Critical Role of Tyrosine 277 in the Ligand-Binding and Transactivating Properties of Retinoic Acid Receptor α†

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ABSTRACT: Retinoic acid receptors specifically bind *all-trans*-retinoic acid (RA) and function as RA-inducible transcriptional regulatory factors. Binding of RA to RARα, *β*, and *γ* is sensitive to nitration with tetranitromethane, a tyrosine-specific modifying reagent. To identify tyrosine residue(s) that are important for RA binding, we carried out chemical modification experiments with purified RARα ligand-binding domain (RARα-LBD) subjected to partial acid hydrolysis and selective proteolysis. The chemically modified peptides containing each of the three Tyr residues present in the RARα-LBD sequence were then analyzed and identified by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC/ESI-MS). We found that RA binding to RARα-LBD protected Tyr^{277}-containing peptides from nitration. Protection of Tyr^{277} could result either from direct masking by the bound ligand or from ligand-induced changes in receptor conformation and tyrosine accessibility. The role of Tyr residues was further documented by site directed mutagenesis using three site-specific RARα mutants: Y208A, Y277A, and Y362A. The affinity for RA of these mutant receptors was in the range of that of the wild-type protein, except for the Y277A receptor mutant, which displays a 15−20-fold reduction in affinity and transactivation activity for RA. Whereas mutation of Tyr^{277} into alanine had a variable effect on different agonists and antagonists binding, it caused a dramatic decrease of retinoid-dependent transactivation activity. This later effect was also observed with mutation of Tyr^{277} into phenylalanine. It is unlikely that major conformational changes are responsible for the lower affinity of RA binding and RA-dependent transactivation since these mutants displayed wild-type dimerization and DNA-binding activities. Limited proteolysis revealed that upon ligand binding, the Y277A mutant induced a conformational change slightly different from that obtained with the wild-type protein. These data could suggest that Tyr^{277} play a critical role in the ligand-induced conformational changes required for the activation of RARα.

Retinoic acid (RA)[1] and synthetic retinoids have important effects on a wide spectrum of biological processes, including cell growth, cell differentiation, and vertebrate development (*1*). These effects are mediated by two distinct families of receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which belong to the steroid/thyroid hormone superfamily of nuclear receptors (*2*). These two families of receptors are each made up of three receptor subtypes, designated α, *β*, and *γ* (*3*−*11*). These receptors are ligand-inducible transcription factors able to bind, as hetero (RAR−RXR) or homo (RXR−RXR) dimers, to specific retinoic acid response elements (RAREs) located in the promoter region of target genes. Two different isomers, *all-trans*-RA (RA) and 9-*cis*-RA, are the natural ligands for the RARs, whereas only 9-*cis*-RA is the natural ligand for the RXRs (*12*, *13*). The RARs and RXRs exhibit a conserved modular structure, divided in six different domains (A−F), according to their homology with the other nuclear receptors (*14*). Unique functions have been devoted to some of these domains. The ligand-independent transactivation function (AF-1) is located in the A and B domains, whereas the C domain constitutes the highly conserved DNA-binding domain also required for receptor dimerization (*15*). The E domain is functionnally complex and has been identified as important for ligand binding, receptor dimerization, and ligand-dependent activation of transcription (AF-2).

Recently, the X-ray crystal structures of the ligand-binding domains of apo-RXRα and of holo-RAR*γ* complexed to t-RA, 9-*cis*-RA, and a RAR*γ*-selective synthetic agonist have

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[1] Abbreviations: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HPLC/ESI-MS, high-performance liquid chromatography coupled to electrospray ionization mass spectrometry; LBD, ligand-binding domain; LBP, ligand-binding pocket RA or t-RA: all-trans retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element RXR; retinoid X receptor; TNM, tetranitromethane; Tyr, tyrosine.

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been reported (*16*−*18*). According to the structural and functional similarities between the ligand-binding domains of the different RAR subtypes, a common folding pattern has been proposed (*19*), and putative amino acid residues responsible for the selectivity of some synthetic retinoids have been identified (*17*, *19*). The role of several amino acid residues of the LBD in the binding of RA, 9-*cis*-RA, and synthetic retinoids to the RAR subtypes has been also highlighted by site-directed mutagenesis (*17*, *20*−*29*). Our group contributed to the identification of some structural requirements of the ligand-binding site of the human RARα. We demonstrated the role of the AF-2 domain in the differential recognition of natural and synthetic retinoids (*30*). The importance of residues 403−410 in the binding and transactivating properties of the receptor was further documented by site-directed mutagenesis (*31*, *32*). Moreover, we resorted also to the chemical modification of protein side chains with group-specific reagents to demonstrate that cysteine residues are essential for ligand binding (*33*) and that lysines 360 and 365 are critical for the DNA-binding and dimerization properties of RARα (*34*). In the current report, we show that the affinity of RA to RARα is significantly decreased by previous treatment of the receptor with tetranitromethane, a tyrosine-specific modifying reagent, suggesting that tyrosine residue(s) could be located in the ligand-binding pocket (LBP) of this receptor. To identify the tyrosine residue(s) important for RA binding, we resorted both to the chemical characterization of the modified polypeptide by electron spray ionization−mass spectrometry (ESI-MS) and to the site-directed mutagenesis of each of the three tyrosine residues present in the hRARα-LBD sequence. Taken together, our data demonstrate that Tyr^{208} and Tyr^{362} play no direct role in the ligand receptor interaction, whereas Tyr^{277} is a specific determinant of the ligand binding and transactivating properties of the receptor. Mutation of Tyr^{277} resulted in altered binding affinity and specificity for RA and various synthetic retinoids, with a dramatic decrease in ligand-dependent gene transactivation.

MATERIALS AND METHODS

Materials. Synthetic retinoids and [^{3}H]CD367 [52.5 Ci/mmol (*35*)] were provided by U. Reichert (CIRD-Galderma, Valbonne, France). The RARα-specific antagonist was obtained from Hoffman-La Roche Inc., Basel, Switzerland. [^{3}H]t-RA (55.6 Ci/mmol; 1 mCi/mL) was obtained from DuPont/NEN, France, and *all-trans*-RA was purchased from Sigma (St Louis, MO). Restriction enzymes were from Promega (Madison, WI); isopropylthio-β-galactopyranoside (IPTG), ampicillin, and kanamycin were from Appligene (Strasbourg, France). Oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium). The PCR site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Trypsin, chymotrypsin, and endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) were from Boehringer (Mannheim, Germany), and all other chemicals were from Merck (Darmstadt, Germany).

Tyrosine Modification Procedures in Crude Bacterial Extracts. Wild-type RARα, β, and γ inserted into bacterial expression vector pET3a, generous gifts from Prof. P. Chambon (IGBMC, Illkirch, France), were expressed in *Escherichia coli* BL21(DE3). Transformed bacteria were grown overnight in LB broth supplemented with 200 μg/mL ampicillin and 30 μg/mL chloramphenicol. These precultures were grown in 1 L of LB broth to an OD_{580} of 0.8, and then 0.5 mM IPTG was added. Derepression proceeded for 3 h after which time cells were pelleted and resuspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10% saccharose, 1 mM PMSF, 20 μg/mL leupeptin, and 20 μg/mL aprotinin. To obtain the bacterial lysate, lysozyme was added to a final concentration of 0.5 mg/mL, and the cell suspension was incubated on ice for 30 min. Triton was added to a final concentration of 0.1%, and the extract was sonicated on ice three times for 20 s. Then, the lysate was brought to 0.4 M NaCl. The homogenate was centrifuged for 15 min at 20000*g*. The supernatant was precipitated using polyethyleneimine (0.2% final concentration) to remove most of the DNA. The bacterial extracts were then adjusted to 10% glycerol. To determine the ligand-binding susceptibility of the receptor to tetranitromethane (TNM), aliquots of crude bacterial extracts were diluted to 1:20 in 20 mM Tris-HCl pH 9.00, and treated with TNM (0.2−200 μM) for 2 h at 0 °C. The chemical reaction was stopped by the addition of stoechiometric concentrations of DTT, and samples were assayed for retinoid-binding activity with tritiated RA, as described below.

Expression, Chemical Modification, and Purification of His_6-hRARα ΔN186-462. The RARα cDNA was introduced into the pQE-9 vector as described previously (*30*). Transformed M15 bacteria were grown and protected from light in LB broth supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin with or without 10^{-6} M RA. Following 5 h after IPTG induction, cells from 500 mL cultures were pelleted and resuspended in 1 mL of cold 20 mM Tris-HCl, pH 8.00, 1 mM PMSF, 20 μg/mL leupeptin, and 20 μg/mL aprotinin. The samples were then sonicated on ice three times for 1 min. Samples were then incubated or not with 10^{-6} M RA before the nitration step. For nitration, 10 μL of 500 mM TNM (in ethanol) was added, and then samples were incubated for 5 h on ice. Nitration was stopped by the addition of 10 μL 500 mM DTT. A total of 5 mL of denaturing buffer (6 M GuCl, 0.1 M NaH_2PO_4, 20 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 8.00) was then added, and samples were incubated for 1 h. After centrifugation for 45 min at 20000*g*, the supernatant was recovered. The recombinant receptor was then purified by affinity chromatography using the NiTA-resin system (Diagen, Düsseldorf, Germany) as recommended by the manufacturer. The protein concentration in the final eluate was determined by the Bio-Rad protein assay and 20 μg of total protein was loaded on a sodium dodecyl sulfate (SDS)−10% polyacrylamide gel to control the expression of His_6-hRARα ΔN186-462 in both the crude protein extract and in the eluate. The presence of the receptor was detected on western blot using a polyclonal anti-RARα antibody as previously described (*33*).

Receptor Proteolysis, Liquid Chromatography and ESI-MS. In each experiment, 100 nmol of purified His_6-RARα ΔN186-462 (10 nmol/mL) was dialyzed for 7 days against HCl 0.01 N and lyophilized before hydrolysis by 0.25 N acetic acid at 105 °C for 24 h. Following lyophilization, the hydrolysate was further dissolved in 0.5 mL of 50 mM ammonium acetate, pH 4.00, submitted to *Staphylococcus aureus* V8 protease hydrolysis using an enzyme-to-receptor ratio of 1/50 for 16 h at 37 °C and then dried under vacuum.

Receptor fragments (12 nmol) were separated by HPLC on a microbore reversed-phase column from Brownlee-Applied Biosystems (C-4, 5 μm particles, 2.1 mm i.d., 100 mm long), at a flow rate of 200 μL/min. The binary solvent system used in the analysis of peptide fragments was solvent A (H_2O, 0.01 vol % CF_3COOH) and solvent B (CH_3CN, 0.01 vol % CF_3COOH). Peptides were eluted by a linear gradient of 10 to 100% solvent B in 60 min. An API-1 (simple quadrupole) mass spectrometer system (Perkin-Elmer Sciex, Thornhill, Canada) was used for the analysis of the receptor fragments. Data aquisition and processing of the molecular mass of receptor peptides were controlled by the MAC Biospec data system. This program was used to select peptide fragments from the known sequence of the receptor and match them with the masses observed for the receptor fragments. The ion spray voltage was operated at 5.5 kV, and the spectra were recorded at an orifice voltage of +90 V. Collection of all electrospray data was performed on samples coupled through an HPLC/ESI MS interface with an Applied BioSystems microbore HPLC (see above). A calculated split ratio of 1:5 was established such that 40 μL/min HPLC eluent was directed to the ESI-MS probe for nebulization. A polypropyleneglycol solution, PPG 425, 1000 and 2000 in 50/50/0.1 H_2O/methanol/formic acid (v/v/v) was used for external calibration of the ESI/MS from *m*/*z* 400 to 2400 in the positive mode. Typical ESI data aquisition was performed using 5.5 s scans; the resulting total ion chromatograms (TIC) cover *m*/*z* 400−2400 with a step size of 0.1 Da and a dwell time of 0.5 ms.

Plasmid Constructs and Site-Directed Mutagenesis. Points mutations were introduced in the RARα cDNA using the ExSite PCR-based site-directed mutagenesis kit, and all reactions were carried out as suggested by the manufacturer (Stratagene). The hRARα full-length cDNA was cloned into the *Bam*HI and *Hin*dIII restriction sites of pQE-9 as described (*30*) and used as template for the preparation of the mutants. The GCC codon was used to encode the mutant Ala residue indicated in bold in the mutagenic primers. For the preparation of Y208A, a specific PCR fragment was prepared using the primer pairs Y208A 5′-s (5′ CTCTGCCAGCTAGGCAAA**GCC**ACTACGAACAACAG 3′) and Y208A 3′-as (5′ GGCAGGGAAGGTTTCCTGGTGCGCTTTGCGCAC 3′), respectively. For the Y277A mutant, the following primers were used: Y277A5′-s (5′ CTGGATATCCTGATCCTGCGGATCTGCACGCGG**GCC**A 3′) and Y277A 3′-as (5′ GCAGGCAGCCTTGAGGAGGGTGATCTGGTCGG 3′). Likewise, the Y362A mutant was constructed using the following primers: Y362A 5′-s (5′ CCGCTGCTCGAGGCGCTAAAGGTC**GCC**GTGCG 3′) and Y362A 3′-as (5′ CTCCTGCAGCATGTCCACCCGGTCCGGCTGCTCC 3′). For the preparation of the Y277F mutant, the TTC codon was used to encode the Phe residue, using the two following primers: Y277F 5′-s (5′ CTGGATATCCTGATCCTGCGGATCTGCACGCGG**TTC**ACG 3′) and Y277F 3′-as (5′ GACGCAGGCAGCCTTGAGGAGGGTGATCTGGTC 3′). The *Sac*I−*Bcl*I fragment that contained the desired mutation was exchanged with that of pSG5-hRARα wild-type to create each of the mutant DNA constructs. In all cases, the presence of the specific mutation and the lack of random mutations were verified by DNA sequence analysis.

Retinoid Binding Assays. The receptor extracts were diluted in binding buffer (50 mM Tris/HCl, pH 8.00, 100 mM NaCl, 1 mM EDTA, 10% glycerol) to a final concentration of 100 μg of total protein. Total RA binding was determined in the diluted extracts by adding tritiated RA in the concentration range 2.5−20 nM in most experiments except for the TNM-modified receptor and Y277A receptor mutant (10−200 nM); and incubating for 16 h at 4 °C. Dilutions of RA were made in ethanol. Nonspecific binding was determined in the presence of 100-fold molar excess unlabeled RA. Bound RA was separated from free by charcoal-dextran adsorption as described (*36*). Specific RA binding was determined by subtracting the nonspecific binding from the total binding. Apparent equilibrium dissociation constants (K_d) were determined for the wild-type and each mutant protein by Scatchard analysis. K_i values of retinoids for RARα and Y277A RARα mutant were determined by competition experiments using 10 nM of tritiated RA or CD367 and with increasing concentrations of the unlabeled competitor, ranging from 10 nM to 1 μM.

Electrophoretic Mobility Shift Assay (EMSA). The wild-type RARα, RXRα, and mutant receptors were all in vitro translated proteins prepared from rabbit reticulocyte lysates as described (*34*). Briefly, 20 μg of lysate protein (10 μg of wild-type or mutant RARα lysate protein and 10 μg of RXRα lysate protein) was preincubated with 1 μM RA or CD367 in 20 mM Hepes, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol for 20 min at 4 °C. The DNA-binding reactions were then set up in 20 μL with 1 μg of salmon sperm DNA, 0.04 pmol of ^{32}P-labeled βRARE probe, and, if indicated, a 50-fold molar excess of unlabeled probe for 15 min at 4 °C as described previously (*34*). When required, 2 μL of monoclonal antibodies directed against hRARα or against hRXRα (RARα C-20 and RXRα D-20 monoclonal antibodies, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added for a further 2 h incubation at 4 °C. Protein-DNA complexes were then resolved on a 5% nondenaturing polyacrylamide gel containing 1% glycerol in 1× TBE run at 150 V for 3 h at 4 °C. The gel was dried and exposed to Amersham hyperfilm MP at −70 °C overnight.

Transactivation Assay. COS-7 cells (5.0×10^5 cells/60 mm dish) were transiently transfected via PEI precipitation (*37*) with 0.3 μg of the reporter plasmid pTRE-pal Luc, 0.1 μg of the β-galactosidase expression plasmid (pCH110, Pharmacia), 0.03 μg of wild-type pSG5-RARα or mutant-pSG5-RARα plasmids, and 0.03 μg of pSG5-hRXRα. Twenty-four hours after introduction of the DNA precipitants, cells were fed with Dulbecco's Modified Eagle's medium containing 2.5% fetal bovine serum. Transfected cells were incubated for an additional 24 h with various concentrations of ligands (10^{-10}−10^{-5} M). After rinsing with phosphate-buffered saline, cells were lysed and luciferase activity was measured as described previously (*38*). Luciferase values represent the mean ± SE of triplicate determinations normalized to β-galactosidase activity.

In Vitro Transcription, Translation, and Limited Proteolytic Digestion. Wild-type RARα and mutants in pSG5 were transcribed and in vitro translated in the presence of [^{35}S]methionine by using rabbit reticulocyte lysates as specified by Promega. Aliquots of reticulocyte lysates containing the labeled receptors were incubated with RA at 10^{-6} M or CD367 at 10^{-6} M for 20 min at room temperature. Then, different aliquots were treated with trypsin at final

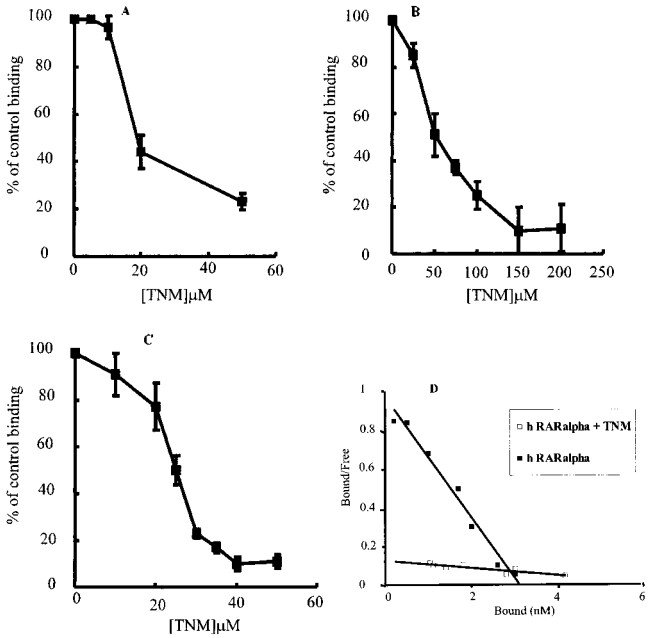


FIGURE 1: RA-binding activity of RARs with and without treatment by tetranitromethane. Bacterial extracts containing the full-length wild-type hRARα (A), hRARβ (B), and hRARγ (C) were treated with the indicated concentrations of TNM for 2 h on ice. Following reduction with DTT, RA binding was determined using 20 nM [^{3}H]t-RA. The specific RA binding was expressed as the percent of RA binding in the absence of TNM for each receptor (% of control binding). Values are the mean ± SE for three independent experiments performed in duplicate. For affinity measurements, bacterial extracts containing the wild-type hRARα were treated or not with 60 μM TNM for 2 h and assayed for RA binding. Specific RA binding was determined by subtracting the nonspecific binding from the total binding, and apparent dissociation constants (K_d) were determined by Scatchard analysis (D) performed in duplicate.

concentrations of 50 μg/mL and 300 μg/mL or chymotrypsin at a final concentration of 50 μg/mL. After 10 min at 20 °C (trypsin) or 30 min at 37 °C (chymotrypsin), the digestion was stopped by adding 5 μL of sodium dodecyl sulfate (SDS) sample buffer and boiling for 3 min. SDS−polyacrylamide gel electrophoresis and autoradiography were carried out as described previously (*39*).

RESULTS

Effects of Tyrosine Modification of RARα, RARβ, and RARγ on RA Binding. Figure 1 (A−C) shows the concentration-dependent effects of TNM on retinoid binding. Full-length RARα, β, and γ exhibit a dramatic reduction in RA binding upon treatment with TNM, giving $IC_{50\%}$ values of 18, 53, and 25 μM for RARα, β, and γ, respectively. Scatchard analysis of RA binding to the wild-type RARα with or without TNM modification (Figure 1D) clearly reveals that the TNM-modified receptor has reduced affinity for RA (K_d = 44 ± 5 nM) as compared to the unmodified receptor (3.5 ± 0.4 nM). These results strongly suggest that one or several essential Tyr residues lie in the retinoid-binding site. As depicted in Figure 2, only three Tyr residues are located inside the RARα-LBD: Tyr^{208} between H1 and H3, Tyr^{277} at the end of H5 and Tyr^{362} in helix H9. Interestingly, all these tyrosines are conserved in RARα, β, and γ, and none of them belong to the ligand-binding pocket defined in the crystal structure of the RARγ-LBD bound to RA (*17*). However Tyr^{277} is contiguous to Arg^{276} (Arg^{278} in RARγ), a very important residue of RARγ-LBP.

Analysis of the TNM-Modified RARα-LBD by HPLC/ESI-MS and Selective Protection by the Ligand. To address the role that the three Tyr residues play in the ligand-binding domain, we had to answer two questions: how many of these tyrosines were modified (and therefore accessible) in the native LBD and which ones were protected from chemical modification by the previous binding of the ligand. Therefore and in order to avoid the interference of the Tyr residues present in the other domains of the receptor, recombinant His$_6$-hRARα ΔN186-462 was produced in bacteria and used as starting material for chemical modification with TNM. It was already demonstrated in our laboratory that this deletion mutant, which encompasses most of the LBD depicted in Figure 2, with the exception of the first six amino acids of helix 1, displayed binding properties comparable to that of the wild-type receptor (*30*). Crude receptor extracts, incubated or not with RA, were submitted to TNM modification. After purification by affinity chromatography on the NiTA resin system (*30*), receptors were subjected to a partial acid hydrolysis (resulting in cleavages at the N- and C-termini of Asp residues, and of some deamidated forms of Asn and Gln residues) and to a selective proteolysis with endoproteinase Glu-C (resulting in a selective cleavage at the C-terminus of Glu residues). A control sample corresponding to the native receptor not incubated with TNM was also analyzed. Each sample was analyzed by HPLC/ESI-MS, and the chemically modified peptides were identified (Table 1). In the absence of preincubation with RA, the three receptor fragments containing each one of the three Tyr residues had an observed mass higher than the mass predicted for the unmodified peptide. The difference (Δmass) between the observed and the predicted mass was close to 45 Da for all peptides, in agreement with the selective nitration of one tyrosine residue (44.98 Da). Thus, in the native LBD, all tyrosine-containing peptides were modified by treatment with TNM. On the contrary, following preincubation with RA, only Tyr^{208} and Tyr^{362} peptides were modified as before, whereas Tyr^{277} peptide was protected as judged by the absence of difference between the observed and predicted masses. Therefore, only one Tyr residue, Tyr^{277} displayed the expected chemical characteristics of a residue lying in the LBP, and its modification is probably responsible for the observed inhibition of RA binding after TNM treatment.

Effect of Tyrosine Point Mutations in the E Domain of RARα on RA Binding and RA-Dependent Transactivation. Three site-specific mutants of RARα in which Tyr^{208}, Tyr^{277}, and Tyr^{362} were individually replaced with an Ala residue (Y208A, Y277A, and Y362A) have been prepared. Western blot analysis of receptor extracts containing wild-type and mutant RARα proteins detected a major band that migrated at the same position (approximate molecular mass of 53 kDa). In addition, the wild-type and mutant receptors displayed a similar level of expression (data not shown). As it can be seen in Table 2, the RA K_d values for wild-type and both Y208A and Y362A mutants were similar. Interestingly, the Y277A mutant displayed a K_d value of 53 nM, which is 15-fold higher than that of wild-type RARα. This difference in K_d is reflected by the similar 21-fold decrease observed in the transactivation assays. The Y208A mutant displayed only a small reduction in activity in the transac-

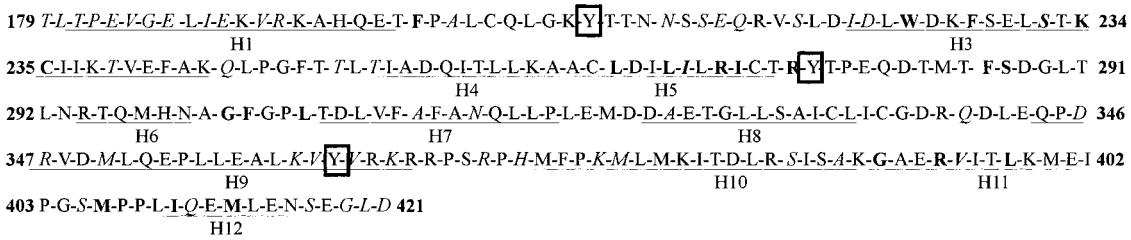


FIGURE 2: Sequence of the ligand-binding domain of hRARα showing respective positions of Tyr[208], Tyr[277], and Tyr[362]. The three tyrosines present in the hRARα-LBD are boxed. Helices 1−12, deduced by homology to RARγ (*17*), are underlined. The amino acids which are not conserved among all the three RARs are in italics. Residues supposed to belong to the RARα-LBP (4.5 Å cutoff for van der Waals interactions with RA in the holo RARγ-LBD crystal) are in bold (*17*, *18*).

Table 1: Detection by HPLC/ESI-MS of hRARα Tyrosine-Containing Peptides Chemically Modified by Tetranitromethane[a]

	Receptor fragment	Mass predicted (Da)	Mass observed (Da)	Δ Mass
- RA	L_{205}-G-K-**Y**$_{208}$-T-T_{210}	682.4	728	45.6
	F_{242}-A-K-.....-**Y**$_{277}$-T-P-E_{280}	4364.4	4410	45.6
	A_{358}-L-K-V-**Y**$_{362}$-.....-K-I-T_{382}	3083.7	3128	44.3
+RA	L_{205}-G-K-**Y**$_{208}$-T-T_{210}	682.4	728	45.6
	D_{256}-Q-I-....-**Y**$_{277}$-T-P-E-Q-D_{282}	3118.6	3118	0.6
	A_{358}-L-K-V-**Y**$_{362}$-.....-I-T-D_{383}	3198.8	3244	45.2

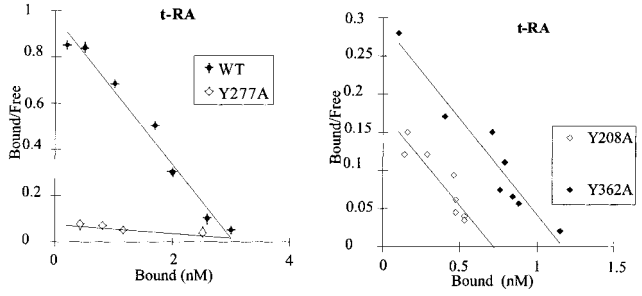
[a] The RARα-LBD incubated or not with t-RA was subjected to nitration by TNM treatment and then to acetic acid and endoproteinase Glu-C hydrolysis. The peptide mixture obtained was analyzed by microbore reversed-phase HPLC coupled to electrospray mass spectrometry (HPLC/ESI-MS). For each peptide, the mass predicted from the native sequence and the observed mass are reported. The theoretical Δmass resulting from the nitration of one tyrosine residue is 44.98. The size of the receptor fragments obtained in the two sets of samples (−RA/+RA) was not exactly the same, a fact explained by our mild conditions of acid hydrolysis and proteolytic digestion (*42*, *43*). Cleavage at Gln[204] and Asn[211] probably resulted from deamidation of these residues into glutamic acid and aspartic acid residues, whereas cleavage at Asp[256] and Glu[280] was not always obtained. However the relevant peptides presented here were identified without ambiguity.

Table 2

(A) Comparison of K_d and EC_{50} for Wild-Type and Mutant hRARα

	all-trans RA	
hRARα	K_d[a] (nM)	EC_{50}[a] (nM)
wild-type	3.5 ± 0.4	39.3 ± 4
Y208A	5 ± 0.7	54.5 ± 6
Y277A	53 ± 5	848 ± 47
Y277F	4.2 ± 0.4	100 ± 5.6
Y362A	6 ± 0.8	485 ± 24

(B) Scatchard Plots



[a] Mean ± SD of three independent experiments.

tivation assay, whereas the EC_{50} value for Y362A was 12-fold higher than that of the wild-type receptor.

Effect of Tyrosine Point Mutations on DNA Binding and Dimerization. The ability of the wild-type and the RARα mutants to bind a RARE and dimerize in vitro was then examined using the EMSA (Figure 3). The three RAR mutants produced a gel shift pattern similar to that of the wild-type RARα. In all cases, the retarded receptor−DNA complexes were upshifted by both the RAR and RXR-specific antibodies. This demonstrates that the different EC_{50} values in the transactivation assays and the apparent discrepancy between the ligand-binding and transactivating properties of the mutant Y362A were not due to an inability of the mutants to bind the DNA sequence of the RARE or to form heterodimers in vitro. The presence of the ligand (RA or CD367) did not interfere with these processes in these experimental conditions.

Mutation of Tyr[277] Results in the Alteration of the Retinoid-Binding Specificity of RARα. Among the three mutants studied, only one, Y277A displayed a decrease in RA-binding affinity (Table 2). As a significant reduction in RA binding was observed for mutant Y277A, this mutant was tested for its ability to bind the RARα agonist CD367. Using direct [^{3}H]CD367 binding assay, we observed a K_d value of 2.5 nM, which is similar to that obtained with the wild-type receptor (data not shown). Therefore, the K_i values of several natural and synthetic retinoids were determined using competition experiments with tritiated CD367 (Table 3). To further explore the importance of the phenolic group of Tyr[277], the binding properties of the Y277A receptor mutant were compared to those of the Y277F mutant. The retinoids tested included 9-*cis*-RA, a natural agonist ligand for RARs,

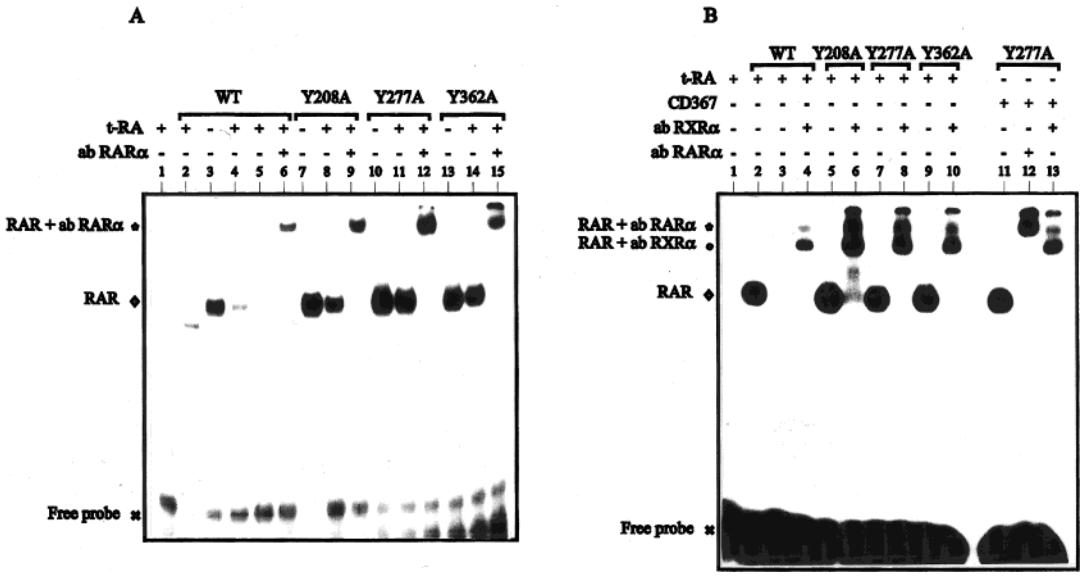


FIGURE 3: DNA binding of wild-type RARα and mutant receptors. EMSA was performed using reticulocyte lysate extracts containing in vitro translated RXRα, wild-type hRARα (WT), or one of its tyrosine mutants as described in the Materials and Methods. Equal amounts of total reticulocyte lysate protein (lanes 2−6 in panel A and lanes 2−4 in panel B), mutant Y208A (lanes 7−9 in panel A and lanes 5−6 in panel B), mutant Y277A (lanes 10−12 in panel A, lanes 7−8 and 11−13 in panel B), and mutant Y362A (lanes 13−15 in panel A and lanes 9−10 in panel B) were analyzed for DNA binding activity in the absence or in the presence of RA or CD367 as indicated in a gel retardation assay using a ^{32}P-labeled β-RARE-DR5 probe. Lane 1 in panels A and B contains unprogrammed lysate as a control. In lane 2 of panel A, a ^{32}P-labeled oligonucleotide unrelated to the β-RARE probe was used as nonspecific control. In lane 5 of panel A and lane 3 of panel B, a 50-fold excess of unlabeled probe was added to samples. The protein composition of the retarded complexes was determined by supershift experiments using monoclonal antibodies directed against RARα (ab RARα) or RXRα (ab RXRα). Supershifted complexes are indicated.

Table 3: Ligand Binding and Transactivating Properties of Wild-Type, Y277A, and Y277F−RARα Mutants[a]

	wild-type		Y277A		Y277F	
	K_I (nM)	EC_{50} (nM)	K_I (nM)	EC_{50} (nM)	K_I (nM)	EC_{50} (nM)
t-RA	10 ± 1.5	39 ± 4.5	80 ± 8.3	848 ± 47	12 ± 1.5	100 ± 5.6
9c-RA	22 ± 2.7	30 ± 4.0	25 ± 3.0	1000 ± 58	60 ± 5.7	20 ± 2.5
retinol	>1000	>10000	502 ± 27	>10000	>1000	>10000
CD367	14 ± 1.8	33 ± 3.5	35 ± 3.8	>10000	63 ± 6.5	>10000
Ch55	33 ± 3.7	70 ± 7.4	26 ± 3.0	>10000	52 ± 5.5	>10000
Am580	12 ± 1.5	10 ± 1.3	259 ± 16	>10000	52 ± 6.0	>10000
CD3105	108 ± 11	>10000	26 ± 3	>10000	89 ± 9.5	>10000
CD3106	29 ± 3.1	>10000	32 ± 3.5	>10000	243 ± 15	>10000
Ro41−5253	25 ± 2.8	>10000	2 ± 0.7	>10000	35 ± 4.0	>10000

[a] The K_I Values (nM) were obtained by competition experiments. K_I and EC_{50} values are the mean ± SD of at least three independent experiments.

retinol, the metabolic precursor of RA that is unable to bind and transactivate RARs, three synthetic RARα agonists (CD367, Ch55, and Am580), and three RARα antagonists (CD3105 or AGN193109, CD3106 or AGN192870, and Ro 41-5253), Figure 4. The RARα-binding and transactivating properties of these compounds have been already reported in the case of the wild-type receptor (*23*, *30*, *41* and references therein). The effect of the mutation of Tyr^{277} into alanine on the ligand-binding affinity appeared variable according to the structure of the ligand tested. Whereas the affinity for RA decreased 15-fold, no change was observed for 9-*cis*-RA. The affinity of the synthetic agonists tested was either unchanged (Ch55) or decreased (Am580). Interestingly and contrary to Ch55 and CD367, Am580 is a RARα-selective ligand supposed to interact in a very specific way with some structural determinants of this receptor (*26*). The affinity of the antagonists belonging to the CD3105 (AGN 193109) series was either unchanged (CD3106) or slightly increased (CD3105), whereas a more significant increase in affinity was observed in the case of Ro 41-5253. Interestingly, the binding data obtained with the Y277F mutant look like those of the wild-type receptor, particularly for retinoid agonists: RA, CD367, and Am580. None of the ligands tested, with the exception of *all-trans*- and 9-*cis*-RA which were weakly active, appeared able to induce ligand-dependent transactivation with the Y277A mutant. This result suggest that Tyr^{277}, besides its limited and variable role in ligand binding, is probably more involved the ligand-induced conformational changes of the receptor leading to the transcriptionnally active form. A specific role of the phenolic group is suggested by the impaired activity of the Y277F mutant.

Impaired Ligand-Dependent Protection of Y277A Receptor Mutant Against Proteolytic Digestion. Limited proteolysis allows the detection of the conformational changes induced in wild-type RARα by ligand binding (*39*). We used this technique to investigate the possible effects of the Y277A mutation on this process. As shown in Figure 4, in the absence of ligand, both wild-type receptor and Y277A mutant were almost completely digested to peptides smaller than

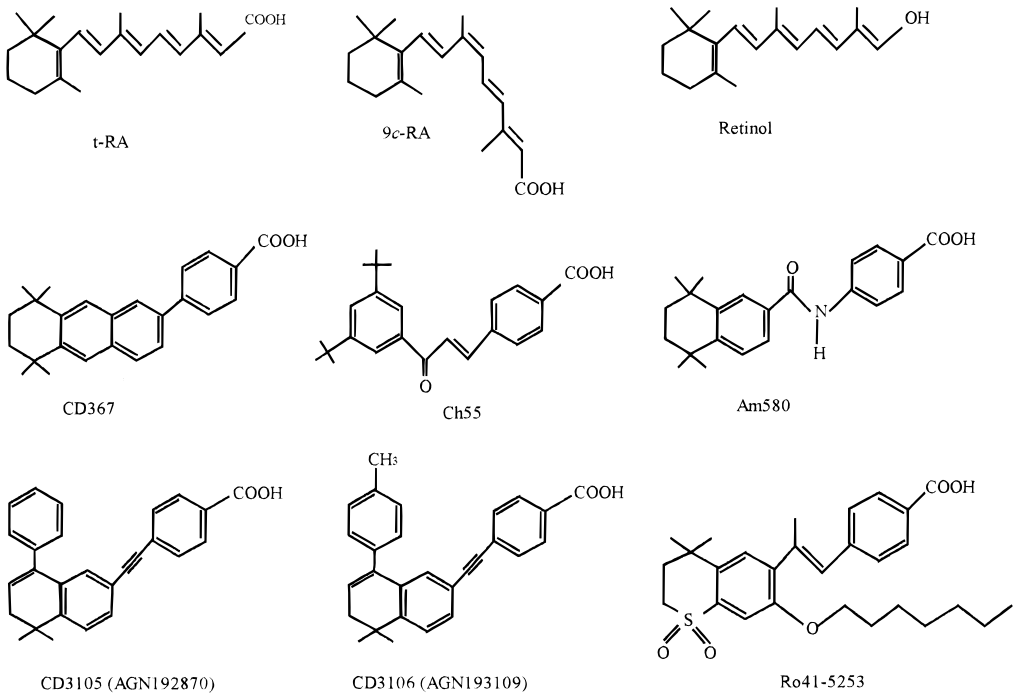


FIGURE 4: Formulas of the natural and synthetic retinoids.

14 kDa by trypsin (panel A, lanes 2 and 8) or chymotrypsin (panel B, lanes 2 and 6, and panel C, lanes 2 and 3). Preincubation of wild-type RARα with RA or CD367 resulted in a clearly reduced proteolysis with the appearance of both trypsin (panel A, lanes 3−6)- and chymotrypsin (panel B, lanes 3 and 4)-resistant peptides. A main 30 kDa and a weaker 26 kDa protected bands were obtained with trypsin, whereas 28 kDa and 26 kDa bands were obtained with chymotrypsin. The digestion pattern of the liganded Y277A mutant was characterized by the appearance of the same trypsin-resistant bands as those observed with the wild-type receptor (panel A, lanes 9−12). However, the 30 kDa band seemed to resist slightly less efficiently at high trypsin concentrations (panel A, lanes 11 and 12). Interestingly, the chymotrypsin digestion pattern revealed a clear difference between the Y277A mutant and the wild-type receptor. In the standard conditions used, RA binding did not afford protection against proteolysis (panel B, lane 7), whereas a significant protection was observed with CD367, generating a 28 kDa band (panel B, lane 8). This difference in the proteolytic resistance could be explained by the differences in the binding affinities of these two ligands to the Y277A mutant. Incubation of the wild-type and mutant receptors were performed with RA or CD367 at 1 μM. Indeed, this concentration is high enough to obtain a saturation of the receptor-LBD even with RA in case of the Y277A receptor mutant. These results suggests that slightly different ligand-induced conformational changes occurred in the Y277A mutant when compared to the wild-type receptor.

DISCUSSION

This work documents the functional importance of the tyrosine residues present in the ligand-binding domain of the RARs. Enzymatic and partial acid hydrolysis of the chemically modified hRARα LBD demonstrated that the various peptides containing tyrosine, corresponding each to one to the three tyrosines of the RARα LBD, were all modified by TNM in the native LBD, whereas the peptide containing Tyr^{277} was the only one protected by preincubation of the receptor with the ligand. TNM is the most frequently used chemical reagent for the modification of tyrosyl residues in native proteins (*40*). Therefore, the functional importance of this specific residue was further documented by site-directed mutagenesis.

This approach, combining chemical modification of specific amino acid residues and mutagenesis, has already been used by Wolfgang et al. (*24*), who further extended our previous observation that RA binding to the three RAR types was blocked by sulfhydryl-modifying agents (*33*). By using site-directed mutagenesis, Wolfgang et al. (*24*) identified two cysteine residues responsible for the loss of RA binding presented by RARβ after chemical modification with sulhydryl-specific reagents. One of these residues, Cys^{228} (Cys^{235} in RARα), is located in helix 3 and belongs to the LBP of the crystallized holo-RARγ (Figure 2), whereas the other one, Cys^{267} (Cys^{274} in RARα), is located in helix 5 and like Tyr^{277} does not belong to the LBP, but lies next to Ile^{273} which belongs to the LBP (Figure 2). Interestingly, mutation of these cysteines into alanine did not alter the affinity of RARβ for RA, whereas chemical modification did, suggesting a steric hindrance mechanism rather than a direct role of these residues in RA binding.

In fact, as shown by the RA-RARγ crystal structure, Tyr^{279} (Tyr^{277} in RARα), despite being next to Arg^{278} (Arg^{276} in RARα), an essential residue that plays a major role by interacting with the carboxyl group of RA, does not interact with RA and appeared turned toward the opposite direction (Figure 6). Interestingly, the side chain of Tyr^{279} is buried inside the LBD of RARγ and unaccessible to the solvent (Figure 6). It can be supposed that, in the apo RARα, Tyr^{277} is accessible to the solvent and can be modified by TNM, yielding a receptor structure that can neither bind properly its ligand nor undergo the conformational change concomitant to binding. However, as shown in Figure 5, panels B

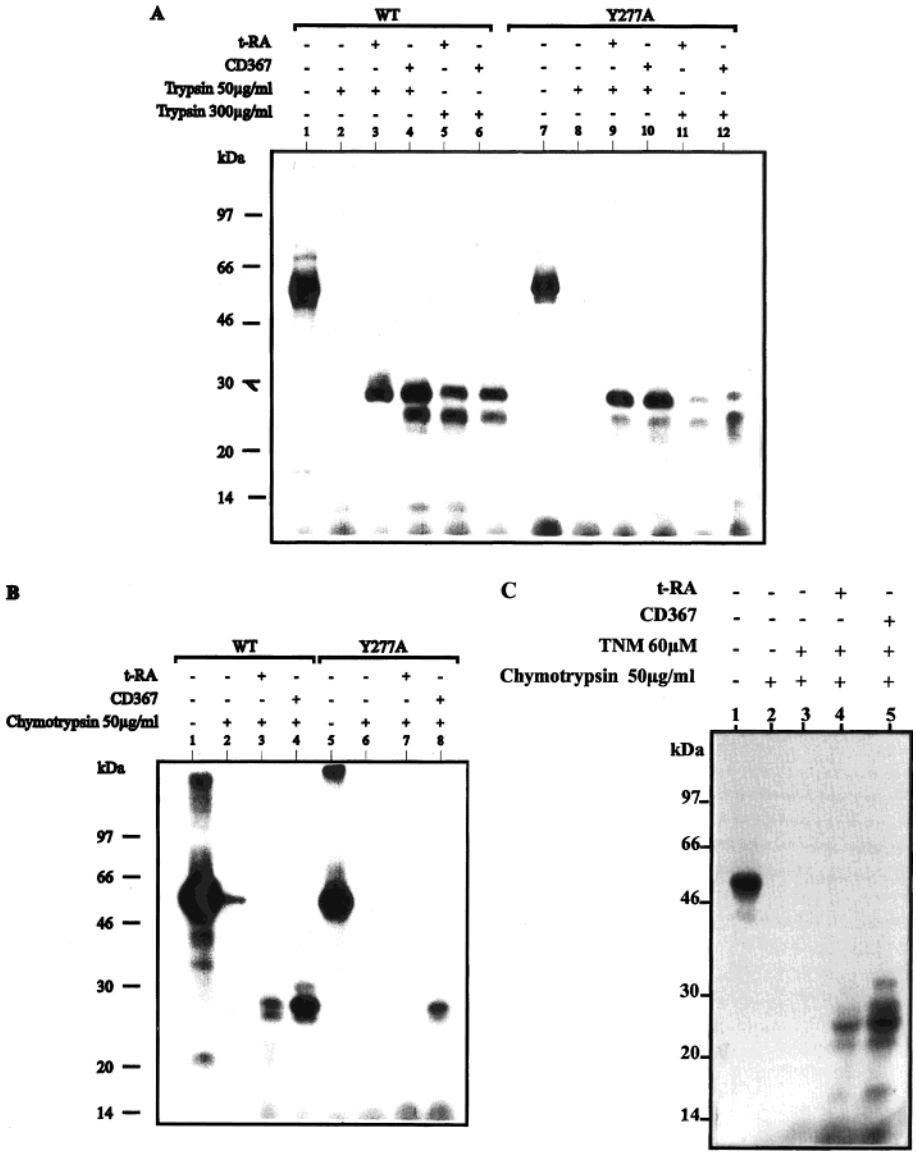


FIGURE 5: Effects of *all-trans*-RA and CD367 on limited trypsin (A) or chymotrypsin (B, C) digestion of wild-type and Y277A-hRARα. The in vitro-translated wild-type RARα (WT) and the Y277A mutant were preincubated with DMSO alone or with 10^{-6} M RA or CD367. Samples were then digested with the indicated concentrations of trypsin (A) or chymotrypsin (B, C) and incubated for 10 min at room temperature (A) or 30 min at 37 °C (B, C). In panel C, the in vitro-translated wild-type RARα was digested with chymotrypsin following or not TNM modification. Samples were analyzed by electrophoresis in a 10% SDS−polyacrylamide gel, and the dried gel was autoradiographed. The sizes of molecular weight markers are indicated.

and C, the chymotrypsin sensitivity of the unmodified and the TNM modified receptors are not different. Conversely, in the liganded receptor, Tyr^{277} becomes unaccessible to chemical modification because it is buried in a pocket delimited by H5 and H7 like in RARγ. Moreover, in the RA−RARγ complex, both Tyr^{210} and Tyr^{364} (corresponding to Tyr^{208} and Tyr^{362}, respectively, in RARα) appear accessible to the solvent (Figure 6). The phenolic group of Tyr^{210} lies on the surface of the receptor and is the most exposed to chemical attack, whereas Tyr^{364}, despite being half-buried in the LBD core, is yet partly exposed to the solvent and, therefore, to chemical nitration by TNM. These structural characteristics are in perfect agreement with our experimental data. Tyr^{208} in RARα easily accessible and displaying no obvious role in the receptor structure can be chemically modified or mutated without any consequence on the binding and transactivating activities of the receptor. Tyr^{362} in RARα is accessible; however, its chemical modification or mutation probably results in a conformational change affecting the LBD core structure and the ability of the liganded receptor to interact with corepressors and/or coactivators, explaining the decrease in transcriptional activity.

The Y277A mutant displayed an altered pattern of retinoid binding. The effect on the affinity was variable and depended on the structure of the retinoid tested. From our binding experiments, Tyr^{277} does not appear to be essential for RA binding but may be important in the adoption of the final active conformation of the protein. The most striking differences were observed with synthetic retinoids. The Y277A mutation could somehow modify the ability of the receptor to wrap around compounds such as Am580, a RARα selective ligand, and certain antagonists such as Ro 41-5253. This hypothesis was strengthened by the binding and transactivating data obtained with the Y277F mutant. The phenolic hydroxyl group in tyrosine 277 appeared essential to trigger ligand-dependent transactivation in the presence

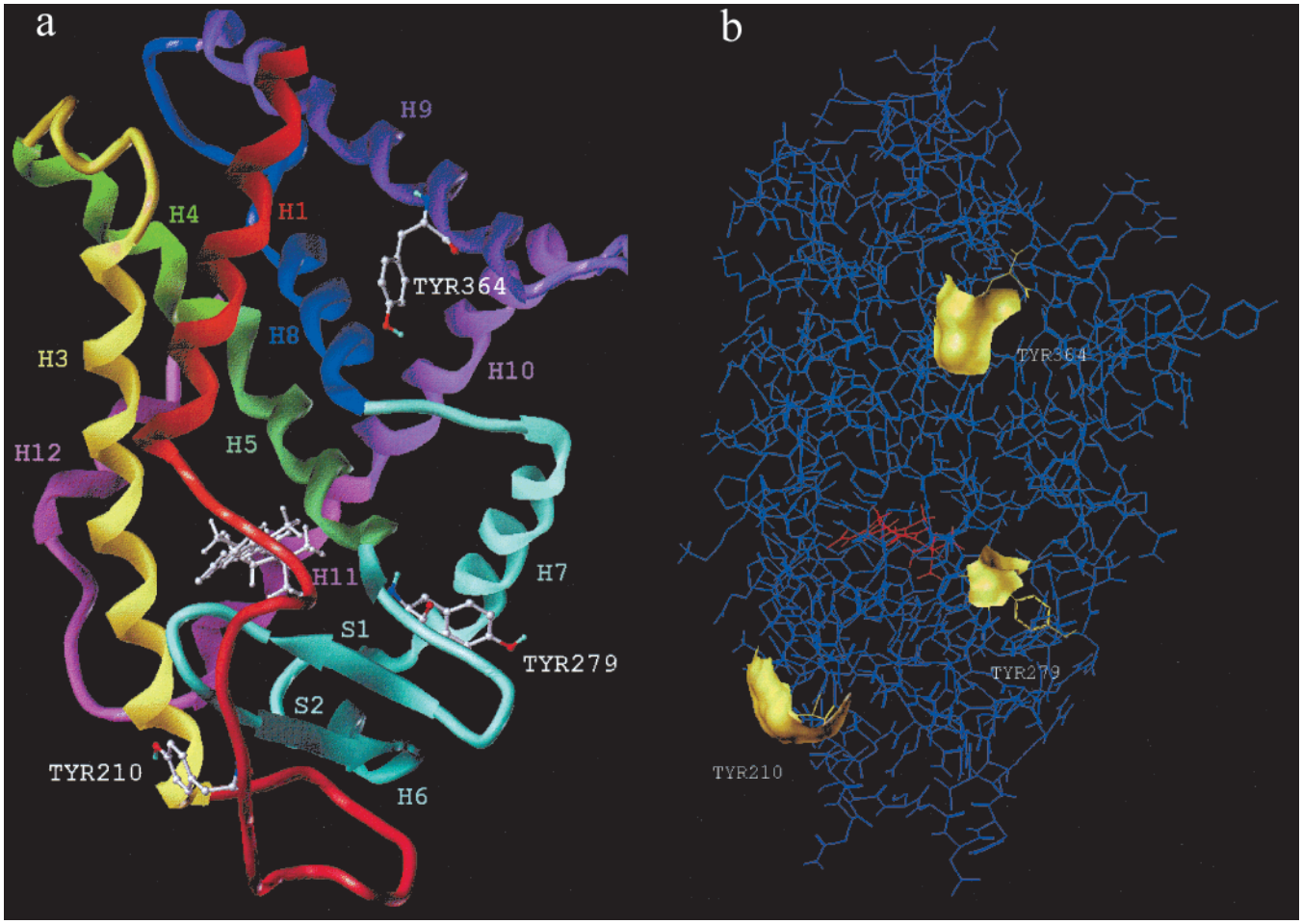


FIGURE 6: Accessibility and orientation of tyrosines in the RARγ-LBD structure. (a) Ribbon drawing showing the holo RARγ secondary structure. The coordinates are from Renaud et al. (ref *17* and Brookhaven Protein Database entry 2LBD). Residues Y210, Y279, and Y364 (corresponding to Y208, Y277, and Y362 in RARα) are represented. Note that Y277 is orientated opposite to the ligand. (b) Solvent-accessible surfaces in the holo RARγ-LBD of Tyr^{210}, Tyr^{279}, and Tyr^{364} (in yellow). Phenol group is accessible in Tyr^{210} and Tyr^{364} whereas only backbone atoms are accessible in Tyr^{279}. Surfaces were calculated using the Quantum Chemistry Program Exchange no. 429 with five points per square angström and a probe radius of 1.4 Å (*44*). Molecular modeling was performed using a Silicon Graphics Indigo 2 station and Sybyl 6.4 software (Tripos, St Louis, MO).

of the synthetic agonists tested, whereas the aromatic ring of tyrosine could be involved in the ligand-binding properties of the receptor, as suggested by the differences in the binding pattern of Y277A and Y277F receptor mutants.

There are other examples of mutations involving residues that, like Tyr^{277}, do not lie in the LBP and yet resulted in some modifications of the ligand specificity of the binding and/or transactivating properties of RARs. Their effects are very often restricted to certain synthetic retinoids. Recently, Lamour et al. (*23*) reported that the R217A and R294A RARα mutants displayed an increased affinity for the RARα selective antagonist Ro 41-5253. Similarly, Ostrowski et al. (*26*) observed that the R212Q RARβ mutant (a residue located in the omega loop and corresponding to Ser^{219} in RARα and Gln^{221} in RARγ, respectively) presented wild-type properties in the presence of various retinoids, with the exception of BMS-185411, a selective RARβ agonist and RARα antagonist. BMS-185411 transactivated very poorly the R212Q mutant. The same effect was observed with the I232T and T246S RARβ mutants (corresponding to Thr^{239} and Thr^{253} in RARα, two residues located in the H3 helix and the H3−H4 linker, respectively).

A conformational effect was also detected in the Y277A mutant by protease mapping. The impaired protection observed in the presence of RA is in agreement with the decrease in affinity and suggests that the conformational change of the mutant induced by the ligand was slightly different from the wild-type. Protease mapping has already been used with success by Keidel et al. (*39*) to document the specific mode of interaction of Ro 41-5253, a RARα antagonist, with the wild-type receptor and by our group to detect alterations in the ligand-induced structural transitions in various RARα mutants (*32*). In conclusion, we have evidenced the role of one of the three tyrosines of RARα-LBD in its binding and transactivating properties. Tyr^{277}, suspected to be essential for ligand binding from the chemical modification experiments, was demonstrated by site-directed mutagenesis and molecular modelization to be involved in the ligand-induced structural changes of the receptor and in the stabilization of its transcriptionnally active conformation. However, further work is clearly needed to understand the specific mode of interaction between synthetic retinoids and RARs.

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