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Monitoring of the Retinoic Acid Receptor - Retinoid X Receptor dimerization upon DNA binding by native mass spectrometry

Nha-Thi Nguyen-Huynh^a, Judit Osz^b, Carole Peluso-Iltis^b, Natacha Rochel^b, Noëlle Potier^{a},
Emmanuelle Leize-Wagner^a*

^aLaboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS), UMR 7140
CNRS/Université de Strasbourg - "Chimie de la Matière Complexe", 1 Rue Blaise Pascal, 67008
Strasbourg, France

^bInstitut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Institut National de
Santé et de Recherche Médicale (INSERM) U964 / Centre National de Recherche Scientifique
(CNRS) UMR 7104 / Université de Strasbourg, 1 rue Laurent Fries, 67404 Illkirch, France

Corresponding Author

* Noëlle Potier

Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS)

UMR 7140 CNRS/Université de Strasbourg - "Chimie de la Matière Complexe"

1 Rue Blaise Pascal, 67008 Strasbourg, France

Tel: +33 (0) 3 68 85 16 41. Email: npotier@unistra.fr

ABSTRACT

Identifying protein-DNA interactions is essential to understand the regulatory networks of cells and their influence on gene expression. In this study, we use native electrospray mass spectrometry (ESI-MS) to investigate how the heterodimerization of retinoic acid receptor-retinoid X receptor (RAR-RXR) is mediated by DNA sequence. In presence of various RAR response elements (RAREs), three oligomeric states of RAR-RXR DNA binding domains (DBDs) bound to RAREs (monomer, homo- or heterodimers) were detected and individually monitored to follow subunit assembly and disassembly upon RAREs' abundance or sequence. In particular, a cooperative heterodimerization was shown with *RARb2* DR5 (5 base pair spaced direct repeat) while a high heterogeneity reflecting random complex formation could be observed with the *RARb2* DR0, in agreement with native gel experiments or molecular modeling. Such MS information will help to identify the composition of species formed in solution and to define which DR sequence is specific for RAR-RXR heterodimerization.

KEYWORDS

Native mass spectrometry; Dynamics study; Protein-DNA interaction; Nuclear receptors; RAR-RXR complexes; Retinoic Acid Receptor (RAR); Retinoid X Receptor (RXR)

ABBREVIATIONS

NR: nuclear receptor; RAR: retinoic acid receptor; RXR: retinoic X receptor; DNA: deoxyribonucleic acid; RA: all-trans retinoic acid (RA); DR: direct repeat; RARE: RAR response element; ESI-MS, electrospray ionization mass spectrometry; DBD: DNA binding domain; LBD: ligand binding domain; K_d: dissociation constant; ITC: isothermal titration calorimetry; MST: microscale thermophoresis.

INTRODUCTION

Proteins, such as many transcription factors that bind to specific DNA sequences, are essential for the proper regulation of gene expression. Identifying the specific sequences that each factor binds can help to elucidate regulatory networks within cells and how genetic variation can cause disruption of normal gene expression, which is often associated with diseases. Various techniques have been reported for the study of protein-DNA interactions such as chromatin immunoprecipitation (ChIP) [1], electrophoretic gel mobility shift assay (EMSA) [2], DNA pull-down assay [3], microplate capture and detection assay [4], reporter assay [5] or isothermal calorimetry, but several new high-throughput methods can provide comprehensive binding information much more rapidly. Combined with *in vivo* determinations of transcription factor binding locations, this information provides more detailed views of the regulatory circuitry of cells and the effects of variation on gene expression.

All-trans retinoic acid (RA), the naturally active vitamin A metabolite, exerts a wide range of effects on vertebrate development and plays a critical role in the homeostasis and physiopathology of adult tissues [6,7]. RA exerts most of its pleiotropic effects through interaction with three members of the nuclear receptor (NR) superfamily, the all-trans retinoic acid receptors (RAR) α , β , γ that heterodimerize with the retinoid X receptors (RXR) α , β , γ . Heterodimer RAR-RXR acts as RA-dependent transcriptional regulators that either activate or repress target genes [8]. The RAR-RXR heterodimer regulates gene expression by binding to DNA response elements or RAREs, the best characterized of which comprise direct repeats of the consensus 5'-RGKTCA-3' (where R = A or G, and K = G or T) separated by 1, 2 or 5 nucleotides (DR1, DR2 or DR5) [9]. Genome wide analysis of RAR binding sites in mouse embryo fibroblasts (MEFs), embryonic stem (ES) and F9 embryonal carcinoma cells have

revealed a variety of RARE motifs consisting of the classical DR1, DR2 and DR5 and also of direct repetition of the core motif without spacer (DR0), direct repeat of the core motif with 8 nucleotides space (DR8) or inverted repeat without spacer (IR0) elements [10]. In undifferentiated cells, high prevalence of the non-canonical DR0-containing RAREs are observed while in differentiated cells, the canonical DR5 motif is more prevalent [10]. In vitro EMSA analysis indicated that flanking and spacer sequence both influence RAR-RXR binding. Of particular interest are DR0 elements since RAR-RXR bound to DR0s are not able to modulate gene expression in transcriptional reporter assay [10]. The molecular basis of RAR-DNA recognition of non-canonical elements and their transcriptional specificities remain unknown. Current atomic level understanding of DNA recognition by RAR-RXR and RXR homodimer is limited to complexes with DR1 elements [11,12] and low resolution solution structures of RAR-RXR on the classical DR1 and DR5 elements [13].

While traditional methods for determining the specificity of DNA-binding proteins are slow and laborious, mass spectrometry (MS) - due to their high sensitivity, selectivity and speed [14] - has emerged as a complementary method for characterizing intact assemblies [15]. Through improvements in instrumental technology and methodology, nondenaturing or native MS is now emerging as a new approach for getting insights into the existence, the stoichiometry or the architecture of protein complexes [16]. In particular, MS is a proven technique for following the different oligomeric states present in solution. This allows to assess the study of protein complex dynamics by monitoring protein subunits assembly or disassembly over time or solution composition [17]. An increasing number of studies were reported since the last decade in the literature [18–20], including very large multiprotein complexes such as intact ribosomes [21] or proteins in interaction with a wide variety of partners including protein subunits, drug molecules,

lipids, oligonucleotides. However, examples of protein-DNA interaction studies by MS are under-represented in the literature [22–25]. The major problem encountered with the analysis of these complexes may lie in the very high polarity and polyanionic nature of the DNA. Due to its polyphosphate backbone, the oligonucleotides have a tendency to catch alkaline cations present in the medium, leading to formation of oligonucleotide-sodium or -potassium adducts that persist after ionization and desolvation. Quite broad m/z signals, if even a signal, are then observed preventing precise mass measurement and stoichiometry unambiguous assignment [26]. Moreover, particular care should be taken in the interpretation since oligonucleotides may undergo nonspecific binding to basic proteins [27].

In this paper, we describe how ESI-MS has been successfully used to monitor the composition of RAR-RXR DNA binding domains (DBD) complexes with various RAREs. In particular, classical DR5 were compared to non-canonical DR0 elements. This method proved to be very efficient for checking the stabilization of heterodimers on RARE sequences. We discovered a correlation between the protein/DNA molar ratios and the dynamics of assembly of binding complexes. To understand how non-canonical DR0s elements are recognized by RAR-RXR and to decipher the relationship between the RAR binding element type and the transcriptional response, molecular model building was used in complement to mass spectrometry characterization.

MATERIALS AND METHODS

Constructs, expression and purification

The HsRXR α -DBD (130-212) and HsRAR α -DBD (82-167) were expressed in fusion with thioredoxine and hexahistidine tags. Purifications of the RXR and RAR DBDs were

performed as previously described [12] and included an affinity chromatography, fusion tag removal by thrombin proteolysis and gel filtration. The HsRAR α DNA binding domain-ligand binding domain DBD-LBD (82-462), and HsRXR α DBD-LBD (130-462) were expressed and purified as described elsewhere [13]. The oligonucleotide strands were purchased from SIGMA and annealed.

TBE native PAGE

The proteins were run on a continuous 6% polyacrylamide gel with constant current of 6 mA for 2 hours at 4°C in the TBE (Tris-Borate-EDTA) migration buffer. The unloaded gel was pre-run before the electrophoresis. The TBE buffer contained 0.089 M Tris base, 0.089 M boric acid (pH 8.3) and 2 mM Na₂EDTA. Approximately 5 μ g of protein was loaded per each lane.

Microscale thermophoresis

Microscale thermophoresis (MST) measurements were performed with a Monolith NT.115 instrument (NanoTemper Technologies GmbH, Munchen, Germany). The purified RAR α /RXR α DBDs-LBDs heterodimer was labelled with NT-647-NHS fluorescent dye following the manufacture protocol (Nanotemper Technologies). After the labeling, the excess of the free dye was eliminated by Illustra NAP-5 desalting column (GE Healthcare) and at the same time, the buffer was changed for either 100 mM ammonium acetate pH 7.8 or 50 mM HEPES pH 8.0, 100 mM NaCl, 2% glycerol. Each measurement consists of 16 reaction mixtures where the fluorescent-labeled RAR α /RXR α DBDs-LBDs concentration was constant and RAR β 2 DR5 concentrations were in serial dilution (1:1) through the 16 reactions. Depending on the

measurements, the RAR α /RXR α DBDs-LBDs concentrations varied between 37 – 94 nM and the RARb2 DR5 serial dilution between 2 nM – 55 μ M.

The measurements were performed using premium type glass capillaries (Nanotemper) at 40%-100% LED excitation and 40-80% MST power, with a laser-on time of 30 s and a laser-off time of 5 s. NanoTemper Analysis 1.4.27 software was used to fit the data and to determine the Kd.

MS analysis

Before analysis, the RXR DBD, RAR DBD and DNAs were subjected to buffer exchange to 100 mM ammonium acetate pH 7.8 in 4°C (Micro Bio-Spin chromatography column, exclusion limit 6 kDa). Particular care was taken to the desalt DNAs separately twice. MS data were acquired in the positive ion mode on an electrospray time-of-flight mass spectrometer (MicroTOF, BrukerDaltonic, Germany). Denaturing conditions measurements were conducted by diluting the proteins to 10 μ M in H₂O/ACN (50/50) acidified with with 1% formic acid to verify their purity and sequence integrity. Mass spectrometric analyses of the noncovalent complexes were carried out in 100 mM ammonium acetate pH 7.8. Mixture of proteins at 32 μ M and DNA at various equivalents (0.1, 0.25, 0.5, 1.2 equivalents) were infused at 4 μ L/min through a KDS100 syringe pump (KD Scientific, Holliston, MA, USA). The source and transfer parameters of the instrument were optimized in order to obtain the best spectrum without dissociating the complexes. Especially, the capillary exit voltage, which accelerates the ions in the middle pressure interface, was increased from 100 V to 200 V. The pre-pulse time, which represents a delay between transfer time and TOF pulser on for injection of ions into the TOF analyzer, was increased from 30 μ s to 40 μ s to optimize the transmission of high m/z ions (for

more details see Results and Discussion part). Heterodimers were best obtained at the capillary exit of 150 V and the pre-pulse time at 40 μ s.

RESULTS AND DISCUSSION

MS method to follow the association dynamics of RAR-RXR-DNA complexes

To clarify our understanding of the specificity of the RAR and RXR binding to DR0s, we compared the formation of RAR-RXR complexes by various techniques on 2 types of DR0s – the DR0 from *Msi* gene and the pseudo-DR0 motif from *Hoxb13* gene – to those formed on DR5s from *RARB2* and *F11r* genes (Figure 1A). All experiments were performed using the DNA binding domains of RAR and RXR. By isothermal titration calorimetry (ITC), we previously have determined the DNA binding affinities of RAR-RXR DBD-LBDs constructs [10] and have shown that RAR-RXR have affinities values for DR0s in the same range as for DR5s (Supplementary Table 1). As MS experiments are performed in ammonium acetate buffer and ITC could not be performed in this buffer, we use another method, the microscale thermophoresis (MST) [28]. Using a titration approach, MST allowed us to determine the affinity constants of interactions in the binding equilibrium. In MST experiments, one of the binding partners is fluorescently labeled at a fixed concentration (a few nM), while the concentration of the unlabeled partner is varied from a high concentration, much above the expected dissociation constant (K_d), down to substoichiometric concentrations with respect to the labeled protein. Unlabeled *RARB2* DR5 was thus titrated into a fixed concentration of labeled RAR-RXR (Supplementary Figure 1). Remarkably, the apparent K_d values calculated from MST experiments compare very well with K_d values obtained independently by ITC and are in the same range in the 2 buffers (Supplementary Table 1). The formation of the RAR-RXR DBDs-

DNA complexes was also followed by TBE native PAGE electrophoretic mobility (Figure 1B). It shows that for DR5, only heterodimers are observed, while for *Hoxb13* DR0, we suspect that a mixture of species is present in the smeared bands. Similar results were observed for other DR0s tested. Mass spectrometry was then chosen to identify which complexes were formed under which conditions.

Denaturing MS was first performed to check the protein homogeneity and integrity. Mass spectra revealed that the RAR DBD was present under 2 forms: a major form at 10 607.1 Da and a minor one at 9 940.8 Da \pm 0.5 Da which may correspond to the loss of the remaining residues of the tag GSHM plus two amino acids (proline and arginine). RXR DBD was detected at 10 236.8 Da \pm 0.5 Da, which is in good agreement with the theoretical molecular mass. For detection of non-covalent complexes, solution as well as instrument conditions were carefully optimized (see Materials and Methods). In particular, the interface voltages implied in collision activation and ion transmission were adjusted using a well-characterized heterodimer complex formed between RAR-RXR and *RAR β 2* DR5. For this, an increasing ratio of *RAR β 2* DR5 (0.1, 0.25, 0.5, 1.2 equivalents) was added to a RAR-RXR mixture and the MS spectra recorded for each equivalent. Figure 2 shows that different oligomeric states were observed upon solution conditions. At 0.1 and 0.25 equivalents of DNA, unbound RAR and RXR were observed in concomitance with a third species at 33 954.6 Da \pm 0.4 Da corresponding to a RAR-RXR-DR5 heterocomplex bound to DNA. Although no zinc was added to our ammonium acetate buffer, 2 Zn²⁺ remain bound to the free or bound proteins, which indicates that at least part of the protein conformation has been retained in vacuo during analysis. No species revealing the existence of RAR-RAR-DR5 or RXR-RXR-DR5 homodimer complexes were furthermore observed. As shown previously [29], MS spectra revealed that the heterodimeric interface is greatly favored in presence of DNA over

homodimeric ones. In particular, heterodimerization of RAR-RXR DBD-LBD bound to DR5 elements was precedently shown by ESI-MS [30], but in the present work we used only DBD to avoid any bias by the LBD dimerization interface. These results strongly support structurally-specific interactions and revealed the different populations of complexes present in solution rather than unspecific aggregation in the gas phase. As the relative protein abundance decreased (0.5 and 1.2 eq. of DNA), RAR-DR5 and RXR-DR5 monomeric complexes emerged in the spectra while signal due to free proteins became negligible. Because of instrument effects, the relative abundance of free protein versus protein-DNA complexes could not be extracted nor evaluated from MS spectra (Supplementary Figure 2). Indeed, both species display different m/z ratios and different conformations, two parameters that are known to affect the ion intensities. Light or heavy m/z ions will then have different trajectories inside the MS interface and thus different transmission rates. Transmission rates were optimized by modifying the interface voltage settings, in particular, the pre-pulse voltage and the transfer time (these 2 settings are responsible for the storage of the ions and their transmission inside the mass spectrometer). This might also explain the apparently low intensity of free proteins at 0.1 – 0.5 equivalents of DNA. However, the spectra along the DNA titration could be compared as they were acquired under the same interface parameters.

To further investigate whether both DNA binding sites were able to bind to retinoic receptors independently of their nature (RAR or RXR), experiments consisting of RAR-only or RXR-only incubation with the *RAR β 2* DR5 were carried out. In each case, the spectra reveal that both DNA binding sites could be occupied by RAR or RXR (Figure 3). Molecular masses of 34 319.0 Da and 33 584.7 Da \pm 0.3 Da corresponding to RAR-RAR-DR5 and RXR-RXR-DR5 homodimers complexes respectively were measured. The complexes formed by DNA with the major and

minor forms of RAR show that the N-terminal degradation of the protein did not seem to affect the DNA recognition. From these data, it is not possible to determine whether both sites are bound with similar affinities, but this is beyond the scope of this study.

Impact of RAREs on heterodimerization

To determine whether the preformed homodimer-DNA complex could prevent the formation of the heterodimer-DNA complex, experiments consisting of addition of RAR and RXR to DNA sequentially were carried out. In particular, RAR was pre-incubated with the DR5 before addition of RXR (Fig. 4A and 4B) or in the opposite way, RXR-DR5 complex was preformed before addition of RAR (Fig. 4C and 4D). The spectra clearly show that all homodimer-DNA species are totally displaced in favor of heterodimer-DNA complex. Indeed, the occupancy of the sites is a dynamic process as already shown [31]. These results confirm that, thanks to the cooperativity of heterodimerization and the stabilization of subunits interactions by DNA, the formation of heterodimer-DNA is favored compared to homodimer-DNA species, as shown for classical DR5 and DR2 elements [32]. The same experiments were performed with *Hoxb13* DR0 yielding the same conclusion, *i.e.* the species present at equilibrium are identical independent of the order of partner addition (Supplementary Figure 3). Because each species present in solution displays a different molecular mass, all oligomeric states could be followed individually. MS appeared in our case to be a powerful technique for monitoring the disappearance of homodimer-DNA forms and the concomitant formation of the corresponding heterodimer-DNA species. It is well suited to follow all fluctuations in subunit assembly or disassembly, and thus possesses a great potential to assess to binding dynamics or even to the assembly pathway if a hierarchy is required [17].

In order to find out whether the length of the spacer between the two DNA binding sites was a determining factor, formation of the RAR-RXR-DNA complexes was followed after incubation of the proteins with various RAREs displaying the same sequence of the two half-sites (*RARβ2*) but differing by the number of nucleotides between the two direct repeat sites (DR0, DR1, DR2, DR5). All experiments were performed under the exact same conditions in terms of relative abundance in solution and mass spectrometer interface parameters. Mass spectra revealed the formation of different oligomeric states according to the considered DNA (Figure 5). When DR0 was added to the solution, the spectrum showed a high level of heterogeneity and many stoichiometries could be measured. Monomer-, homodimer- and heterodimer-DNA complexes coexist without an evident favored stoichiometry, which would have been an indication of specificity in the binding interfaces. On the contrary, the relative abundance of the RAR-RAR and RXR-RXR homodimer-DR0 complexes versus the RAR-RXR heterodimer-DR0 (about 40% and 50 % respectively) rather suggests random filling of the DNA binding sites in agreement with the observed species in the native gel (Figure 1). Similar results were observed with another DR0 RE (*Msi*, Supplementary Figure 4).

When DR1 is added, no signal corresponding to either monomer-DNA or to RAR homodimer-DNA complexes were observed. Only RXR homodimer- and RAR-RXR heterodimer-DR1 were detected. This result can be explained by the fact that for DR1, homodimer of RXR-DR1 is formed preferentially to the detriment of RAR-DR1 [33]. In presence of DR2 and DR5, a minor signal corresponding to RAR and RXR homodimers-DNA is observed while the main species corresponds to the heterodimer-DNA complex. From these results, an observation immediately emerges: while the heterodimeric form is always present, for *RARβ2*, the longer is the spacer, the more abundant is the formation of the corresponding RAR-RXR-DNA complex. As a

consequence, the heterodimeric interface appears to be stabilized with a 5 base pair spaced DNA. Model building of the RAR-RXR heterodimer in complex with DR5 or DR0 (Figure 6) indicates that for RAR-RXR in complex with a DR5 element, interactions between the 2 monomers will be formed, indicative of a cooperative dimer, as already modeled [34]. In contrast, in the complex with DR0 element, no interactions between the 2 monomers are formed; this is consistent with the binding to DR0 element as non-cooperative dimer providing an explanation of MS results. This non-cooperative dimer of RAR-RXR-DR0 may explain the inability of RAR-RXR bound to DR0 to activate gene expression in transcriptional reporter assay, as shown for glucocorticoid receptors with negative response elements [35].

To further investigate how the heterodimerization is mediated on DNA, similar experiments were performed with RAREs displaying different sequences. Table 1 summarizes ratios of homodimers- and heterodimer-DNA complexes depending on the sequence and spacer length. These proportions were deduced from the relative intensities of the m/z ions with the approximation that all homodimer- and heterodimer-DNA complexes had similar response factors. As it can be noticed, heterodimer-DNA seems to be the major form regardless of the considered RAREs. This result suggests a poor selectivity in RARE targets when forming a stabilized favorable heterodimer interface. Except for *RAR β 2* DR5, homodimers- and heterodimer-DNA coexist in solution, the homodimer/heterodimer ratio varies according to the DNA. This suggests that the sequence of DNA has influence over the repartition and the specificity of complexes formed with retinoic receptors, and this influence can be compensated by the difference in the sequence of the half sites.

CONCLUSION

This work illustrates the potential of MS to obtain insight into different species present in solution in a fast, robust and low sample-consuming way. The cooperativity of heterodimerization of RAR-RXR bound to DR5 elements was unambiguously highlighted, whereas the binding to DR0 elements is non-cooperative. The influence of protein/DNA molar ratios, sequence and length of DNA spacer on the relative abundance of RAR-RXR-DNA complexes was also highlighted. ESI-MS was crucial in allowing the study of retinoic receptor heterodimerization as a function of DNA. Thanks to its mass precision, assembly and disassembly of the different oligomeric states could be monitored as a function of the experimental conditions. This method is complementary to structural methods and will contribute to our understanding of the critical role the spacer plays in the specificity of the dimer. Although the first crystal structure of NR DBD was reported in 1991 [36], the number of available crystal structures with diverse or natural REs remains very low, highlighting the need to find alternative structural techniques. The MS information about the dynamics and composition of dimers will help to define conditions favoring heterodimer for crystallization and structural analysis. Considering the strong heterodimerization interface in the RAR-RXR-LBDs that promotes the binding of the full-length receptors on these RAREs, similar MS analyses on the entire protein (and not only the DBD) will now be planned, possibly with co-regulators that may modulate the composition of the dimers, as already shown for PPAR-RXR heterodimer and RXR homodimer bound to PPRE [37]. This might help to better detect and understand the recruitment of co-activators and/or co-repressors, and determine the influence of LBD on the NR-DNA heterodimerization. In combination with complementary techniques, MS data will help

to understand allosteric effects, follow changes in the heterodimerization surfaces and hence the nature of formed complexes.

FIGURES

Figure 1. (A) Sequences of the DNA response elements used in this study with the first hexanucleotide half-site shown in red and the second hexanucleotide half-site in red and underlined. The interspacer nucleotides are shown in black and lowercase; (B) TBE native PAGE gel of RAR and RXR DBDs. The free proteins do not migrate in the conditions of the gel. For DR0 REs the smeared bands and the presence of several bands indicate a mixture of species that cannot be resolved.

Figure 2. Mass spectra of retinoic receptors RAR-RXR bound to DNA depending on different equivalents of DNA. (A) 0.1 equivalent of *RARβ2* DR5; (B) 0.25 equivalent of *RARβ2* DR5; (C) 0.5 equivalent of *RARβ2* DR5; (D) 1.2 equivalent of *RARβ2* DR5.

Figure 3. Mass spectra of retinoic receptors bound to DNA. (A) RAR incubated with *RARβ2* DR5; (B) RXR incubated with *RARβ2* DR5.

Figure 4. Mass spectra of retinoic receptors bound to DNA depending on order of incubation. (A) RAR incubated with *RARβ2* DR5; (B) RAR incubated with *RARβ2* DR5 followed by an addition of RXR; (C) RXR incubated with *RARβ2* DR5; (D) RXR incubated with *RARβ2* DR5 followed by an addition of RAR.

Figure 5. Mass spectra of retinoic receptors RAR-RXR bound to DNA depending on the spacer length. (A) 0.25 equivalent of *RARβ2* DR0; (B) 0.25 equivalent of *RARβ2* DR1; (C) 0.25 equivalent of *RARβ2* DR2; (D) 0.25 equivalent of *RARβ2* DR5.

Figure 6. 3D models of RAR-RXR DBDs bound to Hoxb13DR0 (left) and bound to *RARβ2* DR5 (right). The same color code of the DNA is used as Figure 1. The RAR-RXR complexes were modeled based on crystal structures of RXR complexes (data not shown). On DR0, the 2 monomers lie on each side of the DNA making no protein-protein contact, while on DR5, the 2 monomers are side by side.

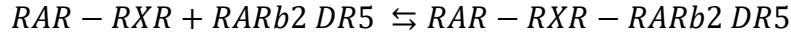
TABLES

Table 1. Relative quantity of homodimers and heterodimer of RAR-RXR bound to 0.25 equivalent of DNA with regard to the total quantity of existing dimers, depending on the sequence and spacer length: X = less than 20 %, XX = between 20 % and 50 %, XXX = between 50 % and 75%, XXXX= between 76 % and 99%, XXXXX = 100 %.

SUPPLEMENTARY MATERIAL

Supplementary Figure 1. DNA binding to RAR-RXR DBD-LBD measured by MST. Unlabeled *RARβ2* DR5 was titrated into a fixed concentration of labeled RAR-RXR. The representative MST curves are showed in (A) 50 mM HEPES, pH 8.0, 100 mM sodium chloride, 2% glycerol and (B) 100mM ammonium acetate, pH 7.8 buffers. ΔF_{norm} = normalized fluorescence change.

Three measurements were averaged and the standard deviation was determined. The K_d values were calculated by directly fitting the curve using the Hill function as described in [28]. In this study:



$$Kd = \frac{[RAR-RXR]_{free} \cdot [RAR\beta 2 DR5]_{free}}{[RAR-RXR-RAR\beta 2 DR5]}$$

$$Kd = \frac{([RAR-RXR]^{\circ} - [RAR-RXR-RAR\beta 2 DR5]) \cdot ([RAR\beta 2 DR5]^{\circ} - [RAR-RXR-RAR\beta 2 DR5])}{[RAR-RXR-RAR\beta 2 DR5]}$$

Supplementary Figure 2. Mass spectra of retinoic receptors RAR-RXR bound to 0.25 equivalent of *RARβ2* DR5 illustrating the influence of the pre-pulse time of the mass spectrometer on the detected abundance of free protein versus protein-DNA complexes. (A) Capillary exit at 150 V and pre-pulse time at 40 μs; (B) Capillary exit at 150 V and pre-pulse time at 30 μs.

Supplementary Figure 3. (A) RAR incubated with *Hoxb13* DR0; (B) RAR incubated with *Hoxb13* DR0 followed by an addition of RXR; (C) RXR incubated with *Hoxb13* DR0; (D) RXR incubated with *Hoxb13* DR0 followed by an addition of RAR.

Supplementary Figure 4. Mass spectra of RAR-RXR bound to 0.25 equivalent of *Msi* DR0.

Supplementary Table 1. K_d values (nM) for apparent binding affinities of RAR-RXR and different RAR binding element oligonucleotides from MST. Buffer A: 50 mM HEPES, pH 8.0, 100 mM sodium chloride, 2% glycerol. Buffer B: 100mM ammonium acetate, pH 7.8. * Measured by ITC in Moutier *et al.* 2012.

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