Engineering Orthogonal Ligand−Receptor Pairs from "Near Drugs"

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Abstract: Cell-permeable small molecules are powerful tools for unraveling complex cellular pathways. We demonstrate that nuclear hormone receptors can be engineered through mutagenesis to create orthogonal ligand−receptor pairs to control transcription. Mutated residues in the retinoid X receptor (RXR) were chosen from structural analysis of RXR and the retinoic acid receptor (RAR) ligand binding domains. The potential ligands screened for activation of variant receptors are "near drugs"—compounds synthesized during structure−activity studies that are structurally similar to an approved drug yet inactive on the wild-type receptor. One variant, Q275C;I310M;F313I, is poorly activated by ligands for the wild-type receptor but is activated by a "near drug", fulfilling the criteria of an orthogonal ligand−receptor pair. These experiments demonstrate that nuclear hormone receptors are well suited to supply orthogonal ligand−receptor pairs for experimental biology, biotechnology, and gene therapy. Our findings also demonstrate the general principle that inactive compounds synthesized during drug discovery can be combined with mutant proteins to rapidly create new tools for controlling cellular processes.

Introduction

Completion of genome sequencing projects has placed renewed emphasis on detailed understanding of how protein networks function within cells. These networks are composed of interdependent and often redundant components that combine to form a complex system. This complexity poses severe challenges to exploring the function of newly discovered genes. To reduce cellular complexity to a level that can be addressed experimentally, there is a need for simplifying approaches that can allow the cellular roles of individual proteins to be determined. One general strategy is to use genetic knockouts (or to turn the gene expression off). An alternate "chemical genetic" strategy is to manipulate protein activities by using small molecules derived from natural sources, combinatorial libraries, or design.[1,2] It has recently been shown that genetic knockouts can produce different phenotypes relative to those produced by inhibiting the intact protein,[3] suggesting that chemical genetics may provide a finer level of detail about protein function and regulation than can be achieved by genetic means alone.

Nuclear hormone receptors are a superfamily of ligand inducible transcription factors that activate gene expression in response to the binding of small molecule ligands.[4−6] These receptors contain a DNA binding domain composed of two zinc modules and a ligand binding domain that is predominantly α-helical.[7−11] The ligand binding and DNA binding domains are modular. Their ability to function independently offers important advantages for engineering the control of gene expression because the ligand binding domain can activate transcription when grafted onto an exogenous DNA binding domain. Thus, many different chimeric DNA and ligand binding domains can be produced and used to direct ligand-inducible regulation to different genes.

One obstacle to using nuclear hormone receptors to simplify the study of cellular pathways is that putting genes under control of a native mammalian ligand binding domain requires the use of ligands that will also activate the endogenous pathways that are normally controlled by the receptor within a cell. This unwanted gene activation would make it impractical or impossible to accurately evaluate the physiological role of the intended target gene. For a receptor−ligand pair to overcome this obstacle they must be orthogonal to the contents of the cell,[2] i.e., the receptor cannot be activated by endogenous small molecules and the ligand cannot activate endogenous receptors. One strategy to achieve this goal is to employ ligand−receptor pairs that are not found in mammals, the only known examples being the insect hormone ecdysone and the ecdysone receptor from

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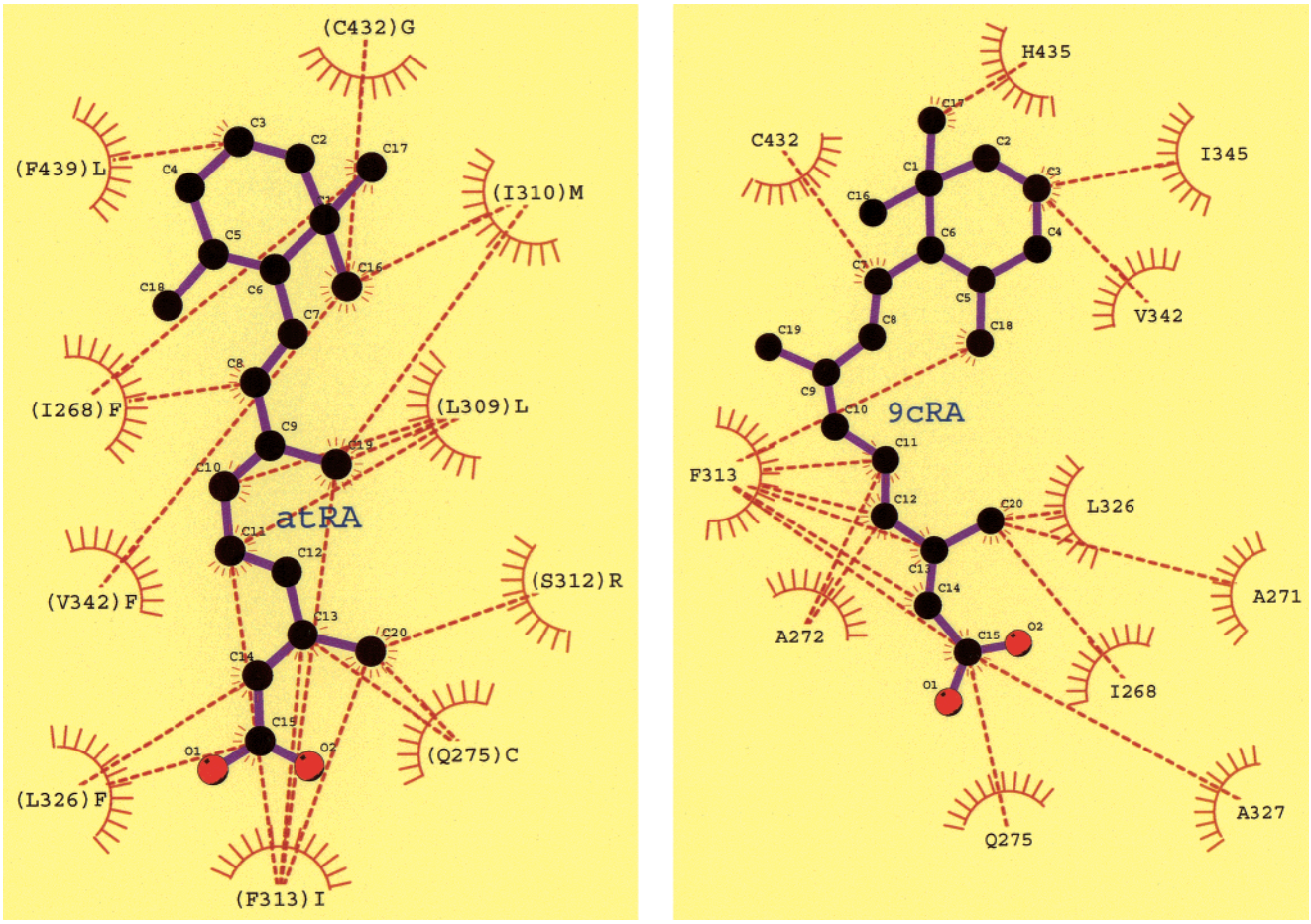

Figure 1. (A, left) RAR residues with any atom within 3.9 Å of the ligand atRA carbon atoms in the atRA-hRARγ crystal structure.[9] The RAR residues are represented by red semicircles. Dashed red lines indicate contacts between the residue and the ligand carbon atoms. The hRARγ residue is outside the parentheses and the corresponding RXR residue and number is indicated inside the parentheses. Except for (S312)R, all of the RAR residues shown were substituted for the corresponding residues in RXR (Table 2). Additionally, valine was substituted for the conserved leucine at position 309 (L309V). Note that the conformation of atRA in the figure does not reflect the bound conformation but has been altered to show the residue−atom interactions more clearly. (B, right) Residues within 3.9 Å of the ligand 9cRA carbon atoms in the 9cRA-hRXRα crystal structure.[10] The RXR residue and number are indicated. The figures were drawn by LIGPLOT 4.0.[25]

Drosophila[12] and the modified tetracycline repressor system from E. coli.[13]

An alternate strategy that does not depend on identifying orthogonal ligand−receptor pairs from other organisms is to modify the receptor to be activated by compounds that do not activate the wild-type protein. This strategy has been used to selectively modulate the function of engineered immunophilins,[14] kinases,[3,15] and SH3 domains.[16] We have previously observed that the nuclear hormone receptor RXR can be mutated to alter its activation in response to various ligands.[17] Koh and colleagues have shown that the related receptor RAR can be mutated to permit activation by neutral and basic ligands,[18] and in very recent work have demonstrated that the same strategy can be applied to the estrogen receptor.[19] Katzenellenbogen has also used mutagenesis of the estrogen receptor in combination with chemical synthesis to obtain new hormone−receptor combinations.[20] These results suggest that nuclear hormone receptors provide a promising protein scaffold for the discovery of ligand−receptor pairs.

Here we describe mutation of RXR and identification of an orthogonal ligand−receptor pair. The orthogonal ligand is a compound that had been found to be inactive during research aimed at developing RXR agonists, suggesting that the chemical diversity of "near drugs" generated during drug development represents a rich source of orthogonal ligands.

Results

Structure-Based Selection of Amino Acid Substitutions. At the time this project was initiated the structure of RXR bound to its ligand 9-*cis*-retinoic acid (9cRA) had not been solved.[10] Therefore, to identify residues to mutate we examined the X-ray crystal structure of *all-trans*-retinoic acid (atRA) bound by hRARγ[9] and focused on the 10 amino acids within 3.9 Å of any ligand carbon atom (Figure 1A). A structure-based alignment identified the analogous residues in RXR.[9] We reasoned that conversion of RXR residues to their RAR counterparts

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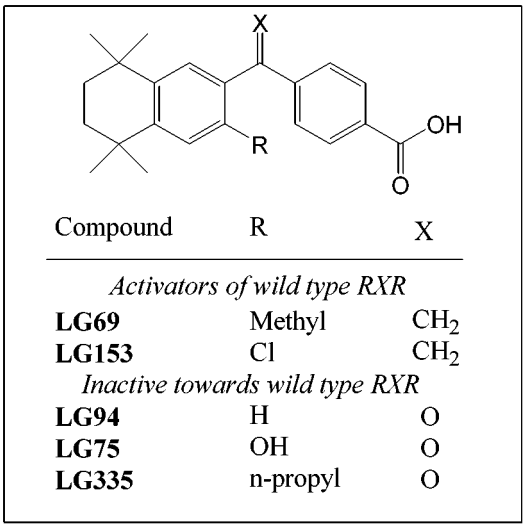
might produce distinct changes in specificity for ligand without altering the global structure of RXR. We did not change the three residues within 3.9 Å of the carboxylate oxygen atoms because the carboxylate is common to all the compounds tested in this study.

One of the 10 contact residues in RAR, L309, is also a leucine in RXR. Although this leucine is conserved we reasoned that it might be able to substantially alter specificity because it contacts C19-, C10-, and C11-carbon atoms on both sides of the double bond that isomerizes between 9cRA and atRA (Figure 1A). Therefore, this residue was changed by using a conservative substitution valine (L309V). Another contact residue, S312, corresponds to an arginine in hRARγ. This residue was not altered to arginine (Figure 1A) because only Cβ is within 3.9 Å of atRA and this atom is common to both residues. The remaining eight residues were changed to the corresponding residues in hRARγ. In addition, we also mutated L436, a residue that lies within 4.5 Å of the β-ionone ring, and corresponds to an alanine in hRARγ. Changing this residue was previously shown to alter ligand specificity.[17] L436 was changed to a valine because valine occurs in the other two isotypes of hRAR. Nine of the ten changed residues were expected to be in contact with ligand in the RXR holo structure.

Availability of the 9cRA-RXR crystal structure coordinates[10] permits analysis of residues actually in contact with the 9cRA ligand (Figure 1B). Six residues (I268, Q275, F313, L326, V342, and C432) contact the ligand as expected. Four residues (L309, I310, L436, and F439) are not within 3.9 Å of 9cRA and five additional residues (A271, A272, A327, I345, and H435) contact 9cRA.

Selection of Potential Orthogonal Ligands. The defining criterion for an orthogonal ligand is that it not be an efficient activator of the wild-type receptor. We wanted a general method for discovery of orthogonal ligand/receptor or enzyme/inhibitor pairs, so we imposed the additional criterion that potential ligands be readily obtainable. The pursuit of RXR agonists and antagonists has been active and many potential ligands have been synthesized and tested. Many of the compounds found to be inactive are closely related to known synthetic activators.

We reasoned that these inactive compounds discovered during drug development might activate mutant receptors. The compounds LG94, LG75, and LG335 are inactive analogues of the synthetic activators LG69 and LG153[21−23] (Figure 2). The inactive analogues show no detectable binding, or weak binding ((10−100)-fold less than the active compounds) to RXR.[21] Because the compounds had already been synthesized they were easily obtained to screen for the activation of transcription by RXR variants. LG69 is a drug (bexarotene, trade name Targretin) approved in the United States and Europe for topical treatment of AIDS-related Kaposi sarcoma and in capsule form for cutaneous T-cell lymphoma.[24] As an approved drug it has withstood stringent requirements for safety, efficacy, oral availability, metabolism, and pharmacokinetics. It is hoped that these properties would also apply to the structurally similar "near drugs" LG94, LG75, and LG335. If true, these compounds would have a head start in making the transition from an inactive compound to a compound suitable for experimental use in mammalian systems or for application to human gene therapy.



Compound	R	X
Activators of wild type RXR		
LG69	Methyl	CH_2
LG153	Cl	CH_2
Inactive towards wild type RXR		
LG94	H	O
LG75	OH	O
LG335	n-propyl	O

Figure 2. Synthetic retinoids that activate (LG69, LG153) or do not activate (LG94, LG75, LG335) wild-type RXR.

Effect of Single Mutations on Transcriptional Activation and Specificity. Plasmids encoding the wild-type and variant proteins were transfected into mammalian cells and analyzed for the ability to activate transcription of an RXR response element linked to a luciferase reporter gene in response to a panel of potential ligands. A plasmid encoding β-galactosidase was transfected simultaneously to serve as an internal standard for transfection efficiency. The single variants at the key contact residues identified by structural analysis were screened for activation of transcription.

An orthogonal ligand−receptor pair should show greatly reduced activation by the wild-type RXR ligands 9cRA or the synthetic ligands LG69 or LG153, and high activation by any of the compounds LG94, LG75, or LG335. None of the single-substitution variants were sufficient to achieve this goal (Table 1). RXR variants L309V and L436V show decreased activation and no broadened specificity. RXR variants I268F, Q275C, and C432G have activation profiles that are similar to wild-type RXR. The RXR variant L326F also has a specificity profile similar to wild-type RXR, with the significant exception that it has lost its ability to be activated by atRA. The remaining four RXR variants, I310M, V342F, F313I, and F439L, have an increased ability to be activated by LG94, LG75, or LG335. I310M and V342F behave most like an orthogonal ligand−receptor pair because they show decreased activation by ligands for the wild-type receptor. However, the levels of activation by LG94, LG75, and LG335 are low. In contrast, RXR variants F313I and F439L are efficiently activated by the wild-type receptor ligands *and* by the previously inactive target compounds.

Combining Mutations to Engineer an Orthogonal Ligand−Receptor Pair. To fully explore the potential for ligand recognition by the RXR scaffold, all combinations of the 10 contact residues should be made and screened for activity. If just two residues are considered for each of the 10 positions, this strategy would require that 1023 variants be made and tested. We felt that the discovery of orthogonal ligands could be achieved without resorting to the need to make and assay so many variants. Instead, determination of which combinations to make was based largely on the effect of the single substitutions and partially based on experimental convenience that enabled rapid generation of some variants for testing.

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Table 1. Activation of Transcription by Wild-Type RXR and Variant Receptors that Contain a Single Mutation with 10^{-5} M Compounds[a]

variant	9cRA	atRA	LG69	LG153	LG94	LG75	LG335
wild-type	+++++	+++	+++	+++	++	−	−
variants with activities similar to wild-type							
I268F	+++++	+++	++++	+++++	+	−	−
Q275C	++++++	+++	++++	+++++	−	−	−
L326F	++	−	++++	+++++	−	−	−
C432G	++++++	+++	+++	+++	−	−	−
variants with reduced activation by all compounds							
L309V	+++	−	+	+	−	−	−
L436V	+	−	++	++	−	−	−
variants with increased activation by LG94, LG75, or LG335							
I310M	++++	++	+++	++	+	-	+
F313I	++++++	++	+++++	+++++	+++++	+++++	++++
V342F	++	++	++	++	++	++	−
F439L	++++++	++++	++++	+++	++++	++	+++

[a] Key: ++++++ >110% of wild-type activation by 9cRA; +++++ 90−110% of wild-type activation by 9cRA; ++++ 70−90% of wild-type activation by 9cRA; +++ 50−70% of wild-type activation by 9cRA; ++ 30−50% of wild-type activation by 9cRA; + 10−30% of wild-type activation by 9cRA.

Table 2. Activation of Transcription by Wild-Type RXR and Variant Receptors that Contain Multiple Mutations with 10^{-5} M Compounds[a]

variant	9cRA	atRA	LG94	LG75	LG335
wild-type	+++++	+++	++	−	−
double mutants					
Q275C;I310M	+++	+	+	−	+
Q275C;F313I	+++	-	+++++	++++	+++++
I310M;F313I	++++	+	+++++	+++++	+++++
F313I;L436V	+	-	-	-	+++
F313I;F439L	++++++	+++	+++++	++++++	++++++
triple mutants					
Q275C;I310M;F313I	+	−	+++++	+++	++++
Q275C;F313I;V342F	+++	−	++++	++++	+++
Q275C;L436V;F439L	+++	−	−	−	+
I310M;F313I;F439L	+++++	+	++++	++++	+++++

[a] Key: same as Figure 1.

We made five double variants:, Q275C;I310M, Q275C;F313I, I310M;F313I, F313I;L436V, and F313I;F439L (Table 2). The I310M, F313I, and F439L mutations were included because they showed an enhanced ability to activate transcription in response to LG94, LG75, and LG335. L436V and Q275C were included to investigate the effects of mutations that either reduced activation by wild-type ligands (L436V) or left activation largely unchanged (Q275C). All of these double mutants have interesting properties. In all cases, some activation by LG94, LG75, or LG335 is observed. RXR variants Q275C;I310M, Q275C;F313I, I310M;F313I, and F313I;L436V also show decreased activation by 9cRA, atRA, or both.

These results encouraged us to explore the properties of a series of triple variants (Table 2). Once again, each variant has interesting properties, displaying reduced activation by wild-type ligands or increased activation by the target synthetic ligands. Remarkably, out of this relatively small pool, one variant met the criterion for an orthogonal receptor. Q275C;I310M;F313I is not activated by atRA and is activated by 9cRA to a small degree (Table 2, Figure 3). In contrast, 10 μM concentrations of LG64, LG75, and LG335 each activate Q275C;I310M;F313I (Table 2). Activation by LG64 and LG75 rapidly diminishes with decreasing concentration, but activation by LG335 is observed at concentrations of 1 μM and below, with a dose response curve that comes within 10-fold of that for 9-cisRA and wild-type RXR (Figure 4).

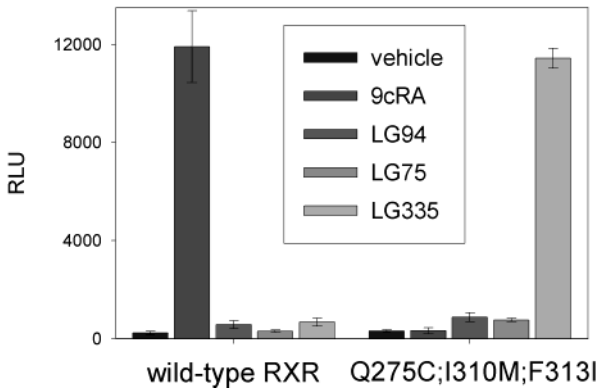

Figure 3. Activation of wild-type RXR and RXR containing the triple mutant Q275C;I310M;F313I by compounds that either activate (9cRA) or do not activate (LG94, LG75, LG335) wild-type RXR. All ligands are 1 μM. Activity is measured in relative light units (RLU) derived from the measurement of luciferase activity and subsequent normalization against a β-galactosidase standard.

Discussion

Orthogonal Ligand−Receptor Pairs as an Approach to Controlling Cell Biology. Completion of the human genome project has revealed the identity of thousands of genes. This historic advance has shifted the emphasis of research from the discovery of new genes and proteins toward understanding how proteins function within the cell. What signals cause protein−protein interactions to occur and then end or an enzymatic activity to begin and then stop? Small molecules that modulate the activities of cellular proteins offer one class of tools for probing the roles of proteins in cellular pathways and signaling. This realization is not new; chemists have been synthesizing enzyme inhibitors and receptor agonists and antagonists for many years. However, the sheer number of newly discovered proteins suggests that new approaches for the identification of bioactive molecules are needed.

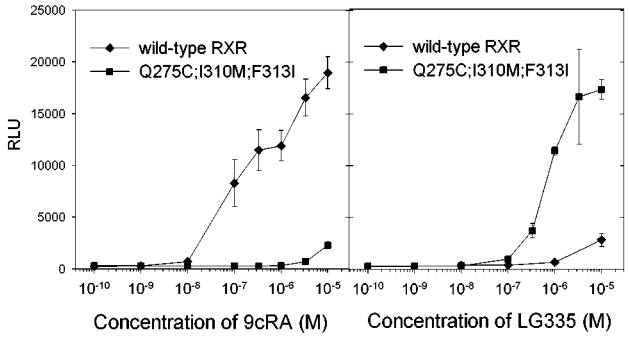

Figure 4. Dose response curves for activation of wild-type RXR and Q275C;I310M;F313I RXR by 9cRA and LG335. Activity is measured in relative light units (RLU) derived from the measurement of luciferase activity and normalization against a β-galactosidase standard.

One goal for the identification of bioactive small molecules is the ability to turn gene expression on or off. Nuclear hormone receptors are excellent targets for a strategy that uses small molecules because they activate transcription in response to binding small ligands. This activation depends on the presence of a response element within the promoter region of the gene of interest, so the expression of almost any gene can be put under the control of a nuclear hormone receptor simply by introducing a response element upstream from the start codon. Thus, the potential exists for activation of nuclear hormone receptors by small molecules to be a general strategy for protein expression to be spatially and temporally controlled. The drawback is that addition of native ligands will activate endogenous wild-type receptors and produce effects that might obscure the intended observations. Our results, together with similar data from the Koh and Katzenellenbogen laboratories,[19,20] indicate that orthogonal ligand−nuclear receptor pairs can be rapidly generated, and that these can allow the activation of genes without activation of wild-type pathways.

"Near Drugs"−A Reservoir of Chemical Diversity for Discovering Orthogonal Ligand−Receptor Pairs. Discovery of an orthogonal ligand−receptor pair requires a mutant protein and a small molecule whose volume and electrostatic characteristics are similar to the native ligand or substrate.[2,3,14,15] Candidate proteins can be readily generated by mutagenesis, with design of mutants often facilitated by knowledge of the three-dimensional structure of the protein. Synthesis of candidate ligands will usually require much more effort. Here we take advantage of the fact that RXR is a target for drug design and that structurally related but inactive small molecules had been synthesized previously during the development of RXR agonists.[21−23] Many proteins have been targets for drug discovery, and many other collections of inactive compounds have been generated during the development of structure−activity relationships. Our results indicate that the compounds in these collections are excellent candidates for orthogonal ligands, and may be able to supply useful reagents for probing the details of cellular processes.

Engineering an orthogonal ligand−receptor pair in RXR succeeded despite being based on inaccurate assumptions from the atRA-RAR structure. Understanding the structural basis of the orthogonal pair requires solving the structure of LG335 bound to Q275C;I310M;F313I and comparing it to the LG69-RXR structure which hasn't been solved and is beyond the scope of this paper. The success of this project in the absence of a high-quality structure indicates that generating orthogonal ligand−protein pairs may be fairly easy, even when based on a rough model structure.

Conclusions

The orthogonal ligands examined in these studies were synthesized during investigations aimed at development of potent agonists of wild-type RXR. However, like many compounds synthesized during the optimization of potential therapeutics, these compounds possess little or no activity for activating the wild-type receptor. We find that these compounds do activate mutant receptors effectively. This result leads to the general conclusion that inactive "near drugs" developed during research programs are a reservoir of chemical potential that can be exploited in conjunction with mutant proteins that possess altered specificities. Many orthogonal ligand−receptor pairs may already exist and their identification may merely require the testing of existing compounds with existing or easily obtained mutants.

Experimental Section

Ligands. 9-*cis*-Retinoic acid and *all-trans*-retinoic acid were purchased from Sigma. LGD69, LG153, LG94, LG75, and LG335 (respectively, compounds **6b**, **6g**, **5a**, **5i**, and **5e** in ref 21) were obtained 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.[26,27] The internal standard plasmid pCMX-βGAL constitutively expresses β-galactosidase under control of the CMV promoter. RXRα mutants containing substituted amino acids were generated in pCMX-RXRα with use of a QuikChange (Stratagene) site-directed mutagenesis kit and confirmed by sequencing.

Transfections. Transfections of CV-1 cells were performed in 48-well plates with Lipofectamine cationic lipid (Gibco BRL/Life Technologies) as recommended by the manufacturer. Briefly, 20 ng of pCMX-hRXRα expression plasmid, 40 ng of pTK-CRBPII-LUC reporter plasmid, and 40 ng of pCMX-βGAL expression plasmid (used as an internal standard) were mixed with 0.3 μL of lipofectamine in 40 μL of Optimem reduced serum media per transfected well. After 15 min a further 160 μL of Optimem was added and the 200 μL mixture was added to a well previously washed with 250 μL of Optimem. After 8 h of transfection the wells were aspirated and ligands diluted in 300 μL of super-stripped CV-1 media (5% lipid stripped, heat-treated CBS; 95% Cell-Gro DMEM; 1% penicillin/streptomycin) were added to the wells. Cells were harvested after 36−40 h and assayed 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.

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