possible to use structure-based design to produce even more dramatic changes in ligand specificity. The ease with which substitutions can generate new selectivities also suggests that screening techniques and genetic selection can be used to identify variant nuclear hormone receptors for 'designer' ligands.

Materials and methods

Ligands

9-*cis*-[20-methyl-^{3}H]retinoic acid (72 Ci/mmol) and all-*trans*-[11,12-^{3}H]retinoic acid (74 Ci/mmol) were obtained from DuPont NEN. Unlabeled 9cRA and atRA were from Sigma. LGD1069 [23], LG100153 (compound 6g in [23]), LG100268 [24], LG100754 [27] and TTNPB [29], 3-methyl-TTNPB [23], ALRT1550 [28], and [^{3}H] LGD1069 were gifts from Ligand Pharmaceuticals. Ligand stocks (10 mM) were dissolved in 80% ethanol/20% DMSO (v/v) and stored under N_2 at −20°C. All ligand manipulations were performed under yellow light.

Plasmids

pCMX-hRXRα and pTK-CRBPII-LUC have been described [38,39]. pET16b-hRXRα was a gift from K.J. Petty [40]. RXRα mutants containing substituted amino acids for residues F313 and L436 were generated in pCMX-RXRα using the Morph (5Prime/3Prime) or QuikChange (Stratagene) site-directed mutagenesis kits and confirmed by sequencing. The reporter plasmid pTK-APOAI-LUC was generated by ligating three copies of site A from the ApoAI gene enhancer [41] in tandem into pTK-LUC.

Transfections

Transfections of CV-1 cells were performed in 48-well plates using the calcium-phosphate precipitation method as described [42] with 25 ng of receptor, 50 ng of reporter (pTK-CRBPII-LUC), 50 ng of pCMX-βGAL as an internal control, and pGEM carrier to give a total of 375 ng of DNA per well. Ligands were added to the cells 8 h after transfection, and cells were harvested 36 h after addition of ligand and analyzed for luciferase and β-galactosidase activities. All data points represent the mean of triplicate experiments normalized against β-galactosidase activity. Error bars represent the standard deviation of the mean. Similar results were also observed with the pTK-APOAI-LUC reporter gene (data not shown). Western blots of transfected CV-1 cells indicate that all variants are expressed at levels similar to wild-type (data not shown). Additionally, western blots of untransfected CV-1 cells failed to detect any endogenous RXR, indicating that the contribution of endogenous RXR to transcription in transfected cells is low or negligible. This is confirmed by the lack of activation observed in the dose–response curves from cells transfected with only the reporter gene (Figure 3). All wild-type EC_{50} values (Table 1) are similar to those previously reported for hRXRα [23–26].

Ligand binding

Histidine-tagged proteins were expressed from the pET16b-hRXRa vectors in *Escherichia coli* BL21(DE3)pLysS cells as described [40]. After harvesting by centrifugation, the bacterial pellet from a 500 ml culture was resuspended in 6.25 ml of 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% glycerol, 1 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM imidazole, 0.1% CHAPS, and lysed by three cycles of freeze-thawing followed by sonication (4 times 10 sec bursts on ice). After centrifugation for 30 min at 10,000 g the supernatant was divided into aliquot and stored at −85°C. All samples were analyzed by SDS–PAGE [43] and western blotting to verify the presence of RXR, and total protein concentrations were determined using a Bio-Rad protein assay. Each protein was titrated in binding assays to determine the optimal amount of extract to use. The extracts were added directly to binding buffer (120 mM KCl, 8 mM Tris.HCl, pH 7.4, 8% glycerol, 4 mM dithiothreitol (DTT), 10 mM CHAPS, 0.5 mg/ml leupeptin, 10 TIU/ml aprotinin, 0.25 mM PMSF) on ice, mixed and then put into 96-well filter plates at 50μl per well (Multiscreen plates with 0.65 mm Durapore membranes, Millipore). The radiolabeled ligands were serially diluted on ice in binding buffer containing 20% ethanol, mixed and added to the filter plates (50 ml per well). To determine nonspecific binding, a 200-fold molar excess of unlabeled 9cRA was included. The filter plates were covered in foil, shaken for 5 min at room temperature and stored for 12–16 h at 4°C. Binding was determined using a modification of the hydroxylapatite separation method [44,45]. Fifty microliters of a 6.25% hydroxylapatite slurry in wash buffer (0.1 M KCl, 10 mM Tris.HCl, pH 7.4, 10 mM CHAPS) was added to each well, and the plates were shaken at room temperature for 30 min. The liquid was removed by filtration using a vacuum manifold (Millipore), and each well was rapidly washed four times with 200 ml of cold wash buffer under low vacuum. The plates were dried under full vacuum for 5 min and the base of each blotted dry and sealed using a Topcount plate adapter (Packard). Scintillant (25 μl, Microscint20, Packard) was added to each well and the plates were sealed and shaken for 1 h at room temperature. Each plate was counted using the Topcount microplate counter (Packard). All data points represent the mean of triplicate values, and error bars represent standard errors. Reported Kd values are the mean and s.e. of at least duplicate experiments. All wild-type Kd values (Table 2, Figure 5) are similar to those previously reported for hRXRα [23–26].

Western blotting

Nuclear extracts were prepared using approximately 3×10^6 transfected cells according to the method of Schreiber *et al.* [46]. Cell extracts containing 5 μg of total protein were subjected to SDS–PAGE [43] and transferred to nitrocellulose membranes (Hybond ECL, Amersham). Membranes were blocked in 5% Carnation instant nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h at room temperature. The rabbit antiserum against hRXRα was provided by Elizabeth Allegretto [45]. This primary antibody was incubated for 1 h at room temperature in 1% milk in TBS-Tween. Anti-rabbit secondary antibody conjugated to horseradish peroxidase was incubated for 30 min at room temperature in 1% milk in TBS-Tween. Washes and color development were as per the Hybond ECL protocol.

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