Purification and Functional Characterization of the Ligand-Binding Domain from the Retinoic Acid Receptor α: Evidence That Sulfhydryl Groups Are Involved in Ligand–Receptor Interactions†

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ABSTRACT: The pGEX-2T expression vector was used to produce the ligand-binding domain from the human retinoic acid receptor α (hRARα LBD) in *Escherichia coli*. The resulting fusion protein, containing the glutathione *S*-transferase separated from the truncated receptor (hRARα 186-462) by a thrombin cleavage site, was purified with use of affinity chromatography on immobilized glutathione. A 90% homogeneity was obtained, with a specific activity of 100 pmol/mg and an overall 10% yield. Following purification and thrombin cleavage, a predominant monomeric (stokes radius = 2.3 nm, molecular mass of 32 kDa) [^{3}H]retinoic acid hRARα LBD complex was characterized by high-performance size-exclusion chromatography.The purified hRARα LBD bound retinoic acid with an apparent K_d of 9 nM, a value close to the K_d of the full-length hRARα expressed in COS cells. Kinetic studies at 0 °C demonstrate that the association of [^{3}H]retinoic acid and [^{3}H]CD367, a synthetic retinoid, to the overexpressed receptor was extremely rapid (complete in less than 3 min), whereas their dissociation from the receptor was slower, with half-lives of about 40 min at 0 °C. Experiments performed at various subzero temperatures allowed a more accurate assay of the association rate constant and indicate that the entropy of activation ($\Delta S^{\ddagger}$) is positive, which is characteristic of hydrophobic interactions. The ligand-binding activity was markedly decreased by pretreatment with various sulfhydryl modifying agents. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) appeared to be the most potent, whereas iodoacetamide was the least active.Furthermore, a series of *N*-alkylmaleimides was shown to inactivate the recombinant receptor. Comparison of these agents revealed a striking increase of receptor inactivation with increasing chain length of the maleimide derivative. Full protection against inactivation was afforded by previous [^{3}H]retinoid-binding on the receptor. The receptor binding activity was insensitive to arsenite, a reagent able to preferentially oxidize vicinal dithiols. Taken together, these results demonstrate that one or several sulfhydryl groups but probably no vicinal dithiols are involved in the retinoid-binding activity of hRARα, lying most probably in the retinoid-binding site itself.

All-trans retinoic acid (RA),[1] a biologically active vitamin A derivative, and its synthetic analogues affect processes as diverse as growth, differentiation, and morphogenesis (Brockes, 1990; Summerbell & Maden, 1990; Eichele, 1989). RA has profound effects on the differentiation of various tissues, including the hematopoietic and nervous system (Amatruda & Koeffler, 1986; Durston *et al.*, 1989), skin, and cartilage (Shapiro, 1980; Paulsen *et al.*, 1988; Asselineau *et al.*, 1989). It can suppress and reverse malignant transformation induced by either chemical carcinogens or ionizing radiation (Sporn *et al.*, 1976; Moon & Mehta, 1990). Many retinoids have now been described, and some are used for the treatment of dermatological disorders (Peck, 1983) and acute promyelocytic leukemia (Chomienne *et al.*, 1989), for cancer chemoprevention, and as immunomodulators (Bollag & Hartmann, 1983; Lippman *et al.*, 1987). A number of retinoids have been tested in various *in vivo* systems, inducing very often a differentiation response (Sporn & Roberts, 1984).

It is now well established that the ultimate targets of retinoids are the nuclear protein receptors termed RARs, which belong to the steroid/thyroid hormone receptor superfamily (Evans, 1988; Chambon *et al.*, 1991). Three RAR subtypes (RARα, RARβ, and RARγ) have been cloned in humans and mice (Petkovitch *et al.*, 1987; Giguere *et al.*, 1987; de Thé *et al.*, 1987; Brand *et al.*, 1988; Benbrook *et al.*, 1988; Zelent *et al.*, 1989; Krust *et al.*, 1989). Another receptor class named RXR, which displays a rather different sequence and which binds specifically to 9-*cis*-retinoic acid, has been identified (Hamada *et al.*, 1989; Mangelsdorf *et al.*, 1990; Levin *et al.*, 1992). RARs bind to their target cognate DNA sequences, termed retinoic acid response elements, in the promoters of responsive genes and upon ligand binding activate or repress the transcription of the target. However, despite the identification of various genes regulated by RARs (Vasios *et al.*, 1989; de Thé *et al.*, 1990; Nicholson *et al.*, 1990; Sucov *et al.*, 1990; Schüle *et al.*, 1991; Umesono *et al.*, 1991), the precise molecular events that are triggered following RA binding to

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[1] Abbreviations: CRABP, cellular retinoic acid binding protein; DMEM, Dulbecco's modified eagle medium; DTNB, 5′,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GST, glutathione S-transferase; hRAR, human retinoic acid receptor; IA, iodoacetamide; IPTG, isopropyl β-D-thiogalactopyranoside; LBD, ligand-binding domain; βME, β-mercaptoethanol; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; RA, retinoic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; K_d, equilibrium constant of dissociation; k_a, rate constant of association; k_d, rate constant of dissociation.

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the receptor are not well understood. RARs and RXRs, like others members of the steroid/thyroid hormone receptor superfamily, are organized in several domains (Green & Chambon, 1988). Two main receptor functions, i.e., ligand binding and DNA binding, are fulfilled by the E and C domains, respectively, which are also the most conserved domains. Owing to the natural low abundance of RARs in target tissues and to the frequent colocalization in the same tissues of different RAR subtypes with various isoforms (Leroy *et al.*, 1991), an absolute requirement for the study of the ligand-binding specificity of each RAR subtype was the resort to recombinant RARs overexpressed either in *E. coli* or in eukaryotic cells. First results in this field have been recently reported, affording the critical information that some of the existing retinoids displayed a partial RAR subtype-binding specificity (Lehmann *et al.*, 1991; Delescluse *et al.*, 1991). These differences in binding specificity are probably related to the limited but significant differences in the sequences of the E domain of RARα, -β, and -γ. The need for RAR subtype-specific ligands, which could be more efficient and less toxic in human therapy, renews the interest in retinoid medicinal chemistry and also greatly stimulates the basic study of the structural and functional properties of RAR ligand-binding domains.

Several successes in the *E. coli* expression of hRARα and -β, either as full-length protein or as ligand-binding domain, have already been reported (Crettaz *et al.*, 1990; Yang, *et al.*, 1991; Keidel *et al.*, 1992). But in all these cases, work was focused on the study of the DNA and ligand-binding specificity of the hRARs, and no attempt was made to purify the recombinant RAR and to study more precisely its biochemical properties. Moreover, very little information is available about the kinetic parameters governing the association and dissociation of retinoid–receptor complexes, and no thermodynamic study has been reported so far. Such a study could allow a better understanding of the respective contributions of hydrophobic interactions and the involvement of other kinds of bonding to the high affinities observed for retinoid–receptor complexes. In this paper, we report the complete purification and describe some biochemical properties (hydrodynamic properties and kinetics parameters of ligand binding) of a recombinant hRARα ligand-binding domain. To gain further insights into the mechanisms involved in retinoid–receptor interactions, the thermodynamic parameters of association were measured, and we have also studied the ligand-binding activity and its sensitivity toward various sulfhydryl reagents.

EXPERIMENTAL PROCEDURES

Materials. The plasmid pHK1 containing the full-length hRARα coding sequence and the pRSV-GR expression vector were obtained from Dr. R. M. Evans, Howard Hughes Medical Institute, La Jolla, CA. The expression vector pSVM-dhfr was obtained from Dr. G. Ringold, Stanford University School of Medicine, Stanford, CA. The expression vector pGEX-2T was obtained from Pharmacia-LKB, France. [^{3}H]*All-trans* retinoic acid ([^{3}H]RA, 55.7 Ci/mmol) was from New England Nuclear. [^{3}H]CD367 (52.8 Ci/mmol) and unlabeled CD367 (4-[5,6,7,8 tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl] benzoic acid) were obtained from Dr. B. Shroot, CIRD, Sophia Antipolis, Valbonne, France.

A rabbit polyclonal antibody (IS39 antiserum) directed against a synthetic peptide (hRARα 425-443) was prepared in our laboratory (Sablonnière *et al.*, in press). Restriction endonucleases and T4 DNA ligase was purchased from Boehringer or Biolabs. The affinity gel glutathione Sepharose 4B was from Pharmacia-LKB. Human thrombin (3000 IU/mg), antiproteases, *N*-ethylmaleimide (NEM), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), methyl methanethiosulfonate (MMTS), and iodoacetamide (IA) were obtained from Sigma. All other chemicals were of the highest available purity grade and were purchased from Merck. *N*-Propyl-, *N*-hexyl-, and *N*-nonylmaleimides were prepared from maleic anhydride and the appropriate amine through a two-step procedure according to the method of Heitz *et al.* (1968), whereas *N*-benzylmaleimide was obtained by the two-step procedure of Cava *et al.* (1961).

Construction of the Expression Vector pGEX-2T-hRARα LBD. The pHK1 vector was digested with *Sac*I, and a *Bam*HI site was inserted for subsequent cloning into the pGEX-2T expression vector. Positive clones were then amplified, and the plasmid was digested with *Bam*H1 and *Aat*II. The excised sequence was introduced into the pGEX-2T vector, giving the resulting pGEX-2T-hRARα LBD plasmid containing the cDNA sequence coding for the E and F domains of hRARα in frame with the sequence coding for glutathione S-transferase (GST). *E.* 4coli JM109 cells were then transformed with this plasmid. The recombinant clones were screened by restriction analysis using *Pst*I digestion.

Expression of the Fusion Protein GST-hRARα LBD in Bacteria and Extract Preparations. Transformed JM109 cells were screened for the expression of fusion protein by analysis on SDS–PAGE. Following an overnight preculture, each culture sample was diluted to 1:20 with fresh medium plus ampicillin and incubated at 37 °C until an OD 600 nm of 0.8 was reached. IPTG was then added to a 0.2 mM final concentration, and the culture was further incubated for 1–4 h. Two-milliliter aliquots were harvested and centrifuged, and the pellet was analyzed on SDS–PAGE (10% acrylamide gels). For large-scale production of recombinant protein, typical experiments were conducted as follows. An overnight culture of transformed JM109 cells was diluted to 1:50 in 2000 mL of fresh medium and grown at 37 °C before IPTG was added to 0.2 mM. Three hours later, cells were pelleted by centrifugation and lysed in 50 mL of 50 mM Tris-HCl, pH 8.00/10 mM EDTA/10% (w/v) saccharose/0.4 mg/mL lysozyme, including a protease inhibitor cocktail:1 mM PMSF, 20 μg/mL leupeptin, and 20 μg/mL aprotinin. The lysis was then completed by a cycle of freezing/thawing and by addition of Triton X-100 (0.1% final concentration). Homogenization was performed on ice by mild sonication, and 10% glycerol (v/v) was added. After centrifugation, the supernatant was saved and referred as crude bacterial extract for subsequent purification of the GST-hRARα LBD fusion protein.

Ligand-Binding Assays. Bacterial extracts or nucleosol samples were incubated with 1–50 nM [^{3}H]RA or [^{3}H]CD367 in binding buffer (50 mM Tris-HCl, pH 8.00/150 mM NaCl/1 mM EDTA/1 mM DTT). After 16 h incubation at 0 °C, 0.1 mL of chilled charcoal–dextran suspension (3% Norit A/0.3% dextran T70 in 50 mM Tris-HCl, pH 8.00/10 mM KCl/1 mM DTT) was added to 0.2 mL of incubates, mixed vigorously, and left for 15 min at 0 °C. The tubes were then centrifuged at 6000*g* for 10 min, and 0.15-mL supernatant samples were counted for radioactivity. Nonspecific binding measured in the presence of 200-fold excess of nonradioactive retinoids was 10–15% of the total binding. Scatchard plots were obtained using a "Scatchard-computed" program (LKB Wallac, Sweden).

Kinetics of [^{3}H]Retinoid Binding to the Recombinant GST-hRARα LBD. Determination of Association Rate. Bacterial extracts were submitted to 20-fold dilution with binding buffer

containing 40% (v/v) glycerol. To 0.5 mL duplicate aliquots of diluted bacterial extracts was added, at time zero, 30 nM tritiated retinoids ([^{3}H]RA or [^{3}H]CD367) in the absence or presence of a 1000-fold excess of the corresponding unlabeled retinoids. At times varying from 1 to 8 min, 0.2-mL aliquots were pipetted, and bound retinoid was measured by dextran–charcoal adsorption as described above. At each time point, the radioactivity observed in the sample containing an excess of unlabeled retinoid was substracted from that obtained in the absence of nonradioactive retinoid, giving the specifically-bound [^{3}H]retinoid. The total concentration of specific-binding sites, R_0, was determined in bacterial extracts incubated to equilibrium with 30 nM of the corresponding tritiated retinoid in the presence or absence of an excess of unlabeled retinoid. R_0 was equal to 3 nM and D_0 to 30 nM. At the various times indicated after the addition of the ligand, the concentrations of free binding sites R_t and free retinoid D_t were calculated. The rate constant of association k_a was calculated from the equation

$$t = \frac{2.303}{k_a(D_0 - R_0)} \log \frac{R_0 D_t}{D_0 R_t}$$

Determination of Dissociation Rate. The measurement of the rate constant of dissociation was performed at the indicated temperatures. Duplicate aliquots (0.5 mL) were incubated with 30 nM [^{3}H]retinoids for 3 h at 0 °C. At time zero, a 1000-fold excess of the corresponding unlabeled retinoid in 0.02 volume of ethanol was added. At various time intervals thereafter, varying from 0 to 180 min, 0.2-mL aliquots of each tube were withdrawn, and bound retinoid was measured by dextran–charcoal adsorption.

Affinity Purification and Cleavage of the Fusion Protein GST-hRARα LBD. A modified protocol of the procedure described by Smith and Johnson (1988) was used. A 55-mL sample of crude bacterial extract was adjusted to pH 7.30 and mixed with 6 mL of glutathione Sepharose 4B for 16 h at 4 °C on a rotating shaker. The affinity gel was rapidly washed with 30 mL of buffer 1 (20 mM Tris-HCl, pH 8.00/150 mM NaCl/10% glycerol (v/v)), followed by 30 mL of buffer 2 (20 mM Tris–HCl, pH 8.00/10% glycerol (v/v)). The fusion protein was eluted by competition for 5 h at 4 °C with free reduced glutathione in 6 mL of eluting buffer:50 mM Tris–HCl, pH 8.00/30 mM reduced glutathione/150 mM NaCl/5% glycerol (v/v). The affinity gel eluate was then concentrated in an Amicon microconcentrator. To ensure an efficient thrombin cleavage of the fusion protein without loss of receptor binding activity, the concentrated eluate was incubated with 300 IU of human thrombin for 16 h at 4 °C, in 50 mM Tris–HCl, pH 7.50/150 mM NaCl/2.5 mM $CaCl_2$/5% glycerol (v/v). At the end of the incubation period, thrombin cleavage was stopped by the addition of 2 mM PMSF.

Size-Exclusion HPLC. Analytical size-exclusion chromatography was carried out with a LKB 2150 liquid chromatography system. The samples were fractionated over a TSK G 3000 SW column (7.5 × 300 mm) equipped with a guard column (7.5 × 100 mm) at a flow rate of 0.5 mL/min using 10 mM Tris–HCl, pH 7.20/150 mM NaCl/1 mM DTT as eluent. Fractions of 0.25 mL were collected and assayed for GST activity and radioactivity. The column was calibrated with the following standard proteins:catalase (R_s = 5.2 nm), bovine serum albumin (R_s = 3.6 nm), ovalbumin (R_s = 2.9 nm), and chymotrypsinogen (R_s = 2.0 nm). The distribution coefficient K_d [$K_d = (V_e - V_0)/(V_t - V_0)$] was plotted as $K_d^{1/3}$ versus the Stokes radii of standards according to Porath (1963).

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed in 10% or 12% acrylamide gels according to Laemmli (1970). Proteins bands were visualized by Coomassie blue staining. Molecular mass markers were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa).

Western Blot Analysis. Proteins separated by SDS–PAGE as described above were electroblotted onto nitrocellulose filters as previously described (Sablonnière *et al.* 1988). The filters were then treated with a blocking buffer and incubated for 2 h at 20 °C with anti-hRARα polyclonal antibodies. The filters were then washed and incubated for 1 h with peroxidase-conjugated goat anti-(rabbit antibody) (Institut Pasteur, Paris, France). Following four washes, the nitrocellulose filters were incubated with luminol as substrate (ECL western blotting kit, Amersham, France) for 2 min, rinsed rapidly, and autoradiographed.

Sulfhydryl Modification Procedures. To determine the ligand-binding susceptiblity of the fusion protein to sulfhydryl reagents, aliquots of crude bacterial extracts were diluted to 1:20 in 50 mM Tris-HCl, pH 7.40, and 150 mM NaCl was incubated with four different reagents as follows. The first aliquot was treated with NEM (0–2 mM) for 5 min at 0 °C; NEM activity was then stopped by the addition of 20 mM β-mercaptoethanol, and specific retinoid-binding activity was assayed with 20 nM [^{3}H]RA. The second, third, and fourth aliquots were treated with IA (0–1 mM), DTNB (100 nM to 100 μM), and MMTS (0–1 mM), respectively, in the same conditions and further assayed for retinoid-binding activity with [^{3}H]RA. To test the reversibility of the receptor inactivation, aliquots incubated for 30 min with DTNB were rapidly mixed with β-mercaptoethanol (20 mM final concentration) and further assayed for retinoid-binding activity with [^{3}H]RA. In another experiment, an aliquot subjected to MMTS treatment for 30 min was then mixed with DTT (10 mM final concentration) and assayed for retinoid-binding activity. In each series of experiments, a control sample which was not treated with the sulfhydryl-modifying reagents was assayed for ligand-binding activity in the same conditions. The ligand-binding susceptibility of the fusion protein to various N-substituted maleimides was tested as follows. Aliquots of crude bacterial extracts were diluted to 1:20 in 50 mM Tris-HCl, pH 7.40, and 150 mM NaCl and incubated with 10–100 μM of the different maleimides (*N*-ethyl-, *N*-propyl-, *N*-hexyl-, *N*-benzyl-, and *N*-nonyl-substituted maleimides) for 5 min at 0 °C. The maleimide activity was then stopped, and retinoid-binding activity was measured.

The ligand-exchange assay was performed with a diluted bacterial extract previously incubated with 1 μM RA for 16 h at 0 °C. The 0.5-mL aliquots were then treated or not with 2 mM NEM for 5 min at 0 °C, and the alkylation was stopped by the addition of 20 mM β-mercaptoethanol. Excesses of both RA and NEM were further eliminated by desalting the incubates on a small Ultrogel ACA 202 column. The excluded fractions were then pooled, left for 30 min at 0 °C, and finally incubated with 50 nM [^{3}H]RA for 4 h at 0 °C prior to specific retinoid-binding activity measurement.

Construction of the pSVM-hRARa Expression Vector. The pHK1 vector was digested with *Eco*RI, and the resulting 2.9-kb fragment containing the entire hRARα coding sequence was inserted into the pSVM-dhfr plasmid as follows. The unique *Bgl*II site of pSVM-dhfr separating the SV40 origin fragment from dhfr sequences was transformed into an *Eco*RI site. pSVM-dhfr was then partially digested with *Eco*RI,

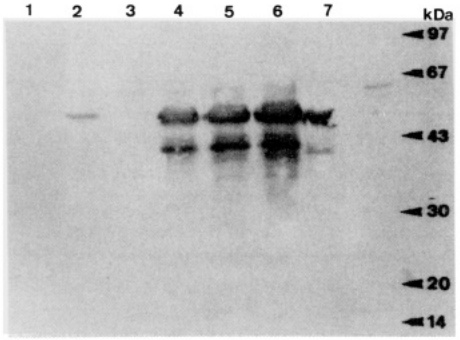

FIGURE 1: Expression of the fusion protein in *E. coli*. Before (lane 2) and after a 1-, 2-, 3-, or 4-h IPTG induction (lanes 4–7, respectively), JM109 cells transformed with pGEX-2T hRARα LBD were lyzed in SDS loading buffer; proteins were then resolved on a 10% SDS-polyacrylamide gel, blotted on a nitrocellulose filter, and then immunorevealed with the IS39 antiserum. The proteins on lane 1 were obtained from a lysate of *E. coli* transformed with pGEX-2T and submitted to a 3-h IPTG induction. Lane 3 corresponds to a lysate of the same transformed cells without IPTG induction. Molecular weight markers are indicated on the right side of the gel.

and the 2.9-kb fragment of pHK1 was inserted, giving the pSVM-hRARα expression vector. Colonies were screened to isolate the DNA containing the hRARα sequence in the correct orientation by restriction analysis.

COS Cell Transfection and Nuclear Extract Preparation. For transient expression of hRARα, COS-7 cells were grown in DMEM containing 10% fetal calf serum (complete medium) and plated at 50% confluence (2.5×10^6/dish) the day before transfection. Cells were then cotransfected with use of the calcium phosphate coprecipitation technique (Chen & Okayama, 1987) with the pSVM-hRARα expression vector (10 μg of plasmid/dish) together with 5 μg of pRSV-GR. Sixteen hours after transfection, cells were washed and refed with complete medium containing 10^{-6} M dexamethasone. Twenty-four hours later, cells were washed again, refed for 48 h, and collected in cold PBS with use of a rubber policeman. Cell nuclear extracts were then prepared according to the procedure described by Nervi *et al.* (1989). These extracts were subjected to ultrafiltration through an Amicon YM 30 membrane to eliminate protein contaminants of molecular mass lower than 30 kDa.

Miscellaneous. Glutathione *S*-transferase (GST) activity was assayed at 30 °C using 1-chloro-2,4-dinitrobenzene as substrate (Simons & Vander Jagt, 1977). Protein contents of the bacterial and cell nuclear extracts were determined with use of the Bradford assay (Bradford, 1986). Radioactivity was measured in a 1214 Rack β liquid scintillation counter (LKB-Wallac, Sweden) with Aqualyte as scintillation cocktail. Subzero temperatures were generated by a Huber HS40 cryostat.

RESULTS

Expression, Purification, and Characterization of the Fusion Protein. JM109 cells transformed with the pGEX-2T-hRARα LBD were induced by IPTG, and bacterial extracts were thereafter produced at various times. The expression of a protein with the 56-kDa molecular mass expected for the GST-hRARα LBD fusion protein was observed between 1 and 4 h following induction. Western blot analysis of the various bacterial extracts clearly demonstrated that the IPTG-inducible, 56-kDa protein contained the hRARα LBD, as it was recognized by the IS39 antiserum (Figure 1). However, another immunoreactive protein with an apparent M_r of 43 000 was observed in most experiments. This protein, representing 5–30% of the immunorevealed material, corresponded probably to a proteolytic degradation of the full-length fusion protein.

The results of a representative purification experiment are summarized in Table I. About 86% of the fusion protein (quantified here by its retinoid-binding activity) was adsorbed to the affinity gel. Washing steps did not result in a significant loss of binding activity (data not shown). Even though a high and quite reproducible adsorption yield was obtained in most experiments, the elution yield was low, varying in a 3-16% range in five separate experiments, and resulted in an overall purification yield of 2–14%. Attempts to preserve the maximum ligand-binding capacity of the hRARα LBD throughout the purification procedure appeared unsuccessful; in particular, no significant improvement was observed when the adsorption and the elution steps were performed in the presence of 40 nM RA (data not shown). The 45% yield of functional fusion protein expressed in terms of enzymatic activity contrasts with the 15-fold lower yield of retinoid-binding activity, suggesting a marked functional instability of the hRARα LBD moiety of the fusion protein. This suggestion is in agreement with the calculated purification yield, which rises 8.5-fold when expressed by the retinoid-binding activity of the fusion protein, whereas it reaches 74-fold when expressed by its enzymatic activity. As indicated by Smith and Johnson (1988), who purified the GST by using the same glutathione-immobilized matrix, optimal yield elution (~90%) could be achieved by performing the elution at pH 9.6. However, an experiment performed at this pH with the GST-hRARα LBD resulted in complete loss of retinoid-binding activity (data not shown). Analysis by SDS–PAGE and Coomassie blue staining of the affinity gel eluate revealed an apparently homogenous protein band (Figure 2) with the expected molecular mass of 56 kDa. This band was also revealed by the IS 39 antiserum, which, here again, detected the presence of a minor putative proteolytic fragment (lane 4).

Thrombin Cleavage and Characterization of the Purified hRARa LBD. In all experiments, cleavage by thrombin was obtained, but its efficiency was variable. In the data reported in Figure 2, the cleavage was complete, giving a main protein species with an apparent M_r of 28 000 (Figure 2B, lane 1). Depending on the purification experiment, some minor protein contaminants were inconstantly observed in the affinity gel eluate. Despite the use of a 12% polyacrylamide gel, separation of the two cleaved protein moieties from the fusion protein (with calculated molecular masses of 27 and 29 kDa, respectively) could not be observed. The purified truncated receptor was analyzed by size-exclusion HPLC on a TSK G 3000 SW column. As shown in Figure 3, two [^{3}H]RA-hRARα LBD species with respective Stokes radii of 3.7 (peak I) and 2.3 nm (peak II) were observed. These two eluted peaks were further analyzed by SDS–PAGE and western blotting (Figure 2B and C). Here again, an apparent unique protein component of ~28 kDa was seen. As shown by western blotting (Figure 2C), both peaks contained the hRARα LBD. The apparent M_r of 68 000 of the receptor species eluted in peak I, the absence of GST activity in this peak (Figure 3), and the absence of 56-kDa immunorevealed protein (Figure 2C, track 2) can suggest that the purified hRARα LBD forms homodimers. Such homodimers are not observed in culture cells *in vivo* (Nagpal *et al.*, 1993) but could correspond here to a facilitated *in vitro* dimerization of the purified hRARα LBD at high concentration.

Both the GST-hRARα LBD and the Purified hRARα LBD Are Functional Proteins. The ligand-binding activity of the fusion protein obtained from a crude bacterial extract and

Table I: Purification and Quantitative Measurement of the GST-hRARα LBD

step	volume (mL)	protein (mg/mL)	functional activity[a]		yield (%)	
			[^{3}H]RA binding (total, pmol)	GST activity (total, IU)[b]	[^{3}H]RA binding	GST activity
crude bacterial extract	55	4.6	3080	55.6	100	100
effluent	51	4.4	428	8.4	14	15
affinity gel eluate	2.8	0.55	154	25	5	45

[a] The functional activity of the fusion protein is measured as the retinoid-binding activity expressed in pmol or as the GST enzymatic activity expressed in international units. [b] IU, one unit of enzyme transforms one micromole of substrate per minute.

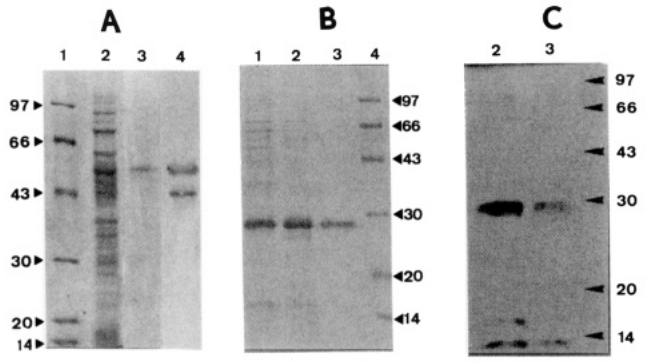

FIGURE 2: Characterization of the purified GST-hRARα LBD and hRARα LBD by SDS–polyacrylamide gel electrophoresis and western blotting. (A) The crude bacterial extract from JM109 cells transformed with pGEX-2T hRARαLBD and the affinity gel eluate obtained following purification of the fusion protein GST-hRARαLBD were submitted to SDS–PAGE. Lane 2: crude bacterial extract analyzed on a 10% polyacrylamide gel and stained by Coomassie blue. Lane 3: affinity gel eluate analyzed in the same conditions. Lane 4: western blot analysis of an affinity gel eluate with use of the IS39 anti-hRARα antibody. (B) An affinity gel eluate submitted to thrombin cleavage (300 IU/mL for 16 h at 0 °C) was analyzed on a 12% polyacrylamide gel and stained with Coomassie blue (lane 1). The eluted fractions corresponding to peaks I and II described in the experiment presented in Figure 3 were also analyzed (lanes 2 and 3, respectively). (C) Western blot analysis of the samples corresponding to peaks I and II (lanes 2 and 3, respectively). Molecular weight markers were analyzed in A (lane 1) and B (lane 4) and indicated on the right side of C.

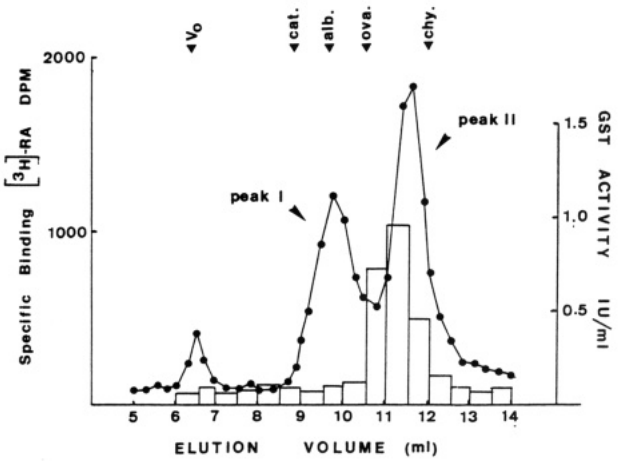

FIGURE 3: Analytical characterization of the purified hRARα LBD by size-exclusion HPLC. A 0.16-mL aliquot of the thrombin-cleaved affinity gel eluate containing the [^{3}H]RA-labeled hRARα LBD was brought to 0.6 M KCl and injected onto a TSK G 3000 SW column equilibrated with 10 mM Tris-HCl, pH 7.20/150 mM NaCl/1mM DTT. Fractions of 0.25 mL were collected at a flow rate of 0.5 mL/min and assayed for both GST activity (histogram) and radioactivity content (●). Arrows indicate the positions of protein standards. Catalase, cat, R_s = 5.2 nm; bovine serum albumin, alb., R_s = 3.6 nm; ovalbumin, ova., R_s = 2.9 nm, and chymotrypsinogen, chy., R_s = 2.0 nm.

from the purified cleaved hRARα LBD were compared (Figure 4A and B). The deduced K_d values were similar (7 and 9 nM, respectively). A comparison was done by studying the equilibrium parameters of RA-binding to the full-length hRARα overexpressed by transient transfection in COS cells. A K_d value of 15 nM was measured, indicating that the ligand-binding domain of the receptor had retained the full binding properties of the native receptor and that production of this domain as a fused protein to GST did not affect its ligand-binding properties. The possible interference of CRABP in the equilibrium parameters of RA-binding from COS cells, as mentioned by Cavey *et al.* (1990), was eliminated by ultrafiltration of the nuclear extracts through an Amicon YM30 membrane. Binding experiments were also performed with [^{3}H]CD367, a chemically stable retinoid compound whose affinity toward hRARα, -β, and -γ has been reported to be particularly high (Delescluse *et al.* 1991). Similar K_d values in the range 2–3 nM were obtained for both the intact and the cleaved fusion protein.

Binding Kinetics Parameters. At 0 °C, the association between the GST-hRARα LBD and retinoids appeared extremely rapid. Therefore, experiments at subzero temperatures were undertaken to allow accurate assay of the rate of association of [^{3}H]RA and [^{3}H]CD367 to the recombinant receptor (Figure 5A). At all three temperatures studied, the reaction followed second-order kinetics. This was verified by measuring the rate at three different ligand concentrations (3, 10, and 20 nM). In all cases, second-order kinetics were observed (data not shown). Calculated values for the second-order rate constants of association derived from these data are given in Table II. The lower value observed for [^{3}H]-CD367 may have been due to some differences in its molecular mechanisms of interaction with the receptor ligand-binding site. The dissociation reaction of the ligand from the recombinant receptor at 0 °C follows first-order kinetics and proceeds rapidly, with $t_{1/2}$ of 35 and 45 min for retinoic acid and CD367, respectively (Figure 5B and Table II). At higher temperatures, this rate was too rapid to be measured accurately. The contribution of the rate constant for the loss of retinoid-binding capacity (inactivation rate) of the recombinant receptor was negligible (4.33×10^{-4} min^{-1} at 0 °C). The k_d values were approximately 3-fold lower at −10 °C than at 0 °C, and we can observe that the acceleration of the rate of dissociation caused by increasing temperature was higher for CD367 than for RA (Table II). The same phenomenon could be observed when we compare the variation of the rate of association of the receptor with the two ligands.

The transition-state thermodynamics of the forward reaction (association) were calculated as described before (Eliard & Rousseau, 1984). The free-energy change (ΔG^a) of the transition state can be expressed as $\Delta G^a = \Delta H^a - T\Delta S^a = RT \log_n k_a + RT \log_n (bT/h)$, where b and h are the Boltzmann and the Planck constants, respectively. When calculated at −5 °C, these values were slightly different for the two ligands: ΔG^a = +27.6 and +29.7 kJ/mol, ΔH^a = +119 and +150 kJ/mol, and ΔS^a = +339 and +447 J/mol for RA and CD367, respectively. In both cases, the enthalpy barrier was partially compensated for by the favorable entropy change. Apparently

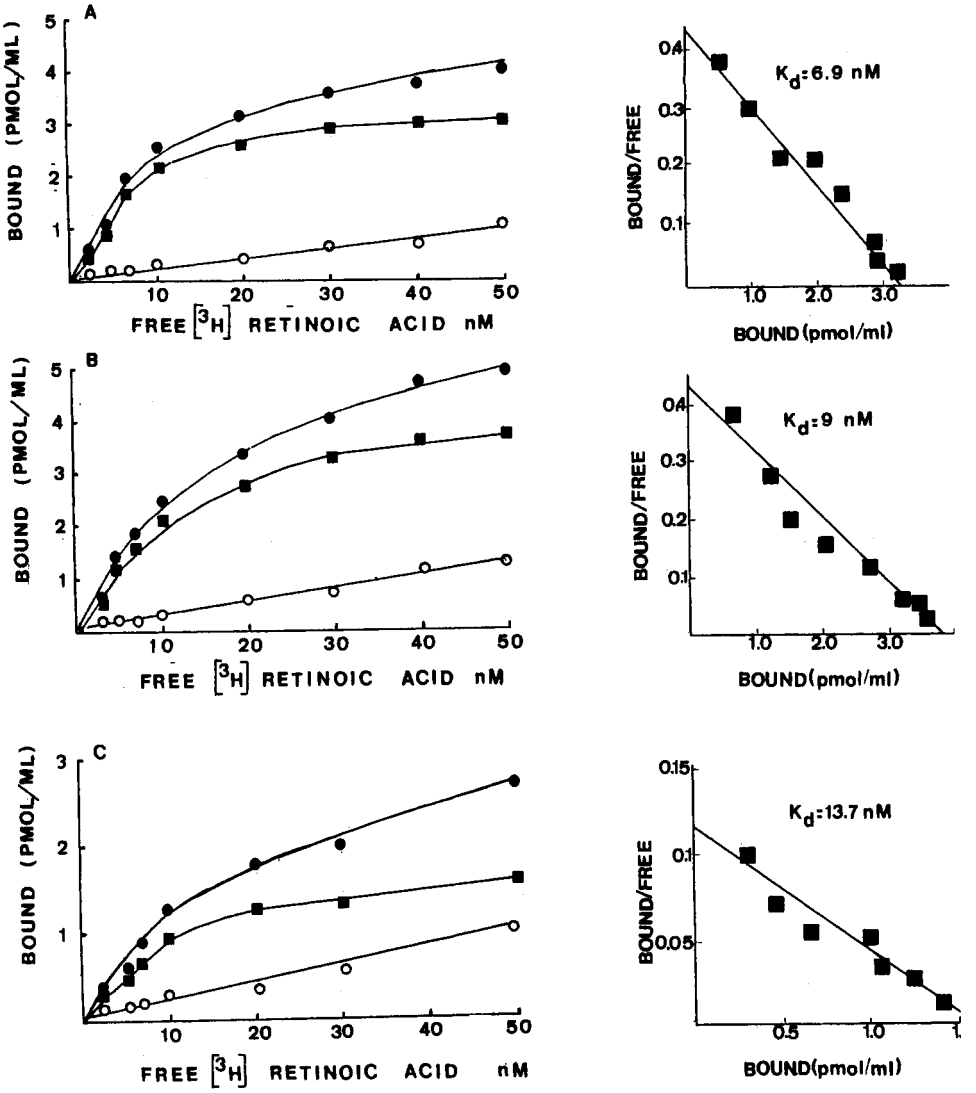

FIGURE 4: [^{3}H]RA binding activity. [^{3}H]RA saturation binding curves (left) and Scatchard plots (right) were determined (A) on the entire GST-hRARα fusion protein, (B) on the purified hRARα LBD polypeptide, and (C) on the full-length hRARα obtained by transient transfection of COS cells with the pSVM-hRARα vector. Total [^{3}H]RA-binding activity (●), nonspecific binding activity (○), and calculated RAR-specific binding activity (■) are represented.

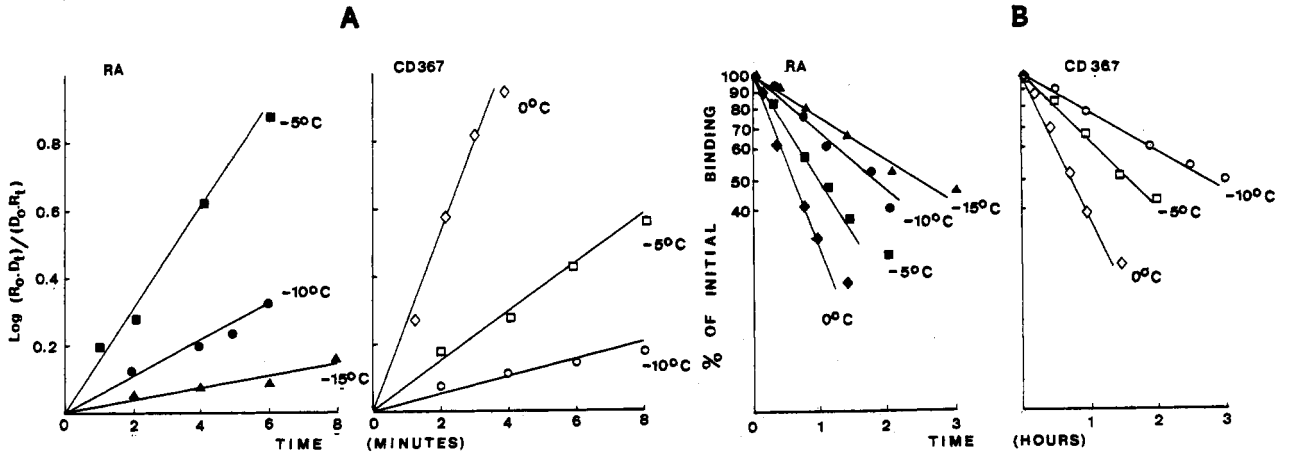

FIGURE 5: Kinetics of [^{3}H]retinoid binding to the recombinant GST-hRARα LBD at different temperatures. (A) Rate of association. Aliquots of bacterial extracts were incubated with 30 nM of the tritiated retinoids (RA or CD367). At times varying from 1 to 8 min, the concentration of specifically bound [^{3}H]retinoid was determined by charcoal assay and used to calculate the concentration of free binding sites R_t and free retinoid D_t at the corresponding times t. (B) Rate of dissociation. Aliquots of the same extracts were incubated with 30 nM [^{3}H]retinoids before addition of a 1000-fold excess of unlabeled retinoids. At various times thereafter, aliquots were withdrawn to determine the amount of specifically bound [^{3}H]retinoid with use of the dextran–charcoal assay.

linear van't Hoff plots with negative slopes were obtained between 0 °C and −15 °C for both ligands (data not shown). Highly positive values were obtained at equilibrium for ΔH (+69 and +57 kJ/mol for RA and CD367, respectively) and ΔS (+433 and +386 J/mol, respectively), indicating that the interaction with the receptor was mainly entropy driven.

Table II: Kinetic Parameters of Retinoid Binding to the GST-hRARα LBD[a]

retinoid	T (°C)	$10^{-6} \times k_a$ ($M^{-1}\cdot min^{-1}$)	$10^2 \times k_d$ (min^{-1})	k_d/k_a (nM)
RA	0	ND[b]	1.98	ND[b]
	−5	22	1.38	0.63
	−10	6.5	0.70	1.08
	−15	2.6	0.47	1.80
CD 367	0	38.4	1.54	0.40
	−5	9.5	0.73	0.77
	−10	3.2	0.40	1.25

[a] k_a and k_d values were calculated from the kinetic data reported in Figure 5. [b] Not determined.

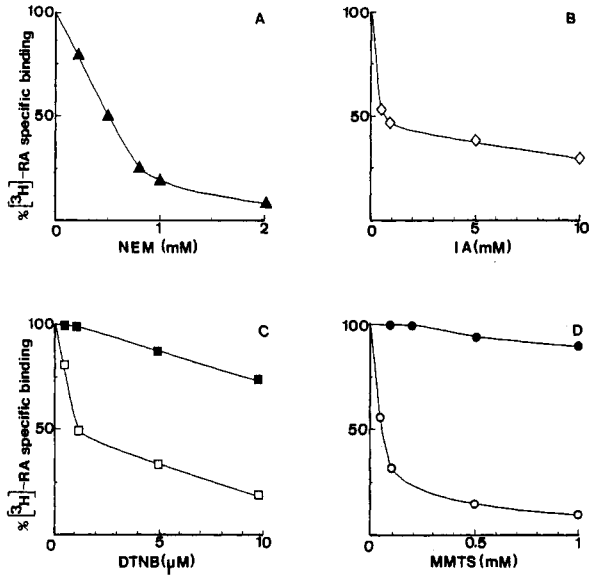

FIGURE 6: Inhibition of [^{3}H]RA binding activity with sulfhydryl reagents. Crude bacterial extracts were incubated for 5 min at 0 °C with varying concentrations of sulhydryl-modifying reagents: NEM (▲), IA (◇), DTNB (□), or MMTS (○). [^{3}H]RA (20 nM) was then added, and specific [^{3}H]RA binding was assayed by charcoal adsorption after a further 16-h incubation at 0 °C. Reversal by βME (■) or by DTT (●) of the DTNB- and MMTS-mediated [^{3}H]-RA binding inhibition is represented on panels C and D, respectively. In both cases, the reducing agent was added at the end of the 5-min preincubation in the presence of DTNB or MMTS.

Exploration on a larger temperature range was precluded by practical limitations: at temperatures higher than 0 °C, association rates were too fast to be accurately measured, and below −15 °C, binding assays gave nonreproducible results, a problem probably related to the increasing viscosity of the incubation medium at very low temperatures.

Effects of Sulfhydryl-Modifying Agents on [^{3}H]RA Binding in Crude Bacterial Extracts. Figure 6 shows the concentration dependence effects of four sulfhydryl reagents on retinoid binding. Among these reagents, DTNB appeared to be the most efficient (50% reduction in RA-specific binding at a 1 μM concentration), whereas IA was less active. The maximal effect was obtained at a DTNB concentration of 100 μM (data not shown) Since IA and NEM are not highly specific for the sulfhydryl groups of cysteine but can also react with the amine groups of lysine, we also used two other reagents, DTNB and MMTS, which are very specific for sulfhydryl groups. The pronounced effect obtained with DTNB and MMTS and its almost complete reversal with reducers like β-mercaptoethanol and dithiothreitol acting through thiol–disulfide interchanges strongly suggests that one or several SH groups are involved in the retinoid-binding activity of hRARα. Moreover, sodium arsenite was also tested in the 0–100 mM range but was inefficient at inactivating the retinoid-binding activity of the receptor (data not shown), suggesting that vicinal dithiols may not be involved in the interaction of the receptor with its retinoid ligand. When crude bacterial extracts were incubated with 20 nM [^{3}H]RA for 2 h and treated with increasing concentrations of NEM (0–2 mM) for 5 min at 0 °C, we did not observe any significant reduction in the retinoid-binding activity. Thus, preformed [^{3}H]RA- hRARα LBD complexes appeared insensitive to NEM treatment, suggesting that the ligand could protect the essential sulfhydryl group(s) from alkylation. This protection effect was further documented by experiments using a retinoid exchange assay as described in experimental procedures: [^{3}H]-RA could be exchanged with unlabeled RA in preformed RA-hRARα LBD complexes, even when these complexes had been submitted to NEM treatment (data not shown). These results suggest that the essential sulfhydryl group(s) could lie in the retinoid binding site itself.

A further indirect evidence sustaining this hypothesis was afforded by preliminary experiments performed with *N*-aryl- and *N*-alkylmaleimides of various chain lengths.

Among them, *N*-nonylmaleimide appeared to be the most efficient (50% reduction in RA-specific binding at about 15 μM), whereas *N*-propyl-, *N*-hexyl-, and *N*-benzylmaleimides were less active, i.e., the same inactivation effect was obtained at about 35 μM for *N*-hexyl- and *N*-benzylmaleimides or at about 200 μM for *N*-propylmaleimide. The higher efficiency of *N*-nonylmaleimide could be explained by hydrophobic interactions between the surroundings of the sulfhydryl group(s) to be alkylated and the side chain of the maleimide (Formstecher *et al.* 1984). This result is compatible with a location of these sulfhydryl group(s) in the retinoid binding groove, which is expected to be globally hydrophobic. However, a precise interpretation of these data needs further work.

DISCUSSION

Availability of substantial quantities of RAR protein appears to be essential for investigating thoroughly their structure and function, particulary their DNA- and ligand-binding domains. Purification of RARs from natural tissues or cells is difficult to achieve because of their low natural abundance (Nervi *et al.* 1989). Therefore, only limited attempts have been reported (Kagechika *et al.* 1988; Sani *et al.* 1990). Recent reports have demonstrated that functional RARs displaying intact DNA and ligand-binding properties (Crettaz *et al.* 1990; Yang *et al.* 1991; Keidel *et al.* 1992) could be expressed in *E. coli*. However, no purification data were reported. Using a fusion expression vector, we have produced in *E. coli* and purified a truncated hRARα including the E and F domains. This choice offers two advantages: (i) the reasonable size of the fusion protein limits problems of insolubility and (ii) a functional truncated receptor represents a valuable tool for further structural studies on the retinoid-binding site. Purification was achieved by using the properties of the affinity tail GST which could be retained on an immobilized glutathione affinity matrix. This system was already used to produce and purify recombinant proteins, but the published data concerning purification and production yield are scarce and incomplete (Smith & Johnson, 1988; Zhu *et al.* 1989; Guan & Dixon, 1991). The hRARα LBD can be cleaved from the fusion protein and still retains its ligand-binding capacity.

The functional properties of the hRARα LBD were examined to determine whether the bacterially-expressed truncated receptor could bind its cognate ligand with the same

affinity as the native receptor. The data obtained are close to those found by others, who expressed the hRARα LBD with a different bacterial expression system (Crettaz *et al.* 1990); but K_d was higher than the values reported by Yang *et al.* (1991) and Keidel *et al.* (1992), who expressed the full-length hRARα in *E. coli*. Thus, the conditions used for ligand-binding measurements which differ throughout these studies can explain these discrepancies. A systematic study of the various technical parameters governing the dextran charcoal assay efficiency confirms this assumption (B. Sablonnière *et al.* unpublished data). Moreover, K_d values calculated with use of the rate constants k_a and k_d determined in kinetic experiments were lower than those obtained by measurement at equilibrium. We therefore believed that k_d/k_a values represent a better estimation of the actual dissociation constant. Of course, the binding kinetic parameters have been measured on the entire GST-hRARα LBD and could not reflect correctly the behavior of the truncated receptor. This choice has been made owing to the high instability of the purified truncated receptor and to the very low yield of the thrombin cleavage of the crude recombinant protein. The K_d value of the full-length hRARα in COS cells was in the same range as that in *E. coli* and was in agreement with previously published values determined from the cloned hRARα expressed in eukaryotic cells (Brand *et al.* 1988; Cavey *et al.* 1990; Delescluse *et al.* 1991). Thus, the differences in posttranslational modifications that occurred in *E. coli* versus eukaryotic cells were not detrimental to the expression of intact receptor molecules.

The fact that ΔS^a is positive for the two tested ligands is further evidence that the driving forces for the formation of the transition state are mainly hydrophobic. Moreover, the higher value for ΔS^a observed in case in CD367 as compared to RA clearly suggest, as expected, that stronger hydrophobic interactions would be involved in CD367 receptor binding. At low temperatures, the retinoid-receptor interaction is mainly entropy driven, as already described with the glucocorticoid receptor (Wolff *et al.* 1978; Eliard & Rousseau, 1984). Binding data reveal that only 1.5 nmol of functional GST-hRARα LBD could be produced per liter of bacterial culture. This production can be compared to those described for the ligand-binding domains of other members of the nuclear receptor family: it is lower than that of the progesterone receptor (Power *et al.*, 1990; Eul *et al.* 1989) but in the same range as those of the androgen (Young *et al.* 1990) and the thyroid hormone receptors (Lin *et al.* 1990). These discrepancies in the expression level could be due either to the expression vector chosen or to differences in posttranslational modifications of the receptor. The peculiar lability of the ligand-binding activity of purified recombinant RARα has already been stressed by others (Keidel *et al.* 1992). However, the expression level obtained here corresponds to a specific-binding activity of 12 pmol/mg from the crude bacterial extract, which is 40–50-fold more than the level found from natural cells (Nervi *et al.* 1989).

Interestingly, hRARα ligand-binding activity appears to be sensitive to sulfhydryl-modifying reagents. These results clearly suggest that cysteines are involved in the binding of RA by the hRARα ligand-binding domain. RAR susceptibility toward sulfhydryl-modifying reagents has already been mentioned by Sani *et al.* (1990), who reported that the [^{3}H]-RA binding activity of RARs from chick skin could be completely inhibited by *p*-(chloromercuri)benzenesulfonic acid. Sulfhydryl-modifying agents have also been reported to inhibit the ligand-binding activity of CRABP (Sani & Banerjee, 1978) and of several members of the steroid receptor superfamily (Jansen *et al.* 1967; Young *et al.* 1975; Coty, 1980). The involvement of vicinal dithiol (Simons *et al.* 1990) and the role of a putative intramolecular disulfide bond (Bresnick *et al.* 1988) have been evoked for the glucocorticoid receptor. Direct identification of modified cysteine residues in the ligand-binding domain has been achieved by affinity labeling for the estrogen and glucocorticoid receptors (Simons *et al.* 1987; Carlstedt-Duke *et al.* 1988; Harlow *et al.* 1989) and further documented by the study of receptor mutants (Byravan *et al.* 1991). Contrasting with this clear demonstration of the paramount importance of thiol groups in the ligand-binding activity of steroid receptors, the study of the role of these groups in RAR function is just beginning. Our data suggest that an overall hydrophobicity of the sulfhydryl-modifying agent is necessary to improve its binding to the receptor. Thus, an essential thiol group of the receptor located in a short proximity or inside the ligand-binding site is probably responsible for the observed results. These results are reminiscent of those previously observed with the glucocorticoid receptor (Formstecher *et al.* 1984). The hRARα LBD contains six cysteine residues, and work is now in progress to identify further their respective implication in RA binding.

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