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Effects of Immunosuppressants FK506 and Rapamycin on the Heterooligomeric Form of the Progesterone Receptor

Jack-Michel Renoir, Stéphane Le Bihan, Christine Mercier-Bodard, Alex Gold, Merhdad Arjomandi, Christine Radanyi and Etienne-Emile Baulieu

Lab. Hormones, INSERM U33, 80 rue du Général-Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France

The non-DNA binding form of the rabbit uterus cytosol progesterone receptor (PR) contains, in addition to the hormone binding unit and heat shock protein M_r 90kDa (hsp90), a Heat shock protein Binding Immunophilin (p59/HBI) which interacts with hsp90. P59/HBI binds the immunosuppressants FK506 and Rapamycin (RAP) and belongs to the FK506 binding protein family. A recombinant p59/HBI–glutathione-*S*-transferase fusion protein, purified by Sephadex LH-20 filtration of tritiated drug–p59/HBI complexes, binds FK506 and RAP with apparent K_d values of 75 ± 40 and 40 ± 15 nM, respectively. Immunopurification from cytosol of [^{3}H]steroid-labeled tungstate-stabilized PR with anti-PR immunoadsorbent yielded "9S"-PR species in which hsp90, hsp70 and p59/HBI were present. In the absence of tungstate ions, only the 4–6S PR was eluted, and Western blot analysis demonstrated the absence of hsps and p59/HBI. In contrast 30 to 50% of the original 9S-PR species containing hsps and p59/HBI, was eluted in the absence of tungstate ions but after exposure of cytosol to 5 μM FK506 or RAP. Other experiments showed that cytosol fractions incubated for 2 h at 25°C with 0.05 to 10 μM FK506 or RAP, then with [^{3}H]steroids (the agonist [^{3}H]Org 2058 or the anti-progestin [^{3}H]RU486), contains greater amounts of 9S-PR species than that detected in non-immunosuppressant exposed control cytosol. Scatchard analysis showed an up to 2-fold decrease of the K_d value for both hormones following exposure to drugs, without modification of the number of steroid binding sites. Purification of cytosol PR on immobilized FK506 yields a 9S form still containing hsp90, hsp70 and p59/HBI associated to PR units. Altogether, these results suggest that binding of immunosuppressants to p59/HBI does not promote hsps dissociation from the receptor and, as a consequence, that inhibition of peptidyl-prolyl isomerase activity of p59/HBI by immunosuppressants binding does not transform (activate) PR *in vitro*. However, given the assumption that hsp90 binds to receptor and that p59/HBI binds hsp90 but not directly to receptor, immunosuppressants affect hormone binding by an unknown mechanism involving receptor associated proteins. In addition, we show that the chick oviduct cytosol 9S-PR, not displaced with the EC1 antibody specific for several mammalian p59/HBI, also binds to FK506 columns and can be eluted by exchange with either FK506 or RAP, suggesting that there is an avian HBI homolog. These findings extend the notion of immunophilin–steroid receptor association and suggest that, besides the already described molecular chaperone hsp90, other associated proteins might be involved in steroid receptor function.

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*Correspondence to J.-M. Renoir.
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Abbreviations: [^{3}H]Org 2058, 16α-ethyl-21-hydroxy-19-nor [6,7-^{3}H] pregn-4-ene-3,20-dione; [^{3}H]RU486,11β-(4-dimethyl-aminophenyl)-17β-hydroxy-17α-(prog-1-ynyl) estra-4,9-dien-3-one; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBST, phosphate buffered saline, containing 0.5% Tween 20; GST, glutathione-*S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside

INTRODUCTION

The non-DNA binding form of steroid hormone receptors is known to contain, besides the hormone binding unit(s), at least one well characterized heat shock protein, hsp90 (see [1, 2] for reviews). In addition to hsp90, and crosslinkable to it *in vitro* [3] as well as *in vivo* [4], a 59,000 Da protein (p59) is detected

by the EC1 monoclonal antibody [5] in several mammalian [3, 6] non-transformed steroid receptor complexes. This p59 was recently cloned [7] and both biochemical experiments [8, 9] and hydrophobic cluster analysis of its amino acid sequence [10] identified p59 as a new member of the immunophilin family. Immunophilins form a class of ubiquitous proteins which bind immunosuppressants such as cyclosporin A (CsA) and FK506 or Rapamycin (RAP). Two distinct subclasses of immunophilins, cyclophilins which bind CsA and FK506 binding proteins (FKBPs) which bind FK506 and RAP are actually the matter of much research because they are thought to mediate the biological function of immunosuppressants (see [11] for review). All immunophilins identified so far possess peptidyl-prolyl-isomerase (or rotamase) activity [11], but the inhibition of this rotamase activity following immunosuppressant binding is not responsible for the immunosuppressive function of the drug [11–13]. Since p59 binds directly to hsp90 [3] and not to the receptor(s) itself, we named it Heat shock protein Binding Immunophilin (p59/HBI) [10].

It is not known if the mechanism of immunosuppression involves p59/HBI and what the consequences of drug binding to p59/HBI on the structure of associated proteins are. Since p59/HBI was originally identified as one of the non-hormone binding proteins associated with steroid hormone receptors in their non-DNA binding 9S-form [3, 6, 14], we have studied the effects induced by FK506 and RAP upon the heterooligomeric form of the rabbit uterus progesterone receptor (PR).

Preliminary *in vitro* experiments indicated that both drugs do not modify the composition of the 9S-PR, but increased its binding activity for agonist and antagonist steroids [15]. We show in this work that this is mainly due to a decrease of the apparent dissociation constant of ligands rather than an increase of the number of binding sites. In fact, exposure of rabbit uterus 9S-PR to FK506 affinity resin, does not modify the protein composition of the heterooligomer and both hsp90 and hsp70 are still present together with p59/HBI in the PR eluted from affinity gel by FK506 or RAP. Similar data have been obtained with chick oviduct 9S-PR suggesting that it contains a p59/HBI homolog, not detected with the monoclonal antibody EC1. For the first time, we show that the PR eluted from such an immunosuppressant affinity gel migrates at the 9S level in density gradients. The putative function of p59/HBI with regard to steroid receptors will be discussed on the basis of its multifunctional features; binding of immunosuppressant, [8, 9 and this work], binding to hsp90 and not to receptors themselves [3, 14], binding to calmodulin [16] and to ATP (or GTP) [17].

MATERIALS AND METHODS

Chemicals

[^{3}H]Org 2058; sp. act. = 44.6 Ci/mmol was from the Radiochemical Centre (Amersham, Bucks., England), [^{3}H]RU486; sp. act. = 44 Ci/mmol was a gift from Roussel-Uclaf (Romainville, France). Affigel-10 was purchased from Biorad (Richmond, CA) and FK506–Affigel-10 (0.5 mg FK506/ml of gel) was a gift from Dr M. D. Deibel (Upjohn Lab., Kalamazoo, MI). The immunoadsorbant anti-rabbit PR was a gift from Dr E. Milgrom (Le Kremlin-Bicêtre, France) and contains 1.1 mg of the specific monoclonal antibody (MI60 [18]) per ml of protein A–Sepharose. Its synthesis was performed as described previously [19, 20]. Mouse monoclonal anti-p23 (R3) was a gift from Dr D. Toft (Mayo Clinic, MI). Tritiated [^{3}H]dihydro-FK506 (50.3 Ci/mmol) was synthesized by Amersham, and [^{3}H]RAP (17.4 Ci/mmol) was obtained from Dr S. R. Howell (Wyeth-Ayerth). FK506 and RAP were gifts from Dr K. Murato (Fujisawa) and Dr S. Sehgal (Wyeth-Ayerth), respectively. CsA was a gift from Sandoz (Bâle, Switzerland). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden) and all other reagents were purchased from Merck (Darmstadt, Germany).

Buffers

Buffer A contained 10 mM Hepes, 10% glycerol, 2 mM dithiotreitol (DTT), 20 mM Na_2WO_4 pH 7.6. Buffer B was identical to buffer A but lacked Na_2WO_4. Buffer C was 0.1 M sodium phosphate plus 20 mM Na_2WO_4 pH 7.4. Buffer D was only used in Sephadex LH-20 filtration and contained 20 mM phosphate, 2 mM DTT pH 7.4.

Cytosol preparation

Cytosol fractions from the uterus of estradiol stimulated rabbit [3] and from oviducts of 5-week-old estradiol stimulated chicks [21] were prepared in buffers A or B supplemented with a mixture of protease inhibitors [3]. The cytosol fraction of Jurkat T cells was prepared in buffer A as described previously [9]. In order to label the PR, rabbit uterus and chick oviduct cytosols were incubated with 50 nM of either [^{3}H]Org 2058 or [^{3}H]RU486 for 16 h at 0°C.

Sephadex LH-20 filtration

The gel (1.8 ml) was equilibrated in buffer D and the technique described by Handschumacher *et al.* [22] was followed. Purified aliquots of recombinant rabbit p59/HBI, isolated from transformed *E. coli* (see below), were incubated with increasing amounts of [^{3}H]immunosuppressants (from 0.5 to 500 nM) for 60 min at 20°C in siliconized tubes and transfered to Sephadex columns. Since radioactive drug–p59/HBI complexes, as well as drugs themselves, stick to the walls of columns and capillary tubing, the complexes were diluted in 0.3 ml of Jurkat cell cytosol (final protein concentration 1 mg/ml) that had previously been chromatographed through FK506 affinity resin in order to remove all FKBPs capable of FK506 (or RAP) binding. Fractions eluted from the LH-20 columns were collected and portions (0.1 ml) were counted for radioactivity in duplicates. The values for

bound drug represent the ^{3}H-drug eluted in the void volume. Total incubated ^{3}H-drug was directly measured in duplicate aliquots (20 μl) of incubates. Scatchard plot analysis was performed by a computer program [23].

Affinity purifications

FK506–Affigel chromatography. Cytosol fractions (5 ml) incubated with radioactive steroids were rotated for 3 h at 4°C with 0.5 ml FK506–affinity resin. The flow-through fraction was then collected and the columns were washed with buffer C, followed by reequilibration in buffer A. Bound proteins were eluted by 3 successive column volumes of buffer A containing 1 mM FK506 (or RAP) for 30 min at 4°C. Similarly, FK506–affinity gel purification of FKBPs from Jurkat cytosol was performed. The remaining content of FK506 binding proteins was assayed by Sephadex LH-20 filtration after incubation of the flowthrough with [^{3}H]dihydro-FK506 (0.5 to 500 nM for 60 min at 20°C).

Anti-PR immunoadsorbent chromatography. Aliquots of the rabbit uterus cytosol fraction (5 ml) were rotated overnight at 4°C with 1 ml of protein A–Sepharose-MI60. After flowthrough collection, the resin was washed as described previously [20] and bound proteins were eluted by 30 min incubation with 50 mM diethylamine, pH 10.5, containing 20 mM Na_2WO_4. Three successive elutions were performed, and immediately neutralized with 20 mM Na_2HPO_4 [19]. The eluates containing most bound radioactive steroid, as measured by the dextran-charcoal adsorption technique, were pooled and further analyzed for protein content and sedimentation constant measurement.

Denaturing electrophoresis and immunoblotting

The proteins contained in the eluates of the FK506–affinity and immunoadsorbent columns were analyzed in 10 or 12.5% SDS polyacrylamide gels according to Laemmli [24]. Following electrophoresis, they were transferred to PVDF membranes (Immobilon, Millipore) with a horizontal × blot apparatus (Cera Labo, France). Non-specific sites were saturated with 10% non-fat milk in PBST, and membranes were incubated with specific antibodies used at appropriate dilutions. The antibodies used were EC1, a mouse monoclonal specific for p59/HBI of several mammals [3, 6, 14]; 7C10, a mouse monoclonal antibody specific for rabbit hsp90 [25]; BF4, a rat monoclonal antibody specific for chicken hsp90 [26] and N27, a mouse monoclonal antibody specific for hsp70 [27] obtained from Stress Gen (Victoria, Canada). EC1 and N27 were used at 10 and 20 μg/ml, respectively, and 7C10 and BF4 at a 1:1000 dilution of ascites fluid. The mouse monoclonal antibody anti-p23, R3 was used at 1:500 dilution, and 173, a rabbit C-terminal anti-p59/HBI [7] was used at 100 μg of purified IgGs/ml.

Exposure of cytosol to immunosuppressants

Cytosols were exposed at various temperatures to different amounts of either FK506 or RAP or CsA (10 nM to 10 μM) for at least 2 h, followed by incubation with 50 nM [^{3}H]steroid overnight at 0°C. Specific steroid binding was measured by subtracting the amount of bound [^{3}H]steroid measured after incubation including 5 μM of radioinert analog steroid from that obtained with the tritiated steroid following dextran-charcoal adsorption.

Recombinant expression of p59/HBI in E. coli

The rabbit p59/HBI cDNA [7] subcloned at the EcoRI site of pGEM-7ZF (Promega), was digested with XhoI and EcoRI, treated with mung bean nuclease, and subcloned into the SmaI site of pGEX-2T (Pharmacia). According to this subcloning procedure, p59/HBI cDNA lacking the N-terminal portion encoding the first 18 amino acids [7] was inserted, in frame, with the glutathione-*S*-transferase (GST) coding sequence and expressed, after induction with IPTG, as a fusion protein in the *E. coli* strain UT5600. The expressed recombinant protein was then purified as described previously [28].

Sucrose density gradient analysis

Sedimentation analyses were performed at 2°C in 5–20% sucrose density gradients in buffer A in a SW60 rotor (Beckman) for 16 h at 48,000 rpm, as described previously [20]. Incubation with antibodies was performed at least 2 h at 4°C prior to loading samples on preformed gradients.

Protein determination and radioactivity counting

Proteins were measured according to the method of Schaffner and Weissman [29] with bovine serum albumin as standard and radioactivity was measured in a Scintillation Minaxi Counter (Packard Instruments) with 55% efficiency.

RESULTS

Binding of [^{3}H]dihydro-FK506 and of [^{3}H]RAP to recombinant p59/HBI

In preliminary experiments, rabbit uterus p59/HBI purified on EC1-immunoaffinity columns [3, 20] did not bind FK506 or RAP, probably as a result of a loss of structural integrity due to the elevated pH used for elution. For this reason, recombinant p59/HBI [78 kDa according to SDS–PAGE, see Fig. 1(A)], was used for binding studies using the Sephadex LH-20 filtration technique. Purified recombinant p59/HBI was introduced to Jurkat T cell cytosol (1 mg/ml total protein concentration) previously depleted of FK506 binding protein [see Materials and Methods and Fig. 1(B), right panel], and incubated with various amounts of tritiated drugs. As shown in Fig. 1(B), the amount of [^{3}H]dihydro-FK506 flowing through the column is proportional to the amount of drug

present in the incubate. Similar experiments were performed with [^{3}H]RAP instead of [^{3}H]dihydro-FK506. The response to bound tritiated drugs reached a plateau beyond 100 nM [Fig. 1(C)]. Scatchard plot analysis indicated an apparent K_d for [^{3}H]dihydro-FK506 of 75 ± 40 nM and for [^{3}H]RAP of 40 ± 15 nM.

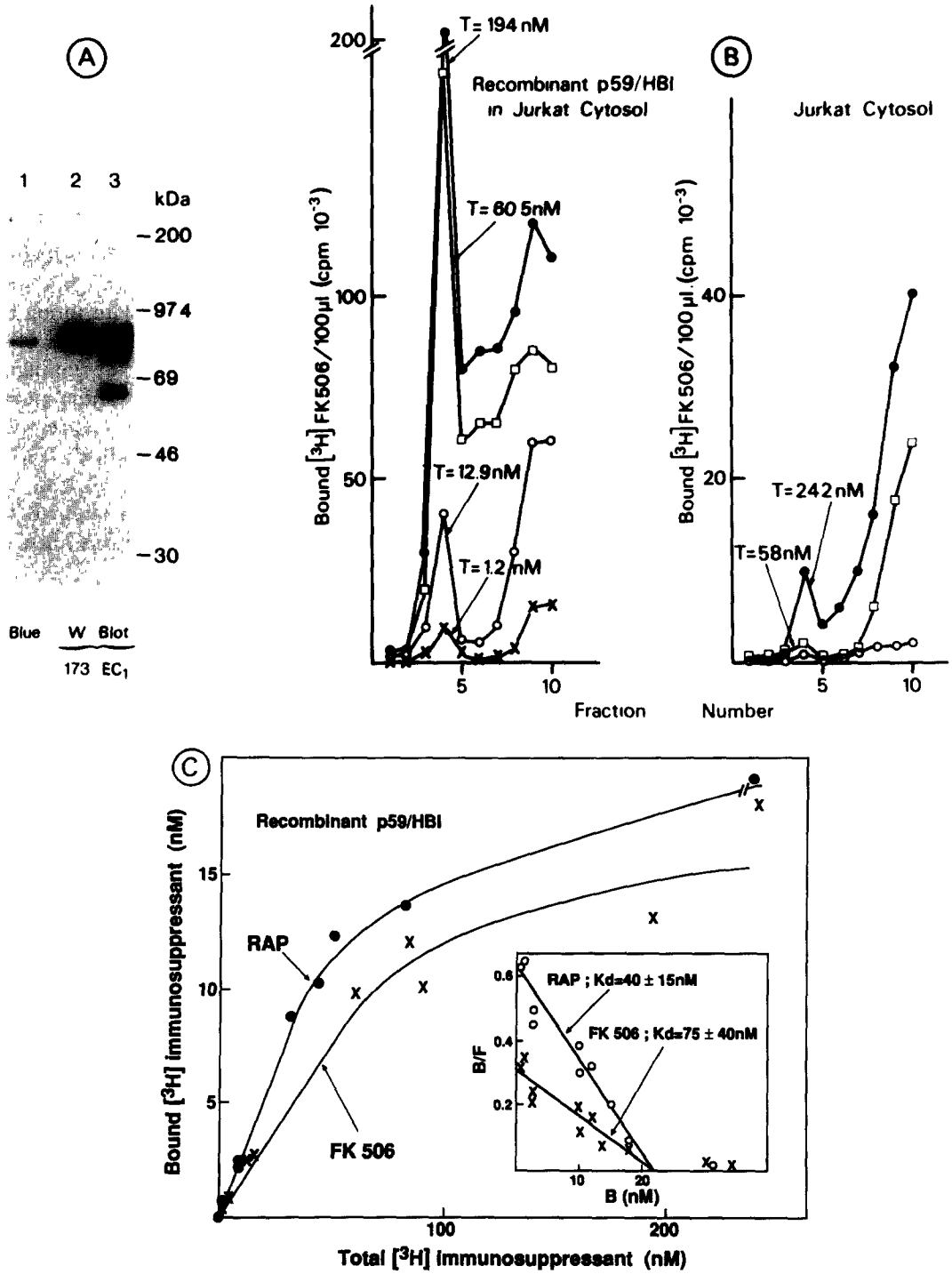

Fig. 1. Binding of [^{3}H]dihydro-FK506 and [^{3}H]RAP to the recombinant GST-p59/HBI. The purified fusion protein (1.5 µg) was analyzed by 10% SDS-PAGE (A) and Coomassie blue stained (lane 1) or blotted with 173 anti-peptide antibody (lane 2) or with EC1 (lane 3). The purified fusion protein migrates at M_r 78 kDa and contains only limited proteolytic fragments. For the ^{3}H-drug binding assay (B) 1.5 µg of purified recombinant protein was diluted in 1 ml of Jurkat cell cytosol (1 mg/ml final protein concentration), depleted of FK506 binding proteins by FK506-affinity chromatography. A control experiment depicted the remaining FK506 binding proteins in 0.3 ml diluted and FKBPs depleted Jurkat T cells cytosol is shown in B, right panel. Aliquots (0.3 ml) of recombinant p59/HBI diluted in depleted Jurkat T cell cytosol were incubated for 1 h at room temperature with various amounts (1 to 500 nM) of [^{3}H]dihydro-FK506 or [^{3}H]RAP, then loaded on to Sephadex LH-20 columns in buffer D. Fractions were collected and the bound radioactive drug flowing through was quantitated as described in Materials and Methods. (C) Represents the increase of [^{3}H]dihydro-FK506 (x—x) and [^{3}H]RAP (○—○) versus the total (T) incubated ^{3}H-drug. Insert in C represents the Scatchard plot analysis. Each point is the mean of two different experiments.

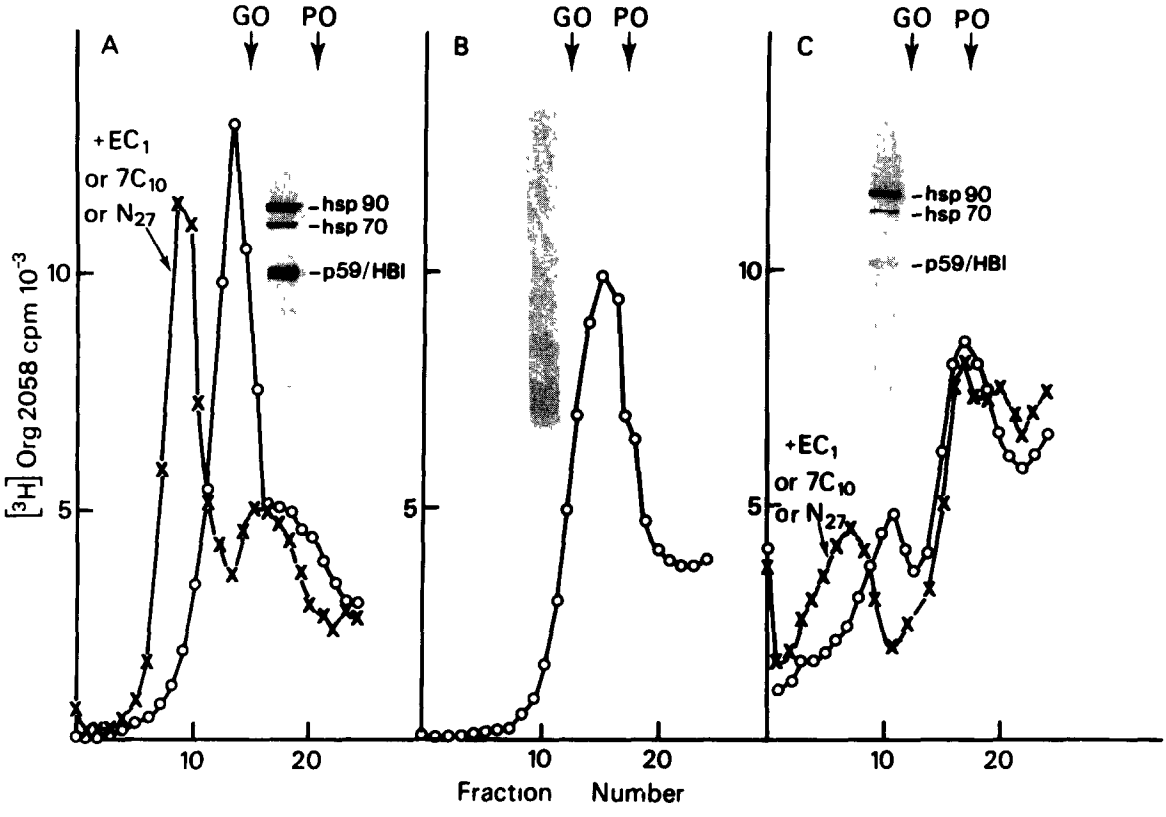

Fig. 2. Purification of 9S-rabbit uterus PR by anti-PR immunoadsorbent following exposure of cytosol to FK506. Rabbit uterus cytosol was prepared in buffer A in order to stabilize the heterooligomeric structure of the PR against the salt induced dissociating effect (A). In B and C, cytosol was prepared in buffer without tungstate, but incubated in the presence (C) or absence of (B) 10 μM FK506 (or RAP, not shown, same results). Each cytosol (5 ml) was further incubated at 4°C with 50 nM [^{3}H]Org 2058, then rotated for 18 h at 4°C with 1 ml of anti-PR (MI60) -immunoadsorbent. Following washing with 10 column volumes of buffer containing (A) or not containing (B and C) Na_2WO_4 and reequilibration in buffer with Na_2WO_4, bound proteins were eluted by 50 mM diethylamine plus tungstate (20 mM), pH 10.5. The size of the eluted labeled PR was analyzed in 200 μl aliquots of the eluates by 5–20% sucrose gradient in buffer containing Na_2WO_4, in a SW60 rotor after 48,000 rpm × 16 h centrifugation, with glucose oxidase (GO = 7.9S) and peroxidase (PO = 3.6S) as internal standards, before (○—○) and after (x—x) incubation with EC1 (50 μg) or 7C10 (50 μg) or N27 (100 μg). Control experiment made with non-immune mouse IgGs (50 μg) gave a profile similar to that obtained in (○—○). Western blotting of each eluted sample was also performed with the mixed three monoclonals and results are shown in the inserts of A to C. Positions of hsp90, hsp70 and p59/HBI are indicated, and the amounts of protein loaded on the gels were 42 μg in A, 29 μg in B and 26 μg in C.

According to the calculated number of binding sites, a single FK506 or RAP molecule appears to be bound to one molecule of p59/HBI fusion protein. This suggests that, in agreement with prediction of structure, p59/HBI binds only one molecule of immunosuppressant although it possesses three FKBP12 related domains [10].

Effects of FK506 and RAP on the heterooligomeric PR structure

Anti-PR immunopurification. We have already shown that tungstate-stabilized rabbit uterus PR [3] can be isolated from anti-PR immunoadsorbent in a form migrating at 9S in sucrose density gradient [20]. This form contains hsp90 and p59/HBI as revealed by Western blots [20]. Here, we also identified hsp70 in the eluted 9S[^{3}H]Org 2058–PR structure, since there is a shift into 11S after incubation with N27 [Fig. 2(A)] and a band at 70 kDa was specifically revealed by this antibody in blotting experiments [Fig. 2(A) insert]. Similarly, both hsp90 and p59/HBI are also present [Fig. 2(A)]. It is interesting to note that a protein migrating at 23 kDa, previously identified in the non-transformed chick oviduct PR [30] is also present in the eluate (not shown), but we have no evidence of its association in the affinity purified 9S-PR.

An identical experiment performed with buffer B, containing no tungstate ions, does not allow purification of 9S-[^{3}H]Org 2058–PR complexes, but only 4–6S species in which neither hsps nor p59/HBI are detectable [Fig. 2(B)]. This is explained by high salt exposure during the washing steps of the columns [1–3, 20]. However, hsps and p59/HBI are present in eluates of the anti-PR immunoadsorbant, if the cytosol was incubated for 2 h at 4°C with 10 μM FK506 (or RAP) prior to incubation with tritiated steroid [agonist, Fig. 2(C), or antagonist, RU486, not shown]. The yield of 9S-PR form is 2 times higher in the case of [^{3}H]RU486 labeling, than that obtained with [^{3}H]Org 2058 (not shown), suggesting an *in vitro* stabilization of rabbit uterus PR interaction with hsp90 as observed previously [31].

FK506–Affigel affinity chromatography. Figure 3 shows that rabbit uterus PR can be eluted by FK506 or RAP from FK506–Affigel columns. The eluted PR migrates at 9S in density gradients [Fig. 3(B)], suggesting the presence of hsps and p59/HBI in association with the eluted PR. This is confirmed by the shifts observed following incubations with either 7C10, or N27 or EC1 antibodies, respectively. These results demonstrate that, together with the PR hormone binding unit, hsp90, hsp70 and p59/HBI, also detected in Western blotting [Fig. 3(A)], are eluted from the FK506–affinity resin in a structure in which they are

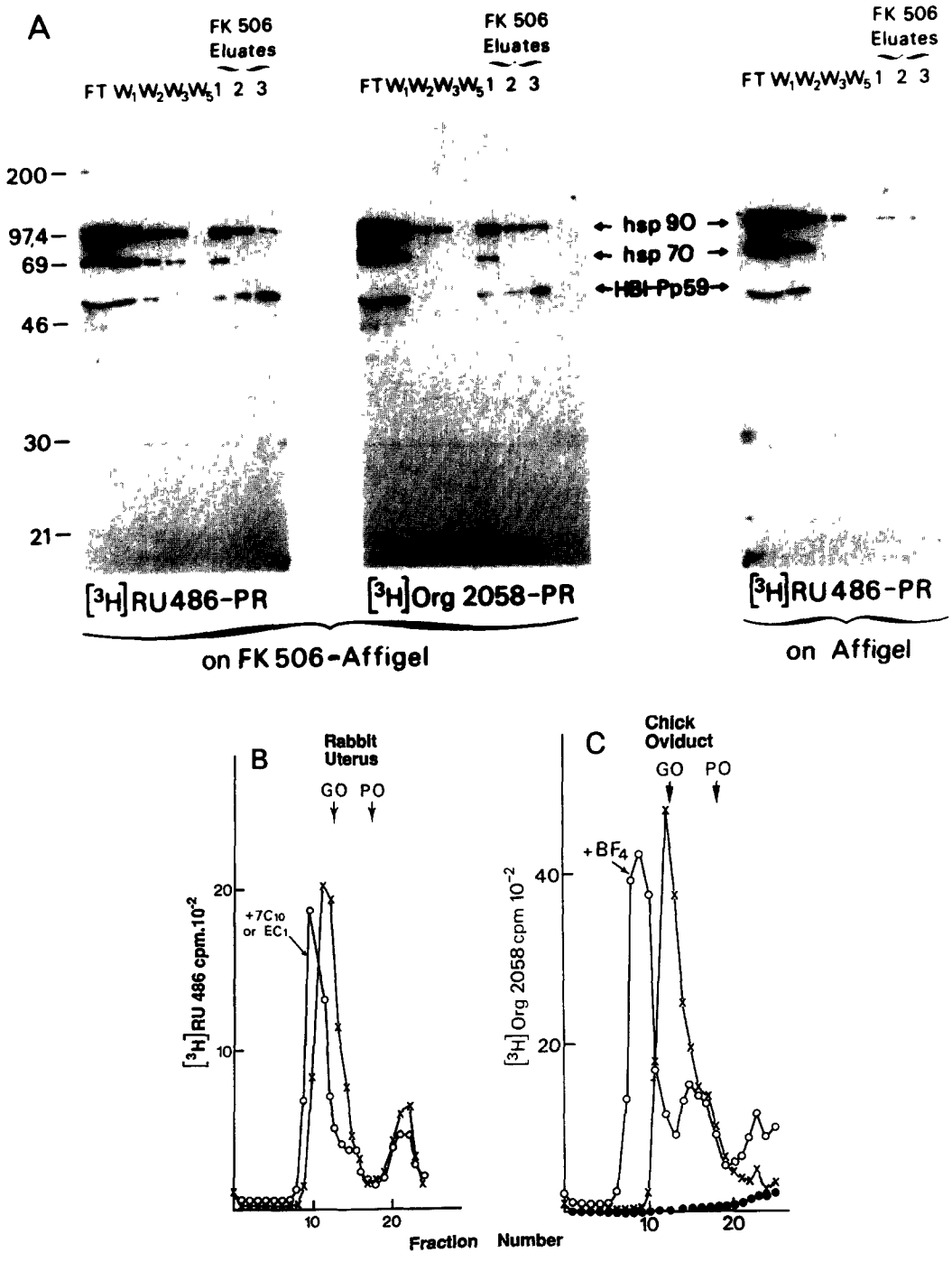

Fig. 3. Purification of 9S-PR from rabbit uterus and chick oviduct by FK506–affinity chromatography. Rabbit uterus cytosol (5 ml) prepared in buffer A was incubated with 20 nM [^{3}H]RU486 or [^{3}H]Org 2058, then rotated overnight with 0.5 ml FK506–Affigel resin at 4°C, or 0.5 ml Affigel alone acting as the control. The flowthrough (FT) and 0.4 M NaCl washing (W_1 to W_3) fractions and reequilibration fractions (W_4, W_5) were collected. The bound proteins were eluted by exchange with 1 mM immunosuppressive drugs (see Materials and Methods). Aliquots (100 μl) of the FT, the washings and the eluates were analyzed by 12.5% SDS–PAGE and Western blotting with the mixed 7C10, N27 and EC1 monoclonal antibodies (A). Similar results were obtained with both FK506 and RAP eluates. In B, 25 μl aliquots of the three eluates obtained for [^{3}H]RU486 incubated rabbit uterus cytosol, were mixed and analyzed in sucrose gradients, prior (x—x) or after incubation (○—○) with 7C10 (25 μg) or EC1 (30 μg). C shows the radioactive profile of the eluates (100 μl) of FK506–Affigel affinity purified chick oviduct PR by the same procedure, before (x—x) or after (○—○) incubation with BF_4 (20 μg).

associated. Neither PR (as revealed by sucrose gradient experiment), nor hsps and p59/HBI are eluted by 10 mM CsA (not shown).

A similar experiment was performed with the chick oviduct cytosol prepared in buffer A. As demonstrated by the radioactive peak migrating at 9S following ultracentrifugation analysis of the FK506 (or RAP—identical results) eluate(s) [Fig. 3(C)], it is suggested that an equivalent of p59/HBI is present in chicken. Up to now, a p59/HBI homolog was not detected in this species, due to the lack of cross-reactivity observed with the previously well described anti-p59/HBI, EC1 [3] and 173 [7].

Exposure of cytosol to immunosuppressants. Cytosol prepared in buffer A or B was treated with increasing amounts (10 nM to 10 μM) of either FK506 or RAP (Fig. 4) or CsA (not shown), then the PR was labeled with [^{3}H]steroid as described in Materials and Methods. Under these *in vitro* experimental conditions, the PR always migrates at the same 9S position A (Fig. 4). This, together with the visualization of hsps and p59/HBI following FK506 (or RAP) elution from affinity columns, suggests that binding of immunosuppressants to 9S-PR does not promote *in vitro* transformation of the receptor. However, *in vivo*, it is possible that similar exposure may alter the heterooligomeric complex in a way that could increase the PR-mediated gene(s) expression. Such a potentation was recently observed for the GR-mediated expression of the murine mammary tumor virus chloramphenicol acetyl transferase (MMTV-CAT) reporter plasmid [32].

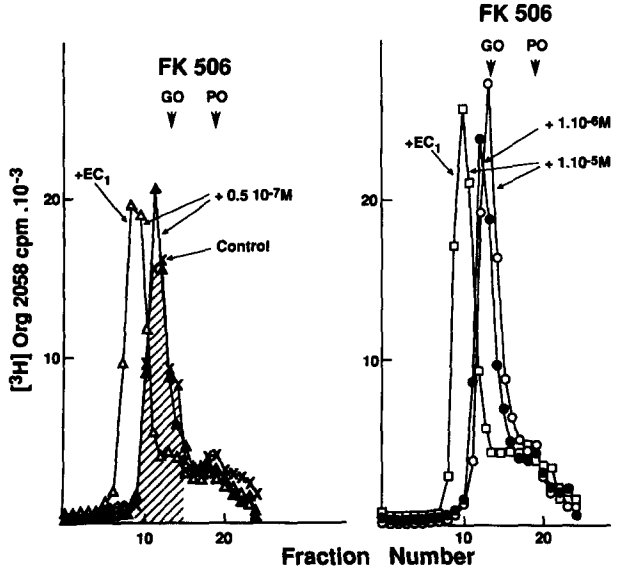

Fig. 4. Effect of FK506 on agonist binding to 9S-rabbit uterus PR. Rabbit uterus cytosol aliquots (100 μl) in buffer B were incubated for 2–4 h at 0°C with or without (x—x) various amounts of FK506 [0.5-10^{-7} M,(▲—▲ and △—△) left panel; 1 μM (●—●) and 10 μM (○—○) and (□—□) right panel] then with 50 nM [^{3}H]Org 2058 (18 h at 0°C) before analysis on sucrose gradients as described in Materials and Methods. The presence of p59/HBI in the 9S complex is revealed by the shifts at 11S observed after 4 h incubation at 0°C with 20 μg EC1 (△—△) in A and (□—□) in B. The hatched area in A depicts the height of the control (without FK506). Positions of internal standards (GO = 7.9S and PO = 3.6S) are indicated by arrows.

Effects of FK506 and RAP upon hormone binding to steroid receptors

Rabbit uterus PR. Despite the fact that there is no change in the size of the PR exposed to immunosuppressive agents (Figs 4 and 5), a small, but significant dose-dependent increase of PR binding of [^{3}H]Org 2058 is observed. Dextran-charcoal measurements (not shown) indicated an up to two times increase and identical results were obtained with the anti-progestin [^{3}H]RU486 (not shown). Analysis by ultracentrifugation demonstrated that this increase occurs only with the 9S-species (tungstate stabilized), but not with the tungstate stabilized 8S-complexes treated with 0.3 M NaCl (not shown) nor with the 4–6S species [activated receptor, Fig. 5(C)].

Since we have previously shown that tungstate ions stabilize PR–hsp90 interaction(s) but not the hsp90–p59/HBI complex against high salt dissociating processes [3], and since p59/HBI is responsible for immunosuppressant binding in the heterooligomeric receptor [8, 9, 15, 33], it is suggested that the modification of the hormonal binding capacity is mediated via p59/HBI. This interpretation is strengthened by the lack of any effect on the p59/HBI-free PR [Fig. 5(C)].

In order to check if this phenomenon affects the steroid affinity or the number of binding sites of the PR, we incubated aliquots of rabbit uterus cytosol in buffer A in the absence or presence of various amounts (0.05 to 5 μM) of either FK506 or RAP for 2 h at 22°C. The receptor was then labeled with tritiated ligands (50 nM for gradient experiments; 0.1 to 500 nM for Schatchard experiments). Ultracentrifugation analysis revealed that the PR binding is higher after drug exposure (Fig. 6). In fact, and as revealed by Scatchard plots, this reflects a higher resistance of PR complexes to thermal inactivation since the apparent K_ds measured following exposure to either FK506 or RAP at 22°C (Fig. 7) are two times smaller than those measured in control experiment, without any significant modification of the number of binding sites.

Other receptors in other species. Preliminary experiments (not detailed here) have indicated that chick oviduct 9S-PR is not only purified as such by FK506–affinity chromatography [see Fig. 3(C)], but it is sensitive to both FK506 and RAP binding similarly to the 9S-rabbit PR. An increase in the apparent binding capacity of the MCF7 estradiol receptor has also been observed (C. Mercier Bodard and J. M. Renoir, work in progress).

CONCLUSION

Steroid hormone receptors were shown to contain, in addition to hsp90 [1, 2], a 59,000 Da protein [6] which associates with hsp90 [3, 4] in the heterooligomeric, non-DNA binding receptor complex. Since the rabbit

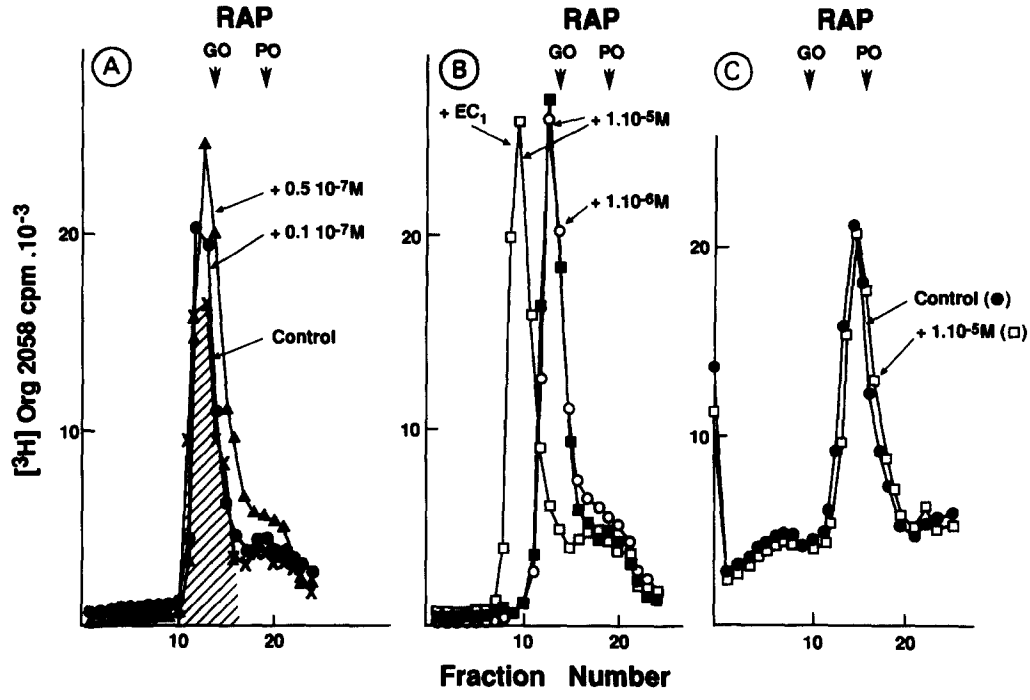

Fig. 5. Effect of RAP on agonist binding to rabbit uterus PR. Rabbit uterus cytosol aliquots (100 μl) in buffer B were incubated for 2–4 h at 0°C with or without (x—x) various amounts of RAP [10 nM, (●—●); 50 nM (▲—▲) in A; 1 μM (○—○) and 10 μM (■—■) and (□—□) in B], then with 50 nM [^{3}H]Org 2058 as indicated in Fig. 4. The presence of p59/HBI in the 9S complex is revealed by the shift at ~ 11S observed after incubation with EC1 (20 μM,□—□ B). In C, cytosol prepared in buffer B was treated overnight at 0°C with 0.3 M NaCl then incubated (□—□) or not (●—●) with 10 μM RAP before labeling of the receptor with [^{3}H]Org 2058 as indicated above. Similar data were obtained with 10 μM FK506 instead of RAP. The hatched area in A depicts the height of the control (without RAP).

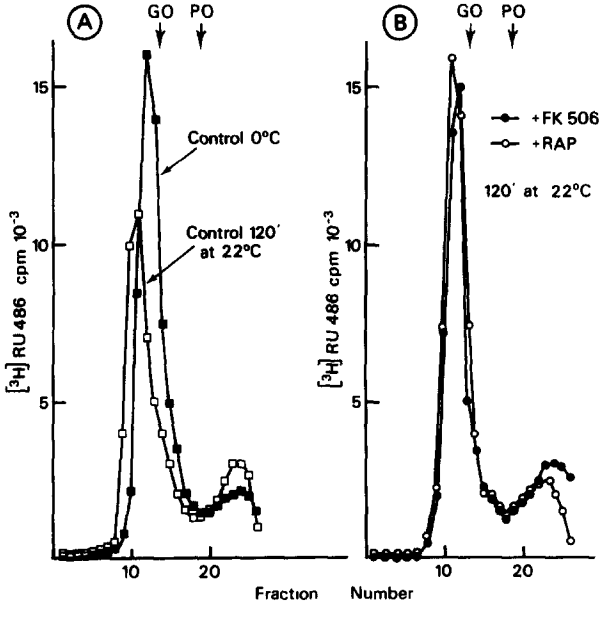

Fig. 6. Effect of FK506 and RAP on the binding capacity of rabbit uterus PR. Effect of temperature. Rabbit uterus cytosol was prepared in buffer containing 20 mM Na_2WO_4, then aliquots were incubated with 10 μM of FK506 (●—●) or RAP (○—○) for 2 h at 22°C (B) or were maintained 2 h at 22°C (□—□) or on ice (■—■) (A) prior to incubation with 50 nM [^{3}H]RU486. Aliquots (100 μl) were loaded onto 5–20% preformed sucrose gradients and analyzed as in Fig. 2.

p59/HBI recently cloned in our laboratory [7] was described as a new immunophilin [8–10] capable of binding to the immunosuppressive agents FK506 and RAP, we investigated the effects of these drugs on the heterooligomeric form of PR. In a preliminary report [15], we showed that *in vitro* exposure of 9S-rabbit uterus PR does not dissociate p59/HBI from the rest of the PR structure. However, a slight increase for either agonist or RU486 binding was observed. Here, we show that this increase is immunosuppressant dose-dependent (see Figs 4 and 5), and occurs only when the PR still contains p59/HBI. This observation is consistent with the property of p59/HBI containing 9S-PR to be retained and specifically eluted from immobilized FK506 resins, by either FK506 or RAP (Fig. 3). PR eluting from such resin still migrates at 9S in density gradients in association with hsps90 and -70 and also with p59/HBI, strengthening the non-dissociating or non-transforming effect induced by immunosuppressant binding *in vitro*.

Similar conclusions were recently obtained for the human and mouse glucocorticosteroid receptor (GR) [32, 34], but contrary to what was speculated for the mouse GR [34], we noted that binding of both immunosuppressive agents to p59/HBI provokes a slight modification of the hormone binding features of the PR. The change in binding capacity of the PR is likely to be due to a conformational change in the ligand

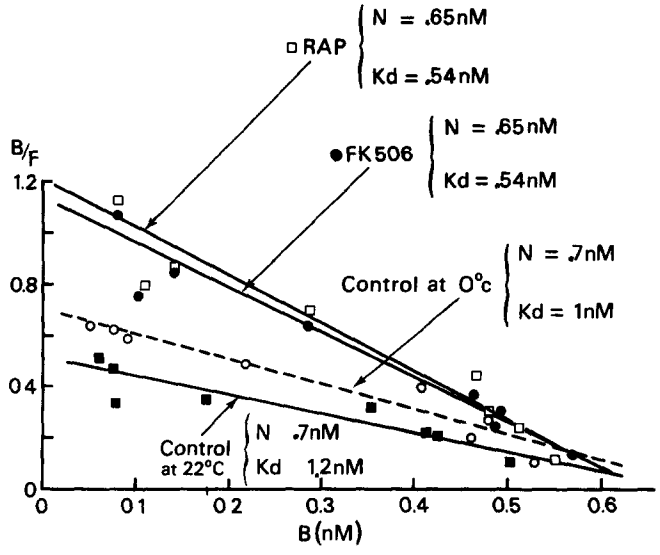

Fig. 7. Scatchard analysis of rabbit uterus 9S-PR following incubation of cytosol with immunosuppressants. Samples of cytosol identical to those described in Fig. 6 were incubated with various amounts of [^{3}H]RU486 (0.1 to 500 nM) for 16 h at 2°C. Duplicates (200 μl) were then treated by the classical dextran-charcoal technique in order to eliminate the free steroid. Bound steroid was deduced from counting the supernatant obtained after centrifugation of the cytosol/dextran-charcoal cytosol suspension. Results are plotted according to Scatchard. The control curves at 0°C (○—○) and 22°C (■—■) have $K_d = 1$ and 1.2 nM, respectively, without any change of the number of specific binding sites. The curves obtained for similar experiments performed in the presence of 10 μM FK506 (●—●) and 10 μM RAP (□—□) indicate a 50% decrease of the K_d values.

binding domain of the receptor, conducting to a greater affinity [as reflected by the decrease of the apparent K_d (Fig. 7) for the steroid]. Binding of immunosuppressants to immunophilins inhibit their rotamase activity [11, 35] which may be involved in protein folding [36], and it is possible that this modifies the function of the hsp90–PR complex.

A similar explanation can be made for the apparent stabilization of the 9S-PR structure observed *in vitro* following incubation of cytosol with FK506 (or RAP). Heterooligomeric 9S-PR-[^{3}H]Org 2058 complexes are recovered from anti-PR immunoadsorbent following preincubation of cytosol with FK506 and high salt washing, while the control experiment (no FK506) gives only hsp90, hsp70 and p59/HBI free receptor (Fig. 2). It is possible that both stabilization of the heterooligomeric structure of the receptor(s) and of their hormone binding capacity increase the hormonal response. In fact, a potentiation of the GR-mediated gene expression by FK506 and RAP was recently reported [32]. The authors concluded that FK506 increased transcriptional activation caused by low (less than saturating) concentrations of dexamethasone because it increased the rate of translocation. An alternative explanation is that FK506 increased the affinity of the GR complex for dexamethasone; this lower concentration of dexamethasone were effective in causing GR translocation and transcriptional activation. It would have been interesting to assay the dexamethasone binding affinity with and without immunosuppressant. This has been done here with progestin affinity for the PR (Fig. 7).

A further possibility could be that, following exposure to immunosuppressants, the receptor becomes differently accessible to enzymes involved in the heteromeric complex assembly/disassembly function [ATPase, phosphatase, kinase(s) activities]. It has been observed that hsp70 is an ATPase [36 for review] and hsp90 may possess such an activity [37]. We have shown that p59/HBI binds ATP and GTP [17], and it has been suggested that assembly of hsps in the heterooligomeric chicken PR structure as well as receptor activation are ATP mediated events [38]. In addition, not only p59/HBI itself binds immunosuppressants [8–10, 33 and this work], but hsp70 also could be an immunosuppressant binding protein [39, 40]. The complexity of the question increased since it has been observed that a 40 kDa protein found in the heterooligomeric structure of the estradiol receptor is in fact a CsA binding immunophilin with homologies with p59/HBI [41, 42].

In conclusion, we found that the binding of immunosuppressants FK506 and RAP to p59/HBI, in agreement with recent reported values [8, 34], is two orders of magnitude smaller than that of both drugs for FKBP12. We also observed that these two drugs affect PR by stabilizing (1) the heterooligomer without activating transformation and (2) steroid binding against heat inactivation. We believe that an avian p59/HBI homolog may exist in chick oviduct 9S-PR structure. It is emphasized that exposure to FK506 and RAP affects the conformation of p59/HBI–hsps complexes by inhibiting the rotamase activity of p59/HBI, then by a putative cascade mechanism, rendering the heterooligomeric receptor structure more accessible to enzymatic activities.

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