A Monoclonal Antibody to the Estrogen Receptor Discriminates between the Nonactivated and Activated Estrogen- and Anti-Estrogen–Receptor Complexes†

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ABSTRACT: An IgM-class monoclonal antibody (B_{36}) [Greene, G. L., Fitch, F. W., & Jensen, E. V. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 157–161] raised against the calf uterine estrogen receptor (R) was used to probe the structure of R bound to estradiol or to 4-hydroxytamoxifen, a nonsteroidal anti-estrogen which displays a high affinity for R. This antibody does not noticeably modify the interaction of R with these ligands, but R, when bound to B_{36}, is markedly displaced in both low- and high-salt sucrose gradients. We found the following: (1) The B_{36} antibody interacts more strongly with activated cytosol estradiol- and 4-hydroxytamoxifen–R complexes than with nonactivated (molybdate-stabilized) complexes. (2) This antibody also interacts strongly with the nuclear forms of R bound to estradiol or to the anti-estrogen. (3) The affinity of B_{36} for the nonactivated R–4-hydroxytamoxifen complex is 3-fold greater than for the nonactivated R–estradiol complex. The difference is slightly less pronounced for activated complexes. (4) Preincubation of activated R with saturating amounts of B_{36} partially (≤60%) inhibits the binding of R–ligand complexes to DNA adsorbed onto cellulose. These results suggest that the B_{36} and DNA binding domains of R are related and strengthen the hypothesis that R has different external structures when activated or nonactivated and when bound to an anti-estrogen or to estradiol.

The hormone-induced process which transforms the estrogen receptor (R) into an "activated" form able to be retained in the nucleus and trigger hormonal responses is not understood in molecular terms. This is mainly because native R has not been purified, which would allow physicochemical studies to be performed in the presence or absence of estradiol. However, several approaches have been used recently to study the activation of R. Sodium molybdate is known to stabilize steroid receptors in forms that are unable to undergo activation or to bind DNA and nuclei (Leach et al., 1979; Nishigori & Toft, 1980). High-affinity anti-estrogens such as 4-hydroxytamoxifen are partial (Jordan et al., 1977) or full estrogen antagonists (Westley & Rochefort, 1980) in mammalian systems even though their affinity for R is similar to that of estradiol (Borgna & Rochefort, 1980). On the basis of modification of the hormone (Weichman & Notides, 1977) and DNA (Yamamoto & Alberts, 1972; Andrē & Rochefort, 1973) binding sites of R during activation, we have proposed that R has a different conformation when bound to anti-estrogen rather than estradiol (Rochefort & Borgna, 1981; Evans et al., 1982; Rochefort et al., 1983). One important advance in the study of R has been the preparation of specific monoclonal antibodies (Greene et al., 1980a,b; Montcharmont et al., 1982). These antibodies allow R to be characterized not only in terms of its steroid or DNA binding but also in terms of its specific antigenic determinants. Antibodies to the calf uterine R displace the R–4-hydroxytamoxifen complex in sucrose gradients (Garcia et al., 1982), and these antibodies can therefore be used to compare R bound to estrogens or to anti-estrogens. In this study, we have used the B_{36} monoclonal antibody prepared against the purified calf uterine nuclear R (Greene et al., 1980a) in an attempt to answer the following questions: (1) Is this antibody able to discriminate between the nonactivated (molybdate-stabilized) and activated R? (2) Is it able to discriminate between the R–estradiol and R–4-hydroxytamoxifen complexes? (3) Does this antibody interfere with the binding of R to DNA?

Materials and Methods

Materials. (*Z*)-[^{3}H]-4-Hydroxytamoxifen (specific activity 42 Ci/mmol; radiochemical purity >90%) was purified (J-L. Borgna, unpublished results) from the *Z*–*E* mixture donated by Dr. A. H. Todd (ICI Laboratories, Macclesfield, England). The *Z* and *E* isomers of [^{3}H]-4-hydroxytamoxifen were acetylated, separated on a Merck analytical thin-layer silica gel plate, and subsequently hydrolyzed by K_2CO_3 in methanol. [6,7-^{3}H]Estradiol (specific activity 50 Ci/mmol; radiochemical purity >95%) was purchased from CEA (Gif-sur-Yvette, France). Preparations of B_{36} monoclonal antibody (40% ammonium sulfate precipitate from the culture medium of a secreting hybridoma cell line) raised against the purified calf uterine nuclear R (Greene et al., 1980a) were kindly provided by Drs. G. L. Greene and E. V. Jensen (Ben May Laboratory, Chicago, IL). They were used either immediately or after purification on Bio-Gel A 1.5m (Greene et al., 1980a).

Cytosol Estrogen Receptor: Preparation and Assay. Cytosol from fresh or frozen (−80 °C) immature calf or rat uteri was prepared in buffer T [10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.4] as described previously (Capony & Rochefort, 1975). Cytosol was diluted with 5% buffer T or with 200 mM Na_2MoO_4 in buffer T, and then 2 nM [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen was added with or without 1 μM nonradioactive estradiol. Stock solutions of ligands were in ethanol (final ethanol concentration in cytosol = 1%). Aliquots were incubated for 2–4 h at 0 °C and then for 0.5 h at 25 °C. In some cases, the 25 °C incubation was omitted. Aliquots were then treated with dextran-coated charcoal at 0 °C for 0.5–2 h as described previously (Borgna & Rochefort, 1980). The radioactivity in the charcoal-treated samples was measured, and the specific

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binding of ^{3}H-labeled ligands was determined according to Blondeau & Robel (1975).

Nuclear Estrogen Receptor. KCl nuclear extracts were prepared after incubation of fresh calf uterine slices in Dulbecco's modified Eagle's medium [containing 10 nM [^{3}H]-estradiol or 75 nM [^{3}H]-4-hydroxytamoxifen] for 2 h at 37 °C in an O_2/CO_2 (95:5) atmosphere. The tissues were first rinsed with fresh medium and then with buffer T. They were homogenized in buffer T. The crude nuclear pellets (10^3g for 10 min) were washed with buffer T, resuspended in buffer TK (10 mM Tris-HCl–400 mM KCl, pH 7.4), left 0.5 h at 0 °C, and then centrifuged (10^5g for 1 h) to obtain the KCl nuclear extracts. Radioactivity was measured before and after 1 h of charcoal treatment at 0 °C.

Interaction of the Estrogen Receptor with B_{36} Antibody: Sucrose Gradient Analysis. Aliquots of uterine cytosol and nuclear extracts labeled with ^{3}H-labeled ligands that had been treated (cytosol) or not treated (nuclear extracts) with charcoal were incubated for 2–4 h at 0 °C with varying amounts of the B_{36} monoclonal antibody. Samples were mixed with ^{14}C-labeled marker proteins (bovine serum albumin and bovine globulin) and layered onto 10–30%, 10–40%, or 10–50% sucrose gradients prepared in TE buffer [10 mM Tris-HCl–1.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] or TKE buffer (10 mM Tris-HCl, 400 mM KCl, and 1.5 mM EDTA, pH 7.4) with or without 5 mM Na_2MoO_4. Low-salt gradients were centrifuged for 13.5 h at $150000g_{av}$. High-salt gradients were centrifuged for 14 h at $190000g_{av}$. The gradients were fractionated from the bottom of the tube, and the radioactivity present in each fraction and on the bottom of the tube was counted in 3 mL of Bray's mixture.

DNA–Cellulose Binding. Aliquots (300 μL) of charcoal-treated cytosol incubated with or without B_{36} were agitated with 200 μL of cellulose or DNA–cellulose [prepared according to Alberts & Herrick (1971)] for 12–17 h at 0 °C. After centrifugation, the supernatants were discarded, and the pellets were washed twice with 3 mL of TE buffer. The radioactivity in the final pellets was extracted with 2 × 1.5 mL of ethanol and counted. Specific binding to DNA was defined as the difference between binding to DNA–cellulose and to cellulose alone as described elsewhere (Evans et al., 1982).

Miscellaneous. Protein concentrations were measured spectrophotometrically (Layne, 1957). DNA was measured according to Burton (1956). The radioactivity of the samples was counted either in 3 mL of Bray's mixture or in 3 mL of ethanol + 10 mL of a 2,5-diphenyloxazole (PPO)–1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP)–toluene mixture by using a Kontron-Intertechnique SL 30 scintillation spectrophotometer with efficiencies of 40% (Bray's mixture) and 25% (PPO/POPOP/toluene mixture). The statistical comparisons of the K_A's calculated for the interaction of the various forms of R with B_{36} and of the percentages of inhibition for R–DNA binding by B_{36} were performed by using the nonparametric test of Mann & Withney (1947).

Results

B_{36} Antibody Interacts More Strongly with the Activated Cytosol Estrogen Receptor Than with Its Molybdate-Stabilized Form. To study the effect of the B_{36} monoclonal antibody on the interaction of ligand with R, kinetic and equilibrium studies were performed using calf uterine cytosol preincubated with or without saturating amounts of B_{36}. B_{36} did not noticeably affect either the kinetics of binding (Figure 1a) or the binding at equilibrium (Figure 1b) of 4-hydroxytamoxifen to R since the number of binding sites and

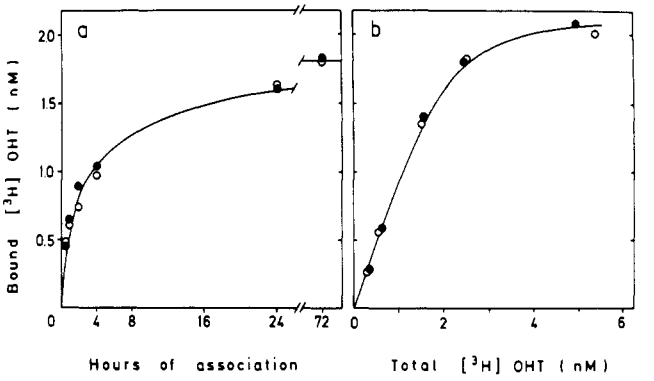

FIGURE 1: Binding of 4-hydroxytamoxifen to the estrogen receptor in the presence or absence of B_{36} antibody. Calf uterine cytosol was incubated with (○) or without (●) 200 μg of B_{36}/mL for 3 h at 0 °C. Then aliquots were incubated at 0 °C with [^{3}H]-4-hydroxytamoxifen in the presence or absence of 1 μM radioinert estradiol. Binding to R was assayed by charcoal adsorption after a 1.5-h incubation at 0 °C and calculated according to Blondeau & Robel (1975). (a) Time course of saturable binding with 2.5 nM [^{3}H]-4-hydroxytamoxifen. (b) Saturation experiment performed at binding equilibrium (72 h) with 0.3–5 nM concentrations of [^{3}H]-4-hydroxytamoxifen.

the affinity as measured by the charcoal assay were similar in the presence and absence of B_{36}. The same results were obtained for estradiol (not shown).

The reactivity of the activated or molybdate-stabilized calf uterine cytosol R–estradiol complexes with the B_{36} monoclonal antibody was studied by sucrose gradient analysis. The IgM-class B_{36} antibody markedly displaced the R–ligand complex, thus allowing easy separation and quantification of the free and B_{36}-bound R–estradiol complexes in low- and high-salt sucrose gradients (Figure 2). In low-salt gradients, the sedimentation coefficient of the 8S activated or molybdate-stabilized R–estradiol complexes (Figure 2a) was increased to 14–15 S (Figure 2b) while in high-salt gradients the sedimentation coefficient of the 4.5S R–estradiol complexes (Figure 2c) was increased to 12–13 S (Figure 2d). We conclude that the heavy peaks resulted from a specific interaction of the R–estradiol complex with the B_{36} antibody because of the following observations: (1) The sedimentation of the rat R–estradiol complex which does not interact with B_{36} (Greene et al., 1980a) was not altered by the B_{36} antibody (not shown). (2) The shift was not the result of aggregation of R (Figure 5d). Using unlabeled estradiol, we verified that the nonspecifically bound estradiol did not interfere with the different peaks found in the sucrose gradients since it was recovered only as free estradiol at the top of the gradient. B_{36} interacted more with the activated R than with the molybdate-stabilized R (Figure 2b,d): the same concentration of the antibody increased the sedimentation coefficient of almost all the activated R whereas only a small proportion of the molybdate-stabilized R was affected. Higher concentrations of B_{36} were required to displace all the molybdate-stabilized R (not shown). The reactivity of the R–estradiol complex from unwarmed cytosol was similar to that of the molybdate-stabilized warmed complex, and the activation of R appeared to be irreversible since molybdate did not alter the degree of interaction with the B_{36} antibody when added after warming (not shown). Whether KCl was added to the incubation medium or only to the sucrose gradients did not affect the interaction of B_{36} with R. Comparison of panels b and d of Figure 2 suggests that KCl decreases the interaction of B_{36} and R–estradiol complexes. These two experiments, however, were performed separately; analysis of the B_{36}–R–estradiol complexes in single experiments did not confirm this result. We conclude that the

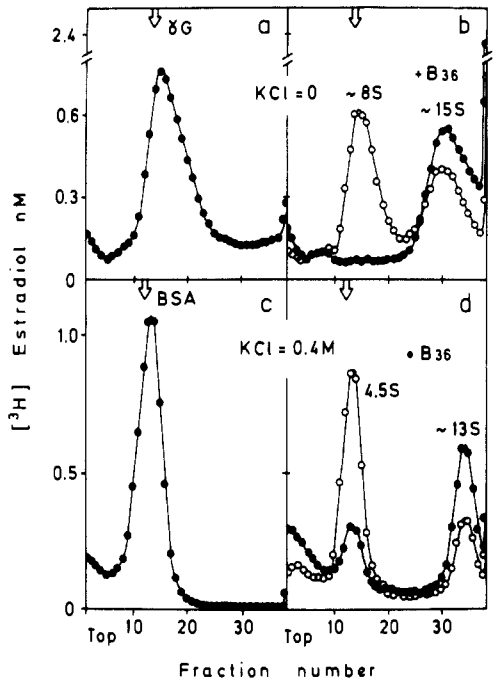

FIGURE 2: Effect of estrogen receptor activation on its interaction with B_{36} antibody. Calf uterine cytosol [(a,b) 3.3 mg of protein/mL; (c, d) 4.7 mg of protein/mL] with (○) or without (●) 10 mM Na_2MoO_4 was incubated with 2 nM [^{3}H]estradiol for 3 h at 0 °C and then warmed at 25 °C for 0.5 h. After treatment with charcoal (1 h at 0 °C), 200-μL aliquots were incubated for 2 h at 0 °C with 300 (b) or 250 μg/mL (d) B_{36} antibody, or without antibody (a, c), and subsequently centrifuged in 10–30% sucrose gradients prepared in TE (a, b) or TKE (c, d) buffer containing 5 mM Na_2MoO_4. Gradient fractions were counted in Bray's mixture. After charcoal treatment, the saturable binding of [^{3}H]estradiol accounted for more than 95% of the radioactivity in the samples. ^{14}C-Labeled bovine γ-globulin (γG, 7.7 S) and bovine serum albumin (BSA, 4.5 S) were used as internal markers.

B_{36} antibody reacts more strongly with the activated R–estradiol complex than with the molybdate-stabilized complex.

Cytosol and Nuclear Estrogen Receptor–4-Hydroxytamoxifen Complexes Interact with the B_{36} Antibody. Both the molybdate-stabilized and the activated R–4-hydroxytamoxifen complexes prepared from calf uterine cytosol reacted with B_{36}. As observed for the R–estradiol complexes, the sedimentation coefficient of the R–4-hydroxytamoxifen complexes was increased by the B_{36} antibody from 8 to 14–15 S (low-salt gradient, Figure 3) and from 4–5 to 12–13 S (high-salt gradient, not shown). The 12–15S forms observed after the addition of B_{36} corresponded to B_{36}–R–4-hydroxytamoxifen complexes, since they were not observed in the presence of an excess of nonradioactive estradiol. The 4-hydroxytamoxifen that was bound nonspecifically sedimented exclusively as a 4.5S peak in both low- and high-salt sucrose gradients in the presence and absence of B_{36}. The interaction of B_{36} with the R–4-hydroxytamoxifen complex was specific for the calf R since the sedimentation of the rat R–4-hydroxytamoxifen complex was not affected by the B_{36} antibody (not shown). As observed for estradiol, the R–4-hydroxytamoxifen complex reacted more strongly with B_{36} when heat activated than when stabilized by molybdate (Figure 3b). All of the R–4-hydroxytamoxifen complexes could be displaced by the B_{36} antibody, indicating that the totality of the saturable antiestrogen binding was to R under these conditions. We then measured the ability of B_{36} to react with nuclear R formed by incubation of uterine slices with [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen in vitro. Low concentrations of B_{36} increased the sedimentation coefficient of all the 4.5S R com-

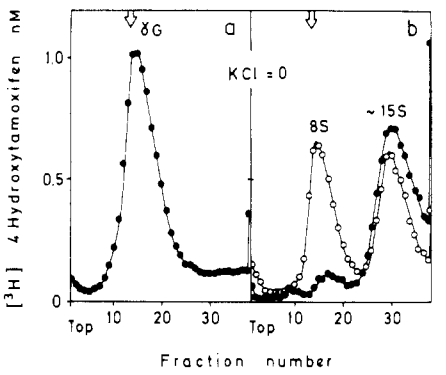

FIGURE 3: Interaction of the estrogen receptor–4-hydroxytamoxifen complexes with B_{36} antibody: effect of receptor activation. Experiments similar to those described in Figure 2a,b were performed in low-salt sucrose gradients using 2 nM [^{3}H]-4-hydroxytamoxifen instead of [^{3}H]estradiol. (a) Cytosol incubated without B_{36}. (b) Cytosol incubated with 300 μg/mL B_{36}. (●) Cytosol without molybdate. (○) Cytosol with molybdate. After charcoal treatment, the saturable binding of [^{3}H]-4-hydroxytamoxifen accounted for more than 95% of the radioactivity in the samples.

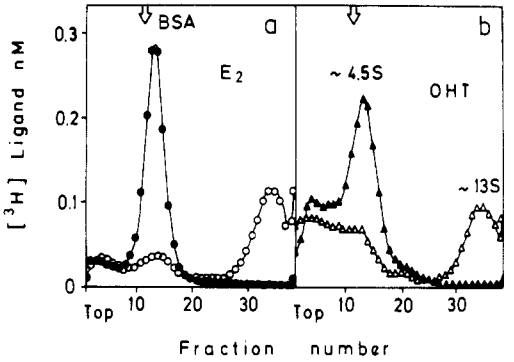

FIGURE 4: Interaction of the nuclear estrogen receptor with B_{36} antibody. KCl nuclear extracts (1.4 mg of protein/mL) prepared after in vitro incubation of calf uterine slices with 10 nM [^{3}H]estradiol (a) or 75 nM [^{3}H]-4-hydroxytamoxifen (b), as described under Materials and Methods, were then incubated with (○, △) or without (●, ▲) 100 μg/mL B_{36} antibody for 2 h at 0 °C. Aliquots were analyzed in 10–30% sucrose gradients prepared in TKE buffer. On the basis of the charcoal-resistant radioactivity, the specific binding in nuclear extracts was about 70% for [^{3}H]estradiol and 55% for [^{3}H]-4-hydroxytamoxifen. The nonspecific binding of 4-hydroxytamoxifen is located only in the 4–5S region (see Figure 5).

plexes to ~13 S (Figure 4a,b), suggesting that both estradiol– and 4-hydroxytamoxifen–nuclear R complexes react better with B_{36} than the corresponding activated cytosol complexes.

Differences between the Interactions of the B_{36} Antibody with the Estrogen Receptor Bound to Estradiol or to 4-Hydroxytamoxifen. The R–estradiol and R–4-hydroxytamoxifen complexes were then compared by using the B_{36} antibody as a probe (Figure 5). The reactivities of activated and nonactivated estradiol– and 4-hydroxytamoxifen–R complexes were estimated from the ratio B_{36}-bound R/unbound R. Since the B_{36}–R complexes sedimented nearly at the bottom of the tube in 10–30% sucrose gradients (Figure 5a,b), we also used 10–40% and 10–50% sucrose gradients (Figure 5d,e) to show that there are no nonspecific aggregates. In all cases, similar differences were found between the reactivities of the R–estradiol and R–4-hydroxytamoxifen complexes. B_{36} reacted with higher affinity with the nonactivated 4-hydroxytamoxifen–R complex than with the corresponding estradiol–R complex. The difference persisted but was less marked for the activated complexes. The 13–15S peak clearly represents the B_{36}–R–4-hydroxytamoxifen complex since there is no nonspecifically bound 4-hydroxytamoxifen in this region

Table I: Relative Affinities of Estrogen Receptor for B_{36} Antibody: Effect of Activation and Binding to Estradiol or 4-Hydroxytamoxifen[a]

expt	B_{36} concn (μg/mL)	rel affinities[b]				factor of increase by activation[c]	
		R_i-E_2	R_i-OHT	R_a-E_2	R_a-OHT	E_2	OHT
1	25	1		5.3	6.9	5.3	
2	80*	1	4.4	4.3	14.2	4.3	3.2
3	80*	1	2.9	4.9	16.5	2.9	5.7
4	80*	1	2.8	3.5	9.5	3.5	3.4
5	200	1	2.7	5.1	8.0	5.1	3.0
6	250	1		4.8		4.8	
7	250	1	2.6				
8	250	1	3.0				
9	250	1	2.6				
10	300	1		4.5		4.5	
		1[d]	3.0[d,e]	4.6[d,f]	11.0[d-f]	4.6[d]	3.7[d,e]

[a] Cytosols with or without molybdate were incubated with 2 nM [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen at 0 °C and then for 0.5 h at 25 °C. After charcoal treatment, aliquots were incubated with varying amounts of crude or purified (*) B_{36} antibody for 2–3 h at 0 °C. The concentrations of B_{36}-bound and unbound R-^{3}H-labeled ligand complexes were then measured in 10–30% (experiments 1, 2, and 5–10), 10–40% (experiment 3), or 10–50% (experiment 4) sucrose gradients prepared in TKE buffer and containing 5 mM Na_2MoO_4 as described in Figures 2 and 3. For the different R-^{3}H-labeled ligand complexes, the ratio of B_{36}-bound R to unbound R was calculated and taken as the measure of the relative affinity of the R complexes for B_{36}. [b] Relative affinities of the different forms of R complexes for B_{36} were standardized by taking the relative affinity for the molybdate-stabilized R-[^{3}H]estradiol complex (R_i-E_2) as 1 in the same experiment. R_i (nonactivated R) and R_a (activated R) correspond, respectively, to the cytosol R warmed in the presence or absence of 10 mM Na_2MoO_4. [c] Factor of increase in the affinity of R complexes for B_{36} antibody elicited by activation. It was calculated as the ratio of the relative affinities of B_{36} for R_a and R_i as measured in the same experiment. [d] Mean values. [e] According to Mann and Withney's test, this value was significantly ($p < 0.01$) greater than the value for the corresponding R-E_2 complex. [f] Significantly ($p < 0.01$) greater than the values for the nonactivated complexes.

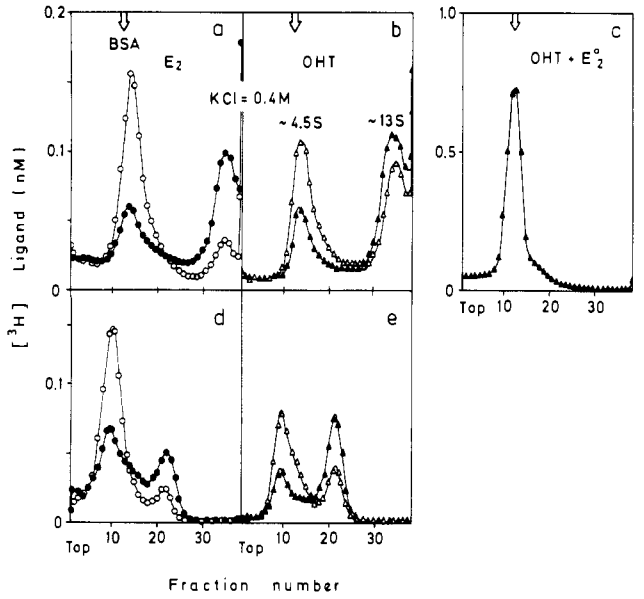

FIGURE 5: Relative reactivities to B_{36} antibody of the estrogen receptor bound to estradiol or to 4-hydroxytamoxifen. Calf uterine cytosol [(a–c) 5.1 mg of protein/mL; (d, e) 1.7 mg of protein/mL] with (○, △) or without (●, ▲) molybdate was incubated for 2 h at 0 °C with 2 nM [^{3}H]estradiol or 2 nM [^{3}H]-4-hydroxytamoxifen in the presence or absence of 1 μM radioinert estradiol, and R was activated for 0.5 h at 25 °C. Aliquots treated (a, b, d, e) or untreated (c) with charcoal (0.5 h at 0 °C) were incubated with 200 (a–c) or 80 μg/mL (d, e) B_{36} antibody for 3 h at 0 °C and then analyzed in 10–30% (a–c) or 10–50% (d, e) sucrose gradients prepared in TKE buffer containing 5 mM Na_2MoO_4. (a, b, d, e) Saturable binding or [^{3}H]estradiol (a, d) and [^{3}H]-4-hydroxytamoxifen (b, e). After charcoal treatment, the concentrations of the R–ligand complexes were 0.95 (a, b) and 0.72 nM (d, e) for both ligands, and the percentages of nonspecific binding were inferior to 10% and 20% for estradiol and 4-hydroxytamoxifen, respectively. (c) Unsaturable binding of [^{3}H]-4-hydroxytamoxifen measured in the presence of radioinert estradiol.

(Figure 5c). In high-salt gradients, the amount of specifically bound 4-hydroxytamoxifen sedimenting at 4.5 S was determined by subtracting the amount of 4-hydroxytamoxifen bound nonspecifically from the total bound 4-hydroxytamoxifen.

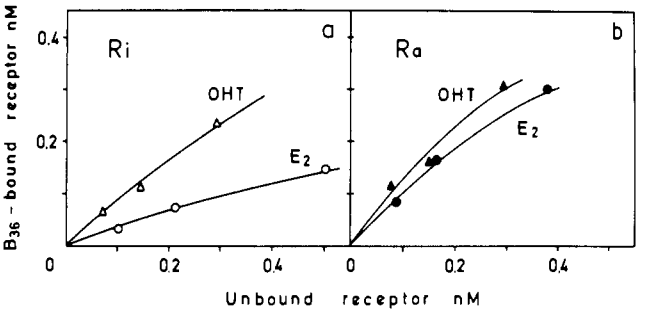

FIGURE 6: Initial saturation of B_{36} antibody by the estrogen receptor bound to estradiol or to 4-hydroxytamoxifen. Calf uterine cytosol [(a) 4.3 mg of protein/mL; (b) 5.0 mg of protein/mL] in the presence (a) or absence (b) of 10 mM Na_2MoO_4 was incubated for 3.5 h at 0 °C with 2 nM [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen and then warmed at 25 °C for 0.5 h. After charcoal treatment (1 h at 0 °C), increasing volumes (50, 100, and 200 μL) of cytosol were incubated for 2 h at 0 °C in a final volume of 220 μL with a constant concentration of B_{36} [(a) 250 μg/mL; (b) 25 μg/mL]. Aliquots were analyzed in 10–30% sucrose gradients prepared in TKE buffer containing 5 mM Na_2MoO_4 as in Figure 5. The radioactivity of the 13S peak was taken as the B_{36}-bound R. The radioactivity of the 4.5S peak as corrected for nonsaturable binding of ligands (determined with 1 μM radioinert estradiol) was taken as the unbound R. This nonspecific binding accounted in both cases for less than 6% of the total radioactivity. The concentrations of B_{36}-bound R-^{3}H-labeled ligand complexes are plotted against the concentrations of unbound R-^{3}H-labeled ligand complexes. (a) R_i = molybdate-stabilized R. (b) R_a = activated R. (○, ●) R-[^{3}H]estradiol complexes. (△, ▲) R-[^{3}H]-4-hydroxytamoxifen complexes. The relative affinities calculated from these experiments are given in Table I [(a) experiment 7; (b) experiment 1].

To estimate the association constant (K_A) for the interaction of B_{36} with the various complexes, increasing concentrations of R–ligand complexes were incubated with a constant amount of the B_{36} antibody. The amount of complex bound to B_{36} increased with the concentration of unbound complex. Figure 6 shows the saturation curves obtained from two different experiments using either molybdate-stabilized (a) or activated complexes (b). For the nonactivated complexes (R_i), the slope was steeper for the R–4-hydroxytamoxifen complex than for the R–estradiol complex. For the activated complexes (R_a),

Table II: Decrease of the Estrogen Receptor-DNA Interaction by B_{36} Antibody[a]

expt	preactivation at 25 °C	[B_{36}] (μg/mL)	[DNA] (mg/mL)	R cpm input		DNA-bound R cpm (% input)		rel DNA binding	
				R-E_2	R-OHT	R-E_2	R-OHT	R-E_2	R-OHT
1	−	0	1.20	4070		2370 (58)		100	
	−	80	1.20	4070		1890 (46)		79	
2	+	0	0.30	7180		1120 (16)		100	
	+	200	0.30	7180		400 (6)		38	
3	+	0	0.70	9730		2120 (22)		100	
	+	240	0.70	9730		920 (9)		43	
	+	210*	0.70	9730		910 (9)		43	
4	+	0	0.66	9030		2460 (27)		100	
	+	260	0.66	9030		1260 (14)		52	
5a	+	0	0.70	8830		1860 (21)		100	
	+	210*	0.70	8830		960 (11)		52	
5b	+	0	0.70	10230		1370 (13)		100	
	+	210*	0.70	10230		1380 (13)		100	
6	+	0	0.12	15710	12260	1890 (12)	1480 (12)	100	100
	+	180	0.12	15710	12260	1100 (7)	1100 (9)	58	75
7a	+	0	0.85	17630		3850 (22)		100	
	+	240	0.85	17630		1430 (8)		57	
7b	−	0	0.85	16020	12420	4860 (30)	3150 (25)	100	100
	−	240	0.85	16020	12420	1640 (10)	2360 (19)	37	76
8	+	0	0.10	10890	13010	2480 (12)	1620 (12)	100	100
	+	260	0.10	10890	13010	1570 (8)	1220 (9)	66	75
		0[b]						100[b]	
		+[b]						47[b,d]	
		0[c]						100[c]	100[c]
		+[c]						49[c]	75[c,e]

[a] Calf or rat (experiment 5b) uterine cytosols labeled with 2 nM [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen either for 3 h at 0 °C or for 2.5 h at 0 °C and then 0.5 h at 25 °C were treated with a charcoal suspension for 1 h at 0 °C. The specific binding of ^{3}H-labeled ligands accounted for more than 95% (estradiol) and more than 80% (4-hydroxytamoxifen) of the radioactivity of charcoal-treated cytosols. Aliquots (300 μL) were incubated with or without varying amounts of crude or purified (*) B_{36} antibody (80–260 μg/mL) for 2–3 h at 0 °C, then added to a 200-μL suspension of cellulose or DNA-cellulose (0.10–1.20 mg of DNA/mL), and agitated at 0 °C for 12–17 h. After centrifugation, the cellulose and DNA-cellulose pellets were washed twice with TE buffer (3 mL), and then their radioactivity was extracted with 2 × 1.5 mL of ethanol and counted. Residual DNA was measured in the pellets by Burton's method. Specific binding to DNA was calculated as the difference between the radioactivities bound to DNA-cellulose and to cellulose. The amount of R-[^{3}H]estradiol (R-E_2) and R-[^{3}H]-4-hydroxytamoxifen (R-OHT) bound to DNA is expressed as cpm and also as a percentage of the amount of complex incubated with DNA. The relative binding to DNA of R-^{3}H-labeled ligand complexes preincubated with B_{36} is also calculated by taking the value for DNA binding to complexes not incubated with B_{36} as 100%. [b] Mean percentage of the binding of calf uterine complexes to DNA for experiments 1–8 (R-E_2). [c] Mean percentage of the binding of calf uterine complexes to DNA for experiments 6–8 (R-E_2 and R-OHT). [d] According to Mann and Withney's test, this value was significantly ($p < 0.01$) less than the 100% value corresponding to the binding of the R-E_2 complex to DNA in the absence of B_{36}. [e] Not significantly greater than the 49% value corresponding to the binding of the R-E_2 complex to DNA in the presence of B_{36}.

the mean difference calculated from a series of experiments (Table I) was more marked than that shown in Figure 6b. The relative affinities of the R complexes for B_{36} were calculated from the initial slopes of the saturation curves (Figure 6). These calculations assume the following: (i) The binding sites of B_{36} for the R are equivalent and independent; thus, the slope at the origin of the saturation curves is equal to K_aN (N being the binding capacity of B_{36} for R). (ii) For a constant ionic strength, N is the same for the activated and molybdate-stabilized R and for estrogen- and anti-estrogen–R complexes; thus, the slopes of the saturation curves are relative measures of the K_A values. We have further verified by gel filtration and ultracentrifugation in sucrose gradients that the Stokes radius and the sedimentation constant of the calf uterine R were not altered during the activation process. This suggests that dimerization of R did not occur under the conditions used in these experiments and therefore that N is the same before and after activation. To normalize the values calculated from the different experiments, the affinity of the molybdate-stabilized R–estradiol complex was taken as 1.

As shown in Table I, the affinity of B_{36} for activated R (R_a) was significantly greater than that for molybdate-stabilized R (R_i) in high-salt sucrose gradients, whatever ligand bound to R. The affinities of the two R–4-hydroxytamoxifen (OHT) complexes (R_i–OHT and R_a–OHT) were significantly higher than those of the corresponding R–estradiol (E_2) complexes (R_i–E_2, and R_a–E_2). The affinities for B_{36} increased in the order R_i–$E_2 < R_i$–OHT $< R_a$–$E_2 < R_a$–OHT, the mean values for the relative affinities being 1, 3.0, 4.6, and 11.0, respectively. The affinity of the molybdate-stabilized R–4-hydroxytamoxifen complex was approximately 3.0-fold higher than that of the corresponding R–estradiol complex. The affinity of the activated anti-estrogen complex for B_{36} was ~2.4-fold higher than that of the estradiol complex.

B_{36} Antibody Inhibits the Binding of Receptor–Estradiol and Receptor–4-Hydroxytamoxifen Complexes to DNA. In an attempt to localize the relative positions of the B_{36} and DNA binding domains on the R, we studied the interaction of R complexes with double-stranded calf DNA in the presence or absence of B_{36} antibody. The R–DNA complex was measured by adsorption of the R onto DNA–cellulose with or without pretreatment of the cytosol R with the B_{36} antibody at binding equilibrium (≥12 h at 2 °C) as described previously (Evans et al., 1982). In the case of the R–E_2 complex, a marked and significant ($p < 0.01$) decrease of the proportion of complex bound to DNA was observed after preincubation with crude or purified preparations of B_{36} (Table II). Preincubation of the R–estradiol complex by heating the cytosol at 25 °C decreased binding of the complex to DNA as observed previously (Andrê, 1977). The B_{36} antibody decreased binding of the R–estradiol complex to DNA whether or not the complex was preactivated. The inhibition was not

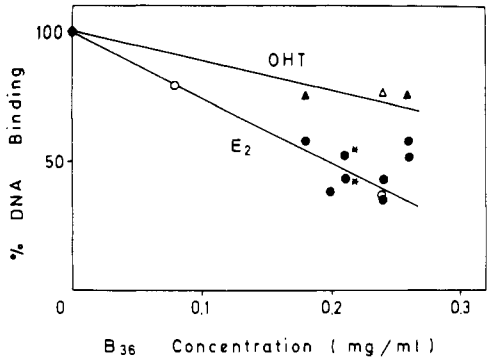

FIGURE 7: Effect of B_{36} antibody on DNA binding of the estrogen receptor. The relative DNA binding of the R-^{3}H-labeled ligand complexes after interaction with varying concentrations of crude or purified (*) preparations of B_{36} was evaluated by the DNA-cellulose assay as described in Table II and is plotted against the concentration of B_{36} used. The 100% value corresponds to the DNA binding of R complexes preincubated without the B_{36} antibody. (●) Preactivated and (○) nonpreactivated R-estradiol complexes; (▲) preactivated and (△) nonpreactivated R-4-hydroxytamoxifen complexes.

overcome by increasing the time of incubation, suggesting that it was not due to a delay in reaching equilibrium. This inhibition was dependent on the concentration of B_{36} and appeared slightly more pronounced when R had been activated in the presence of estradiol rather than 4-hydroxytamoxifen (Figure 7). However, the difference observed between the two complexes is not statistically significant because of the limited number of experiments performed with 4-hydroxytamoxifen. B_{36} antibody concentrations which altered the sedimentation of all R complexes in sucrose gradients only partially inhibited ($\simeq$60%) DNA binding, indicating that a quaternary complex, B_{36}-(R-ligand)-DNA, could be formed. The inhibition appeared to be specifically due to the B_{36} antibody, since the addition of equal concentrations of nonspecific antibodies (IgG) did not decrease the binding of the R-estradiol complex to DNA. In addition, the B_{36} antibody, which does not interact with the rat uterine R (Greene et al., 1980a), did not affect the DNA binding of the rat R-estradiol complex (Table II). These results indicate that B_{36} did not prevent binding of R complexes to DNA by a nonspecific mechanism, for instance, partial proteolysis. We, therefore, conclude that the B_3 antibody inhibited DNA binding as a result of a specific interaction with R-ligand complexes.

Discussion

This study, using the B_{36} monoclonal antibody that was raised against the calf uterine R-estradiol complex, has provided three series of results: (1) The antibody interacts more strongly with the activated cytosol R than with the molybdate-stabilized R. This is not surprising as this antibody was raised against an activated nuclear R and is consistent with the finding that the B_{36} antibody interacts more strongly with the nuclear R than with the cytosol R (Greene et al., 1980a). (2) The antibody specifically recognizes the cytosol R and the nuclear R whether bound to estrogen or anti-estrogen, thus allowing a comparison between the reactivities of the two types of complexes to be made. Two differences were found between the interaction of B_{36} with the R-estradiol and the R-4-hydroxytamoxifen complexes. First, the molybdate-stabilized cytosol R interacts more strongly with B_{36} when bound to 4-hydroxytamoxifen than when bound to estradiol. The reason for this greater interaction of B_{36} antibody with the R-4-hydroxytamoxifen complex is not known but is probably related to specific antigen-antibody recognition since the rat R-4-hydroxytamoxifen complex does not interact with B_{36}. Second, in high concentrations of salt, the activation step increases the reactivity of the R-4-hydroxytamoxifen complex for B_{36} slightly less ($\simeq$ 3.7-fold) than that of the R-estradiol complex ($\simeq$4.8-fold). (3) The B_{36} antibody inhibits the binding of R to DNA. This inhibition is not observed with other antibodies which do not react with R (nonspecific IgG) or with cytosol R that does not react with B_{36} (rat uterus). B_{36} did not completely inhibit the binding of complexes to DNA; a maximum of 60% inhibition was obtained for B_{36} concentrations that displaced all the R in sucrose gradients. This partial inhibition by B_{36} suggests either a decreased affinity of R for DNA or a heterogeneous population of R-DNA complexes, only some of which are affected by B_{36}. Our results contrast with those observed for the DNA binding of the glucocorticoid receptor, which was unaffected by both IgM-class monoclonal (Westphal et al., 1982) and polyclonal (Carlstedt-Duke et al., 1982) antibodies.

The relative localizations of the DNA and B_{36} binding domains of the R cannot be ascertained from these experiments. These two binding domains may partially overlap, or they may be distinct but functionally related, the binding of B_{36} to the R resulting in a decreased affinity of R for DNA. These two domains may also be distinct and functionally independent, the lower reactivity of R for DNA in the presence of B_{36} being due to steric hindrance of the DNA binding site of R by the bulky IgM antibody. A relationship between the DNA and B_{36} binding domains is, however, the most likely, since the activation step alters the degree of interaction of both R with DNA (Yamamoto & Alberts, 1972) and R with the B_{36} antibody (this paper). While molybdate totally prevented the interaction of R with DNA (J.-L. Borgna, unpublished results), it decreased but did not suppress the R-B_{36} interaction. However, even though the R-4-hydroxytamoxifen complex interacted more strongly with the B_{36} antibody than the R-estradiol complex, the effect of this antibody on the DNA binding of the R-4-hydroxytamoxifen complex appeared weaker. Our results suggest that the conformational changes of R induced by estradiol or by 4-hydroxytamoxifen differ at the hormone site (Rochefort & Borgna, 1981), the DNA binding domain (Evans et al., 1982), and the B_{36} binding domain (this paper). The present study illustrates that monoclonal antibodies can be used to discriminate different forms of R and to characterize some of their binding domains. Further studies with purified native R are required to map the binding domains located at the surface of R and to discriminate between those which are altered when R interacts with estrogens or anti-estrogens.

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Oxygen Equilibrium Studies on Carp–Human Hybrid Hemoglobins†

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ABSTRACT: Hybrid hemoglobins have been made in which one chain is derived from human hemoglobin and the other from carp hemoglobin. Both hybrid hemoglobins show low cooperativity in oxygen binding. Hybrid I (α carp:β human) has a very small Bohr effect, whereas hybrid II (α human:β carp) has a Bohr effect nearly as large as that for human hemoglobin. Both hemoglobins have P_{50}'s more closely resembling carp hemoglobin than human hemoglobin in the region of pH 7, and for both hybrids, as for carp, cooperativity virtually disappears at acid and alkaline pHs. Since both hybrids are formed from chains derived from cooperative parent hemoglobins, it is difficult to account for the low cooperativity in terms only of the T-state salt bridges and the α_1–β_2 contacts involved in the R–T switch region. We suggest that the F9 Ser in the carp β-chain as well as α_1–β_1 interactions is important in controlling the allosteric transitions in these hybrids.

Oxygen binding equilibria have been extensively studied in both human and carp hemoglobins (Roughton & Lyster, 1969; Imai & Yonetani, 1975; Imai, 1973; Noble et al., 1973; Tan et al., 1973; Chien & Mayo, 1980; Parkhurst et al., 1983). In human hemoglobin, the cooperativity is virtually unchanged over the pH range 6–9, and the ligation has frequently been interpreted to first order in terms of R and T quaternary conformations (Shulman et al., 1975; Baldwin, 1975). Within this framework, the Bohr effect corresponds to a shift of the R–T equilibrium toward T as the pH is decreased from 9 to 6. In many fish hemoglobins, the pronounced Bohr effect is known as the "Root effect" (Root, 1931; Root & Irving, 1941, 1943). At pH 6 (+IHP),[1] carp hemoglobin, in both deoxy and liganded forms, is in the T state; at pH 9, both deoxy and liganded forms are in the R state (Tan et al., 1972, 1973). At these extremes of pH, the hemoglobin is noncooperative in oxygen or CO binding. At intermediate values of pH, an equilibrium exists between the R and T forms, and cooperativity is observed in ligand binding. The importance of the β-93 Ser in carp and its interaction with His-147β have recently been discussed (Parkhurst et al., 1983; Perutz & Brunori, 1982). We were interested in observing the ligation properties of hybrid hemoglobins made by combining complementary chains from these two cooperative parent hemoglobins. We report in this paper the oxygen equilibrium binding properties of the two human–carp hybrid hemoglobins and discuss the ligand equilibria in terms of allosteric models and substitutions at selected sites.

Materials and Methods

Protein Preparation. Human hemoglobin was prepared from whole blood and separated into α- and β-chains by the methods described by Geraci et al. (1969). The separated chains were stored frozen as small pellets under liquid nitrogen. Carp hemoglobin was prepared from red blood cells (also stored in liquid nitrogen) as described by Tan et al. (1972). The carp hemoglobin was then separated into α- and β-chains as described in the following paper (Goss & Parkhurst, 1984). For these preparations, the CO form of the protein was used to prepare carp hemoglobin chains. Polyacrylamide gel electrophoresis was carried out in slabs following Brewer &

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[1] Conventional three-letter codes are used for the amino acids: Ser, His, Glu, Asp, Val, Gln, Pro, Ala, Lys, Arg, Trp, and Thr refer respectively to the amino acids serine, histidine, glutamic acid, aspartic acid, valine, glutamine, proline, alanine, lysine, arginine, tryptophan, and threonine. Results referred to with +IHP are for experiments in which the buffer contained 1 mM inositol hexaphosphate (IHP).

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