Identification of Amino Acids Critical for the DNA Binding and Dimerization Properties of the Human Retinoic Acid Receptor α

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Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) activate target genes by binding to retinoic acid response elements (RAREs) as heterodimeric, asymmetrical complexes, and display a high degree of cooperativity in binding to RAREs. We have examined here the effect of lysine, cysteine, arginine, histidine, and tyrosine side chain chemical modification on the DNA binding, homo- and heterodimerization properties of the full-length human retinoic acid receptor α (hRARα). Lysines are the only residues to be engaged in the dimerization with human retinoid X receptor α (hRXRα) in the absence of DNA, whereas histidines are selectively involved in the homodimerization of hRARα in the presence of a RARE. Arginine modification affected the DNA binding activity of each type of dimer, whereas cysteines and tyrosines were primarily involved in the homo- or heterodimerization process in the presence of the same RARE. Modified lysines, interfering with the dimerization with hRXRα, were identified by receptor labeling and peptide mapping. They are located in the hormone binding domain eighth heptad repeat, at positions 360 and 365. In keeping with these results, mutation of Lys360, Val361, and Lys365 diminished strongly the DNA binding activity of hRARα as a homodimer or a heterodimer. Our results thus provide direct evidence for the differential involvement of basic, polar, or aromatic amino acids in the DNA binding, homodimerization, and heterodimerization properties of hRARα. Furthermore, they demonstrate the use of distinct dimerization interfaces and identify the type of amino acids involved in these protein-protein interactions.

Six different cDNAs coding for distinct types of retinoid receptors (RXRs and RARs α, β, and γ) have been isolated and display strong sequence homology in their DNA binding domain (DBD) with other members of the nuclear receptor superfamily, whereas the ligand binding domain (HBD) appeared to be poorly conserved (for review, see Refs. 1 and 2 and references therein). Nuclear proteins, initially described as factors able to potentiate the DNA binding activity of RARα by establishing protein/protein contacts in vitro (3), were found to be homologous to RXRα and RXRβ (4, 5). The observed cooperativity in binding to DNA and transactivation is not limited to the RXR-RAR pair, but extend to VDR, T3R, and PR heterodimers (9, 10). RXR/RXR heterodimers bind to retinoic acid response elements (RAREs) that consist, in most cases, of a direct repeat (DR) of the sequence PuGGTCA spaced by five nucleotides (9, 11). However, RXR/RAR heterodimers can bind to RAREs with a spacing ranging from two to five nucleotides (12, 13), and to core recognition sites arranged into palindromic, inverted palindromes (14) and inverted repeats.

DBDs of RARβ (15) and RARα (16) contain, like the glucocorticoid receptor DBD (17), two perpendicular α-helical structures located at the C-terminal end of each zinc finger (noted C1 and C2). The first α-helix maps to the so-called P box, and establishes direct contacts with base pairs in the major groove of the core recognition motif, whereas the second α-helix lies perpendicularly to the first one. The D box, located at the N terminus of the second zinc finger, is involved in the heterodimerization of RAR and T3R with RXR when bound to DR5 and DR4, respectively (18). Spacing restrictions are imposed by the interaction of the D box of RXR and DR boxes of T3R and RAR. The location of DR boxes is different according to the spacing of the two half-sites of the response element (18–21). Although protein-protein interactions between DBDs of each dimerization partner are necessary and sufficient to impose the binding repertoire of the dimer (22), hormone binding domains (HBDs) are also taking a notable part in the dimerization process. Nine heptad repeats were identified in RAR, VDR, and T3R HBDs, and were predicted to be organized in a leucine zipper-like structure (23). The ninth heptad repeat is required for heterodimerization and different interfaces are involved in the absence or the presence of the cognate ligand (24). Amino acids located in this region of RAR, T3R, and RXR have a critical role in regulating the ligand-dependent homo- and heterodimerization properties of these receptors, and may even be determining the binding of RXR/RAR dimers to a DR1 element (22). The first and fifth heptad repeats have also been shown

PyP, pyridoxal 5'-phosphate; PAG, polyacrylamide gel electrophoresis; T3R, thyroid hormone receptor; TNM, tetranitromethane; VDR, vitamin D receptor; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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The abbreviations used are: RXR, retinoic X (9-cis retinoic acid) receptor; RAR, retinoid acid receptor; RARE, retinoid acid response element; DRX, direct repeat retinoic acid response element with a spacer of X bases; DBD, DNA binding domain; DEPC, diethyl pyrocarbonate; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; HBD, hormone binding domain; HPG, p-hydroxyphenylglyoxal; hRARα, human α-trans retinoid acid receptor; NEM, N-ethylmaleimide; NITA, nitritolactric acid; PR, progesterone receptor; PGE, prostaglandin E; T3R, thyroid hormone receptor; T3R HBDs, tetranitromethane; VDR, vitamin D receptor; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
to be required for heterodimerization of hRARα with hRXRα (25, 26).

All of the studies mentioned above demonstrate that multiple interfaces are available to enable homodimerization or het-
erodimerization of RAR, T3R, VDR, and RXR. Chemical modification of proteins by specific reagents is an useful tool to identify critical residues for a given function of a protein (for review, see Ref. 27 and references therein). We and others have used cysteine-specific reagents to assess the function of these residues in the ligand binding activity (28-31) or in the activa-
tion process of the GR (31, 32). We also used a similar approach to study the ligand binding properties of hRARα. In this report, we have assessed the contribution of cysteines, arginines, lysines, histidines, and tyrosines to the DNA bind-
ing, homodimerization, and heterodimerization of hRARα. We found that these residues are involved differentially in the different activities of the receptor. Functional, exposed NH2 groups from lysyl residues were mapped to the hormone bind-
ing domain of the receptor and more precisely to the eighth heptad repeat motif. Our method therefore defines a general strategy to identify amino acids critical for nuclear receptor functions.

EXPERIMENTAL PROCEDURES

Materials

Antiproteases, 2-hydroxy-5-nitrobenzyl bromide, and diethyl pyro-
carbonate were purchased from Sigma. p-Hydroxyphenylglyoxal and N-ethylmaleimide were from Pierce, pyridoxal 5'-phosphate was from Merck (Darmstadt, Germany), and tetratinonethane was supplied by Aldrich. Taq DNA polymerase, isopropyl-1-thio-galactopyranosyl-
ide (IPTG), ampicillin, and kanamycin were from Appligene (Strasbourg, France). Restriction enzymes were from Promega (Madison, WI), and oligonucleotides were purchased from Eurogentec (LeSart-Tilman, Belgium). Acrylamide and bisacrylamide mix (Polytope) were from Na-
tional Diagnostics (Atlanta, GA).

Plasmids and Bacterial Strains

The plasmid pHK1, containing the cDNA of hRARα (34), was ob-
tained from V. Giguerre and R. M. Evans (Salk Institute, H.H.M.I., La Jolla, CA). The pQE-9 vector was obtained from Diagen Gmbh (Dus-
seldorf, Germany). The hRARα cDNA was obtained by polymerase chain reaction amplification and inserted into the pQE-9 vector as a BamHI-HindIII fragment, in order to generate an in-frame fusion pro-
tein made of a histidine tag followed by the sequence coding for the receptor. The identity of the amplified cDNA was confirmed by restric-
tion mapping and sequencing. Furthermore, microsequencing of BrCN-
generated fragments of the purified protein yielded the expected amino acid sequences. Histidine residues do not interfere with the ligand binding properties of hRARα (35). F1-hRXRα was created by inserting in frame the hRXRα cDNA (36) as a HindIII-BglII fragment into the expression vector pF1 (IBI-Kodak, Rochester, NY) containing the Flag epitope.

DH5α (Life Technologies, Inc.) cells were used for routine subcloning procedures, and M15 or SG 13009 (Diagen) bacterial strains containing the Rep4 plasmid coding for the lac repres sor were the host cells for overexpression of His1-tagged hRARα. J M19 cells were used to overex-
press F1-hRARα.

In Vitro Translation and Transcription

The chicken progesterone receptor (form B), the human glucocorti-
coid receptor, the human RARα and the human RXRα were synthesized by coupled transcription/translation using the Promega TnT lysate system (Promega, Madison, WI). hRARα and hRXRα expression vectors were obtained from J. M. Williams (University of California, San Francisco, CA), J. G. Papaioannou (Istituto Superiore di Sanita, Rome, Italy), J. Leiden (University of Texas Health Sciences Center, San Antonio, TX), and R. M. Evans (Salk Institute, La Jolla, CA), respectively.
β-mercaptoethanol, 0.05% Nonidet P-40, and 20% glycerol. Unbound hXRα was removed by four washes with ice-cold binding buffer, and the specifically adsorbed [35S]-labeled hXRα was released by incubation with 50 μl of 0.1% SDS, 0.4 mM HCl. Radioactivity was quantified by scintillation counting.

Alternatively, cross-linking experiments were performed to control for the effect of reagents on the dimerization of His6-hRARα with hXRα in solution. In these experiments, similar amounts of labeled hXRα and purified His6-hRARα were mixed and incubated in conditions similar to that used for EMSA, except that Tris-HCl was substituted for HEPES. Dimethyl suberimidate (Pierce) was added to a final concentration of 1 mM for 1 h at 4°C, and the cross-linking reaction was quenched by bringing the mix to 400 mM Tris-HCl, pH 7.4. Products were then resolved on a 8% SDS-PAGE.

Chemical Modification Procedures

A strategy was devised to assess the functionality of hRARα after modification by amino acid-specific reagents and is detailed in Fig. 1. 150 pmol of purified hRARα (5.8 μm) were incubated in the presence of an increasing molar excess of the reagent, such that the ratio of the concentration of the reagent to the amino acid content of the receptor varied from 0 to 40. Note that the His6-hRARα fusion protein contains 18 Cys, 25 Arg, 27 Lys, 13 His, and 10 Tyr residues. All reactions were carried out in a total volume of 26 μl at 12°C, yielding reagents concentrations ranging from 0.012 mM to 13 mM. In all cases, control reactions were performed by omitting only the modifying reagent.

Arginine Modification—The procedure was essentially that described by Yamashita et al. (37). Briefly, purified hRARα was incubated in the dark for 120 min in the presence of p-hydroxyphenylglyoxal (HPG) in 10 mM Tris-HCl, pH 8.2. The reaction was quenched by bringing the reaction mix to 20 mM arginine.

Lysine Modification—Pyridoxal 5'-phosphate (PyrP) was used essentially as described earlier (27). PyrP was allowed to react with the receptor for 50 min in 20 mM HEPES, pH 6.8, in the dark, and the reaction was quenched by bringing the mix to 400 mM Tris-HCl, pH 7.4, for 1 h with varying concentrations of NEM, and the cross-linking reaction was stopped using 5 mM sodium borohydride (NaBH₄) for 5 min at 4°C.

Cysteine Modification—N-Ethylmaleimide (NEM) was used in conditions adapted from that previously established in our laboratory (33). Briefly, purified hRARα was incubated in 10 mM Tris-HCl, pH 7.4, for 1 h with varying concentrations of NEM, and the reaction was stopped by the addition of 20 mM β-mercaptoethanol.

Histidine Modification—Diethyl pyrocarbonate (DEPC) was used in 50 mM sodium phosphate buffer, pH 6.9, as described in Cheng and Nowak (38). In this case, hRARα was, prior to treatment with DEPC, desalted through a Chromaspin column (2 × 0.5 cm, Clontech) to avoid quenching of the reaction by imidazole, which is used to elute hRARα from the NTA resin. The reaction was for 120 min and was stopped by bringing the solution to 20 mM imidazole.

Tyrosine Modification—Tetranitromethane (TNM) was used according to Sokolowski et al. (39). hRARα was incubated in 20 mM Tris-HCl, pH 7.9, for 120 min, and the reaction was ended by 20 mM dithiothreitol in 20 mM NaOH (PyrP). Upon completion of the chemical modification, the receptor was immediately submitted to the various assays as described above.

Receptor Labeling and Peptide Analysis

To further determine the localization of modified lysyl residues within the receptor, purified His6-hRARα was labeled with [125I]formaldehyde (DuPont NEN, 25–100 Ci/mmol). Labeled formaldehyde was diluted twice with unlabeled formaldehyde, and increasing concentrations of reagent were used to react with the receptor (0.3–12 mM final concentration). Variations in reagent concentration did not affect the qualitative outcome of the labeling (data not shown). Cyanogen bromide (CNBr) was used as a reducing agent and was added at an equimolar concentration just prior to the addition of formaldehyde. The reaction was stopped by addition of NaBH₄, CNBr (0.5–2.0 mM, according to the concentration of formaldehyde used) and glycine (20 mM final concentration) after a 1-h incubation at 12°C. The receptor was then purified to homogeneity by electroelution as described above (see also Fig. 2B). Samples were lyophilized and dissolved in 70% formic acid. CNBr (Silar) was added at a final concentration of 50–100 mM, and incubation proceeded overnight at room temperature. The reaction mix was lyophilized twice to remove formic acid and the excess of CNBr, and peptides were fractionated by reverse-phase high performance chromatography using a C18 Delta-Pack 15-μm column (300 × 7.5 mm; Waters, Milford, MA). Peptides were eluted by a linear gradient of H₂O, 0.1% trifluoroacetic acid to 80% acetonitrile, 0.085% trifluoroacetic acid at a flow rate of 2 ml/min. Fractions were collected every 30 s, assayed for radioactivity by scintillation counting, and subjected to microsequencing analysis when appropriate.

Other Techniques

Western Blotting Procedure—Proteins were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Immunodetection of hRARα was performed as described previously using the 181 Enzymergic Web system (40). The anti-hRARα monoclonal antibody R300 was raised against a synthetic peptide from the F domain of hRARα (41) and purchased from Affinity Bioreagents (Neshanic Station, N J).

Protein Assay—The protein content of receptor preparation was assayed by the Bradford assay (42) using bovine serum albumin as a standard. The percentage of receptor in the preparation was estimated by densitometry of silver-stained gels, using a Hoefer GS300 scanning densitometer.

Oligonucleotides—The following oligonucleotides and their complements were linked by BamHI and HindIII sites, were synthesized: (i) a DRS retinoic acid response element from the promoter P2 of the RAR-α gene (43) and (ii) a consensus half-site (44). The sequences of these oligonucleotides are as follows: (i) DR5: gatcGGTAGGGTTCCGGAGAATCAT, (ii) half-site: aggcGAGAGCTAACATG.

Site-directed Mutagenesis—Point mutations were introduced in the hRARα cDNA using the ExSite polymerase chain reaction-based site-directed mutagenesis kit and all reactions were carried out as suggested by the manufacturer (Stratagene). Oligonucleotides were designed to convert Lys906, Val907, and Lys908 into Thr, Gly, and Thr, respectively (mutations are indicated in bold, underlined characters). In addition, a silent mutation was introduced at position 356, converting the CTG codon (Leu) that has HpaII sites, thereby introducing a Xhol site used for the initial screening of mutants (indicated in bold). The mutagenic primers sequences were: K360T, 5'-CCCGCTGTCGAGGCGCTAAAGGCTTACGTCGGG-3'; pV961G, 5'-CCCGCTCTGGAGGGCTCAAAAGGCTACGGGG-3'; K365T, 5'-CCCGCTGTCGAGGCGCTAAAGGCTACGGGG-3'. The second oligonucleotide (OLIG), used to synchronize the noncoding strand, had the following sequence: OLIG, 5'-CCCTGGACTGATCCTCAGGCCGGCTAAAGGCTACGGGG-3'. Mutations were confirmed by sequencing, and fragments containing the desired mutations were subcloned into pQE9-hRARα. In addition, the identity of the overexpressed proteins was established using the anti-hRARα antibody R300 (data not shown).

Peptide Sequence Analysis—Microsequencing of peptides was performed on a gas-phase sequencer (Applied Biosystems 470A) using the O3PRTH program. Phenylthiohydantoin derivatives of amino acids were identified on-line as described previously (45).

DNA Sequencing—All reactions were carried out using the Sequa-

nase 2.0 sequencing kit as indicated by the manufacturer (U. S. Biochemical Corp.).

RESULTS

Experimental Strategy—Several experimental approaches can be considered to identify and localize functionally important amino acids of hRARα necessary for either DNA binding or dimerization in the presence or the absence of DNA. Chemical modification of amino acids has some advantages over mutational studies, i.e. studying the protein under its native conformation, and it may also turn out to be a prerequisite for obtaining information about the nature and the localization of functionally important amino acids. Therefore, we used side chain-specific reagents to modify accessible groups in the native, purified hRARα, keeping in mind limitations imposed by optimal reaction conditions for each reagent and the stability of the receptor. These conditions were determined in preliminary experiments using crude extracts, and the reversibility of chemical modifications was assessed whenever possible. Indeed, the effects of thiol groups alkylation was reversible upon dithiothreitol treatment when methylmethanesulfonate or di-thionitrobenzoate were used in place of NEM (see also Ref. 33).
PyrP of formaldehyde (lysine modification) and of cyclohexanedione (arginine modification) are known to generate short-lived derivatized products. Omitting stabilizing agents, such as NaBH₄ or NaBH₃CN (lysine) or boric acid (arginine), yielded fully functional receptors in our assays, attesting that such modifications did not cause merely a denaturation of the protein.²

A strategy was designed to assess the effect of amino acid modification on homodimerization, heterodimerization, and DNA binding properties of hRARα and is described in Fig. 1.

His₆-tagged hRARα was partially purified over a Ni²⁺-NTA resin to produce soluble and functional receptor (see below and Fig. 2). The receptor was then treated in the presence or the absence of a side chain-specific reagent as described under "Experimental Procedures." Native or modified receptors were assayed by EMSA for their ability to bind to DNA as a monomer, a homodimer, or a heterodimer with hRXRα, and the "RXR binding assay" was used to test the capacity of hRARα to heterodimerize, in the absence of DNA, with hRXRα (Fig. 3).

hRARα was purified by a single step procedure using immobilized chelate affinity chromatography, which allowed for the recovery of milligram amounts of native receptor. We obtained routinely receptor preparations judged to be 50% pure with a major contaminant at 27 kDa, which accounted for more than 60% of contaminant proteins (Fig. 2A, left panel). A proteolytic product of 48–50 kDa resulted from the truncation of part of the A/B domain and was not retained on the NiTA resin (Fig. 2A, lanes Wash). The N-terminal sequence of the 27-kDa protein was determined and is MYVASDLVMSNAYQXRT. Therefore this protein is not related to hRARα, but displays a strong homology with a bacterial, histidine-rich prolyl-isomerase.³

The identity of the 52-kDa protein as hRARα was confirmed by Western blot analysis using a monoclonal anti-hRARα antibody (Fig. 2A, right panel).

Confirmation of chemical modifications was obtained by SDS-PAGE analysis of hRARα polypeptides purified to homogeneity. Side chain modification could be monitored by an altered electrophoretic mobility of polypeptides, displaying molecular masses in the 50–56-kDa range (Fig. 2B). This electrophoretic mobility shift parameter was thus used to assess the efficiency of side chain modification for each reagent (which was observed reproducibly for all reagents) and confirmed by radiolabeling with tritiated formaldehyde (see Fig. 6) and NEM,⁴ as well as to verify receptor integrity.

Electrophoretic mobility shift assays were used to assess the dimerization properties and the intrinsic DNA binding activity of hRARα.

⁴ C. Rachez, unpublished observations.
of the native receptor. The ability of purified hRARα to bind cooperatively with a tagged hRXRα on the β-RARE probe is shown in Fig. 3 (left panel, lanes 2–4). Binding is specific (lanes 4–6), and shifted complexes contain both RAR and RXR, as shown by the decreased mobility of these complexes observed in the presence of an anti-hRARα antibody (lane 7) or an anti-Flag antibody (lane 8), respectively. At higher hRARα concentrations, binding of RXR homodimers could be observed on the same response element (Fig. 3, middle panel). Again, binding was specific (lanes 10–12), and complexes were super-shifted in the presence of an anti-hRARα antibody (lanes 13). Finally, we were able to refine experimental conditions (low stringency and high receptor concentration (0.6 μM)), so that binding of hRARα to a consensus half-site could be monitored. Complexes migrated faster than homodimers and heterodimers (compare lane 15 to lanes 4 and 10), bound specifically to the half-site (lanes 15–17) and were super-shifted by the anti-hRARα antibody. hRARα is thus able to bind in these conditions to a consensus half-site as a monomer. Quantification of hRARα binding to a DR5 response element as a monomer or a dimeric complex is therefore possible using the assays described above.

To estimate the effect of specific amino acid modification of hRARα on its heterodimerization properties in the absence of DNA, we set up a solid phase assay similar to that described by Kurokawa et al. (12), a system more reproducible and more suitable to statistical analysis, when compared to cross-linking experiments that yielded similar results (see Fig. 4D). The ELISA plate was loaded with purified His6-hRARα and washed in order to produce a hRARα-coated plate. 25 fmol of [35S]methionine-labeled cPR-B, rGR, hRXRα, or hRARα were then incubated with this matrix. As shown in the Fig. 3, panel B, only hRARα bound strongly to the hRARα matrix, whereas, in similar conditions, only a low amount of hRARα remained associated to the matrix, showing that the stability of hRARα binding is higher than that of hRARα homodimers. Neither cPR-B, hVDR, nor rGR were able to bind to the matrix, and the interaction of hRARα with the matrix was dependent on the presence of hRARα (Fig. 3C). This solid phase assay demonstrates the specificity and the saturability (Fig. 3C) of the interaction between hRARα and hRXRα, in the absence of DNA, and mirrors heterodimer formation in solution (Fig. 4D).

Differential Involvement of Thiol Groups in hRARα DNA Binding and Dimerization Properties—The effects of increasing concentrations of NEM on the hRARα ability to bind to DNA as a monomer, homodimer, or heterodimer, as well as to interact
The specific effect of the thiol alkylating reagent NEM described above prompted us to investigate the role of other side chain groups using a similar approach. Compounds known to be selective and active in mild conditions in order to preserve the receptor were selected and tested for their effects on hRARα functions. HPG, PyrP/NaBH₄, TMN, and DEPC were chosen to modify select Arg, Lys, Tyr, and His residues, respectively. Results for each reagent are summarized in Fig. 5A. They are further expressed as the ratio of the concentration of the thiol group in the stabilization of RXR/RAR heterodimers bound to DNA, and to a lesser extent in the non-DNA-dependent formation of heterodimers.

Direct Evidence for the Distinct Contribution of Arg, Lys, Tyr, and His Residues to the Dimerization and DNA Binding Properties of hRARα—The specific effect of the thiol alkylating reagent NEM described above prompted us to investigate the role of other side chain groups using a similar approach. Compounds known to be selective and active in mild conditions in order to preserve the receptor were selected and tested for their effects on hRARα functions. HPG, PyrP/NaBH₄, TMN, and DEPC were chosen to modify select Arg, Lys, Tyr, and His residues, respectively. Results for each reagent are summarized in Fig. 5A. They are further expressed as the ratio of the concentration of the thiol group in the stabilization of RXR/RAR heterodimers bound to DNA, and to a lesser extent in the non-DNA-dependent formation of heterodimers.
Results detailed above establish clearly a major role for lysyl residues in the dimerization of hRARα. Polypeptide—Polypeptide—

Nitration of tyrosine by TNM is highly specific and selective, although some mild reactivity with cysteinyl and methionyl residues has been reported for some proteins (see Ref. 27 and references therein). This compound was poorly active on the DNA binding activity of RARα, with a R50 above 40, but displayed a better efficiency at inhibiting homo- and heterodimer formation in the presence of a β-RARE, with comparable R50 values of 15 and 17 respectively. TNM treatment affected RXR/RAR heterodimer formation in the absence of DNA, whereas they do not appear to be implicated in this phenomenon in the absence of DNA. It is interesting to note that, in opposition to PyrP/NaBH4-modified RAR, HPG-modified receptor does not see its loss in DNA binding activity partially alleviated upon heterodimerization.

O-Carboxethylation by DEPC of histidyl residues located in the active site of various enzymes has been widely used to inhibit their activity. DEPC showed a very strong specificity on the homodimerization of purified His6-hRARα (R50 = 15) but was totally inactive at inhibiting heterodimer formation in the presence of DNA, and showed a mild effect on the DNA binding activity of the receptor to the half-site (R50 = 34). Heterodimerization in the absence of DNA was also barely affected by DEPC treatment, with a R50 above 40. This result thus clearly demonstrate that one or several histidyl residues are involved in the homodimerization process, whereas they appeared to be dispensable for heterodimerization with hRXRα.

Identification of Critical Lysine Residues in the hRARα—Polypeptide—Results detailed above establish clearly a major role for lysyl residues in the dimerization of hRARα with hRXRα, and most notably in the absence of DNA. Formaldehyde also reacts specifically and irreversibly with lysine residues in the presence of NaBH4 or NaBH3CN and can therefore be used in place of PyrP. Indeed, this compound displayed an activity similar to that of PyrP/NaBH4 on the DNA binding and treatment by NaBH4 or NaBH3CN alone had no effect on receptor activities, showing that the observed effect is the result of Schiff base reduction. The derivatization of hRARα by PyrP/NaBH4 yielded a polypeptide that bound poorly to DNA as a monomer and as a homodimer, with observed R50 of 2.0 for both assays. More surprisingly, PyrP/NaBH3CN was found less efficient at inhibiting heterodimer formation on the β-RARE since the measured R50 was around 10. The DNA-independent heterodimerization of hRAR with hRXRα was also strongly affected by PyrP/NaBH4 treatment, since the R50 was around 4.0. These results indicate that heterodimerization of His6-hRARα with hRXRα is able to overcome partially the effect of PyrP/NaBH4 on the DNA binding activity of RXR/RAR heterodimers and consequently suggest that ε-amino groups of lysine residues are less critical for this activity.

The guanidyl group of arginine reacts specifically with HPG between pH 7.00 and 9.00 at 20 °C and modify less than 5% of other residues in these conditions (37). Treatment of hRARα with this reagent affected strongly its ability to bind to a DR5 element as a homodimer, whereas binding to DNA as a heterodimer was affected to a lesser extent, with a R50 of 7.5 and 12.5, respectively. The DNA binding activity of the monomeric receptor was found to be equally affected by arginine modification, with a R50 of 13. Very interestingly, heterodimerization without DNA was much less sensitive under these conditions, since the observed R50 was in the 40–45 range. Thus, it is reasonable to conclude that arginine residues are very important for the DNA binding activity of RAR and for RXR/RAR heterodimer formation in the presence of DNA, whereas they do not appear to be implicated in this phenomenon in the absence of DNA. It is interesting to note that, in opposition to PyrP/NaBH4, modified RAR, HPG-modified receptor does not see its loss in DNA binding activity partially alleviated upon heterodimerization.

Identification of Critical Lysine Residues in the hRARα—Polypeptide—...
they were poorly separated. Three labeled peaks (9, 10, and 11) were identified reproducibly in this region. These fractions were isolated, repurified by reverse phase HPLC, and cleaved by endoproteinase Arg-C. Digested samples were fractionated, yielding profiles shown in Fig. 6B. This second chromatography step showed that peptides present in peaks 9, 10, and 11 could be resolved into two species with similar retention times, but different relative labeling rates. Thus this result shows that peptides 9, 10, and 11 are identical but labeled differentially by tritiated formaldehyde, thereby affecting the hydrophobicity of the molecule. This hypothesis was further confirmed by microsequencing analysis that identified the Leu-Gln-Glu-Pro-Leu sequence as the N terminus of these peptides. This sequence corresponds to a fragment of hRARα mapping from Leu351 to Met373 including the eighth heptad repeat (23) and two lysines separated by an Arg-C cleavage site (Fig. 8).

Mutation of Lysine 360, Valine 361, and Lysine 365 of hRARα Diminishes Its DNA Binding Affinity—The contribution of Lys360 and Lys365 to the dimerization activities of hRARα was further tested by site-directed mutagenesis. These residues were converted into threonine, generating two receptor mutants referred to as K360T and K365T. In addition, structure predictions (see Fig. 8B) showed that Val361 could also form part of the dimerization interface of the receptor, and this was similarly tested by mutating Val361 into a glycine (V361G). As shown in Fig. 7, all mutated receptors displayed, at similar receptor concentrations (Fig. 7A), a lower affinity for the DR5 RARE, whether they were allowed to homodimerize (Fig. 7B) or to form heterodimers with hRXRα (Fig. 7C). Quantification of these results showed that mutant K360T had an affinity decreased by 75% for the DR5 RARE when binding as a homodimer, when compared to the wild type molecule. Similarly, V361G and K365T bound this probe with a 2-fold decreased affinity. The impact of mutations was different when hRARα mutants bound to the same probe as heterodimers with hRXRα: Lys365 appeared to be involved to a similar extent in heterodimer formation than in homodimer formation, since its mutation into a threonine reduced its binding efficiency by 75%. In contrast, mutation of Val361 and Lys365 induced a less pronounced effect on heterodimer binding to the DR5 probe, this property being especially marked for Val361 (Fig. 7C). These data are thus in perfect agreement with chemical modification data and show that Lys360 and Lys365 are equally engaged into homodimer and heterodimer formation on a DR5 response element, whereas Val361 is more prominently engaged in homodimerization. All three mutants bound all-trans retinoic acid with an affinity similar to that of the wild-type receptor (Kd ≈ 1–4 nM) (35), demonstrating that the three-dimensional structure of the HBDs of these mutated receptors is not drastically modified. A full characterization of the biological properties of these mutants is in progress.

**DISCUSSION**

Asymmetrical dimerization interfaces located in the DBD of RARα impose a binding polarity to RXR/RAR heterodimers, such as RXR always binds to the 5'-half-site of DR5, DR4, and DR2 response elements (12, 19). On the contrary, this polarity is inverted on a DR1 response element (18, 20, 22, 44). Other sequences located in the extended C-terminal region of the DNA binding domain (H box) (47) and in the hormone binding domain (24–26) are involved in the dimerization process. However, the contribution of distinct amino acids to this process has been established for only two hydrophobic residues of hRARα (Met373 and Leu384), located at the N and C terminus of the ninth heptad repeat (24) (see also Fig. 7). No data are at present available with respect to the contribution of polar (Cys), charged (Arg, Lys, His), and aromatic (Tyr, Trp) amino acids to the dimerization interface.
Arginine alteration affected equally well the DNA binding and the DNA-dependent dimerization of hRARα. This result is in agreement with their scattered location along the receptor sequence. Indeed, arginine residues are found in the DNA binding domain at sites close to or in P, D, and H boxes and also at the fifth position in the first and sixth heptad repeat of the HBD. The weak activity of HPG on the dimerization in the absence of DNA would rather suggest that critical arginine residues are likely to be found in the DNA binding domain.

Tyrosine nitration appeared to impede preferentially the dimerization of hRARα in the presence of a DR5 RARE, whereas the DNA binding and the dimerization without DNA were affected to a lesser extent. This aromatic amino acid is abundant in the HBD, but is more rarely found in the DBD. Again, Tyr98 and Tyr100 are located in a critical region for dimerization, the DR box mapping from amino acid 90 to 102-104. A third tyrosine has also a potentially critical location, at position 122 and therefore very close to the D box of hRARα. Tryptophan did not display any significant role in the various activities tested (data not shown), in agreement with the location of this unique tryptophanyl residue in the ligand binding pocket (48).

Carbethoxylation of histidines revealed that these residues are specifically involved in the homodimerization activity of hRARα, but not in heterodimerization with hRXRα in the presence of DNA. This finding is particularly interesting when considering the position of these residues. As DEPC was poorly active on other activities (DNA binding, homodimerization), a probable location of the functionally important residue(s) is in region(s) involved in dimerization outside of the HBD. Likely candidates are located at position 99 in the DB box and 125 in the D box, and therefore in close vicinity with the already mentioned tyrosine residues at position 98, 100, and 122.

The use of PyrP/NaBH₃CN and of formaldehyde/NaBH₃CN evidenced a peculiar role of the ε-amino group of lysines. Indeed, these groups appeared to be involved strongly in the stabilization of the interaction of hRARα with hRXRα in the absence of DNA, and were as such unique. Since the hormone binding domain is suspected to play a major role in this case, we scrutinized its sequence to locate lysine residues. Very interestingly, these residues can be divided into two groups: (i) conserved among members of the nuclear receptor family known to dimerize with RXR (VDR, TR, RAR) and (ii) unique to RAR. Another salient feature of these amino acids is their location along the primary sequence of RAR, since they are clustered at the N and the C terminus of the E domain, and are, in the latter case, located in the eighth and ninth heptad repeats, which were proposed as putative structural subdomains of the HBD (27) (see Fig. 8). The two labeled lysines residues were indeed found in the eighth heptad repeat (Lys236) and between the eighth and ninth heptad repeat (Lys237). Very interestingly, the crystal structure of hRXRα-HBD homodimers revealed a crucial role of this particular region. It contains helices 9 and 10, which encompass the eighth and ninth heptad repeat motifs, respectively, in which Lys237 (helix 9) and Lys137 (helix 10) establish salt bridges between the two RXR monomers (49) (see also Fig. 8A). These two RXR lysine residues are located in a position analogous to that of hRARα Lys360 and Lys237 (Fig. 8A).

Molecular modeling of this region, based on the hRARα and hRXRα HBD structures, suggests that these two lysines are located in an α-helical structure and oriented in such a way that they could form a dimerization interface, together with Val361 (Fig. 8B). Thus a role similar to that of hRXRα Lys237 and Lys137 can be envisioned for hRARα Lys360 and Lys237 in the dimerization process, a hypothesis significantly strengthened by mutagenesis data. Indeed, Lys360 ap-
peared to be equally involved into homo- and heterodimerization processes, whereas Lys\(^{365}\), and more noticeably Val\(^{361}\), are contributing more prominently to the homodimerization activity of hRAR\(_{a}\). We cannot, however, rule out allosteric effects due to an interaction of this part of the HBD with the DBD of hRAR\(_{a}\).

Our method allows for the location of potentially important amino acids for the dimerization activities of hRAR\(_{a}\), and these predictions can be confirmed by site-directed mutagenesis. In addition, this approach can now be extended to other response elements (DR2 and DR1) to distinguish and assay the relative contribution of hydrophobic and charge interactions to the dimerization activities of retinoid receptors, when different interfaces (and therefore different amino acids) are used. Finally, it provides direct evidence for the differential involvement of amino acid side chains in homo- and heterodimerization of hRAR\(_{a}\).

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