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**Title and Subtitle**

Toward a New Chemotherapy for Breast Cancer: Structural and Functional Mechanism of the Retinoid Receptors Addressed by a Novel Computer Approach

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**Abstract**

Estrogen receptor modulators represent the most widely employed therapy for breast cancer. However, resistance of some tumor cells to this treatment and other side effects result in the need to improve therapeutic strategies. Retinoids, which are known to inhibit the growth of a wide variety of cancer cells, including breast cancer cells, bind to cognate members of a large family of Nuclear Receptors, which activate or repress transcription in a ligand-dependent manner. Two types of retinoid receptors have been identified: the Retinoid Acid Receptor (RAR) and the Retinoid X Receptor (RXR). Retinoids exert their anticancer activities both in estrogen receptor-positive and -negative cells, mainly through their abilities to modulate the growth, differentiation, and apoptosis of tumor cells. In spite of their therapeutic potential, their clinical use is so far limited, because of toxic side effects.

The identification of structural motifs of small molecule modulators of the retinoid receptors is critical for the development of novel ligands with improved toxicity profiles, which could lead the way towards a new generation of drugs against breast cancer. Our goal is to use state-of-the-art computer modeling technologies to identify the structural determinants of retinoids which confer them their receptor selectivity and their agonist or antagonist activity, and rationally design molecules with various affinities for the RARs and the RXRs.
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Introduction

Estrogen receptor (ER) modulators, such as Tamoxifen, represent the most widely employed therapy for breast cancer. However, the resistance of estrogen receptor-negative tumor cells to this treatment, the possibility that Tamoxifen may increase the chances of endometrial carcinoma and the development of Tamoxifen-resistant breast cancer cells in many patients result in a need to improve therapeutic strategies (see Brown and Lippman, 2000, for an updated comprehensive clinical review of breast cancer prevention studies).

Retinoids, the natural vitamin A derivatives and synthetic analogs, are known to inhibit the growth of a wide variety of cancer cells, including breast cancer cells (Fitzgerald et al, 1997; Giannini et al, 1997; Budd et al., 1998; Tacher et al., 2000), constituting a novel and promising approach in the prevention and treatment of the disease. Retinoids, like steroid hormones and certain vitamins, enter target cells and bind to cognate members of a large family of Nuclear Receptors (NRs) (see Mangelsdorf and Evans, 1995; Weatherman et al., 1999 for review), which activate or repress transcription in a ligand-dependent manner by binding to their cognate response element located in the promoter region of the target gene. Two types of retinoid receptors have been identified: the Retinoid Acid Receptor (RAR) and the Retinoid X Receptor (RXR), each composed of three isotypes α, β and γ. Retinoids exert their anticancer activities both in estrogen receptor-positive and -negative cells, mainly through their abilities to modulate the growth, differentiation, and apoptosis of tumor cells. In spite of their therapeutic potential, their clinical use is so far limited, because of toxic side effects (Rizvi et al., 1998).

Consequently, the identification of structural motifs of small molecule modulators of the retinoid receptors is critical for the development of novel ligands with improved toxicity profiles, which could lead the way towards a new generation of drugs against breast cancer. Our goal is to use state-of-the-art computer modeling technologies to identify the structural determinants of retinoids which confer them their receptor selectivity and their agonist or antagonist activity, and rationally design molecules with various affinities for the RARs and the RXRs.

Body

Task 1: Identify the structural determinants which confer a ligand retinoid X receptor (RXR) or retinoic acid receptor (RAR) selectivity

Using the structure of RAR ligand binding domain (LBD) bound to All-Trans Retinoic Acid (ATRA) we docked 9-cis retinoic acid (9-cis RA) in the ligand binding pocket of the receptor. The ligand and receptor side chains were flexible in our improved docking algorithm (Totrov and Abagyan, 1994; see also Totrov and Abagyan, 2001, for review).

Using the crystal structure of the unligated RXR and the result from the previous step, we were able to build a model of the RXR LBD bound to 9-cis RA, and docked RXR and RAR ligands to the two receptors. We predicted that the ligand binding pocket of RXR is shorter and bulkier than that of RAR, which accounts for receptor selectivity. Our prediction was confirmed by the
crystal structure of the RXR/9-cis RA complex (Gampe et al., 2000). The binding pocket shape
determines the specificity of the interaction, more than the hydrogen-bond network, so steric
parameters are critical for receptor selectivity.

We also docked RXR- and RAR selective ligands into the receptors ligand binding pockets and
showed that our models correlated with experimental observations: non-binders had a positive
calculated Van der Waals energy of interaction, whereas binders had a negative Van der Waals
energy of interaction (see Report 1 for details). This correlation between ligand binding
modeling and experiments was an encouraging indication of the validity of our methodology.

An increasing amount of data suggest that the RARα isoform, which controls the expression of
RARβ, is the most relevant target for breast cancer therapy (Liu et al., 1996; Widschwendter et
al., 1997). A detailed understanding, at the atomic level, of the determinants for isoform
specificity is therefore important to design ligands with appropriate receptor selectivity. We
docked a RARα specific ligand, Am580, in the crystal structure of RARγ, and in a model that
we built of RARα and RARβ, and showed that residue 234, one of the three residues of the
ligand binding pocket which are not conserved among the three RAR isoforms, was responsible
for receptor isoform specificity (Figure 1).

Task 2: Identify the structural properties a ligand must satisfy to be an agonist

Our original strategy was to model the interaction between RXR and the TATA binding protein
(TBP), which is believed to act as a coactivator. However, key data regarding the identification,
activity and structure of NRs coactivators (Freeman, 1999) and the structural mechanism of
agonist and antagonist activity revealed by a series of crystal structures (Moras and Gronemeyer,
1998) have been published since and enabled us to refine our approach: Upon binding of an
agonist, the C-terminal helix 12 of the LBD undergoes a conformational change, closes like a lid
on the ligand, and forms a hydrophobic pocket on the surface of the receptor. This pocket is the
target for several known and unknown coactivator proteins, which bind the LBD through a
conserved LxxLL motif. In the antagonist-bound conformation, the helix H12 is pushed away
from the ligand binding pocket, and occupies the coactivator binding site, preventing coactivator
recruitment.

We collaborated with Dr Herbert Samuel's lab, at NYU Medical Center, to identify a novel
coactivator, NRIF3, mediating functional specificity of nuclear hormone receptors. This novel
protein coactivates RXR but not RAR. Our modeling suggests that NRIF3 binds to liganded
receptors through an LxxIL module contained within the C-terminal domain of the coactivator.
This data was the object of an article published in Molecular and Cellular Biology and is
appended to this report (Article 1).

Very interesting data recently published on the structure-activity relationship of another nuclear
receptor, the estrogen receptor, encouraged us to initiate an additional avenue of investigation on
RAR, with possible important applications in breast cancer therapy. Paige et al. showed that
different agonists of the estrogen receptor (estradiol, estriol, and diethylstilbestrol) could induce
different conformational changes of the receptor LBD, resulting in recruitment of different
coactivators (Paige et al., 1999). Structural analysis of many nuclear receptors suggests that, upon ligand binding, the core of the receptor LBD remains fixed, while the C-terminal H12 helix is relocated and interacts with the ligand, thus generating a binding site for coactivator proteins (Moras and Gronemeyer, 1998; Freeman, 1999). It is therefore reasonable to predict that the different estrogen receptor agonists induce a different rearrangement of the H12 helix.

We built low energy models of the RAR LBD where the H12 helix is relocated onto different docking sites at the surface of the receptor. Only a limited number of rearrangements of H12 are possible (Figure 2). Such molecules could have a different pharmacological profile, compared with existing RAR ligands.

**Task 3: Design rationally and test novel drugs against breast cancer**

1- Use our model to screen libraries of compounds and select ligands with desired structural characteristics:

Based on the crystal structure of estrogen receptor bound to Tamoxifen, we built a model by homology of antagonist-bound RAR and carried out a high-throughput computer screening where over 150,000 ligands were automatically docked into the receptor's binding pocket and assigned a score according to the quality of the fit (see Schapira et al., 1999; Abagyan and Totrov, 2001, for details on the methodology). About thirty RAR antagonist candidates were selected for *in vitro* assay. This work was published in 2000 in the Proceedings of the National Academy of Sciences and is appended to this report (Article 2).

We have also used a benchmark virtual library made of the structure of known ligands for different members of the nuclear receptor family in order to optimize our virtual library screening technology. We could show that after four independent screenings, all the RAR ligands, and only the RAR ligands were selected (Table 1), which constitutes an important validation of our approach. This results were also published (Article 2).

Additionally, we have used the crystal structure of agonist-bound RAR to screen our virtual library of over 150,000 ligands and selected about 30 agonist candidates to be tested in vitro. This work has been published in *BMC Structural Biology*, and is appended to this report (Article 3).

Little is known about the natural ligands to RXR. Crystallographic data of RXRα bound to agonist 9-cis RA were published recently (Egea et al., 2000; Gampe et al., 2000). Also, fatty acids were found in the ligand binding pocket of murine RXR (Bourguet et al., 2000; Urquiza de et al., 2000). Aiming first at the discovery of potential natural ligands of RXRα, we docked 4,000 natural occurring compounds to the ligand binding pocket of RXRα in the agonist conformation. Four different calculations were performed, and the lowest score for each compound is shown in Figure 3a. Control known binders to RXR like 9-cis RA and Targretin and not binders like hydroxytamoxifen and MPA were correctly predicted by our scoring protocol (see Figure 4). According to Figure 3a, we considered that compounds with a score less than −50 may be candidates for experimental testing (see Figure 3b). These results are confidential and they should be protected.
2- Test these ligands in vitro for their receptor selectivity, and their ability to trigger transactivation

Activity tests were performed on the set selected after virtual screening procedure, as described in 1-. Two novel RAR antagonists were discovered, thus validating our modeling strategy (Article 2).

We also discovered novel agonists of RAR (Article 3). One of them, agonist 3, presents very original structural features: (i) penta-methylated benzene linked by a ketone to a second aromatic ring, forms a very large hydrophobic head. (ii) pyridine probably makes a strong interaction with Cys 237 and replaces a carboxylate or ester present in all RAR ligands described so far (Figure 5). These novel structural features may translate into improved toxicity profiles and result in the development of new ligands for breast cancer therapy.

This last finding is extremely encouraging, and we initiated a collaboration with Prof. Stephen Wilson and Prof. Herbert Samuels to discover analogs of agonist 3 with improved activity. Dr Wilson is Professor of chemistry at New York University and the director of the New York University Laboratory for Combinatorial Chemistry. He is a renown expert in solid phase chemistry, and the author of Combinatorial Chemistry: Synthesis and Application, John Wiley & Sons, March 1997; edited by Wilson & Czarnik, one of the first books on combinatorial chemistry and its organic chemistry applications. Prof. Samuels is an internationally recognized expert in endocrinology, and has been collaborating with us since the beginning of our research effort.

RAR ligands described in the literature belong to limited series of related structures, and display similar toxic side effects. The discovery of agonist 3 and the model of its interaction with RAR open an avenue towards new RAR agonist ligands with possibly more favorable specificity and toxicity profiles.

Key Research Accomplishments

- We built a model of the active conformation of RXR, which was recently confirmed by a crystal structure (Gampe et al., 2000), and could derive the structural differences between the RAR and RXR binding pockets responsible for ligand selectivity.

- We modeled the interaction between known RAR- and RXR- ligands, observing a very good correlation between our models and biochemical data.

- We provided structural determinants for RAR isoforms selectivity (Figure 1).

- We modeled the interaction between RAR or RXR and NRIF3, a novel nuclear receptor coactivator (Article 1).

- We discovered novel RAR antagonists (Article 2).
- We optimized our virtual screening technology and demonstrated its efficacy at discriminating between binders and non-binders (Table 1 and Article 2).

- We applied our virtual ligand screening procedure to find candidates among natural occurring compounds which may bind to RXR.

- We discovered an RAR agonist with very original structural features, and initiated a collaboration with chemists (Prof. S. Wilson's laboratory) and biologists (Prof. H. Samuels' laboratory) to design, synthesize and test structural analogs of this very promising molecule (Figure 3 and Article 3).

Reportable Outcomes

Article 1:
NRIF3 is a novel co-activator mediating functional specificity of nuclear hormone receptors
D. Li, V. Desai-Yajnik, E. Lo, M. Schapira, R.A. Abagyan, and H.H. Samuels
Molecular and Cellular Biology 1999, 19 (10), 7191-7202.

Article 2:
Rational discovery of novel nuclear receptor antagonists
M. Schapira, B. Raaka, H.H. Samuels, and R.A. Abagyan

Article 3:
In Silico discovery of novel retinoic acid receptor agonist structures
M. Schapira, B. Raaka, H.H. Samuels, and R.A. Abagyan
BMC Structural Biology 2001, 1, 1.

Abstract 1:
Structural and functional mechanisms of the Retinoid receptors addressed by a novel computer approach
M. Schapira and R.A. Abagyan
Presented at the "Era of Hope" meeting, Atlanta, GA, June 8-11, 2000.

Conclusions

During the three years of our effort, we have accomplished most of the tasks outlined in the approved Statement of Work, despite a difficult move of the laboratory from the NYU Medical Center to the Scripps Research Institute. We have identified the structural determinants which confer a ligand RXR or RAR selectivity, and successfully challenged our models with known ligands for either receptor. We have also put a rational on RAR isoforms specificity which will be useful to develop RARα specific agonists—the biological activity which seems the most promising for breast cancer therapy.
Based on structural data on the estrogen receptor, we also produced models of distinct RAR conformations, which may be used to derive novel types of ligands for this receptor. Such ligands could have interesting activities, mirroring the complexity of small molecule estrogen receptor modulators.

We have developed a strategy to rationally design RAR antagonists, and were also able to discover novel RAR agonists with very original structural features. Analogs of these novel molecules could result in the identification of leads against breast cancer. This task is currently on its way. Virtual ligand screening was also used to find potential natural ligands of RXRα with promising results. Candidates should be synthetized and tested in vitro and in vivo.

"So what":

We have developed a series of approaches to identify new antagonists and new agonists for the retinoid receptors, based on the structure of the receptor, and regardless of existing compounds. This original strategy already resulted in the discovery of active ligands presenting very original features. Collaboration with chemists and biologists to develop analogs of these molecules with improved activity and specificity, which would constitute valuable leads for breast cancer therapy is presently on its way.

References


Freeman LP (1999). Increasing the Complexity of Coactivation in Nuclear Receptor Signaling, *Cell* 97, 5-8

Gampe R, V Montana, M Lambert, A Miller, R Bledsoe, M Milburn, S Kliwer, T Willson, and


morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer, *Cancer Res* 57, 4158-4161.
Figures and Tables
**Figure 1**: Docking of a known RARα specific agonist. The RARα selective agonist Am580 was docked into the modeled ligand binding pocket of RARα. A: The complex(?) ligand (white sticks) superimposes with the crystal structure of bound all-trans RA (green). Hydrogens are not shown for clarity. B: Am580 (CPK display) fits tightly into the receptor's pocket (yellow wire), except for a ketone oxygen, which does not clash with the receptor only because it can share an hydrogen with Ser234 (displayed as stick). In RARβ and γ, Ser234 are replaced by an alanine, which clashes with the ketone oxygen. The receptor in the vicinity of the ligand is shown as a white ribbon. Carbons, hydrogens, oxygens and nitrogen are colored white, grey, red and blue respectively.
Figure 2: Models of RAR LBD where the C-terminal helix H12 (green, magenta) is relocated onto different docking sites of the receptor (surface representation) could provide novel binding pocket conformations for the structure based discovery of agonists (cyan) with an improved pharmacological profile.
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<th>Score2</th>
<th>Score3</th>
<th>Score4</th>
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<th>Binding</th>
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<td>-36.5</td>
<td>-36.7</td>
<td>-35.3</td>
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<td>RARα antagonist</td>
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<td>RAR antagonist</td>
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<td>Tamoxifen</td>
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<td>-38.7</td>
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<td>AGNpartial</td>
<td>RAR partial agonist</td>
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<td>Am580</td>
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<td>-33.8</td>
<td>+</td>
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</table>

**Table 1:** Control screening of known ligands. A similar screening as the one performed on the large library was carried out four times on a small database made of known nuclear receptor ligands: the compounds were automatically docked in the structure of RAR. The molecules which met at least once with the criteria for selection are listed as "Selected". The ligands which are experimentally binding to RAR are listed as "Binding".
Figure 3: Virtual ligand screening scores for natural occurring ligands docked to RXRα. Figure (a) shows the scores of all molecules in the set, and (b) the best scores.
Figure 4: Ligands docked to holo-RXRα. 9cis-RA and Targretin are known ligands to RXR. Hydroxitamoxifen is an Estrogen Receptor binder and MPA an Androgen Receptor binder which do not bind to RXR.
Figure 5: Unlike all retinoid receptor ligands described in the literature, the discovered Agonist 3 (green) does not have carboxylate, but pyridine which makes thiolate salt with cystein (white) of the receptor, and could also interact with neighboring arginine (white). This compound is active at 200 nM, and the rational design of analogs, which conserve this original feature could result in leads against breast cancer with improved specificity and toxicity profiles. We embarked on the development of such molecules in collaboration with renowned chemists and biologists.
Appendices
Toward a new chemotherapy for breast cancer: Structural and functional mechanism of the retinoid receptors addressed by a novel computer approach.

DAMD17-98-1-8133

List of salaried personnel from grant:

Dr. Ruben Abagyan, Ph.D.
Dr. Matthieu Schapira, Ph.D.
Dr. Claudio Cavasotto, Ph.D.
Dr. Andrew Bordner, Ph.D.
Structural and Functional Mechanism of the Retinoid Receptors Addressed by a Novel Computer Approach

Matthieu Shapira and Ruben Abagyan

Era of Hope, Atlanta, GA, June 2000.

Unlike antiestrogens, such as tamoxifen or raloxifene, which can be effective against estrogen-dependent breast tumors, compounds targeted at the Retinoid Receptors (RR), members of the Nuclear Receptors (NR) family, can be active against both estrogen receptor (ER) positive and negative tumors. As a consequence, understanding the structural mechanism of NR in general, and RR in particular, could help design and optimize a novel generation of chemotherapeutic agents against breast cancer.

The RR are divided into two protein subfamilies, the Retinoic Acid Receptor isoforms (RAR-alpha, -beta, and -gamma) and the Retinoid X Receptor isoforms (RXR-alpha, -beta, and -gamma). It is today not clear which receptor and which isoform is the best target for breast cancer therapy. As a result, it is important to test both agonists and antagonists targeted at various isoforms of RAR and RXR. A detailed understanding of (i) the structural mechanism of activation/inhibition of NR and (ii) the structural determinants of RR specificity is important to designing modulators with required activity. Our goal is to use a powerful computer modeling technology to address these two questions.

We hypothesized that the structural mechanism of antagonist activity revealed by the tamoxifen crystal structure could be extended to other members of the NR family, and use this structure as a template to build a model of RAR in an antagonist-bound confirmation. Known RAR antagonists docked into the modeled pocket displayed a perfect fit. This 3D model of inactive RAR was then used to discover novel RAR antagonists: a high throughput virtual screening algorithm was used to dock rapidly a database of 200,000 virtual structures into our modeled RAR-antagonist binding site. 32 compounds were actually tested in vitro and 2 novel antagonists were discovered. Three novel agonists were also discovered using the same approach.

The crystal structure of the ligand binding domain (LBD) of RAR bound to its natural agonist is known, but the structure of active RXR is not. We produced a model of RXR-LBD bound to the agonist 9-cis Retinoic Acid. The comparison of active RAR and RXR Revealed that the two ligand binding pockets are equally hydrophobic; however, the RXR ligand binding pocket is shorter and bulkier, and can accommodate for ligands presenting a more distinct shape than RAR agonists.

The U.S. Army Medical Research and Material Command under DAMD17-98-1-8133 supported this work.
NRIF3 Is a Novel Coactivator Mediating Functional Specificity of Nuclear Hormone Receptors

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Many nuclear receptors are capable of recognizing similar DNA elements. The molecular event(s) underlying the functional specificities of these receptors (in regulating the expression of their native target genes) is a very important issue that remains poorly understood. Here we report the cloning and analysis of a novel nuclear receptor coactivator (designated NRIF3) that exhibits a distinct receptor specificity. Fluorescence microscopy shows that NRIF3 localizes to the cell nucleus. The yeast two-hybrid and/or in vitro binding assays indicated that NRIF3 specifically interacts with the thyroid hormone receptor (TR) and retinoid X receptor (RXR) in a ligand-dependent fashion but does not bind to the retinoic acid receptor, vitamin D receptor, progesterone receptor, glucocorticoid receptor, or estrogen receptor. Functional experiments showed that NRIF3 significantly potentiates TR- and RXR-mediated transactivation in vivo but has little effect on other examined nuclear receptors. Domain and mutagenesis analyses indicated that a novel C-terminal domain in NRIF3 plays an essential role in its specific interaction with liganded TR and RXR while the N-terminal LXXLL motif plays a minor role in allowing optimum interaction. Computer modeling and subsequent experimental analysis suggested that the C-terminal domain of NRIF3 directly mediates interaction with liganded receptors through an LXXIL (a variant of the canonical LXXLL) module while the other part of the NRIF3 protein may still play a role in conferring its receptor specificity. Identification of a coactivator with such a unique receptor specificity may provide new insight into the molecular mechanism(s) of receptor-mediated transcriptional activation as well as the functional specificities of nuclear receptors.

Nuclear hormone receptors are ligand-regulated transcription factors that play diverse roles in cell growth, differentiation, development, and homeostasis. The nuclear receptor superfamily has been divided into two subfamilies: the steroid receptor family and the thyroid hormone/retinoid (nonsteroid) receptor family (51). The steroid receptor family includes receptors for glucocorticoids (GR), mineralcorticoids, progestins (PRs), androgens (AR), and estrogens (ERs) (51). The nonsteroid receptor family includes receptors for thyroid hormones (TRs), retinoids (retinoic acid receptors [RARs] and retinoid X receptors [RXRs]), 1,25-(OH)2-vitamin D (VDR), and prostanoids (peroxisome proliferator-activated receptors [PPARs]) as well as many orphan receptors whose ligands (if any) remain to be defined (49, 51). Members of the nuclear receptor superfamily have common structural and functional motifs. Nevertheless, an important difference exists between the two subfamilies. Steroid receptors act primarily as homodimers by binding to their cognate palindromic hormone response elements (HREs) (77, 78). In contrast, members of the nonsteroid receptor family can bind to DNA as monomers, homodimers, and heterodimers (25, 78). Their corresponding HREs are also complex and can be organized as direct repeats, inverted repeats, and everted repeats (49). Therefore, the combination of heterodimerization and HRE complexity provides the potential of generating enormous diversity in receptor-mediated regulation of target gene expression.

Structural and functional studies indicate that the ligand binding domain (LBDs) of many members of the thyroid hormone/retinoid receptor family harbor diverse functions. In addition to binding to ligands, the LBD also plays roles in mediating receptor dimerization, hormone-dependent transactivation, and, with TR and RAR, ligand-relieved gene silencing (54, 61). The carboxyl-terminal helix of the LBD has been implicated in playing an important role in ligand-dependent conformational changes and transactivation (6, 9, 21, 43). Although it has been suggested that an activation function (AF-2) resides in this C-terminal helix, recent studies indicate that AF-2 results from a ligand-induced conformational change involving diverse areas of the LBD (23, 66). Thus, ligand binding serves to switch the receptor from one functional state (e.g., inactive or silencing) to another (e.g., transactivation).

Although much has been learned from studying the structures and functions of these receptors, the detailed molecular mechanism(s) of transcriptional regulation by these receptors is not well understood. Efforts to understand the molecular mechanism of transcriptional repression by unliganded TRs and RARs have led to the description (12) and isolation of the putative corepressor proteins SMRT and N-CoR, which interact with the LBDs of these receptors in the absence of their ligands (15, 36). The recent discovery that both SMRT and N-CoR form complexes with Sin3 and a histone deacetylase suggests that chromatin remodeling by histone deacetylation may play a role in receptor-mediated transcriptional repression (33, 55).

In a somewhat parallel approach, the identification of coactivators has recently received extensive experimental attention in order to elucidate the molecular mechanism(s) of transcriptional activation by nuclear receptors (27). Identified coactiva-
tor proteins primarily belong to two groups: the SRC-1 family and the CREB-binding protein (CBP)/p300 family. The SRC-1 family includes SRC-1/NCoA-1 (37, 58, 74) and the related proteins GRIP1/TIF2/NCoA-2 (34, 35, 74, 79) and AIB1/p300/ACTR/RAC3/TRAM-1 (2, 14, 44, 73, 74). The second group of coactivators includes CBP and its homolog p300, which not only influence the activities of nuclear receptors (13, 31, 37) but also functionally interact with many transcription factors such as CREB (3, 16, 40, 46), the Stats (10, 87), AP1 (4, 7), and p53 (28, 45). There are also coactivator proteins that do not belong to these two groups, such as ARA70 (85), PGC-1 (60), and the recently reported RNA coactivator SRA (41). Members of both the SRC-1 family and CBP/p300 family have been shown to possess histone acetyltransferase activities (8, 14, 57, 69), suggesting that chromatin remodelling by histone acetylation is an important mechanism involved in transcriptional activation by ligand-bound nuclear receptors.

Interaction of members of the SRC-1 and CBP/p300 families with nuclear receptors occurs through conserved LXXL motifs (32), which interact with a hydrophobic cleft in the receptor LBD formed as a result of conformational changes mediated by ligand binding (19, 23, 56). SRC-1/NCoA-1 and GRIP1/TIF2 contain three LXXL regions or boxes (referred to as LXDs or nuclear receptor boxes) that differentially interact with nuclear receptors so that different nuclear receptors functionally utilize different LXXL boxes (19, 52). Thus, ER uses the second LXXL box of SRC-1/NCoA-1 while PR uses both the first and second LXXL boxes for optimal interaction. In contrast, TR and RAR require both the second and third LXXL boxes for optimal interaction (52). The specificities of receptor recognition by the different LXXL boxes of SRC-1/NCoA-1 are primarily mediated by 8 amino acid residues C-terminal to the LXXL motif rather than by the 2 amino acids (XX) within the motif itself. Thus, while members of the SRC-1 family are capable of interacting with many nuclear receptors, the molecular details of such interactions differ for each receptor in the number or combination of LXXL boxes used as well as in the critical amino acid residues surrounding the LXXL motifs.

While much has been learned from the study of known coactivators, a number of key mechanistic questions remain to be answered. For example, many nuclear receptors can recognize common DNA elements, (25, 49, 51), while not all are capable of regulating genes containing those elements (20, 47, 65). Thus, native target genes containing such elements are selectively regulated by specific receptors is a very important but poorly understood problem. Although the various LXXL boxes of SRC-1 and GRIP1 show differential receptor preference (19, 52), these coactivators are unlikely to play a primary role in mediating effects that are receptor specific since they appear to interact with all ligand-bound nuclear hormone receptors. Thus, the detailed molecular mechanism(s) underlying receptor-specific regulation of gene expression remains to be elucidated. Whether a coactivator(s) contributes to this specificity is currently unknown.

To further our understanding of the molecular events underlying receptor-activated transcription, we sought to identify additional coactivators using a yeast two-hybrid screening strategy (29). In this paper, the novel coactivator for nuclear receptors, designated NRIIF3 (for nuclear receptor-interacting factor 3). Fluorescence microscopy indicates that NRIIF3 is a nuclear protein. The yeast two-hybrid and in vitro binding assays revealed that NRIIF3 interacts specifically with TR and RXR in a ligand-dependent fashion but does not interact with other examined nuclear receptors. Transfection experiments indicated that NRIIF3 selectively potentiates TR- and RXX-mediated transactivation in vivo. The NRIIF3 gene encodes a small protein of 177 amino acids and, other than having an N-terminal LXXLL motif, has no homology with known coactivator genes. The results of a combination of computer modeling and domain and mutagenesis analyses suggest that NRIIF3 interacts with nuclear receptors through its C-terminal domain that contains a novel LXXLL module while another part of NRIIF3 may contribute to its observed receptor specificity. These studies may provide novel insights into the molecular mechanism(s) of receptor-mediated transcriptional activation as well as the functional specificities of nuclear receptors.

MATERIALS AND METHODS

Isolation of NRIIF3 and the yeast two-hybrid assay. The Brent two-hybrid system (29) was employed to isolate candidate cDNA clones containing LexA-TRA in a ligand-dependent fashion. Full-length chicken TRα (cTRα) was fused in frame to the C terminus of the LexA DNA binding domain (DBD) in pEG202 (29). The LexA-TRA bait, the LacZ reporter (pSH18-34), and a pG4-5-based HeLa cell DNA library were transformed into the yeast strain EGY48 (29). The transformants were selected on Gal-Raf-X-Gal (5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside) medium in the absence of leucine and were further screened for the expression of LexA in the presence of 1 μM T3. Blue colonies were picked and renamed for T3-dependent activation of LexA. Positive yeast clones were then selected, and plasmids harboring candidate prey cDNAs were isolated. An individual candidate prey plasmid was then amplified in Escherichia coli and retransformed into the original yeast strain to confirm the interaction phenotype. The cDNA inserts were then sequenced with an automatic sequencer. Four novel clones (NRIIF1, -2, -3, and -4) were obtained. Among them, NRIIF3 was a full-length clone.

Type-specific assay. NRIIF3, the β3-endorphin long form (EnL), and short form (EnS), and the 1.9A RA mutant protein were examined for their interaction with various nuclear receptors in a yeast two-hybrid assay. The following receptor baits were used: the LexA-cTRα LBD, LexA-human TRβ (hTRβ) LBD, LexA-hRARα LBD, LexA-hMR-RARα LBD, hRARα LBD, and hRARα LBD. The TRα LBD (LXXLL) domain (NCD) was fused in frame with the LexA DBD and examined for interaction with receptor LBDs with the following preys: the b4-cTRα LBD, b4-hRARα LBD, and b4-hRXRα LBD expressed from pG4-5. Yeast cells harboring appropriate plasmids were grown in selective media with Gal-Raf in the presence or absence of cognate ligand (1 μM T3 for TR, all trans 9-cis RA for RAR, 9-cis RA for RAR, and 10 μM dexamethasone (Dex) for GR) overnight before β-galactosidase activity was assayed with o-nitrophenyl β-D-galactopyranoside as the substrate. β-Galactosidase units were calculated with the formula (OD420 × 1,000)/(minutes of incubation × OD500 of yeast suspension), where OD420 and OD500 are the optical densities at 420 and 600 nm, respectively.

Fluorescence microscopy. Full-length NRIIF3 was fused into the green fluorescent protein (GFP) fusion protein vector pEGFP (Clontech). The resulting GFP-NRIIF3 vector and the control plasmid pEGFP were transfected into the cells by calcium phosphate precipitation. After incubation at 37°C for 24 h before the examination with a fluorescence microscope to determine the subcellular location of GFP-NRIIF3 or the GFP control.

In vitro binding assay. Full-length NRIIF3 was cloned into pGEX2T, a bacterial glutathione (GST) fusion protein expression vector (Pharmacia). The GST-NRIIF3 fusion protein was expressed in E. coli and affinity purified with glutathione-agarose beads (30). 35S-labeled full-length cTRα, hRARα, hRXRα, hVDR, hNR, hPF, and hER were generated by in vitro transcription and translation with a reticulocyte lysate system (Promega). Binding was performed as previously described (30) with the following buffer: 20 mM HEPES (pH 7.9)-1 mM MgCl2-1 mM dithiothreitol-10% glycerol-0.05% Triton X-100-1 mM ZnCl2-150 mM KCl. Appropriate ligands were added into the binding reaction mixture where indicated in the figures in the following concentrations: 1 μM T3 for TR, 1 μM all-trans RA or 9-cis RA for RAR, 1 μM 9-cis RA for RAR, and 150 mM 1,25(OH)2vitamin D3, dexamethasone, progesterone, or estradiol for VDR, GR, PR, or ER, respectively. After the binding reaction, the beads were washed three times and the labeled receptors bound to the beads were examined by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis followed by autoradiography. Five percent of the 35S-labeled receptor input was also electrophoresed in the same gel.

Transfection studies. Most reporters used in this study, including IR-DMVAT, DR-MDMVAT-CAT, GH-IRE-kCAT, and IR-3 (ERS)-DMVAT-CAT, have been described previously (5, 25, 78). A DR1-DMVAT-CAT reporter responsive to RXX was obtained from Ron Evans. A GRE/IRE-kCAT reporter was obtained from Gunther Schütz. (IR)-2ATA-CAT was constructed in our laboratory by cloning two copies of the inverted-repeat (IR) sequence (AGG TCA TGA CCT) upstream of a TATA element derived from the thymidine kinase (tk) promoter. An hVDR expression vector and VDRE-DMVAT-CAT containing the VDRE from the osteocalcin promoter were obtained from J. Wesley Pike. Vectors expressing cTRA, hRARα, hRXα, or GR (gk), hPR,
and hER have been described previously (15, 25, 26, 50, 53, 81). The NRIF3 expression vector was constructed by cloning full-length NRIF3 into a pExpress vector (25). Appropriate plasmids were transfected into HeLa cells by calcium phosphate co-precipitation with 25 to 100 ng of the receptors, 250 to 500 ng of the chloroformic acetyltransferase (CAT) reporters, and 750 ng of the NRIF3 or control pEG202 vector. After transfection, the cells were incubated at 37°C (with or without cognate ligands) for 48 h before being harvested. CAT assays were carried out as previously described (30). Relative CAT activity was determined as the percent acetylation of the substrate per 30 μg of cell protein in a 15-h incubation at 37°C. The results were calculated from duplicate or quadruplicate samples and the variation among samples was less than 10%.

Domain and mutagenesis analyses. To construct pEG4-5-derived vectors expressing EnL or EnS, the pEG4-5/NRIF3 plasmid was digested with NcoI and XhoI and the resulting vector fragment was gel purified. This fragment was then ligated to an EnL or EnS insert generated from pExpress-EnL or pExpress-EnS by NcoI/SalI double digestion. The resulting pEG4-5/EnL or pEG4-5/EnS plasmid was confirmed by sequence analysis. The L9A mutant form of NRIF3 was generated by site-directed mutagenesis by a PCR-based method, and the mutation was confirmed by sequence analysis. pEG4-5-derived vectors expressing EnL, EnS, or the L9A NRIF3 mutant form were transformed into yeast strains harboring the LacZ reporter (pSH18-34) and appropriate bait plasmids (LexA-TR, LexA-RAR, LexA-RXR, and LexA-GR). Transformants were subjected to quantitative assays of β-galactosidase activity as described above.

Docking of coactivator peptides to receptors. We built a model of the interaction between the 17-residue C-terminal peptide of NRIF3 (KASHRLDYEFLKAILN) and the LBDs of several receptors (TRα was used as an example in the experiment reflected in Fig. 10). An LXXL motif within the NRIF3 peptide is underlined. A similar modeling procedure was carried out on a 20-residue peptide (SILTERKHILLRLOEGGPSD) of the second LXXL box of SRC-1 (32). We hypothesized that the LXXL motif of the C terminus of NRIF3 could interact with the coactivators' LXXL box. The NRIF3 docking procedure was carried out towards this site (71, 75, 76). Two critical features of the interaction between the LBDs of nuclear hormone receptors and their coactivators were used to build the model. (i) One was the "charge clamp," initially observed in the complex between SRC-1 and PPARα (56), where a or without cognate ligands) for 42 h before being harvested. CAT assays were carried out as previously described (30). Relative CAT activity was determined as the percent acetylation of the substrate per 30 μg of cell protein in a 15-h incubation at 37°C. The results were calculated from duplicate or quadruplicate samples and the variation among samples was less than 10%.

RESULTS

Cloning of NRIF3 cDNA. To isolate potential coactivators mediating the transcriptional activation functions of nuclear receptors, we employed a yeast two-hybrid screening strategy (29). A bait expressing a full-length TRα fused to the C terminus of the LexA DBD was used to screen a HeLa cell cDNA library cloned into pEG4-5 (29). Candidate clones were identified by a thyroid hormone (T3)-dependent interaction with LexA-TRα vectors were selected and further examined and sequenced. Four novel clones were identified, and all were found to exhibit levels of interaction with the LBD of TRα similar to the levels they exhibited with the full-length receptor (data not shown). These clones were designated NRIF1, -2, -3, and -4. Not surprisingly, the LBD of TRβ was also found to interact with these NRIFs in a T3-dependent manner (data not shown). Among these four isolated NRIFs, NRIF3 was a full-length clone. As shown in Fig. 1, LexA alone (negative control) did not interact with NRIF3 (as indicated by the low β-galactosidase activity) and incubation with T3 had no effect. Similarly, no interaction was detected between the LexA-TR LBD and B42 alone with or without T3 (data not shown). The LexA-TR LBD also showed little interaction with NRIF3 in the absence of T3. However, incubation with T3 resulted in strong stimulation of the NRIF3-TR LBD interaction (Fig. 1). The extent of T3-dependent interaction between NRIF3 and the LexA-TR LBD was similar to that of Trip1 (Fig. 1), one of the first TR-interacting factors cloned in a two-hybrid screen (42).

Sequence analysis of NRIF3. Sequence analysis of the NRIF3 cDNA revealed a single open reading frame encoding a poly-peptide of 177 amino acids (Fig. 2). NRIF3 has no homology with members of the SRC-1 and CBP/p300 families. The size of NRIF3 is in sharp contrast to the size of CBP/p300 (around 300 kDa) or of SRC-1 family members (around 160 kDa). NRIF3 contains a putative nuclear localization signal (KRKK), as well as one copy of an LXXL motif (amino acids 9 to 13) that was recently identified as being essential for the interaction of a number of putative coactivators with nuclear receptors (32).

A database search identified two highly related homologs of NRIF3, which were previously designated β3-endonexin short and long forms (67). The endonexin short form (EnS) was originally isolated from a two-hybrid screen intended to clone factors that interact with the cytoplasmic tail of integrin β3 (67). The long form (EnL) was then identified as an alternatively spliced product of the same gene. However, the long form does not bind to integrin β3 (67). Nucleotide sequence comparisons between cDNAs of NRIF3 and EnS or EnL indicate that NRIF3 is a third alternatively spliced product of the same gene (alignment not shown). The precise function(s) of the two endonexin proteins is under investigation (reference 66a and see Discussion).
location of the expressed proteins was visualized by fluorescence microscopy. We also studied the interaction of NRIF3 with direct contact with a structurally conserved surface in the LXXLL motif appears to be involved in the tranlation domains. The LXXLL motif appears to be involved in increase in TR binding to GST-NRIF3, confirming that NRIF3 shown in Fig. 3, the control binding assays were performed (30). Interestingly, when LexA-RAR or LexA-GR was used as the bait, no interaction was detected with NRIF3 in the presence or absence of their cognate ligands (Table 1). The finding that NRIF3 interacts with TR but not RAR was surprising in light of previous reports which showed that TR and RAR functionally interact with the same LXXLL nuclear receptors. The LBDs of several nuclear receptors were examined for interaction with NRIF3 in a yeast two-hybrid assay. As shown in Table 1, NRIF3 does not interact with LexA alone (negative control) with or without ligand. LexA-TR and LexA-RXR showed little (if any) interaction with NRIF3 in the absence of their cognate ligands. However, the presence of T3 (for TR) or 9-cis RA (for RXR) resulted in a strong stimulation of their interaction with NRIF3, as indicated by the induction of β-galactosidase activity (Table 1). Most interestingly, when LexA-RAR or LexA-GR was used as the bait, no interaction was detected with NRIF3 in the presence or absence of their cognate ligands (Table 1). The finding that NRIF3 interacts with TR but not RAR was surprising in light of previous reports which showed that TR and RAR functionally interact with the same LXXLL boxes (2 and 3) of SRC-1/NCoA-1 (52). As positive controls, we confirmed that both LexA-RAR and LexA-GR exhibited ligand-dependent interaction with other coactivators that are not receptor specific (data not shown). Taken together, these results suggest that NRIF3 exhibits differential specificities in its interactions with different nuclear receptors.

NRIF3 selectively potentiates TR- and RXR-mediated transactivation in vitro. To further examine the interaction between NRIF3 and various nuclear receptors as to confirm the potential receptor specificity of NRIF3, in vitro GST binding assays were performed (30). "S"-labeled nuclear receptor, generated by in vitro transcription and translation, was incubated with purified GST-NRIF3 or the GST control bound to glutathione-agarose beads. All binding assays were carried out with or without the cognate ligand of the examined receptor. As shown in Fig. 4 (top left), TR and NRIF3 interact poorly in the absence of T3. Addition of T3 resulted in a strong increase in TR binding to GST-NRIF3, confirming that NRIF3 associates with TR in a T3-dependent manner. Using similar binding assays, we also studied the interaction of NRIF3 with six other nuclear receptors. Consistent with our findings from the yeast two-hybrid experiments (Table 1), NRIF3 interacted with RXR in vitro in a ligand-dependent manner (Fig. 4) but showed little or no binding to other nuclear receptors (RAR, VDR, GR, PR, and ER) in the presence or absence of their cognate ligands (Fig. 4). Taken together, the results of the yeast two-hybrid (Table 1) and the in vitro binding (Fig. 4) assays suggest that NRIF3 possesses a distinct receptor specificiy.

**TABLE 1. Interaction of NRIF3 with nuclear receptors in yeast**

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey</th>
<th>Without ligand</th>
<th>With ligand</th>
<th>Fold stimulation</th>
</tr>
</thead>
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<td>0.8</td>
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<td>125</td>
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<td>315</td>
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<tr>
<td>LexA-GR</td>
<td>NRIF3-B42</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
</tr>
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*The LacZ reporter activities were determined for yeast strains harboring the indicated bait and prey plasmids in the presence or absence of cognate ligands as described in Materials and Methods. See the text for detailed explanations.

**FIG. 3.** GFP-NRIF3 fusion protein was expressed in HeLa cells by transient transfection, and the subcellular location of the expressed protein since extensive homology was found between NRIF3 and EnL. EnL consists of 111 amino acids and is 100% identical to the first shown in Fig. 3, the control frame was fused to the C terminus of the entire NRIF3 open reading boxes (boxes 2 and 3) of SRC-1/NCoA-1 (52). As positive controls, we confirmed that both LexA-RAR and LexA-GR exhibited ligand-dependent interaction with other coactivators that are not receptor specific (data not shown). Taken together, these results suggest that NRIF3 exhibits differential specificities in its interactions with different nuclear receptors.

NRIF3 selectively potentiates TR- and RXR-mediated transactivation in vitro. To further examine the interaction between NRIF3 and various nuclear receptors as to confirm the potential receptor specificity of NRIF3, in vitro GST binding assays were performed (30). "S"-labeled nuclear receptor, generated by in vitro transcription and translation, was incubated with purified GST-NRIF3 or the GST control bound to glutathione-agarose beads. All binding assays were carried out with or without the cognate ligand of the examined receptor. As shown in Fig. 4 (top left), TR and NRIF3 interact poorly in the absence of T3. Addition of T3 resulted in a strong increase in TR binding to GST-NRIF3, confirming that NRIF3 associates with TR in a T3-dependent manner. Using similar binding assays, we also studied the interaction of NRIF3 with six other nuclear receptors. Consistent with our findings from the yeast two-hybrid experiments (Table 1), NRIF3 interacted with RXR in vitro in a ligand-dependent manner (Fig. 4) but showed little or no binding to other nuclear receptors (RAR, VDR, GR, PR, and ER) in the presence or absence of their cognate ligands (Fig. 4). Taken together, the results of the yeast two-hybrid (Table 1) and the in vitro binding (Fig. 4) assays suggest that NRIF3 possesses a distinct receptor specificiy.

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FIG. 4. Characterization of the NRIF3 interaction with nuclear receptors in vitro. A 35S-labeled full-length receptor (cTRα, hRARα, hRXRα, hVDR, hPR, hGR, or hER) was incubated with an affinity-purified GST control or GST-NRIF3 linked to glutathione-agarose beads. The binding was performed in the absence (−) or presence (+) of cognate ligands as described in Materials and Methods. After incubation and washing, the bound receptors were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and detected by autoradiography. The input lane in each binding assay represents 5% of the total 35S-labeled receptor used in each incubation. GST-RXR was used as a positive control for RAR binding.

out. HeLa cells, which lack endogenous TR (25), were transfected with a vector expressing TR and a CAT reporter under the control of the ΔMTV basal promoter linked to an idealized IR (AGGTCATGACCT) TRE sequence (IR-ΔMTV-CAT) (25), along with either a control plasmid or a vector expressing NRIF3. As shown in Fig. 5A, NRIF3 significantly enhances TR-mediated activation of the CAT reporter (typically 2.5- to 3-fold). As a control, we also examined the effect of CBP, a reported coactivator for nuclear receptors (13, 37), and found that its expression results in a degree of enhancement similar to that with NRIF3 (around threefold) (Fig. 5A).

We also examined another CAT reporter controlled by the herpesvirus tk promoter linked to native rat growth hormone TRE sequences (5). NRIF3 was found to also enhance TR-mediated activation of this reporter (about 3.5-fold) (Fig. 5B).

In addition, using similar transfection assays, we found that NRIF3 enhances TR-mediated activation of two other reporters, (IR)2-TATA-CAT and DR4-ΔMTV-CAT (data not shown). Therefore, NRIF3 potentiates TR-mediated transactivation in a variety of different TRE and promoter contexts. Taken together, the results of these transfection studies suggest that NRIF3 can function as a coactivator of TR.

To examine whether NRIF3 can also act as a coactivator for RXR, HeLa cells were transfected with the IR-ΔMTV-CAT reporter, whose IR sequence can also function as a strong response element for the RXR(s) and RAR(s) (25, 49, 61). HeLa cells express the endogenous RXR(s) and RAR(s), as the activity of the IR-ΔMTV-CAT reporter was strongly stimulated by their cognate ligands, even without cotransfection of any receptor expression plasmid (Fig. 6A, bars 1, 3, and 5).

FIG. 5. NRIF3 enhances TR-mediated transactivation in vivo. HeLa cells were transfected with a vector expressing cTRα and the IR-ΔMTV-CAT reporter (A) or the GH-TRE-tk-CAT reporter (B) in the presence (filled bars) or the absence (hatched bars) of 1 μM T3. The vector expressing NRIF3 or the empty control vector was cotransfected to examine the effect of NRIF3 on TR-mediated activation. In panel A, the effect of CBP was compared to that of NRIF3. GH, growth hormone.
Cotransfection of NRIF3 enhanced the activation of this reporter by either 9-cis RA, or LG100153 (72), an RXR-specific ligand (Fig. 6A, bars 1 and 2 and bars 3 and 4). In contrast, although the RAR-specific ligand TTNPB (68) also activated the IR-ΔMTV-CAT reporter, cotransfection of NRIF3 had no effect (Fig. 6A, bars 5 and 6). These results indicate that NRIF3 potentiates the activity of the endogenous RXR(s) but not the RAR(s), which is consistent with the distinct receptor specificity of NRIF3 revealed from the yeast two-hybrid assay (Table 1) and in vitro binding experiments (Fig. 4).

To further document that NRIF3 can function as a coactivator for RXR, a vector expressing exogenous RXR was cotransfected with IR-ΔMTV-CAT. Exogenous RXR expression enhanced the activation of this CAT reporter by either 9-cis RA or LG100153 (compare Fig. 6A and B, bars 1 and 3). This RXR-mediated activation of reporter expression was further stimulated by NRIF3 (Fig. 6B). Finally, we also examined the activation of a DR1-ΔMTV-CAT reporter. This DR1 (AGGT CANAGGATCA [where N is any nucleotide]) sequence is thought to be a specific response element for RXR (39, 51). Although we found that this DR1 is a weaker response element than the IR sequence, cotransfection of an RXR expression vector led to ligand-induced activation of this DR1 reporter, which was also further enhanced by NRIF3 (Fig. 6C).

NRIF3 does not potentiate the activities of GR, PR, ER, and VDR in vivo. The selective coactivation of TR and RXR (but not RAR) by NRIF3 is consistent with its distinct binding specificities to these receptors. To further establish that NRIF3 acts as a receptor-specific coactivator, we next examined the effect of NRIF3 on the activities of four additional nuclear receptors, including GR, PR, ER, and VDR, by transfection experiments. HeLa cells were transfected with a GRE/PRE-tk-CAT reporter along with a vector expressing either GR or PR. As shown in Fig. 7A, cognate hormone treatment results in activation of the CAT reporter. However, expression of NRIF3 has little effect (Fig. 7A). Similar experiments were carried out with ER and ERE-ΔMTV-CAT or VDR and VDRE-ΔMTV-CAT. As shown in Fig. 7B and C, NRIF3 was found to have little or no effect on the activities of these receptors as well. Taken together, the combined results of our transfection studies support the notion that NRIF3 is a coactivator with a unique receptor specificity.

**A novel C-terminal domain in NRIF3 is essential for ligand-dependent interactions with TR and RXR.** The LXXLL signature motif has been found to be present in the receptor-interacting domains of many identified coactivators, such as SRC-1/NCoA-1 and GRIP1/TIF-2 (32). The broad spectrum of receptor binding by coactivators such as SRC-1 suggests that the LXXLL-containing interacting domain may recognize structurally similar surfaces of these LBDs. Indeed, recent structural and functional studies revealed that the LXXLL motif and its nearby flanking amino acids are involved in direct contact with a hydrophobic cleft of the target surfaces presented by the ligand-bound LBDs of nuclear receptors (19, 23, 52, 56). The facts that NRIF3 also contains an LXXLL motif (amino acids 9 to 13) (Fig. 2 and 8A) and exhibits a distinct structural similarity to the LXXLL-containing domains of many known coactivators (19, 23, 52, 56, 58) suggest that NRIF3 might be a novel coactivator with a unique receptor specificity.

To explore these issues, we examined whether EnS and EnL, which contain the same LXXLL motif and flanking amino acids as NRIF3, can interact with nuclear receptors in a yeast two-hybrid assay (Fig. 8). EnS consists of 111 amino acids and is 100% identical to the first 111 residues of NRIF3, while EnL consists of 170 amino acids, the first 161 of which are also 100% identical to the same region in NRIF3 (Fig. 2 legend and 8A). Thus, NRIF3 and EnL differ only in their C termini, with NRIF3 having a unique region of 16 amino acids and EnL having a unique region of 9 amino acids (Fig. 8A). Interestingly, despite their extensive identity with NRIF3, the interaction with liganded TR or RXR is completely abolished in EnS and EnL (Fig. 8B). We also examined other nuclear receptors that do not interact with NRIF3 and found that they also do not interact with EnS or EnL (data not shown). These results...
FIG. 7. NRIF3 does not potentiate the activity of GR, PR, ER, or VDR. HeLa cells were transfected with the following CAT reporters and appropriate receptor expression vectors: GRE/PRE-tk-CAT and rGR or hPR (A), ERE-ΔMTV-CAT and hER (B), and VDRE-ΔMTV-CAT and hVDR (C). Cells were incubated in the presence (filled bars) or absence (hatched bars) of 100 nM dexamethasone for GR, progesterone for PR, estradiol for ER, and 1,25-(OH)₂-vitamin D₃ for VDR. Cotransfection of NRIF3 was found to have little effect on the activities of these receptors.

indicate that the unique C-terminal domain in NRIF3 (NCD) (residues 162 to 177) is essential for its specific interaction with liganded TR and RXR while the N-terminal LXXLL motif (amino acids 9 to 13) and its flanking sequences are not sufficient to allow for detectable receptor interactions.

Although the LXXLL motif was found to be insufficient for interaction, we examined whether this N-terminal motif of NRIF3 contributes to the NRIF3-receptor interaction by mutating the first leucine of the LXXLL motif into alanine (L9A) by site-directed mutagenesis. Previous experiments have shown that the three leucine residues are essential for an LXXLL module to interact with receptor LBDs and that the replacement of any of them with alanine abolishes the interaction (32). We examined the L9A NRIF3 mutant form for its interaction with TR and RXR in a yeast two-hybrid assay. As shown in Fig. 9, the L9A mutant was still capable of ligand-dependent interaction with TR and RXR (~25-fold induction by ligand). However, the introduced mutation reduced the interaction by about 4-fold (for TR) or 14-fold (for RXR). These results suggest that although the LXXLL motif is not absolutely essential for NRIF3 interaction with liganded receptors, it plays a role in allowing an optimum interaction to occur.

Computer modeling suggests that the NCD docks into the hydrophobic cleft of the liganded LBDs. Secondary-structure analysis of the C-terminal domain of NRIF3 predicted the formation of an α-helix. Moreover, inspection of the putative C-terminal helix revealed an LXXL motif (amino acids 172 to 176), which is reminiscent of the canonical LXXLL. Although the ultimate elucidation of the molecular basis of the NRIF3-

FIG. 8. The NCD is essential for the interaction with liganded TR or RXR. (A) Schematic comparison of NRIF3 with EnS and EnL. EnS is 100% identical to the first 111 amino acids of NRIF3 and EnL (open boxes). The regions from amino acids 112 to 161 in NRIF3 and EnL (stippled boxes) are 100% identical. NRIF3 and EnL differ in their C termini (16 amino acids in NRIF3 [hatched box] and 9 amino acids in EnL [filled box]). The positions of the LXXLL motif and a putative nuclear localization signal (KRKK) are also indicated. (B) NRIF3 (N), EnS (S), or EnL (L) was examined for interaction with LexA-TR or LexA-RXR in a yeast two-hybrid assay as described in Materials and Methods. The assays were performed in the absence (hatched bars) or the presence (filled bars) of 1 μM T3 (for TR) or 9-cis RA (for RXR).
receptor interaction awaits future studies such as X-ray crystallography, the putative helix structure of the NCD and its LXXIL motif suggest that the NCD may interact with the liganded LBDs in a fashion similar to that of the receptor-interacting domains that employ the canonical LXXLL motif(s). To explore this possibility, we modeled the interaction of the C terminus of NRIF3 with the liganded LBDs, using algorithms developed mainly by the staff of the laboratory of one of the authors (R. Abagyan and coworkers) (1, 63, 70, 74, 75). The background information and procedures used for constructing these models are described in Materials and Methods. The results of our modeling suggest that the NCD fits well into the hydrophobic cleft formed on the LBDs as a result of ligand binding. An example of such a model (NCD-TR LBD) is shown in Fig. 10. In this model, the two leucines and one isoleucine of the LXXIL motif are predicted to be deeply buried in the central cavity of the hydrophobic groove formed by the liganded LBD of the receptor. We also calculated the putative binding energy for the modeled NCD-TR complex, using an improved partitioning binding energy function, with continuum representation of the electrostatics of the system (64). The calculated binding energy for the modeled NCD-TR complex is about \(-21 \text{ kcal/mol}\). As a control, we carried out a similar modeling procedure using the second LXXLL box within the receptor-interacting domain of SRC-1. This LXXLL box has been shown to be required for interaction with TR (52). Our calculated binding energy for this LXXLL box with liganded TR LBD is \(-18 \text{ kcal/mol}\), a value that is very close to the one calculated for the NCD. Altogether, our modeling and calculations suggest a mechanism in which the NCD directly mediates interaction with liganded LBDs through an LXXIL motif.

**Functional interaction of the NCD with liganded LBDs and the essential role of its LXXIL motif.** To explore the possibility suggested from our computer modeling, the NCD (amino acids 162 to 177) was fused to the LexA DNA binding domain and was examined for interaction with the receptor LBDs in a yeast two-hybrid assay. The LexA-NCD fusion protein alone does not activate the LacZ reporter in yeast (data not shown). However, when the LexA-NCD and the LBD of TR or RXR (fused with B42) were used in the two-hybrid assay, a strong ligand-dependent interaction was observed, as indicated by the induction of \(\beta\)-galactosidase activity by their cognate ligands (1 \(\mu\)M T3 for TR; 1 \(\mu\)M 9-cis RA for RXR).

**FIG. 9.** The LXXIL motif of NRIF3 is required for optimum interaction with TR and RXR. Wild-type NRIF3 (WT) or the L9A NRIF3 mutant (L9A) was examined for interaction with LexA-TR or LexA-RXR in a yeast two-hybrid assay as described in Materials and Methods. \(\beta\)-Galactosidase activities were determined in the absence (filled bars) or presence (stippled bars) of cognate ligands (1 \(\mu\)M T3 for TR; 1 \(\mu\)M 9-cis RA for RXR).

**FIG. 10.** Hypothetical model of the interaction of the NCD and the liganded LBD. The docking of the C-terminal helix of NRIF3, which contains an LXXIL motif, to the ligand-bound LBDs was carried out as described in Materials and Methods. The NCD-TR LBD model is shown here as an example. The side chains of the two leucines (green) and one isoleucine (cyan) of the LXXIL core fit within a hydrophobic groove (salmon) on the surface of the liganded LBD (80). A similar modeling procedure was carried out with an LXXLL box of SRC-1 (result not shown). Putative binding energies (\(-21 \text{ kcal/mol}\) for the NCD and \(-18 \text{ kcal/mol}\) for the LXXLL box of SRC-1) were calculated as described in Materials and Methods. See the text for details.

**FIG. 11.** Interaction of the NCD with the receptor LBDs and the role of the LXXIL motif. The wild-type NCD (WT) or the NCD mutant form (Mut) in which the three core hydrophobic residues of the LXXIL motif (two leucines and one isoleucine) are changed into alanines was examined for interaction with the LBDs of TR, RXR, and RAR in a yeast two-hybrid assay as described in Materials and Methods. \(\beta\)-Galactosidase activities were determined in the absence (open bars) or presence (stippled bars) of cognate ligands. The prey expressing B42 alone was used as a negative control.
ligands (Fig. 11). These results suggest that the NCD can directly interact with the LBDs of TR and RXR in a ligand-dependent manner.

Since NRIF3 harbors a distinct receptor specificity in interacting only with TR and RXR and not other receptors (e.g., RAR), we next asked whether the NCD also harbors a receptor specificity. To our surprise, the NCD was found to interact efficiently with the LBD of RAR in a ligand-dependent manner (Fig. 11). Therefore, while our results clearly suggest that the NCD is an important surface for receptor interactions, as the NCD is found to be both essential for (Fig. 8) and sufficient to mediate (Fig. 11) such interactions, it nevertheless does not appear to be (solely) responsible for the receptor specificity of NRIF3. It is possible that another region of the NRIF3 molecule contributes to the observed receptor specificity of NRIF3 and/or that the specificity is determined by the overall three-dimensional structure of NRIF3.

Since our model predicts the importance of the LXXIL motif in the NCD-receptor interaction (Fig. 10), we tested this by changing the three core residues of the motif (two leucines and one isoleucine) into alanines. As expected, interaction with the LBDs is completely abolished in the resulting mutant NCD (Fig. 11), confirming that the LXXIL motif is essential for the interaction.

DISCUSSION

Recent efforts in understanding receptor-mediated transcription have led to the identification of a number of co-activators for nuclear hormone receptors, which can be categorized into two main groups based on overall homology, the SRC-1 family (including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and AIB1/pCIP/ACTR/RAC3/TRAM-1) (2, 14, 34, 35, 37, 44, 58, 73, 74, 79) and the CBP/p300 family (13, 31, 37). Other putative coactivators (e.g., ARA70 and PGC-1) that do not belong to the SRC-1 or CBP/p300 family have also been identified (60, 85). In addition, pCAF may also be involved in receptor action through its association with nuclear receptors as well as with other coactivators (11, 14, 38, 83). Among these known coactivators, CBP/p300, members of the SRC-1 group, and pCAF all possess histone acetyltransferase activities (8, 14, 57, 69, 83).

In this study we report the identification of a novel nuclear receptor protein (NRIF3) which exhibits specific ligand-dependent interactions with TR and RXR but not with RAR, VDR, GR, PR, or ER. Functional experiments indicated that NRIF3 potentiates TR- and RXR-mediated transactivation in vivo but exhibits little or no effect on the activities of other examined receptors. Therefore, NRIF3 represents a novel coactivator with a distinct receptor specificity and, thus, may shed light on clarifying the molecular mechanism(s) underlying receptor-specific regulation of gene expression.

A database search indicated that NRIF3 has no homology with any known coactivators except in a single LXXLL motif. An unusual feature of NRIF3 is its relatively small size, which is in sharp contrast to the sizes of SRC-1 and CBP/p300. A homology search identified two alternatively spliced isoforms of NRIF3 which were previously designated β3-endonexin short and long forms (67). Preliminary studies with these two endonexins indicate that, like NRIF3, they localize to the cell nucleus (43a, 66a). Interestingly, despite their extensive identities with NRIF3, both EnS and EnL fail to exhibit interaction with liganded nuclear receptors (Fig. 8). Consistent with this finding, we found that EnS and EnL have little effect on receptor-mediated transcription in transient transfection experiments (data not shown). Therefore, the precise roles of these two endonexins remain to be elucidated. We suggest two non mutually exclusive possibilities. First, since both EnL and EnS appear to localize to the nucleus, it is possible that they act as cofactors for other transcriptional regulators. Second, since the EnS can interact with the cytoplasmic tail of β3-integrin (22, 67), it may communicate signals generated at the plasma membrane to the cell nucleus. An example of a protein which is involved in both cell adhesion and transcriptional regulation is β-catenin (82).

Previous study of the endonexins identified the presence of NRIF3-related mRNAs (by Northern blotting) in a wide range of human tissues (67). Because NRIF3 and EnL contain almost identical nucleotide sequences and differ only by an alternative splice site which results in the removal of a small exon in NRIF3, it is difficult to specifically identify NRIF3 mRNA by Northern blotting. A search of the expressed sequence tag database indicates that NRIF3 mRNA, as well as both EnL and EnS mRNAs, is expressed. However, the precise determination of cell and tissue distribution of NRIF3, EnS, and EnL will require the development of highly selective antibodies. Nevertheless, the wide expression pattern of NRIF3-related mRNAs is consistent with the role of NRIF3 as a coactivator of the TRs, which are also widely expressed (70), or the RXRs, which are ubiquitously expressed (48).

A key goal concerning the action of nuclear hormone receptors is to understand the molecular events underlying the functional specificities of different receptors in regulating the expression of their target genes. Determinants of specificity include specific ligand binding and selective binding of the receptors to their cognate response elements, as well as specific expression patterns of different receptors. These determinants alone, however, are not always sufficient to explain the extents of specificity observed for members of the nuclear receptor family. For example, several members of the thyroid hormone/retinoid receptor subfamily may bind similarly to common DNA elements while target genes containing those elements are only selectively activated by certain receptors (20, 47). Therefore, it is likely that additional factors (determined by cell and promoter contexts) are involved in determining receptor functional specificity. In this respect, most known coactivators do not appear to be receptor specific. For example, members of the SRC-1 and CBP/p300 families interact with and appear to be involved in the actions of many nuclear receptors (13, 14, 34, 37). Two known coactivators that may be involved in receptor-specific functions are ARA70 and PGC-1. The AR coactivator ARA70 has been reported to potentiate the activity of AR more efficiently than it does the activities of other nuclear receptors (85). However, whether ARA70 can associate with other receptors remains to be thoroughly examined. The expression of PGC-1 is restricted mainly to the brown fat tissue and is thought to be directly involved in the regulation of thermogenesis by PPARγ (60). Nevertheless, PGC-1 exhibits a relatively broad spectrum of binding to different nuclear receptors. Therefore, the identification of NRIF3 represents the first example of a coactivator with such a clearly defined receptor specificity.

The receptor specificity of NRIF3 raises an interesting question about its molecular mechanism. Domain analysis suggests that the LXXLL motif (amino acids 9 to 13) and its flanking sequences in NRIF3 are not sufficient for interaction with liganded nuclear receptors. In fact, such interaction is completely abolished in EnL, an alternatively spliced product which has the same LXXLL motif and contains the first 161 amino acids (of a total of 177 amino acids) of NRIF3. This result suggests that a putative domain consisting of the last 16 amino acids of NRIF3 (residues 162 to 177) is essential for its
interaction with liganded receptors. Inspection of this NCD indicates that it contains an LXXLII motif (amino acids 172 to 176), and secondary-structure analysis predicts the formation of an α-helix. The predicted helix structure and the similarity of LXXLII to the canonical LXXLL raise the possibility that this LXXLII-containing region plays a direct role in NRIF3-receptor interactions.

Our modeling of the NCD-LBD interaction (Fig. 10) suggests that the same hydrophobic groove in the ligand-bound LBD, which has been shown by previous studies to be the binding site for coactivators such as SRC-1/NCoA-1 and GRIP1 (19, 23, 56), may also be a suitable site for the docking of the C-terminal helix of NRIF3. Thus, the utilization of the complementary pair of an α-helix (in the coactivator) and a hydrophobic groove (in the receptor) for interaction seems to be a general scheme that also applies to NRIF3. The binding energy estimated for the NCD and the TR LBD (−21 kcal/mol) is similar to the value calculated for the second LXXLII box of SRC-1/NCoA-1 and the TR LBD (−18 kcal/mol). To explore the mechanisms suggested by the modeling, we found that the NCD can directly mediate interaction with the LBDs in a ligand-dependent manner (Fig. 11). Moreover, the LXXLII motif contained in the NCD was found to be essential for such interactions (Fig. 11). In summary, the results of a combination of a computer modeling and domain and mutagenesis analyses clearly suggest that the NCD is an important surface that is directly involved in interaction with the LBDs of the receptors, where the LXXLII motif of the NCD mimics the function of a canonical LXXLL. The AF-2 helix (which is a critical constituent of the hydrophobic groove formed upon ligand binding) of the LBD has been shown to be important for interaction with the LXXLII boxes of the coactivators (23). Interestingly, we have examined two TR AF-2 mutants (66) and found that in both cases, ligand-dependent interaction with NRIF3 was abolished (43a).

However, the NCD alone does not appear to harbor the same specificity as NRIF3 (Fig. 11). Thus, it seems likely that another part of the NRIF3 molecule contributes to the observed specificity and/or that the specificity is determined by the overall three-dimensional structure of NRIF3. In this regard, the potential role of the N-terminal LXXLII motif is intriguing. Although the N-terminal LXXLII motif (amino acids 172 to 181) is insufficient alone to mediate an interaction with TR or RXR (Fig. 8), it can influence the interaction of NRIF3 with these receptors, as the L9A NRIF3 mutant retains a significant but nevertheless reduced level of association with liganded TR or RXR (Fig. 9). Thus, NRIF3 appears to employ at least two regions in interacting with liganded TR or RXR, with the NCD playing an essential role and the N-terminal LXXLII motif playing a secondary role. A simplified explanation for these findings is that the NCD provides a major surface for receptor binding while the N-terminal LXXLII motif makes some minor contact with either the same receptor molecule or, more likely, with the other partner of a homodimer or heterodimer to further stabilize the NRIF3-receptor interaction. An example of a coactivator molecule employing two separate regions to interact with the two partners of a receptor dimer has been documented for the recently solved crystal structure of liganded PPARγ complexed with SRC-1/NCoA-1 (56). If NRIF3 indeed employs both its NCD and its N-terminal LXXLII motif in receptor interactions, the specificity may result from the intramolecular dialog between the two regions as well as the intermolecular dialog among NRIF3 and the receptors. However, it remains possible that the N-terminal LXXLII plays only a more indirect role and that the overall three-dimensional structure of NRIF3 is responsible for its observed specificity.

Accumulating evidence suggests that the actions of transcriptional activating proteins are (usually) mediated by multiprotein complexes (59), and such a scheme is also likely for nuclear receptors. For example, biochemical evidence suggests that multiprotein complexes associate with liganded TR and VDR (24, 62, 86). Interestingly, many of the proteins identified in these studies are not known coactivators. While the study of known coactivators such as CBP/p300 and members of the SRC-1 family has suggested that histone acetylation may play an important role in receptor-mediated transactivation (8, 14, 57, 69), detailed elucidation of the transactivation mechanism(s) used by these receptors awaits the identification and study of additional cofactors involved in the transactivation process.

Our results with NRIF3 suggest that transcriptional activation by nuclear receptors may employ a receptor-specific coactivator(s) in addition to the generally used coactivators such as CBP and SRC-1. Therefore, coactivators with NRIF3-like properties may contribute to the functional specificities of nuclear receptors in vivo. Based on our results with NRIF3 and the results of previous studies of nuclear receptor action, we suggest a combinatorial specificity model where a coactivation complex is likely composed of two kinds of factors: general factors that interact with and are involved in the action of many nuclear receptors (such as CBP and SRC-1) and specific factors that exhibit receptor specificity (such as NRIF3). In addition to interacting with the liganded receptor, coactivators may also communicate with each other within the coactivation complex through protein-protein interactions (e.g., CBP/p300 can interact with SRC-1/NCoA-1 or p/CIP) (37, 74, 84). An intriguing possibility is that the combinatorial actions of specific factors and other partners involved in the transactivation process facilitate the recruitment of specific coactivation complexes for different receptors (under different cell, promoter, and HRE contexts), which would provide an important mechanistic layer for receptor functional specificity. An advantage of employing such a combinatorial strategy is that a broad array of diversity can be generated from a relatively small number of involved factors. Further study of NRIF3 with known and possibly other yet to be identified coactivators, as well as analysis of the interplay among these coactivators, should provide important insights into the molecular mechanism(s) underlying the specificity of receptor-mediated regulation of target gene expression.

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Rational discovery of novel nuclear hormone receptor antagonists

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Nuclear hormone receptors (NRs) are potential targets for therapeutic approaches to many clinical conditions, including cancer, diabetes, and neurological diseases. The crystal structure of the ligand binding domain of agonist-bound NRs enables the design of compounds with agonist activity. However, with the exception of the human estrogen receptor-α, the lack of antagonist-bound “inactive” receptor structures hinders the rational design of receptor antagonists. In this study, we present a strategy for designing such antagonists. We constructed a model of the inactive conformation of human retinoic acid receptor-α by using information derived from antagonist-bound estrogen receptor-α and applied a computer-based virtual screening algorithm to identify retinoic acid receptor antagonists. Thus, the currently available crystal structures of NRs may be used for the rational design of antagonists, which could lead to the development of novel drugs for a variety of diseases.

Materials and Methods

Building of the Model of Antagonist-Bound RAR. A helical peptide PIREMLENP corresponding to helix H12 of RARγ was docked into the putative coactivator binding pocket of another RARγ molecule. We hypothesized that the LxxML motif contacts the coactivator binding site of the receptor, and an automatic docking procedure was carried out toward this site, with flexible protein and peptide side chains, according to a biased probability Monte Carlo energy minimization procedure (19, 20). Two critical features of the interaction between the LBDs of NRs and their coactivators were used to carry out the docking: (i) The “charge clamp,” initially observed in the complex between SRC-1 and peroxisome proliferator-activated receptor γ (21), where a conserved glutamate (E414 in RARγ) and lysine (K246 in RARγ) at opposite ends of the hydrophobic cavity of the receptor contact the backbone of the coactivator’s LxxLL box, enabled the orientation of the helical peptide. (ii) The finding that the leucines of the LxxLL motif of SRC-1 are buried in the hydrophobic cavity of the receptor determines which side of the helix faces the receptor. Here, the isoleucine, methionine, and leucine of the LxxML motif were buried in the binding site of RARγ. Loose distance restraints were set between the charge

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Abbreviations: NR, nuclear hormone receptor; RAR, retinoic acid receptor; ER, estrogen receptor; LBD, ligand binding domain; RXR, retinoid X receptor; CAT, chloramphenicol acetyltransferase.

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Fig. 1. Modeling of the antagonist-bound structure of RAR. Agonist (white) and antagonist (cyan) superimpose in the binding pocket of ERα, but the antagonist presents an additional protruding arm that pushes helix 12 (H12, green) away (A). As a result, H12 relocates in the coactivator binding pocket of RARγ (red) was docked to the coactivator binding pocket of the RARγ-LBD (critical hydrophobic residues are displayed in magenta) (C), and the C terminus of the protein was remodeled from its agonist-bound conformation (green) to its antagonist-bound conformation (red) (D).

Receptor-Ligand Docking. An initial docking was carried out with a grid potential representation of the receptor and flexible ligand, by an iCM stochastic global optimization algorithm as implemented in the MolSoft iCM 2.7 program (23, 24).

Screening of a Virtual Library of Compounds. The flexible-ligand/grid-potential-receptor docking algorithm (23, 24) was carried out automatically on the Available Chemicals Directory library of 153,000 available chemical compounds (MDL Information Systems, San Leandro, CA). The screening took less than a month on 10 194-MHz IP25 processors. Each compound was assigned a score, according to its fit with the receptor, which took into account continuum as well as discrete electrostatics, hydrophobicity, and entropy parameters (25). The distribution of the compounds according to their score is presented at http://abagyan.scripps.edu/PNAS/MS2000/. All compounds scoring better (i.e., lower) than −32 were screened further for the number of hydrogen bonds engaged with the receptor. The 134 compounds that made at least two hydrogen bonds with the receptor were preselected. The 609 compounds scoring better than −37 also were preselected, regardless of the hydrogen bonding network. This preselection pool then was further minimized with a full atom representation of the receptor, as described above. The quality of the fit of the 500 best-scoring compounds then was visually estimated, and 32 compounds were selected for biological testing. These compounds are not necessarily the ones with the best final scores, but the ones we thought, after careful visual inspection, presented the best characteristics, such as Van der Waals fit or hydrogen bonding (see http://abagyan.scripps.edu/PNAS/MS2000/).

It occurred to us that during the selection by the MolSoft virtual screening procedure, it was preferable to set up an initial cut-off value poorly selective (i.e., −32) to recover a large pool of preselected compounds and to apply to this pool subsequent screens specific for the system, such as number of hydrogen bonds (used here) or presence of a hydrogen bond acceptor (for example) at a specific point of space. As a result, we derived the value −32 as a good initial threshold (this value generates an initial pool of 3,000–4,000 compounds).

Biological Activity of the Antagonist and Agonist Candidates. HeLa cells were transfected by calcium phosphate precipitation using 1 μg of the Gal4-responsive chloramphenicol acetyltransferase (CAT) reporter pMC110 and 1 μg of Gal4-hRARα-LBD or 1 μg of Gal4-hRXRβ-LBD. Studies also were performed with the three wild-type hRAR isoforms (hRARα, hRARβ, and hRARγ) by using a δMTV-IR-CAT reporter as described (26, 27). Cell cultures were supplemented with indicated ligands immediately after addition of the calcium phosphate/DNA precipitate. Media and ligands were replaced after 24 h, and cells were harvested and assayed for CAT activity 24 h later.

Results

Modeling of the RAR Antagonist Binding Pocket. The X-ray structure of RARγ bound to the agonist all-trans RA is available (18); however, the conformation of the receptor bound to an antagonist is not known. We used the observations made from the structure of ERα bound to an agonist, 17β-estradiol (11), and two antagonists, tamoxifen and raloxifene (11, 12), to build a model of antagonist-bound RAR (Fig. 1 A and B). We docked helix H12 of RARγ into the putative coactivator binding pocket of the receptor as described (27) (see Materials and Methods for details) (Fig. 1C) and remodeled the 25 C-terminal residues, starting near the end of helix 11, through an extensive global energy minimization procedure (Fig. 1D).

Docking of Known RAR Antagonists into the Modeled Receptor. A few RAR antagonists have been described in the literature; and
binding pocket could be used to design novel antagonists. which protrudes out of the pocket. Antagonist shared ERα bound to tamoxifen and raloxifene. Therefore, our docking The two RAR antagonists dock into the ligand binding pocket the inactivation mechanism revealed antagonist, was fortuitous. On the contrary, this feature mimics does not result from some nonspecific effect on and 3. Functional assays of the novel antagonists. HeLa cells were transfected with a Gal4-hRARα-LBD expression vector and a Gal4-CAT reporter gene (results were similar in studies using the three hRAR isoforms). The cells were incubated with 5 nM all-trans RA to stimulate CAT activity, and the effect of each antagonist on inhibiting CAT was examined at 2 and 20 μM concentration (the known antagonist RO-41-5253 was used as a positive control).

several of them are serious candidates for cancer therapy (2, 28). A well-characterized ligand is AGN193109, which inhibits the three RAR isoforms at nanomolar concentrations (29). Another very potent antagonist is MX781, which is effective against ERα-positive and -negative breast cancer cells, with no apparent toxicity (2). The activity of these two ligands has been presented in detail, but no structural information has been reported on their mode of interaction with the receptor. We built a model of RARγ complexed either with AGN193109 or MX781, by using our flexible docking algorithm (24) (Fig. 2 A and B). In both cases, the antagonist superimposed with the agonist all-trans RA. As observed for ERα, the antagonists also presented a protruding arm, which was absent in RAR agonists. Very importantly, this protruding arm coincided exactly with the single opening in the ligand binding pocket of our modeled receptor, generated by the displacement of helix H12 (Fig. 2 A and B), and made stabilizing hydrophobic contacts with the protein. It is very unlikely that this perfect fit, observed for both antagonists, was fortuitous. On the contrary, this feature mimics the inactivation mechanism revealed by the crystal structure of ERα bound to tamoxifen and raloxifene. Therefore, our docking results of AGN193109 and MX781 very strongly suggest that: (i) the structural mechanisms of antagonist activity for ERα are shared by other NRs, and (ii) our model of the RAR antagonist binding pocket could be used to design novel antagonists.

Screening of a Virtual Library and Discovery of Novel RAR Antagonists. High throughput functional screening currently is the most used method for the discovery of receptor-specific ligands. Although efficient, it requires the physical availability and management of hundreds of thousands of chemical compounds. In the present work, we used a virtual library composed of the predicted structure of more than 150,000 available compounds (see Materials and Methods). Each compound was automatically docked in a grid representation of the modeled RARα antagonist binding pocket. Five grid potentials carried information on the shape, hydrophobicity, electrostatics, and hydrogen-bonding availability of the receptor, and enabled a rapid docking simulation (24, 25). RARα was selected over the other two isoforms (RARβ and RARγ) because recent data suggests it could be a medically more relevant target (28). After an automatic selection procedure with flexible ligands, and optimization of the selected candidates with flexible protein side chains (see Materials and Methods for details), 32 compounds were considered as potential antagonists of RARα and ordered.

To test these compounds in vitro, HeLa cells were transfected with a Gal4-hRARα-LBD expression vector and a Gal4-CAT reporter gene (26). Studies also were performed with the three wild-type hRAR isoforms and a ΔMTV-IR-CAT reporter (26, 27). These gave similar results as those found with Gal4-hRARα-LBD (data not shown). The cells were incubated with all-trans RA to stimulate CAT activity, and the effect of each antagonist candidate on inhibiting CAT stimulation by all-trans RA was examined. Possible toxicity of the compounds was deduced from the amount of cellular protein extract after 2 days of incubation. Two antagonist candidates inhibited CAT activity by 55% and 33% at 20 μM with no apparent toxicity (Fig. 3). The Gal4-hRARα activity illustrated in Fig. 3 was equivalent for the other two RAR isoforms (data not shown). No inhibition was observed when CAT expression was under the control of a Gal4-mRXRβ-LBD fusion construct, indicating that: (i) the antagonists are specific for RAR, and (ii) the inhibition is caused by an interaction with the Gal4-RAR-LBD fusion protein and does not result from some nonspecific effect on CAT activity (data not shown).

The two RAR antagonists dock into the ligand binding pocket of the receptor (Figs. 2 C and D and 4). As observed for AGN193109 and MX781, they fit in the same binding pocket as the natural agonist all-trans RA, but present an additional arm, which protrudes out of the pocket. Antagonist 1 has a tri-fluoro group where the retinoid receptor ligands usually carry a carboxylate group (in antagonist 2, the corresponding domain is truncated). In our model, antagonist 2 engages in a hydrogen bond with Ser-234 of the hRARα (Fig. 4B). However, the S234A

Fig. 2. RAR antagonists. Two known antagonists (A and B) and two novel antagonists (C and D). (Left) Chemical structure. (Right) Conformation docked into the receptor (part of the receptor is displayed as a ribbon representation, and the binding pocket boundary is displayed in yellow). Cyan, carbons; red, oxygen; blue, nitrogen; magenta, fluorine; yellow, sulfur. Hydrogens are not represented for clarity.
virtual database made up of antagonists and agonists for different members of the NR family (Table 1). We screened this database with our model of antagonist-bound RAR, as we did for the Available Chemicals Directory database. The screening was repeated four times, to test the reproducibility of our method. Table 1 shows that for each ligand the score varies a lot from one screening to the other. This finding reflects the generation of different ligand conformations from one docking simulation to another (data not shown) and represents the limitation of our method, as discussed below.

Table 1 lists as "selected" the ligands that met with the criteria for preselection and final inspection during the Available Chemicals Directory screening (i.e., score better than -37 or score better than -32 and at least two hydrogen bonds with the receptor; see Materials and Methods for details). Seven of the nine known RAR ligands (i.e., >80%) and one of the six non-RAR ligands (i.e., >16%) were selected. The fact that RAR agonists, as well as antagonists, produced good scores was expected, because the binding pocket used for the screening is equivalent to the agonist binding pocket, with an additional opening generated by the remodeling of the C terminus of the receptor. The two false negatives, AGN193836 and Ro415253, were missed because of steric clashes, as discussed below.

Antagonist 1 was not found either, reflecting its rather low affinity for the receptor. It is important to underline here that we do not expect to detect all of the true binders. The algorithm was rather designed to minimize the number of false positives, which correlates with the number of unnecessary in vitro experiments (25).

In that respect, the presence of one false positive of six nonbinders could be alarming, because such a ratio would represent about 25,000 false positives of a database of 150,000 compounds. However, the binding pockets of the NRs represented in this database are close in size and shape; as a result, the database used for this benchmark was composed of molecules presenting strong similarities with RAR ligands. Therefore, we believe this ratio is not representative. The fact that we needed to test only 32 molecules to discover three novel RAR ligands confirms this assumption.

Next, we tried to address why some ligands, such as Ro415253, were repeatedly missed by our screening algorithm (Ro415253 was still not selected after 10 docking simulations, data not shown). We hypothesized that the ligand could not fit into the potential maps generated from our model and carried out a docking simulation with a full atom representation of the receptor, according to a Monte Carlo energy minimization of the complex, with both flexible ligand and flexible receptor side chains (24). This docking simulation produced a solution were the ligand fits into the binding pocket; the core of the ligand (from the carboxylate to the internal sulfone) superimposes with agonists such as all-trans RA, whereas the alkyl arm sticks out of the pocket, as previously described for the other antagonists (data not shown). The conformation of several receptor side chains was modified during the docking simulation, to accommodate the size of the ligand, and this solution would not have been found with rigid side chains. This finding suggests that Ro415253 could not fit into the potential maps generated from the original receptor conformation, which we used for the screening. We generated a new series of potential maps from the optimized receptor structure and screened the small database of known ligands with these maps four times as above (Table 1).

The score assigned to Ro415253 was twice lower (i.e., better) than the threshold. Surprisingly, this new series of potential maps totally eliminated the presence of both false positive and false negative (all RAR ligands and only RAR ligands were selected).

Fig. 4. Novel RAR antagonists. (A and B) Stereo representation of antagonists 1 and 2 docked into the binding site of the receptor. The ligands make extensive hydrophobic interactions with residues from helix 3, helix 5, and helix 11. Antagonist 2 (B) is engaged in an additional hydrogen bond with Ser-234 of helix 3 and contacts the remodeled C terminus (red) at Pro-405. (C and D) The fit of antagonists 1 and 2 into the receptor binding pocket is shown.
Table 1. Control screening of known NR ligands

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Second series

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First series: A similar screening as the one performed on the ACD database was carried out four times on a small database made of known RAR antagonists, agonists, as well as ligands for other NRs and the two novel RAR antagonists. The ligands that met at least once with the criteria for selection used during the ACD screening are listed as Selected. The ligands that are experimentally binding to RAR are listed as Binding. Second series: Screening of known ligands after adjustment of the receptor's binding pocket conformation. The RAR antagonist Ro415253 was docked into our model of antagonist-bound RAR with flexible receptor side chains and ligand. The resulting receptor conformation was used for a novel screening.

Discussion

In this study, we presented a strategy for the discovery of antagonists, as well as agonists, for NRs, which are very important targets for drug design. An important aspect of our approach was to exclude any preconceived pharmacophore bias from our database screening. Most drug design strategies impose chemical constraints on the selected molecule to conserve the functional groups believed to be most important in existing ligands, preventing the discovery of novel ligand types. In the present work, we avoided pharmacophore constraints thanks to a robust flexible docking program and scoring function: the only filters used for screening were a good fit with the receptor and reasonable bioavailability parameters (30). As a result, we discovered novel original ligands that could be further optimized into potent RAR-selective antagonists and agonists.

A limitation of our method, which leaves room for further improvement, is that a compromise must be made between the time allocated for each ligand (less than 2 min on one processor here) and the reliability of the sampling of the conformational space. Indeed, Table 1 shows that four runs for each ligand are necessary to minimize efficiently missed hits (the remaining missed positives were not selected because of inappropriate receptor side-chain conformations and not because of an insuffi cient sampling). Improvement of the computing power, the docking algorithm, and the scoring function all could result in a more robust virtual database screening.

Another drawback is that the conformation of the receptor is not necessarily unique, but can vary from one ligand to another. As a result, a ligand that fits in receptor conformation A will never be found if receptor conformation B is used for the screening. The case of Ro415253 illustrates this issue well: this known antagonist was never selected, even after 10 trials, because the binding pocket used for the screening was too narrow. The potential maps used for the screening have a smoother van der Waals profile than the atomic representation of the receptor; as a result, the maps are more tolerant regarding steric clashes with the ligand. However, the degree of tolerance is limited and cannot accommodate important conformational changes of the receptor side chains (or backbone, obviously). When new potential maps generated from a model of RAR bound to Ro415253 were used for screening, the three RAR ligands missing from the first screening were selected (Table 1). This finding confirms that the initial conformation of the


Research article

**In silico discovery of novel Retinoic Acid Receptor agonist structures**

Matthieu Schapira¹, Bruce M Raaka², Herbert H Samuels² and Ruben Abagyan*¹,³

**Abstract**

**Background:** Several Retinoic Acid Receptors (RAR) agonists have therapeutic activity against a variety of cancer types; however, unacceptable toxicity profiles have hindered the development of drugs. RAR agonists presenting novel structural and chemical features could therefore open new avenues for the discovery of leads against breast, lung and prostate cancer or leukemia.

**Results:** We have analyzed the induced fit of the active site residues upon binding of a known ligand. The derived binding site models were used to dock over 150,000 molecules in silico (or virtually) to the structure of the receptor with the Internal Coordinates Mechanics (ICM) program. Thirty ligand candidates were tested in vitro.

**Conclusions:** Two novel agonists resulting from the predicted receptor model were active at 50 nM. One of them displays novel structural features which may translate into the development of new ligands for cancer therapy.

**Background**

The retinoic acid receptors (RAR-α, -β, and -γ) are transcription factors regulating a variety of endocrine metabolic pathways. Unlike anti-estrogens, such as tamoxifen or raloxifene, ligands targeted against the RAR isoforms can present anticancer activity against both estrogen receptor positive and negative breast tumor cells [1]. As a result, such molecules could constitute a novel generation of drugs against breast cancer. For reasons not yet clear, both agonists and antagonists of RAR can present anti-tumor activity against breast, prostate, lung cancer or leukemia [1,2,3,4,5,6,7]. The development of both types of ligands could therefore have important biomedical implications. We have recently demonstrated that antagonists could be discovered rationally, based on a model of the antagonist-bound conformation of the receptor [8]. Our goal here is to discover innovative molecular structures with RAR agonist activity.

Several retinoid and non-retinoid ligands have been described, which activate one or a combination of RAR isoforms. Some of them, such as the natural hormone all-trans retinoic acid (all-trans RA) (Fig. 1a), have been tested clinically, and display unacceptable side effects, such as skin dryness, cheilitis, hypertriglyceridemia and conjunctivitis [9,10]. However, the compounds tested so
far belong to limited series of related structures. An increasing amount of data suggests that the RAR-β isoform, which is under the transcriptional control of RAR-α, is involved in suppressing cell growth and tumorigenicity [11,12,13,14,15,16]. Innovative molecules with RAR-α and RAR-β agonist activity could therefore present more favorable toxicity profile than pan-agonists.

We applied a flexible virtual screening algorithm (Molsoft ICM, virtual library screening module [17]) which rapidly docks hundreds of thousands of flexible compound structures into the ligand binding pocket of RAR, and discovered two novel RAR-β selective agonists. One of these ligands displays original structural and chemical characteristics, which could be used in the development of novel compounds for cancer prevention and therapy.

Results and discussion
We first built a model of the RAR-α agonist binding pocket from the crystal structure of the RAR-γ ligand binding domain (RAR-γ LBD) / all-trans RA complex [18]. All but three amino acids in the vicinity of the ligand are conserved between the two isoforms. These three non-identical residues -A234, M272, and A397- were changed to the RAR-α isoform -S234, I272 and V397- and the energy of the system was minimized (see "Materials and Methods").

In order to address the accuracy of our model of the RAR-α binding pocket, we docked Am580, an RAR-α specific agonist [19], into the receptor (the chemical structure of Am580 is shown Fig. 1b). A rapid docking procedure with flexible ligand and a grid representation of the receptor was followed by an extensive Monte Carlo energy minimization with both ligand and receptor side...
A high throughput virtual screening was carried out on the Available Chemicals Directory (MDL Information Systems, San Leandro, CA), a compound structure database of over 150,000 molecules. Each compound was automatically docked into a grid representation of RAR-α, as previously described for Am580, and assigned a score according to the quality of the fit [8, 17, 20]. The 5364 ligand candidates which scored better (i.e. lower) than -32 kcal/mol were preselected for a more refined energy minimization procedure, with flexible receptor side chains, and the energy of complexation was predicted as previously described [21] (see "Materials and Methods").

After careful visual examination of the 300 candidates displaying the lowest predicted binding energy, 30 molecules were selected and purchased to be experimentally tested in vitro.

HeLa cells were separately transfected with either of the three wild type hRAR isoforms, and a AMTV-IR-CAT reporter gene [22, 23]. The cells were incubated with each ligand at concentrations from 50 nM to 20 μM to stimulate CAT activity. Possible toxicity of the compounds was deduced from the amount of cellular protein extract after 2 days of incubation. The percentage of conversion induced by 1 μM all-trans RA (RARs) or 1 μM 9-cis RA (RXR-β) was used as a positive control to determine the maximum induction. Fig. 3 shows all-trans RA induced conversion of RAR-α as a function of acid concentration. RAR-β could induce 20% of the maximum CAT activity when activated by 50 nM agonist 1 (chemical structure shown Fig. 1c), while RAR-γ was only 12% active and RAR-α not active at all under the same conditions (Fig. 4). At 200 nM of agonist 1, RAR-β was 50% active, RAR-γ 25% and RAR-α 5% active. Similarly, RAR-β could induce 22% of the maximum CAT activity when activated by 50 nM agonist 2 (chemical structure shown Fig. 1d). RAR-α and RAR-γ were 10% and 14% active at the same concentration of agonist, respectively. At 200 nM of agonist 2, RAR-β displayed 48% of its maximal activity, RAR-α 40% and RAR-γ 17%. At 20 μM, agonist 1 induced full activation of RAR-β and RAR-γ, and 80% activation of RAR-α, showing that this compound is a full agonist. Agonist 2 was toxic for the cells at 20 μM, as shown by protein content of cell culture dishes, which is why little transcriptional activity was observed at this concentration. However, little or no toxicity was observed at 8 μM, a concentration at which agonist 2 induced about 50% maximal activity of RAR-α and RAR-β, and 35% maximal activity of RAR-γ. Agonist 1 was not toxic at 20 μM. Finally, RXR-β was activated only weakly by both agonists 1 and 2 at 2 μM, but was significantly activated by agonist 1 at 20 μM (Fig. 4). Comparison of Figs. 3 and 4 also shows that at 200 nM of agonists 1 and 2, RAR-β exhibits the same activity as the positive control RAR-α induced by all-trans RA at the same concentration.

chains flexible (see "Materials and Methods" for details). The ligand superimposed well with the natural hormone all-trans RA (Fig. 2a). Interestingly, Am580 does not seem to fit in the receptor binding site: the ketone oxygen of the ligand sticks out of the binding pocket, due to too close proximity of residue 234 (Fig. 2b). However, in the complex with RAR-α, this ketone oxygen shares an hydrogen atom with Ser234 of the receptor (displayed as stick). The receptor in the vicinity of the ligand is shown as a white ribbon. Carbons, hydrogens, oxygens and nitrogen are colored white, gray, red and blue respectively. (Image generated with Molsoft ICM)
This compound illustrates the benefits of the \textit{in silico} screening procedure, a rational approach which is based solely on the structure of the receptor, and is not biased towards existing ligands. Indeed, structure-based drug design usually derives novel compound structures from ligands previously described. Our approach enabled the discovery \textit{ab initio} of RAR agonists displaying some activity at 50 nM with a 7\% success rate.

The specificity profile of the two RAR ligands described here are similar to that of Ch55, a compound with ten times higher affinity for RAR-\(\beta\) than RAR-\(\alpha\), and which can block the growth of certain cancer types \cite{4}. It will be interesting to test these molecules for anti-proliferative activity, both \textit{in vitro} and \textit{in vivo}. The observation that agonist 1 does not belong to any of the series of RAR ligands described so far makes this compound particularly interesting for further development.

While a model of RAR-\(\alpha\) was used to conduct the virtual screening, agonists 1 and 2 have an EC-50 of 200 nM for RAR-\(\beta\), and an EC-50 of 4 and 2 \(\mu\)M respectively for RAR-\(\alpha\). This observation emphasizes the primary goal of structure-based screening procedures, which is to significantly accelerate the initial steps of lead identification by automatically discriminating between binders and non-binders, rather than ranking ligands according to their affinity for the receptor. The discovery of 2 RAR-\(\alpha\) agonists out of 30 molecules tested is a good illustration of this approach.

\textbf{Conclusions}

This report details the rapid discovery of RAR agonists with novel structural features, thanks to a powerful virtual ligand screening approach, and a research strategy where considerations on existing ligands are avoided. One of the molecules presented here constitute a good framework for the development of a novel series of RAR ligands very different from all structures described so far. Such ligands could present more favorable specificity and toxicity profiles, and have important applications in cancer therapy.

\textbf{Materials and Methods}

\textit{Modeling of RAR-alpha ligand binding pocket}

The crystal structure of RAR-\(\gamma\)-LBD complexed to all-trans RA was used as a template \cite{18} and the three residues in the vicinity of the ligand which are not conserved between the two isoforms were modified accordingly: A234, M272, and A397 were changed to S234, I272 and V397 respectively. The rotation variables of the side chains within 3.5 \(\AA\) of the modified residues were unfixed and the energy of the system was minimized in the internal coordinate space, according to the ICM method \cite{26, 29}.
Docking of AMS80 into RAR-alpha
The flexible ligand was docked into a combination of five potential map representations of the RAR-α ligand binding pocket, which account for two Van der Waals boundaries, hydrophobicity, electrostatics and hydrogen bonding profiles [20,27]. This rapid docking procedure was followed by a more refined energy minimization of the complex, with a full atom representation of the receptor, and flexible receptor side chains, according to the ICM stochastic global optimization algorithm [26, 27] as implemented in the Molsoft ICM 2.7 program [17].

Virtual screening of the compound structure database
The ICM program [17] was used to perform both receptor modeling and virtual ligand screening. The procedure followed was similar than previously described [8]: each flexible ligand of the Available Chemicals Directory (MDL Information Systems, San Leandro) was docked automatically into the combination of potential maps described above, and assigned a score according to its fit with the receptor. The scoring function included continuum as well as discreet electrostatics, hydrophobicity and entropy parameters [20]. The screening of the database of over 150,000 ligands took less than a month on 10 "194 MHZ IP25" processors. Using the same computing power, the 5364 compounds which scored better (i.e. lower) than -32 were preselected for a second automatic round of selection: they were all docked in two days into a full atom representation of the receptor, with flexible receptor side chains, according to a global energy optimization in internal coordinates [17, 26,27,28]. The structure generated by the initial library screening was used as a starting point for this second, more refined, docking procedure. The binding energy of the compounds was then evaluated with a boundary elements implementation of solvation electrostatics [21] and the 300 compounds showing the lowest binding energy were selected for further examination. After careful visual inspection for shape complementarity, hydrogen bonding network, compound flexibility, and potential Van der Waals clash-
Figure 5
Structure of the novel RAR agonists. Agonists 1 and 2 are shown (respectively A and B). Left: chemical structure of the compounds. Right: Representation of the compounds docked into the binding pocket of RAR (important residues are displayed as sticks: R274, R278, S289), and superimposed with the crystal structure of all-trans RA (green). The receptor is represented as a white ribbon. Hydrogens are not displayed for clarity. Color coding: carbons, oxygens, nitrogens, sulfurs, fluorides and hydrogens are colored white, red, blue, yellow, magenta and gray respectively.

Biological activity of the ligand candidates
HeLa cells were transfected by calcium phosphate precipitation using 1 µg of plasmid expressing the full length receptor isoform and 1 µg of a ΔMTV-IR-CAT reporter gene, as previously described [22, 23]. Ligands were dissolved in DMSO at 20 mM final concentration. Cell cultures were supplemented with indicated ligands immediately after addition of the calcium phosphate/DNA precipitate. Media and ligands were replaced after 24 h and cells were harvested and assayed for CAT activity 24 h later: acetylated and unreacted [14C] chloramphenicol was excised from a thin layer chromatography plate and quantitated in a liquid scintillation counter. The amount of cellular protein extract after two days of incubation was measured to determine the compounds toxicity. Agonist 1 was purchased from Bionet Research (catalog number 1G-433S). Agonist 2 was purchased from Sigma-Aldrich (Sigma Aldrich library of rare chemicals. Catalog number So8503-1).
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