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Characterization of the differential coregulator binding signatures of the Retinoic Acid Receptor subtypes upon (ant)agonist action

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ABSTRACT

Retinoic Acid Receptor alpha (RARα/NR1B1), Retinoic Acid Receptor beta (RARβ/NR1B2) and Retinoic Acid Receptor gamma (RARγ/NR1B3) are transcription factors regulating gene expression in response to retinoids. Within the RAR genomic pathways, binding of RARs to coregulators is a key intermediate regulatory phase. However, ligand-dependent interactions between the wide variety of coregulators that may be present in a cell and the different RAR subtypes are largely unknown. The aim of this study is to characterize the coregulator binding profiles of RARs in the presence of the pan-agonist all-*trans*-Retinoic Acid (AtRA); the subtype-selective agonists Am80 (RARα), CD2314 (RARβ) and BMS961 (RARγ); and the antagonist Ro415253. To this end, we used a microarray assay for coregulator-nuclear receptor interactions to assess RAR binding to 154 motifs belonging to > 60 coregulators. The results revealed a high number of ligand-dependent RAR-coregulator interactions among all RAR variants, including many binding events not yet described in literature. Next, this work confirmed a greater ligand-independent activity of RARβ compared to the other RAR subtypes based on both higher basal and lower ligand-driven coregulator binding. Further, several coregulator motifs showed selective binding to a specific RAR subtype. Next, this work showed that subtype-selective agonists can be successfully discriminated by using coregulator binding assays. Finally this study demonstrated the possible applications of a coregulator binding assay as a tool to discriminate between agonistic/antagonistic actions of ligands. The RAR-coregulator interactions found will be of use to direct further studies to better understand the mechanisms driving the eventual actions of retinoids.

1. Introduction

Retinoic Acid Receptors (RARs) are transcription factors regulating gene expression in response to ligands known as retinoids. There are three different RAR subtypes, namely, Retinoic Acid Receptor alpha (RARα/NR1B1), Retinoic Acid Receptor beta (RARβ/NR1B2) and Retinoic Acid Receptor gamma (RARγ/NR1B3), which have been described to play important roles from both a therapeutic (e.g. breast cancer treatment) as well as a toxicological (e.g. liver toxicity) perspective [1]. In order to modulate gene expression, RARs form heterodimers with Retinoid X Receptors (RXRs) and bind to specific DNA regions called Retinoic Acid Responsive Elements (RAREs) and to different coregulators [2]. Within the genome, the DNA binding regions of the RAR subtypes have been shown to highly overlap suggesting functional redundancy [3]. However, despite their structural and mechanistic similarity, the respective RAR subtypes have been shown to often play different and even opposite roles in both physiological and pathological cellular contexts [4].

Within the RAR genomic pathways, binding of RARs to coregulators (coactivators or corepressors) is a key intermediate regulatory step since these coregulators modulate chromatin structure and accessibility for transcription [5]. Coregulators interacting with RARs can be divided in three major categories, being apo-form-bound corepressors, ligand-induced binding coactivators, or ligand-induced binding corepressors [1] and it has been shown that distinct cellular levels of some of these coregulators (e.g. MAPE) may lead to different biological responses [6].

Abbreviations: MARCoNI, Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction; NR, Nuclear Receptor; RAR, Retinoic Acid Receptor; AtRA, all-*trans*-Retinoic Acid; Am80, 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid; CD2314, 5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic acid; BMS961, 3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8,-tetrahydro-2-naphthalenyl) acetyl]amino]-benzoic acid; Ro415253, 4-[(*E*)-2-(7-Heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid

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In addition, previous studies suggested that the specific effects of the different RAR subtypes may arise from a differential capacity to recruit coregulators to promoter regions [7]. Thus, in order to better understand retinoid-induced biological effects, deeper knowledge on the specific ligand-dependent RAR-coregulator interactions is required [8–11].

In order to study retinoid-driven modulation of RAR-coregulator interactions, several methods such as GST-pull down assays, or yeast two hybrid assays have been used [12]. However, most of these assays only allow studying one specific coregulator while in a biological scenario, many different coregulator proteins could participate in this interaction. Consequently, only a limited number of coactivators and corepressors have been studied so far. The interactive region of coregulators is composed by leucine-rich motifs called Nuclear Receptor boxes (NR-boxes) [5] which are considered to be necessary and sufficient for the interactions with NRs. Coactivators possess one or several consensus sequences LXXLL while corepressors contain LXXI/HIXXXI/L sequences [8] where L is Leucine, I is Isoleucine, H is Histidine and X represents any amino acid. Previous studies have shown differences between the functionality of NR-boxes [13,14] and that residues immediately adjacent to these canonical sequences can play an important role in the affinity and specificity of NR-coregulator interactions [15,16]. Therefore, technologies assessing NR binding to coregulator peptides containing such sequences could add initial insights to test whether these differences may also occur among the different RAR subtypes when activated by a variety of different ligands. The Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction makes use of this concept in a high-throughput manner offering the possibility to rapidly and simultaneously assess ligand-modulated binding of NRs to 154 motifs derived from > 60 different coregulators. Thus, by using this technique, broad coregulator binding profiles can be obtained.

The present study aims at characterizing and comparing the coregulator binding profiles of RARα, RARβ and RARγ in the presence of both generic and subtype-selective agonists in order to get new insights in the possible role of specific coregulators in the eventual action of RAR subtypes. In addition, the coregulator binding signature generated by the RAR antagonist Ro415253 was also characterized. For this purpose, retinoid-driven coregulator motif interactions with purified recombinant ligand binding domains (LBDs) of the respective RAR subtypes were assessed.

2. Materials and methods

2.1. Chemicals

All-*trans*-Retinoic Acid (AtRA) was purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). 4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl] benzoic acid (Am80) and 4-[(*E*)-2-(7-Heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid (Ro415253) were obtained from Abcam (Cambridge, UK). 5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-3-thiophenecarboxylic acid (CD2314) and 3-fluoro-4-[[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8,-tetrahydro-2-naphthalenyl) acetyl]amino]-benzoic acid (BMS961) were purchased from Tocris Bioscience (Bristol, UK).

2.2. MARCoNI coregulator binding assay

In the present study, the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) was used in order to characterize and compare the different coregulator interactions of all RAR subtypes in the presence of the agonists AtRA, Am80, CD2314 and BMS961, and the antagonist Ro415253 at concentrations ranging from 0.1 nM to 200 μM. All samples contained a final concentration of DMSO of 2% which was also used as a solvent control. Assay mixtures containing the respective RAR, the ligand of interest and the detecting antibody, were prepared in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer D (Life technologies, Ltd, UK, #PV4420) supplemented with 5 mM dithiothreitol (DTT) (Sigma, Zwijndrecht, the Netherlands, #43819) and kept on ice before starting the assay. Glutathione S-transferase (GST)-tagged RARα-LBD (RARα-LBD^{GST}, amino acids 176-462, Life technologies, Ltd, UK, #PV4415), glutathione S-transferase (GST)-tagged RARβ-LBD (RARβ-LBD^{GST}, amino acids 168-448, Life technologies, Ltd, UK, #PV4716) or glutathione S-transferase (GST)-tagged RARγ-LBD (RARγ-LBD^{GST}, amino acids 178-454, Life technologies, Ltd, UK, #PV4413) were used for the experiments at a final concentration of 10 nM. For all the subtypes, 25 nM of Alexa 488–conjugated anti-GST (Invitrogen, Breda, the Netherlands, #A11131) was utilized as detecting antibody. Each ligand concentration, was tested in singular (unless otherwise stated) by using PamChip® plates (PamGene International B.V.,'s-Hertogenbosch, the Netherlands) containing 4 or 96 peptide microarrays. Microarrays consist of a three-dimensional porous metal oxide carrier containing 154 spots with different immobilized NR-binding motifs belonging to 65 different coregulatory proteins. The automated platform PamStation® (PamGene International B.V.,'s-Hertogenbosch, the Netherlands) and EvolveHT software (PamGene International B.V.,'s-Hertogenbosch, the Netherlands) were used applying 2 (up-down) pumping cycles per minute at 20 °C. Prior to the addition of the samples to the Pamchip® arrays, incubation for 20 cycles with 25 μl blocking buffer composed of Tris buffered saline (Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609) and 0.01% Tween-20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) was carried out in order to avoid unspecific binding. Immediately after blocking, samples were added to the Pamchip® arrays and incubated for 80 pumping cycles. As last step, unbound receptor was washed away with 25 μl Tris buffered saline and TIFF images were taken by a charge coupled device camera (CCD) installed in the PamStation®.

2.3. MARCoNI data analysis

Image analysis was performed using the software Bionavigator (Version 6.1, PamGene International B.V.,'s-Hertogenbosch, the Netherlands). Basal and agonist-driven binding of the different RAR subtypes to the different coregulator peptides was assessed by quantifying the fluorescence signals. Shortly, boundaries for every spot (peptide) were determined, and fluorescence binding Arbitrary Units (AU) were quantified by subtracting the background signal from the median fluorescence signal. Concentration-response curve fitting was carried out utilizing the DRC package in R (version 2.15.3, www.r-project.org) by means of a sigmoidal, 4-parameter Hill (logistic) model ($response = ((A - D) / (1 + ((concentration / C)B))) + D$, *with parameters* $A = response\ minimum$, $B = Hill\ slope$, $C = EC50$, and $D = response\ maximum$). Unless otherwise stated, coregulators presenting complete concentration-response curves with goodness of fitting (R^2) higher than 0.8 (cut-off value) were considered as ligand-dependent for analysis and discussion. Throughout this work, results were also expressed as a percentage of the solvent control or as Modulation Index (MI), the latter representing the log10 transformed ratio of the binding in the presence of the ligand over the binding in the solvent control. In order to evaluate whether the different RAR-coregulator interactions observed in this work have been previously described in the literature, the databases STRING v9.1 (http://string-db.org/) and BioGRID$^{3.4}$ (http://thebiogrid.org/) were used.

2.4. Pull down assay

Ligand-dependent binding of RARα to the full length coregulator DDX5 was evaluated by adapting and using the Pierce GST Protein Interaction Pull-Down kit (ThermoFisher Scientific, Rockford Illinois,