Signature of the oligomeric behaviour of nuclear receptors at the sequence and structural level

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Nuclear receptors (NRs) are ligand-dependent transcription factors that control a large number of physiological events through the regulation of gene transcription. NRs function either as homodimers or as heterodimers with retinoid X receptor/ultraspiracle protein (RXR/USP). A structure-based sequence analysis aimed at discovering the molecular mechanism that controls the dimeric association of the ligand-binding domain reveals two sets of differentially conserved residues, which partition the entire NR superfamily into two classes related to their oligomeric behaviour. Site-directed mutagenesis confirms the functional importance of these residues for the dimerization process and/or transcriptional activity. All homodimers belong to class I, in which the related residues contribute a communication pathway of two salt bridges linking helix 1 on the cofactor-binding site to the dimer interface. A salt bridge involving a differentially conserved arginine residue in loop H8–H9 defines the signature motif of heterodimers. RXR/USP and all *Caenorhabditis elegans* NRs belong to class I, supporting the hypothesis of an earlier emergence of this class.

Keywords: nuclear receptors; dimerization; evolution; sequence; structure; mutagenesis

EMBO reports (2004) 5, 423–429. doi:10.1038/sj.embor.7400119

INTRODUCTION

Signalling by nuclear receptors (NRs) is one of the major signal transduction paradigms invented by metazoans to regulate gene transcription (Robinson-Rechavi *et al*, 2003). The most prominent feature differentiating them from other transcription factors is their capacity to bind small hydrophobic molecules specifically. Molecular and structural studies reveal that these ligands constitute regulatory signals, which modify the NR transcriptional activity through conformational changes (Renaud & Moras, 2000).

Prototypical NRs share a common structural organization with a variable amino-terminal (Nter) domain, a conserved DNA-

binding domain (DBD) and a C-terminal (Cter) ligand-binding domain (LBD). From a regulatory point of view, the LBD is the most important domain, containing both the ligand-binding pocket and the ligand-dependent transactivation function. It possesses interaction surfaces for the homo- or heterodimeric partner, co-repressors, co-activators and other bridging factors.

Most NRs function as dimers, the LBDs being the longest contributors to the dimer stability through extensive protein–protein contacts. Owing to several crystal structures of LBD dimers, we know the contact points at the interfaces (Table 1). However, the molecular mechanisms that control the functionally important association into either homo- or heterodimers are unknown. To establish the molecular basis of the discrimination between the two states, we performed a structure-based sequence analysis of the LBD domain coupled with mutagenesis experiments.

RESULTS

Sequence alignment

The multiple alignment includes 706 full-length LBDs, 489 of which are from complete genomes (a subset is presented in Fig 1A with position numbering at the top of the figure corresponding to the residues shown; full data are available as supplementary information online). The analysis reveals an extreme divergence exemplified by the *Caenorhabditis elegans* and *C. intestinalis* NRs, which introduce variability in some of the previously reported conserved motifs (Wurtz *et al*, 1996), notably in the LBD-specific signature ((F,W)AKxxxxFxxLxxxDQxxLL, positions 16–36 in Fig 1A) and in the two alternative patterns found at positions 1–8 ((I,L)Ix(A,I)Exxh in the retinoid X receptor (RXR)/steroid receptors and hxcAHxxT in the retinoic acid receptor/thyroid hormone receptor (RAR/TR) subgroup). The only almost invariant residue present in all NRs is the acidic residue at position 50 (E in 95% and D in 3% of all NR sequences) (Fig 1A).

When the two sets of sequences corresponding to the experimentally verified homodimer or heterodimer NRs were compared (Table 1), two residues were observed to be differentially conserved (conserved in more than 90% of one functional group and *strictly* absent in the other). This observation could be extended to all sequences and allowed to partition the entire superfamily into two classes. The class-specific signature residues are the basic amino acids (aa) at position 93 (R93 in 91% and K93

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Received 5 November 2003; revised 12 January 2004; accepted 5 February 2004; published online 19 March 2004

Table 1 | Class partition of nuclear receptors based on our structure-based sequence analysis

Class I	Class II
HNF4*	TR**
DHR96	RAR**
RXR*, USP*	PPAR**
TR2, TR4	RevErb, E75
DHR78	E78
TLL, PNR	ROR, HR3
COUP, SVP, EAR2	EcR**
ER*	LXR**, FXR
ERR*	VDR**, PXR**, CAR
GR*, PR*, AR, MR	NGFIB, Nurrl**, NOR1
SF1, LRH1, FTZ-F1	DHR38**
DHR39	
GCNFI	
<i>Dax1, SHP</i>	
All <i>C. elegans</i>	

Only a representative subset including all human receptors of the entire superfamily is shown here but the partition encompasses all NRs. Experimentally verified homodimers or heterodimers are indicated by (*) or (**), respectively. The known 3D LBD structures are in bold. See supplementary information online. The particular cases mentioned in the text are in italic.

in 5% of all homodimer sequences) and the arginine at position 62 (R in 98% of all heterodimer sequences).

Furthermore, when the *C. elegans* sequences were excluded, we found two additional residues discriminating homodimers from heterodimers: W40 (90%) present in all homodimer members except the hepatocyte nuclear factor 4 (HNF4) relatives, and K55 present in all homodimer NRs except the RXR, where it is replaced by an arginine (K 82% and R 17%) (Fig 1B).

Structural analysis

The results were then examined at the structural level using the known crystal structures (references are available at http://bioinfo-igbmc.u-strasbg.fr/Nuclear_Receptors) (Fig 2). In all homodimer structures, the differentially conserved E5 at the Cter end of H1 marks the beginning of a pathway from H1 to loop H9–H10 (L9–10) via H8 through two salt bridges. The first one links E5 (H1) to K55 (H8), whereas the second bridge associates the superfamily invariant E50 (H8) to the differentially conserved R93 at the Nter part of H10. The differentially conserved W40 at the end of H4 points into the ligand-binding pocket close to H12.

In the LBD structures of class II NRs, the superfamily invariant residue E50 forms a salt bridge with the basic aa 90 at the Cter end of H9. The differentially conserved R62 forms a salt bridge with the acidic residue 42 (E/D42) at the H4–H5 kink (Fig 1B). Located at the dimer interface, this salt bridge tethers L8–9, which is in contact with H7 of the heterodimeric partner. However, in the crystal structure of heterodimers, the arginine residue itself does not form direct contacts with either H7 or H10. The exclusive

presence of R62 in heterodimers makes this salt bridge a structural signature motif.

Site-directed mutagenesis

At the functional level, several RXR residues at the dimer interface, selected on the basis of the crystal structures (Bourguet *et al*, 1995, 2000), have been mutated and tested for homo- and heterodimer stability and transactivation potency (Vivat-Hannah *et al*, 2003). Two main observations emerge from this study: several single mutations (X→A) affect the homodimerization properties of RXR, including R426A (see Figs 1B and 2B), but only one single point mutation at the Cter end of H9 significantly lowers the stability of the RXR/RAR heterodimer (Y402A). In the present study, three hRARα residues of heterodimer signature motifs were mutated and tested for transactivation and dimerization with hRXRα (Fig 3). The effects of mutations on heterodimer interactions were first determined by examining binding of RARs to His-RXR in pull-down assays. The D267A and R367A mutations have a clear impact on the stability of the dimer, whereas the R339A mutation has no obvious effect (Fig 3A). These mutants were also assessed for their abilities to induce ligand-mediated transcription in cultured cells in a context where the interactions occur only through the LBDs with endogenous RXR and RAR not bound to DNA (Fig 3B). The most important effect on transactivation is observed with the D267A (E/D42) and R339A (R62 in Fig 1A) mutants that strongly inhibit the activity, whereas R367A (R90) has a weaker effect. Taken together, these two experiments suggest that the heterodimer stability is not the driving force of arginine conservation.

DISCUSSION

The present structure-based sequence analysis leads to an interesting partition that encompasses all NRs. All homodimer-forming receptors belong to class I, whereas those known to function as heterodimers with RXR/ultraspiracle protein (USP) are members of class II (Table 1). Interactions between COUP-TF and RXR/USP have been suggested, which would make COUP-TF the exception to the partition's functional correlation (Butler & Parker, 1995). Our study would exclude a canonical heterodimer. Interestingly, RXR and USP, which have a central role in the heterodimer family but can form both homo- and heterodimers, clearly belong to class I. These ubiquitous receptors do not exhibit specific signature motifs, except for the K55R mutation in all RXR residues (Fig 1A). The spatial location of the class conserved residues raises the questions of functional significance of the salt-bridge networks and their relationship with dimer stability.

The class-I-specific pathway

The class-I-specific pathway leading from H1 to H9–H10 suggests the existence of a communication channel between a cofactor-binding site adjacent to H1 and the dimer interface (Figs 2B and 4A). The Cter end of H1 is close to the binding cleft for the LXXLL motif common to all cofactors (Heery *et al*, 1997; Shiau *et al*, 1998). For TR, mutations at this position have been shown to hamper NcoR binding (Hoerlein *et al*, 1995). It is tempting to suggest that this part of the helix senses the release of a co-repressor and/or the binding of a co-activator, this information being transferred to L9–10 through a conserved molecular path. This communication could be part of an intermolecular cross-talk.

Fig 1 | Multiple sequence alignment of LBDs and class-specific features. (A) Multiple sequence alignment of a subset of NR LBDs. The complete multiple alignments of the 706 NRs and the 489 NRs from complete genomes are available as supplementary information online and at http://bioinfo-igbmc.u-strasbg.fr/Nuclear_Receptors. NRs and the differentially conserved residues are underlined in blue and green for class I and II, respectively. Numbers between aligned blocks indicate the length of variable inserts; numbers at the beginning of each sequence indicate the position of the first aligned residue; and numbers at the end of each sequence indicate the number of remaining residues. The convention for residue conservation is as follows: 100%, white against black; >80%, white against grey; >60%, black against grey. NR LBD secondary structure elements are indicated. The residue numbering at the top of the alignment is the one used in the text. h, *Homo sapiens*; ci, *C. intestinalis*; dm, *Drosophila melanogaster*. **(B)** Secondary structure diagram showing NR class-specific features. Conserved but not strictly class-specific residues are in black (bold for the most frequent one). Arrows indicate the salt bridges in the 3D structures.

In class II NRs, the sequence variability at the Cter end of H1 and the absence of a conserved bridge between H1 and H8 suggest a more divergent mechanism of communication. H1 and the nearby tip of the LBD β -sheet form one side of the ligand-binding pocket that can accommodate conformational variations without affecting the position of the functionally important H12. The crystal structures of class II NRs confirm the variability of this H1/ β -sheet junction, which is probably due to the functional necessity of binding chemically and structurally different ligands. This in turn explains the absence of a conserved salt bridge between H1 and H8, and suggests the existence of more

NR-specific patterns of recognition and communication transfer for this segment.

The conservation of W40 in class I NRs and the involvement of the conserved E/D42 in the class-II-specific salt bridge at the H4–H5 kink provide an additional argument in favour of a transfer mechanism. This part of the LBD structure is structurally conserved in all LBDs, and constitutes a fixed point of interaction with ligands. In other words, these class-specific features are directly (W) or indirectly (E50 in H8) sensing the presence of a ligand, an event associated with cofactor binding (Ghosh *et al*, 2002).

Fig 2 | Class-specific residues. (A) Consensus NR LBD structure. (B) hRXRα: close-up view of the class-I-specific residues including the two salt bridges forming the class I communication pathway. The residue numbers refer to the full-length receptors (subscripts) or the arbitrary scale (superscripts) of Fig 1. (C) hRARα: close-up view of the two class-II-specific salt bridges.

Helix 8-loop 9-10 salt bridges

Both classes exhibit a conserved salt bridge, close to the dimer interface, connecting H8 to L9-10 (Nter or Cter ends for class II and I, respectively) (Figs 1B and 2). These salt bridges probably act on the strength of the dimeric association through the stabilization and positioning of the loop conformation. This explanation was provided to account for the incidence of two mutations of hNF4α observed in patients with the MODY1 form of diabetes (Dhe-Paganon *et al*, 2002). The point mutation R367A in hRARα confirms this functional correlation affecting both transactivation and dimer formation (Fig 3A,B). Note that this salt bridge is missing in the Nurr1 subgroup. The crystal structure of Nurr1 shows that H9 is bent outwards with a significant shift of its Cter residues, compared with the closely related RAR structure (Wang *et al*, 2003). The correlation between this movement and the absence of an anchoring salt bridge is tempting. Our analysis suggests that this conformational change could weaken the dimeric interaction with RXR, in agreement with the observed weak interaction of the two LBDs (our unpublished data). Similarly, the mutation Y402A in RXRα (Nter of L9-10) affects the heterodimer and not the homodimer stability (Vivat-Hannah *et al*, 2003).

Class II signature motif

The class-II-specific salt bridge (E/D42-R62) contributes the only conserved residue located near the dimer interface (R62). Although homo- and heterodimers exhibit the same canonical structure with two dyad-related H10 helices forming the backbone of the interface, further stabilized by contacts through H7 and

Fig 3 | Pull-down and transactivation assays on hRARα mutants. (A) Pull-down assays of hRXRα LBD with [35S]hRARα LBD wild type (a) or mutants D267A (b), R339A (c), R367A (d) and Gal4 control (e). (B) Transcriptional activity of hRARα wild type and hRARα mutants (D267A, R339A, R367A).

Fig 4 | Stereoviews of homo- and heterodimeric structures. (A) Crystal structure of the heterodimer LBDs of hRXRα (cyan) and hRARα (green) bound to oleic acid and BMS614, respectively. The two class-specific salt bridges are shown. (B) View along the pseudo-two-fold axis. Arrows highlight the dissymmetrical distances between RXRα's H7 and L8-9 (magenta) and RARα's L8-9 and H7 (yellow). The class-II-specific salt bridge is shown. (C) Crystal structure of the symmetrical hRXRα LBD homodimer as seen in the complex with DHA.

L8-9 on one side, and H9 and the Nter of H10 on the other side (Bourguet *et al*, 1995, 2000; Gampe *et al*, 2000; Egea *et al*, 2002; Svensson *et al*, 2003), the crystal structures show that the heterodimers deviate significantly from the perfect two-fold symmetry observed for homodimers (Fig 4). A marked tilt of the dimer interface brings helix H7 of RXR closer to L8-9 of its class II partner. The homologous parts of H7 from the class II partner and H8/H9 from RXR are further apart. The asymmetric heterodimer

association is more stable than the RXR homodimeric one (Bourguet *et al*, 2000), suggesting a role of the class II H5/L8-9 salt bridge as a conformation stabilizer favouring heterodimeric interactions. However, the pull-down assays on our hRARα mutants do not support this interpretation. In keeping with the lack of direct contacts observed in the heterodimer crystal structure, the R339A mutation does not affect the dimer stability. It only reduces the transactivation potency, whereas the E267A

mutation affects both functions. The *in vitro* observed effect on transactivation potency could be due to the loss of a specific recognition of R62 by co-activators. The pull-down assays show that other effects such as eventual conformational changes do not suffice to disrupt the heterodimer. The deleterious effect of the D267 mutation on the dimer stability can be explained by the loss of a compensating charge at the core of the interface, near the H10–H10 contact.

Specificities of the steroid subgroup and *C. elegans* NRs

Our study reveals that the steroid subgroup is characterized by specific features that may affect the homodimer structure and/or stability. (i) The absence of the otherwise conserved acidic residue at the H4–H5 kink (E/D42) probably affects the homodimer stability, via electrostatic effects or inter-LBD interactions. This residue is strictly conserved in all metazoan NRs except for the steroid subgroup (androgen receptor, glucocorticoid receptor (GR), mineralocorticoid receptor and progesterone receptor (PR)), Dax1 and short heterodimer partner. It is not surprising that a point mutation at this location (F602S) in the rat GR affects ligand affinity and protein solubility (Garabedian & Yamamoto, 1992). Whether this mutation alone would induce the formation of a specific dimer interface remains to be proved (Bledsoe *et al.*, 2002). (ii) A characteristic extension at the Cter end of H12 forming a small β -sheet with L8–9 also prevents the constitution of a canonical homodimer as already noticed for PR (Williams & Sigler, 1998). This extension is present in all steroid NRs except oestrogen receptor (ER). (iii) Another difference that should not affect the homodimer stability as it is shared by ER concerns the class-I-specific salt bridge connecting H8 to L9–10. In steroid NRs, the size of L9–10 is shorter, with a concomitant extension of H10 Nter by one turn; however, the salt bridge involves two residues at the same special location.

The multiple alignment analysis of the *C. elegans* genome shows that the canonical motifs for the LBD of NRs are less conserved than previously thought. In spite of this hypervariability, two exceptional features remain: the presence of the class I conserved arginine at the Nter of H10 and the strict absence of the class-II-specific arginine in L8–9. Thus, according to the signature motif concept, all NR sequences from *C. elegans* belong to class I. This observation supports the proposal that ancestral receptors were homodimeric transcription factors (Laudet, 1997).

CONCLUSION

The present analysis based on the distribution of differentially conserved residues in the LBDs leads to the partition of the entire NR superfamily into two mutually exclusive classes and suggests the following proposals. (i) Class I NR LBDs can form RXR-like homodimers. While maintaining most class I signature motifs, the steroid subgroup, Dax1 and SHP probably constitute an exception to this rule. (ii) Class II NRs can form stable LBD heterodimers with RXR/USP or monomers. This has already been verified for most class II members. The salt bridge involving the differentially conserved arginine 62 of L8–9 to the acidic residue 42 at the H4–H5 kink is the signature motif that appears to be associated with this functional behaviour. In this class, a second conserved salt bridge linking H8 to the Cter end of H9 is present in all NRs except for the subgroup of nerve growth factor IB-like (NGFIB). Its absence should affect the LBD heterodimer stability. This is already verified for Nurr1 and AHR38 (an orthologue of NGFIB)

(Zhu *et al.*, 2000). The partition stresses the functional importance of two structural networks and provides a set of key residues to test the specific molecular mechanisms of NR-dependent transactivation. Issues such as heterodimer implication in the case of orphan receptors or cross-talk interference through ligands can now be investigated with a new perspective.

METHODS

Sequence analysis. A set of 48 human NR proteins was retrieved from the NuclearRDB (<http://receptors.ucsf.edu/NR>) and a subset of the variable *C. elegans* NRs. This set was used as probes for searches of the SwissProt, SpTrEMBL and SpTrEMBLNEW databases and the Protein Data Bank using the BlastP program (Altschul *et al.*, 1990), and potential missing sequences were checked in the NuclearRDB. We detected 706 full-length NR proteins (excluding fragments) from early metazoans to humans that were aligned using DbClustal (Thompson *et al.*, 2000). Validation and correction of the alignment were performed using the NorMD (Thompson *et al.*, 2001) objective function and the RASCAL program (Thompson *et al.*, 2003). The complete multiple alignments of the 706 and the 489 NRs from complete genomes are available as supplementary information online and at http://bioinfo-igbmc.u-strasbg.fr/Nuclear_Receptors. Aligned sequences were clustered using DPC (Wicker *et al.*, 2002) and homo- and heterodimer sequences compared.

Pull-down. 35 S-Met-radiolabelled hRAR α LBDs were expressed using the *in vitro* transcription and translation TNT T7 system (Promega). hRXR α LBD (233–462) was expressed in *Escherichia coli* BL21DE3 as a His₆-tagged fusion protein and purified as described (Bourguet *et al.*, 1995). Buffer was exchanged by dialysis to buffer A (5 mM imidazole, 100 mM NaCl, 50 mM Tris (pH 8), 5% glycerol, 0.1% NP-40). Ni-agarose beads were resuspended, washed with buffer A, incubated for 1 h at 4 °C with either 35 S-Met-radiolabelled hRAR α LBD wild type or mutants, or with Gal protein (control), and then incubated overnight with hRXR α LBD in buffer A with 200 μ g/ml BSA in the presence of either 0.25 mM 9-*cis* retinoic acid (RA) or 1% ethanol. The bound proteins were released with SDS sample buffer and analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. Inputs were diluted 1:4 before loading.

Transactivation. hRAR α LBD was subcloned into the pG4M vector as a Gal fusion protein. Mutants (D267A, R339A, R367A) of the hRAR α were generated using the QuickChange™ site-directed mutagenesis kit from Stratagene. Transient transfection assays were carried out in 24-well plates (10⁵ cells per well) using a standard calcium phosphate co-precipitation technique as described in Xiao *et al.* (1991). HEK-EBNA293 cells were co-transfected with 25 ng of pG4M–hRAR vector, 250 ng PAL1(x5)–TK–LUC reporter, 75 ng PCH110 β -galactosidase expression plasmid, treated with either RA or ethanol for 20 h, and cell extracts were assayed for luciferase and β -galactosidase activity. Luciferase values were normalized to β -galactosidase activity. Data points represent the mean of assays performed three times.

ACKNOWLEDGEMENTS

We are grateful to Julie Thompson for a critical reading of the manuscript and Jerome Fagart, Sylvia Eiler, Frederic Plewniak, Isabelle Billas, Thomas Iwema and Pascale Karcher for useful discussion. We thank Luc Moulinier for providing the program ORDALI before publication and Valerie Vivat-Hannah for communicating her results on RXR

dimerization before publication. This work was supported by funds from CNRS, INSERM, the University Louis Pasteur of Strasbourg, Hoechst Marion Roussel, the FNS through the Genopole program and SPINE EEC QLG2-CT-2002-00988.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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